Ethanol-regulated gene expression of Neuroendocrine Specific Protein in mice: Brain region and genotype specificity

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CERTIFICATE OF APPROVAL

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ABSTRACT

Physical dependence on alcohol (ethanol) can develop in humans after long-term use and abuse of alcohol and is characterized by a withdrawal syndrome upon removal of ethanol. In mice, a withdrawal syndrome is characterized by convulsions and can be elicited after short or prolonged exposure to ethanol. Several neurochemical systems have been suggested to mediate seizure or convulsion activity, including $GABA_A$ and NMDA receptors. Effects of ethanol on these systems include changes in gene expression. There is an inherited component to susceptibility to develop severe withdrawal convulsions, and mice have been genetically selected for ethanol withdrawal severity. The underlying genetic factors which differentiate the Withdrawal Seizure-Prone (WSP-1 and WSP-2) mouse lines (exhibiting severe withdrawal convulsions) from Withdrawal Seizure-Resistant (WSR-1 and WSR-2) mouse lines (exhibiting minor or no withdrawal convulsions) have not yet been elucidated. Genetically selected lines are useful tools for identifying alterations of gene expression that contribute to the selected ethanol-induced phenotype.

The purpose of these studies was to identify ethanol-regulated genes and determine the influence of genotype on ethanol regulation of gene expression. It was hypothesized that ethanol interacts with cellular factors to regulate gene expression and these interactions could be differentiated between WSP and WSR mice lines. mRNA differential display was used to identify ethanol-regulated genes in the WSP-2 mice without prior knowledge of gene function.

WSP-2 mice were exposed to either ethanol (ETOH) vapor or control conditions (CTL) for 72 hours. For differential display, RNA was reverse transcribed from whole brain of ETOH and CTL WSP-2 mice and amplified in the presence of arbitrary primers. Although several candidate gene products were isolated, cloned, and used as probes for confirmation on Northern blots. One product was hybridized to two bands on Northern blots containing

whole brain RNA; one transcript was 1.4 kb and the larger transcript was 3.0 kb. Nucleotide sequence analysis of this fragment revealed >85% homology to the 3' region of human Neuroendocrine Specific Protein (NSP); this gene has multiple transcripts of similar sizes to the differential display product. One transcript (3.0 kb) showed a 13% increase in relative abundance in whole brain of WSP-2 mice exposed to ethanol. In hippocampus and cortex, the relative abundance of both of these transcripts was increased 27% - 40% in ethanol-exposed mice when compared to air-exposed mice. In the cerebellum, no effect was observed in the 1.4 kb transcript, but a moderate decrease (25% of air-exposed mice) in expression was observed. This decrease in expression was quite striking compared to the increases observed in other brain regions.

Genetic analysis of ethanol-regulated expression of this product was performed by also examining WSP-1, WSR-1, and WSR-2 selected lines for all these brain regions. In the hippocampus, modest levels of induction in expression was observed in the 1.4 kb transcript in all selected lines while very little change in expression was observed for the 3.0 kb transcript. In cortex, modest increases in expression were observed in expression of the 1.4 kb transcript of WSP-1 and WSR-2. Ethanol had no effect on expression of the 3.0 kb transcript in WSP-1 mice; however, modest increases in expression after ethanol were observed in WSR-1 & -2 mice.

In the cerebellum, a similar difference in expression of the 1.4 kb transcript between control and ethanol treated mice was observed in WSP-1 and both WSR lines. Similar to WSP-2 mice, a decrease in expression of the 3.0 kb transcript was observed in WSP-1 mice while no effect (WSR-1) or very small increase (WSR-2) was observed in the WSR lines. These results suggest that there is a different mechanism underlying ethanol's ability to alter

the expression of this product in cerebella of WSP and WSR mice, which may be determined by the differences in gene frequencies in selected genes.

In addition to the selected lines, two inbred strains that show differential withdrawal severity were compared. C57BL/6J mice exhibit mild withdrawal convulsions, resembling WSR mice. DBA/2J mice exhibit a withdrawal syndrome very similar to the WSP mice in severity. Results from these inbred strains can be used to test further the association of genotype to ethanol-regulated gene expression in the cerebella. Results from the hippocampus indicated moderate increases in expression of both transcripts in both inbred strains exposed to ethanol versus air. In the cortex, modest increases in expression after ethanol were observed for the 1.4 kb transcript but no effect of ethanol on expression was observed for the 3.0 kb transcript. In the cerebellum, differential effects of ethanol were observed between C57BL/6J and DBA/2/J mice. Ethanol increased expression of the 1.8 and 3.0 kb transcripts in C57BL/6J mice but decreased expression of both transcripts in DBA/2J mice. These results were similar to those observed for WSP and WSR selected lines. A decrease in expression of the 3.0 kb transcript was observed in mice that subsequently dipslay a heightened withdrawal response.

Overall, the results indicate complex patterns of ethanol-induced regulation of the putative mouse homolog of NSP. A genotype-influenced difference in expression of the 3.0 kb transcript (the equivalent to NSP-A) was observed in cerebellar tissue. A decrease in expression of this transcript was observed only in mice susceptible to withdrawal convulsions (WSP and DBA/2J mice). These results suggest that the cerebellum, and NSP-A, may play a role in the development of physical dependence and the subsequent withdrawal syndrome.

INTRODUCTION

Physical dependence is a serious health consequence of long-term alcohol use. Alcohol dependence is defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM IV) as a maladaptive pattern of alcohol use and the occurrence of at least 3 symptoms including an impaired control of drinking, neglect of activities, tolerance, or withdrawal (DSM IV, 1994). In humans, physical dependence develops after prolonged exposure to ethanol. This exposure results in mild (nausea, irritability, tremulousness) to severe (delirium tremens) withdrawal depending on several factors, including length of time since last alcohol exposure and duration of alcohol exposure (Victor,1990). In 1953, Victor and Adams described a syndrome in alcoholics who had been admitted to a hospital, and therefore, withdrawn from alcohol. In this study they were able to describe the temporal pattern of the withdrawal syndrome, characterized by central and autonomic nervous system hyperactivity. A continuum of symptoms was observed: an early-presenting tremulous phase, followed by hallucinations and loss of orientation in the next 24 hours, and finally, in the most severe case, convulsions. It has been estimated that between 4-7% of alcoholics have convulsive seizures (Hauser, 1990).

Although it is now accepted that these signs and symptoms are elicited by the withdrawal from prolonged ethanol exposure, in the early part of the century, few groups attributed the withdrawal from alcohol to the severe syndrome that appeared in certain alcoholics. A pioneering study by Isbell et al. (1955) demonstrated that the symptoms were a consequence of decreasing blood alcohol levels. These investigators administered high doses of alcohol to human subjects in a highly controlled environment for up to three weeks, with blood alcohol levels monitored in 3 patients. A withdrawal syndrome began upon cessation of drinking. Various signs and symptoms, described by Victor and Adams

(1953), were monitored during the withdrawal period. Tremor, the earliest detectable sign, appeared after a small reduction in blood alcohol level. There appeared to be a correlation between the duration of the drinking period and the severity of symptoms.

The question of whether the presence of ethanol or the withdrawal from ethanol was the stimulus for the observed signs after cessation of drinking was one of the factors that led to the development of animal models for further characterization of the withdrawal syndrome. The ability to use animals ina controlled environment facilitated investigations into neurophysiological and cellular factors mediating the development and maintenance of the withdrawal syndrome.

Physical Dependence and Withdrawal: Animal Models

The development of animal models for alcohol-related behaviors has assisted investigations of the ontogeny of these behaviors, including the development and susceptibility to withdrawal seizures. The mechanism(s) by which physical dependence develops and withdrawal is elicited have not been clearly resolved. Physical dependence has been defined as an adaptive mechanism consequent with ethanol exposure (Himmelsbach, 1942), and withdrawal represents manifestations of the homeostatic systems returning to "normal" conditions.

An ethanol withdrawal syndrome has been characterized in several animal species: monkeys (Ellis and Pick, 1970), dogs (Essig and Lam, 1968), and0 rats and mice (McQuarrie and Fingl,1958; Goldstein, 1972; Freund, 1969). Falling blood ethanol concentrations were correlated with the onset of withdrawal symptoms in these models. Not all signs and symptoms that have been observed in man were observed in animals. Of the most-studied signs, tremor and convulsions are present in all the species investigated (Friedman, 1980).

Cicero (1980) cautioned against the generalized use of the term "dependence", without distinguishing between psychological and physical dependence. Psychological dependence is not easily quantifiable in animals. Physical dependence presumably develops as a consequence of chronic ethanol exposure; however, the presence of the withdrawal syndrome is used to define physical dependence. Cicero (1980) suggested that withdrawal signs and symptoms be quantitated; the quantitation could then be used to describe the consequence of long-term ethanol exposure and withdrawal. In this document, the terms "physical dependence" and "dependent" will be used to describe animals that have been exposed to ethanol concentrations known to elicit a withdrawal syndrome.

Withdrawal convulsions are a common sign of withdrawal from severe ethanol intoxication and have been observed in mice and rats (Goldstein,1972; Majchrowicz, 1975; Friedman,1980). Other signs of withdrawal include increases or decreases in locomotor activity, increases and decreases in body temperature, changes in startle reactivity, and hallucinations and vocalizations. Withdrawal convulsions represent a variable that can be easily quantitated and evoked by various stimuli. Goldstein developed a method that elicited convulsions from mice during ethanol withdrawal (Goldstein and Pal, 1971). To elicit robust convulsions mice were picked up by the tail and, if handling alone did not elicit a response, gently spun. Convulsions produced this way were called "handling-induced convulsions" (HIC). Handling-induced withdrawal convulsions were scored using a numerical rating scale. In this way, it was possible to quantify the withdrawal syndrome and compare animals showing mild to severe withdrawal. Systematic investigations of the withdrawal convulsions determined that quantitation was reproducible and convulsions were elicited in a dose-dependent fashion.

The importance of blood ethanol concentration (BEC) in determining withdrawal severity has been investigated most thoroughly in mice, where BEC, duration of ethanol exposure, and other parameters can be manipulated more easily than in humans. Goldstein (1972, 1974) had shown specific relationships between duration of exposure, BEC attained, and severity of withdrawal. In quantitating withdrawal over time, it was shown that the peak of withdrawal scores was greater for mice maintained at higher BECs (≥2 mg/ml) than at lower BEC (≤1 mg/ml) for any given length of time (Goldstein, 1974).

Goldstein also determined that ethanol withdrawal convulsion severity had an inherited component (Goldstein, 1973). Using a genetically heterogeneous base population, pairs of mice with HIC scores greater than the average were mated together as were pairs of mice with lower than average HIC scores. When subsequently tested for withdrawal, offspring from high scoring parents exhibited higher HIC scores than offspring from low-scoring parents. These results indicated that there are important genetic differences which predisposed mice to more or less withdrawal severity.

Genetic Animal Models

Ethanol withdrawal severity is a quantitative, or continuously variable trait; that is, it is not an all-or-none phenomenon. Individual variability (phenotypic variance) in sensitivity to ethanol is produced by genetic and environmental factors, and the interaction between them. Goldstein (1973) showed that withdrawal severity had an inherited component, illustrating that the differences in phenotype were determined to some extent by genes. By using genetic animal models, environmental factors can be controlled allowing for precise analysis of genetic components, including potential differences in gene expression. Two

commonly used genetic animal models are inbred strains and selected lines. Both of these are discussed in further detail below with respect to ethanol phenotypes.

Inbred Strains

Inbred mouse strains can be produced by sequential brother-sister matings. After 20 generations of matings, all gene loci should be fixed in the homozygous state. When inbreeding is complete members of a single strain are, in theory, identical twins and in addition allelic variability has been eliminated. When members of a single strain are compared for a given response, differences can be attributed mainly to environmental effects. However, when multiple strains are compared for a given trait, differences among the strain means indicate that there are genetic factors influencing the trait. Thus, inbred strains have been very useful for determining the extent to which there is genetic variability influencing the phenotypic differences, and for estimating genetic correlation between traits. Genetic correlations estimate the degree to which two (or more) traits are influenced by common genes. When inbred strains are tested in the same environment, covariance among strain means for multiple traits suggests that similar genetic factors influence these traits.

Many inbred strains have been characterized for responses to ethanol (Crabbe, 1983). The results indicated that there was strain dependent variation in the degree of sensitivity to ethanol for a given behavioral measure. Although these inbred strains have not been selected for any particular ethanol-relevant trait, these results suggested that there was allelic variability that influenced sensitivity to ethanol. In particular, C57BL/6J and DBA/2J mice differ markedly for many responses to ethanol, e.g., voluntarily consumption of ethanol solutions (McClearn and Rodgers, 1959; Belknap et al., 1993) and sensitivity to the stimulant effects measured in an open-field (Tritto and Dudek, 1994; Dudek and

Phillips, 1990). With respect to ethanol dependence and withdrawal, DBA/2J mice display high HIC scores after withdrawal from chronic ethanol exposure whereas C57BL/6J mice exhibit minor convulsions (Goldstein and Kakihana, 1974; Crabbe et al., 1983). Thus, some of the genetic differences between these strains includes genes that mediate ethanol sensitivity.

Selected lines

The goal of selective breeding is to target a specific phenotype and enrich a population for that character. A popular design for selective breeding studies is a bidirectional selection; that is, the individuals of one or the other of the phenotypic extremes are mated to produce a line showing an increased response and a line showing a decreased response. For example, individuals exhibiting the highest scores are intermated to produce a "high" line and low scoring individuals are intermated to produce the "low" line. In this example, "high" and "low" describe scores with respect to the base population and "scores" refers to the metric character (e.g., ethanol withdrawal severity) being selected.

Selection pressure produces changes in gene frequencies at the loci influencing the selected trait and forces them to homozygosity (Falconer and McKay, 1996). Ideally, by maintaining an otherwise outbred population, only trait-relevant genes are influenced by selection pressure. It is preferable that the selection be performed in replicate, that is two or more high and low lines. Replicated selected lines control for environmental effects and effects from chance fixation of genes on the differential selected response between the lines. The usefulness of selected lines lies in the ability to determine genetic correlations, known as correlated responses to selection. When properly designed, experiments evaluating responses to a nonselected trait determine the degree of pleiotropism. Pleiotropy refers to the property of a gene affecting two or more traits; that is, the variance in two traits is

determined (at least partially) by a common gene or set of genes. With respect to ethanol-related responses, correlated responses are important for elucidating mechanisms of action of ethanol. Correlated responses are determined by comparing divergent selected lines for the nonselected trait of interest. The genetic correlation can be demonstrated by analysis of variance when the experiment has been performed in highly similar environmental conditions; ideally, there should be simultaneous evaluation of the correlated response in both lines (Crabbe et al., 1990).

Proper interpretation of results from selective breeding studies are facilitated by appropriate controls. By avoiding brother-sister matings, effects of inbreeding can be minimized, which decreases the chance of identifying correlated responses that occur by random fixation of genes. Replication of selected lines allows for less ambiguous interpretations of data concerning correlated responses (traits not selected that differ between the lines); observing the same effect in replicate lines more likely implies a causal relationship between the selected genes and the nonselected trait than if an effect is observed in only one replicate pair of lines.

The heritability of a trait is described as the extent to which phenotypes in offspring are determined by genes from the parents (Falconer and McKay, 1996, p. 123). Similarity among relatives for traits can only be inherited if there is sufficient additive genetic variance, that is, variation in the genes from the parent determining the phenotype of the offspring. Genetic variation is also called allelic variation. Estimates of heritabilty (h^2), then, indicate the amount of the phenotypic variance that can be attributed to additive genetic variance; the larger the estimate (up to $h^2 = 1$ or 100%), the greater the genetic influence. The magnitude of the selected response of the new generation (i.e., offspring) can be predicted by how much variance in a selected trait can be attributed to additive genetic variability and

the difference in response of the parental line from the base population. There will be no response to selection if the heritability of the trait is zero. Thus, even if there is phenotypic variance present in a population (represented by a unimodal distribution), selective breeding for extremes will result in the same mean phenotype in the succeeding generations if there is no heritability. As selection pressure changes the gene frequencies, the overall effect of the alleles should change. Successful selection experiments should result in a fairly rapid divergence (within 3-6 generations) between the high and low lines. How fast the selected lines reach the maximum response is related to the number of genes influencing the phenotypes. For example, if the maximal response is reached within one selection generation then there are only one or two major genes involved; the longer it takes to reach the maximum response, the more genes with varying degrees of effect are likely involved (Falconer and McKay, 1996).

Lines of mice selected for differential ethanol responses allow one to investigate allelic differences in genes for candidate neurochemical systems as well as more general effects of ethanol on these systems (e.g., neurotransmitter function or gene expression). Similarities observed between the replicate lines strongly implicate a role for that system (or receptor or gene) in withdrawal susceptibility.

Withdrawal Seizure-Prone and Withdrawal Seizure-Resistant Mice

Mice have been genetically selected for severe ethanol withdrawal convulsions (Withdrawal Seizure-Prone, WSP) or relatively mild withdrawal convulsions (Withdrawal Seizure-Resistant, WSR; Crabbe et al., 1985). Selection of these lines of mice was performed in replicate; two divergent lines were selected from independent breeding pairs

of genetically heterogeneous mice resulting in two "high" lines (WSP-1 and WSP-2) and two "low" lines (WSR-1 and WSR-2).

The selection procedure (Crabbe et al., 1985) involved injecting mice with ethanol, to elevate blood ethanol concentration (BEC), and pyrazole, to inhibit ethanol metabolizing enzymes in the liver. Mice were placed in ethanol vapor inhalation chambers for 72 hours to induce physical dependence. The ethanol chambers provide relatively constant ethanol dosing through forced inhalation of ethanol vapor. Upon removal from the chambers, the mice were scored for handling-induced convulsions (HIC) once an hour for 15 hrs, then at 24 and 25 hrs. The HIC rating scale, modified from Goldstein and Pal (1971), is outlined in Table 1. The scale ranges from no convulsion (with or without handling), to mild convulsions elicited by picking the mouse up by the tail and gently twisting in an 180° arc, to severe tonic-clonic convulsions elicited without handling.

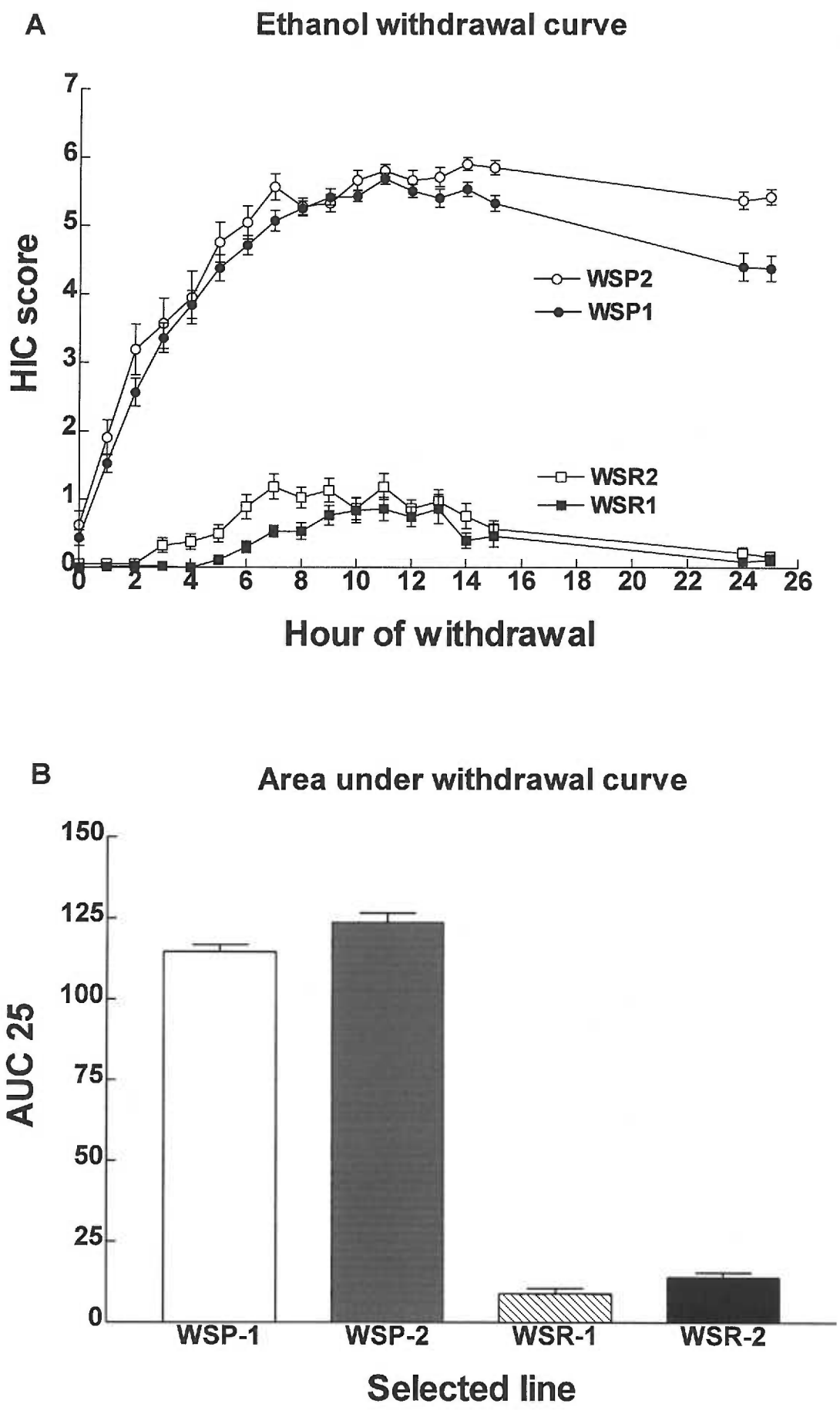
A typical time-course of the withdrawal syndrome, as described by HIC scores over time, is shown in Figure 1A. WSP-1 and WSP-2 mice exhibit severe HIC scores (greater than 3) for an extensive period during withdrawal. Presumably as the BEC levels decline over time that convulsions are elicited; ethanol itself acts as an anticonvulsant. WSR-1 and WSR-2 mice rarely show HIC scores above 2. The area under the withdrawal curve (AUC) is calculated for each mouse for the withdrawal period, and was used as an index of withdrawal severity. Figure 1B shows the AUC for WSP-1, WSR-1, WSP-2, WSR-2 lines from selection generation 22. The greater the AUC, the more severe the withdrawal reaction. This measure clearly shows the difference between WSP and WSR mice in magnitude of response. In addition to the Prone and Resistant selected lines, two control (i.e., nonselected) lines, WSC-1 and WSC-2, were generated through random matings. These lines have intermediate HIC scores (data not shown).

Table 1. Handling-induced convulsion rating scale [1]

Score	Physical sign
0	No convulsion after handling
1	Facial grimace after gentle 180° spin
2	Tonic convulsion include forelimb clasp and facial grimace after being gentle 180° spun
3	Tonic-clonic convulsion (fore- and hindlimb tremor) lasting several seconds after gentle 180° spin
4	Tonic convulsion elicited by picking up the tail without a spin
5	Tonic-clonic convulsion elicited by picking up by the tail (without a spin), often delayed 1-2 sec after being lifted by the tail
6	Severe, tonic-clonic convulsion elicited by picking up by the tail, quick onset and duration of convulsion often lasting several seconds
7	Severe tonic-clonic convulsion (quick onset, long duration) either spontaneous or elicited by a non-handling stimulus (e.g., cage top removal)

[1] Adapted from Crabbe and Kosobud, 1990

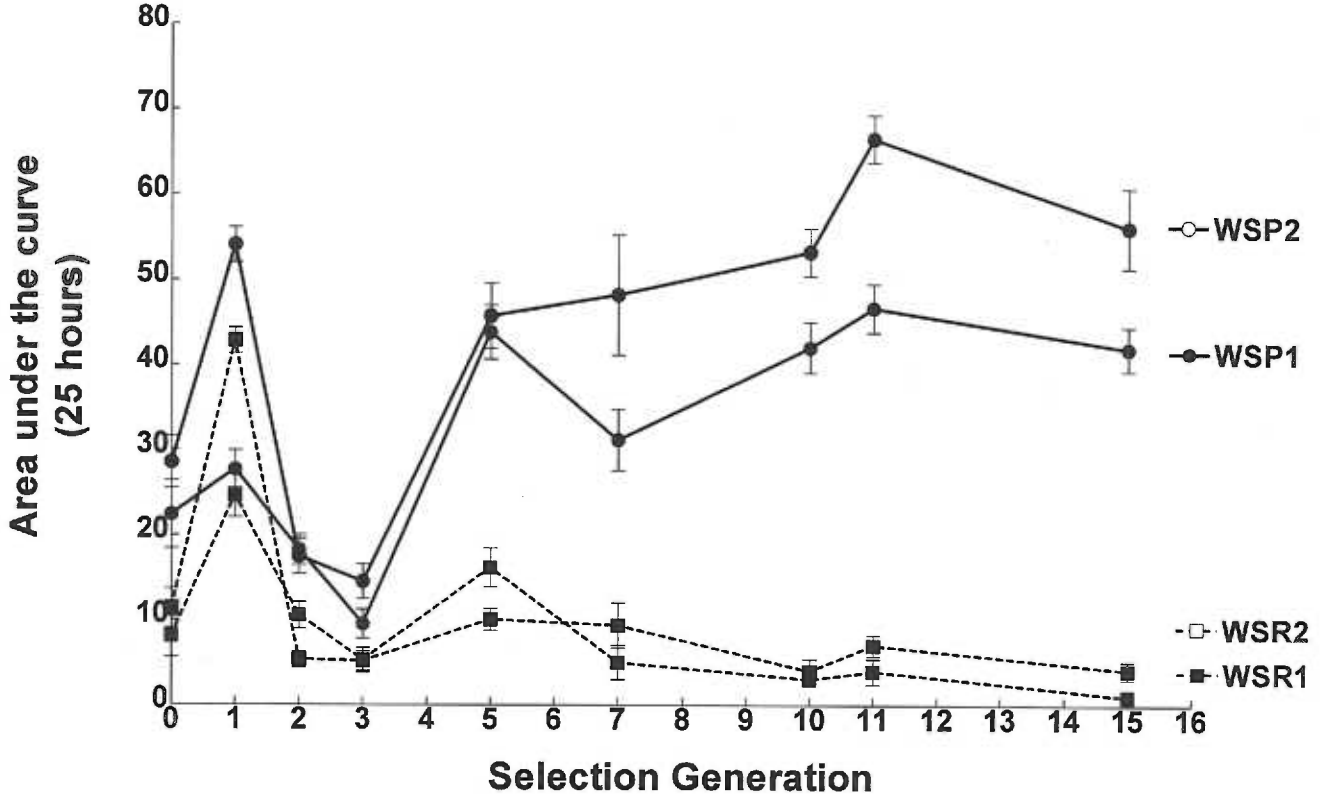
Figure 1. Ethanol withdrawal responses in WSP and WSR selected lines. **A.** HIC scores during withdrawal from ethanol for WSP-1, WSP-2, WSR-1, and WSR-2 mice. Mice were scored hourly for 15 hours and at 24 and 25 hours following removal from ethanol chambers. Data are from mice of selection generation 22. **B.** Area under the withdrawal curve (AUC25) for WSP-1, WSP-2, WSR-1, and WSR-2 mice of selected generation 22. Data are presented as mean ± sem. Data taken with permission from J. Crabbe.


A
Ethanol withdrawal curve
HIC score
Hour of withdrawal
WSP2
WSP1
WSR2
WSR1
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16
18
20
22
24
26
B
Area under withdrawal curve
AUC 25
Selected line
WSP-1
WSP-2
WSR-1
WSR-2
0
25
50
75
100
125
150

The AUC for the 25 hour period was used as the selection criterion: mice with large AUC values were mated to form the WSP lines and mice with smaller AUC values were mated to produce the WSR mice. Figure 2 shows the response to selection, as defined by AUC, for the selected lines. A large divergence between the WSP and WSR lines of mice occurred within 11 generations (Crabbe et al., 1985). Since the 26th selected generation, these lines have been maintained through random mating. The total realized heritability (h^2), calculated after selection generation 11, was estimated to be 0.28. This suggests that 28% of the variance in the observed behavior can be attributed to allelic genetic variability. This value is sufficiently large to suggest there are strong genetic effects, which may be manifested as effects from a few major genes or alternatively many smaller, yet significant, minor gene effects.

The marked difference in withdrawal severity between WSP and WSR mice could have resulted from selection for pharmacokinetic differences (e.g., ethanol metabolism) between the selected lines (Crabbe et al., 1985). In the initial selection procedures, all mice were exposed together at the same ethanol vapor concentrations. By selection generations 10 and 11, WSP-1 males achieved ~30% higher BEC levels than WSR-1 mice, suggesting there could be differences in ethanol elimination. Differences in metabolism could lead to uneven ethanol exposure. However, no differences in ethanol elimination during withdrawal were identified (Kosobud and Crabbe, 1986). Since there were no differences in BEC levels attained during exposure between WSP-2 and WSR-2 mice and the lines did not differ in metabolism, it can be concluded that this difference in BEC was not responsible for the behavioral difference. The difference in BEC levels during exposure was overcome by exposing WSR mice in a separate chamber from WSP mice and by daily monitoring for BEC during the exposure period (Terdal and Crabbe, 1994). The importance of having

Figure 2. Response to selection. Response to selection as determined by AUC (mean ±sem) for the 25 hour withdrawal period up to generation 15. (Maximum responses were obtained by selected generation 15). Divergence between the WSP and WSR lines was detectable by the 5th selected generation.


Area under the curve
(25 hours)
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Selection Generation
WSP2
WSP1
WSR2
WSR1

equivalent BEC levels at the termination of the exposure period was exemplified by Goldstein (1972), who showed that withdrawal severity in heterogeneous mice (determined by HIC) was influenced by BEC. Terdal and Crabbe (1994) demonstrated the effect of varying BEC on the withdrawal response in WSP and WSR mice. At all BEC levels, measured after 72 hrs of ethanol exposure, WSP mice had significantly greater withdrawal scores than WSR mice (Terdal and Crabbe, 1994). Examining the AUC values for WSP mice alone, AUC increased as the BEC levels increased from <1 mg/ml to 1.5 or 2 mg/ml. Equating the selected lines for BEC levels eliminates confounding effects of non-equivalent ethanol exposure on withdrawal severity.

The WSP and WSR selected lines are powerful tools for investigating specific physiological and molecular correlates of withdrawal. However, before results of correlated responses can be discussed, assumptions and interpretations of neurochemical and molecular differences need to be addressed. Since genetic selection alters gene frequencies of trait-relevant genes, the assessment of correlated responses to selection presumes that these allelic differences underlie differences in correlated responses. Determination of molecular correlates (i.e., gene or protein expression differences not necessarily of selected genes) assumes that these allelic differences can lead to altered gene expression since selection does not act directly on the expression of genes. These molecular correlates are very important in determining mechanisms of action of ethanol, as they relate to the functional consequences of selection. The interpretation of studies of neurochemical and molecular correlates must take into account results from both replicate lines. For example, if differences in expression of gene Y are observed between one set of replicated selected lines (WSP-1 and WSR-1) but not in the other set of replicated lines, one cannot conclusively conclude that this alteration is correlated with a selected phenotype; that is,

selection did not result in the segregation of the same alleles in both of the high-responder (or low-responder) lines. In other words, the gene frequencies did not change in both replicate lines of a phenotype and thus are probably not trait-relevant genes.

Before discussing neurochemical and molecular correlates of selection in WSP and WSR lines, the effect of ethanol on candidate systems thought to be involved in ethanol withdrawal will be discussed to accentuate the relevance to WSP and WSR lines.

Effects of chronic ethanol on neurotransmitter function: Pharmacology and gene expression

$GABA_A$ receptor complex

γ-Aminobutyric acid (GABA) is the major inhibitory amino acid neurotransmitter receptor system in the brain. Upon binding of GABA to its site on the receptor, a pore is formed allowing for the passage of chloride (Cl^-) ions into the neuron, leading to a decrease in the release of neurotransmitter(s) from the nerve terminal. Ethanol, at doses known to be intoxicating in man, directly stimulated $GABA_A$ receptor-mediated Cl^- ion flux into the neuron (Suzdak et al.,1986) as well as enhanced GABA- (or $GABA_A$ agonist) mediated Cl^- uptake (Allan and Harris, 1987). Ethanol also potentiates benzodiazepine (BZ)-mediated Cl^- flux. Ro15-4513, a BZ partial inverse agonist, potently antagonized ethanol-stimulated and ethanol-potentiated GABA Cl^- flux (Suzdak et al., 1986). However, chronic ethanol exposure reduces acute ethanol potentiation of GABA-mediated Cl^- uptake in mouse or rat brain preparations (Allan and Harris, 1987; Morrow et al., 1988). Studies characterizing receptor binding affinity and density have yielded variable results (Liljequist and Tabakoff, 1985; Mhatre et al., 1988; Sanna et al., 1993), suggesting that behavioral effects do not necessarily result from decreases in receptor pharmacological function.

$GABA_A$ receptors have been hypothesized to play an important role in modulating ethanol withdrawal seizures (Frye, 1990). GABA agonists, including muscimol, barbiturates, and benzodiazepines, decrease severity of audiogenic seizure symptoms during ethanol withdrawal whereas GABA antagonists potentiate withdrawal syndromes (Frye et al., 1983). Ethanol enhanced inhibition of $GABA_A$ receptor-mediated Cl^- uptake by Ro15-4513 and decreased flunitrazepam potentiation of $GABA_A$ receptor-mediated Cl^- uptake (Buck and Harris, 1990). Mice lacking the γ isoform of protein kinase C (PKC) lack an ethanol-induced enhancement of muscimol-stimulated Cl^- uptake in cerebellum, and the reduction in cortex, and have decreased sensitivity to the sedative properties of ethanol (Harris, et al., 1996), suggesting that phosphorylation of the $GABA_A$ receptor by PKC may mediate ethanol actions at these receptors.

Molecular approaches have been used to determine the level at which ethanol exposure may be exerting its effects on receptor function. Chronic ethanol treatment alters receptor mRNA expression of various subunits of the $GABA_A$ receptor (α, β, γ). It can be argued that changes in gene expression of neurotransmitter receptor constituents may either lead to adaptive changes in behavior or be a consequence of adaptation. Several studies have found decreases in RNA levels of the $\alpha 1$ subunit in cortex (Morrow et al., 1990; Montpied et al., 1991; Mhatre et al., 1993). In one case, an increase in expression of the $\alpha 1$ subunit in whole brain of mouse exposed to chronic ethanol vapor was reported (Hirouchi, et al., 1993). A decrease in $\alpha 2$ subunit expression was observed in rats (Devaud et al., 1995), whereas $\alpha 4$, $\alpha 6$, $\gamma 1$ and $\gamma 2S$ subunit mRNA levels were increased following chronic ethanol exposure (Ticku et al., 1992; Devaud et al., 1995). In agreement with the observed changes in mRNA levels, peptide levels were increased for the $\alpha 4$ and $\gamma 1$ subunits and decreased for $\alpha 1$ subunit (Devaud et al., 1997). $GABA_A$ receptor $\beta 2$ and $\beta 3$ subunit

mRNAs in cortex were increased by chronic ethanol exposure (Mhatre and Ticku, 1994; Devaud et al., 1995). Concomitant increases in peptide levels of the β subunits was observed (Mhatre and Ticku, 1994).

NMDA- glutamate receptors

Ethanol exposure *in vitro* modulates functioning of *N*-methyl-D-aspartate (NMDA) receptors. In primary cerebellar granule cell cultures, it was found that ethanol (acting through modulation of NMDA receptors) inhibited glutamate-stimulated cyclic GMP production (Hoffman et al., 1989). Ethanol exposure inhibited NMDA-stimulated ion current in hippocampal neurons (Lovinger et al., 1989) and cerebellar neurons (Dildy and Leslie, 1989). In contrast, hippocampal-slices prepared from mice chronically exposed to ethanol exhibited an increase in NMDA receptor-mediated voltage dependent-calcium spiking during withdrawal (Whittington, Lambert, and Little, 1995). Morrisett (1994) demonstrated inhibitory actions of ethanol on the duration of NMDA receptor-activated afterdischarges in dissociated rat dentate gyrus and increases in duration and firing frequency after removal of ethanol from the perfusate. These experiments demonstrated cellular adaptation resulting from ethanol exposure and withdrawal, suggesting that there may be behavioral correlates associated with ethanol effects on NMDA receptors.

Withdrawal convulsions may arise due to hyperexcitability in the glutamate receptor system (including NMDA receptors) since other seizure forms, such as epilepsy, have been attributed to overactivity of glutamatergic neurons (Nadler et al., 1978). Rats withdrawn from ethanol for 24 hours exhibited an enhancement of NMDA-induced seizures, indicating an increase in NMDA sensitivity (Davidson, Shanley, and Wilce, 1995). *In vivo*, NMDA potentiated handling-induced convulsions during withdrawal in ethanol-dependent C57BL/6 mice (Grant et al., 1990). Administration of the noncompetitive NMDA

antagonist, MK-801, during withdrawal attenuated HICs (Grant et al., 1990) and audiogenically-produced withdrawal convulsions (Morrisett et al., 1990). An increase in the number of MK-801 binding sites in the hippocampus of mice was observed after chronic ethanol exposure (Grant et al., 1990). The effect of ethanol withdrawal on changes of the NMDA receptor were analyzed by quantitative autoradiography of MK-801 binding (Gulya et al., 1991). The greatest changes were observed in the hippocampus and cortical regions but no changes were observed in the cerebellum.

Alteration of expression of NMDA receptor subunit mRNA and peptides after ethanol exposure has yielded interesting results. Trevisan et al. (1994) found an increase in NMDA receptor subunit 1 (NMDAR1 receptor) immunoreactivity in hippocampus, but not striatum, cortex, or nucleus accumbens after chronic ethanol exposure. AMPA receptor subunit protein (GLUR1 and GLUR2) was not altered by ethanol exposure in the hippocampus, indicating the specificity of ethanol's effects for the NMDA receptor system. Snell et al. (1996) also found an increase in NMDA-R1 receptor subunit protein level in hippocampus and cerebellum. In addition, NMDA-R2A (NR2A) receptor subunit protein level was increased in hippocampus and cortex. Interestingly, mRNA levels for these subunits were not increased in the brain regions where protein levels were increased; expression of NR2A mRNA was decreased in cerebellum with no effect on protein levels. These results suggest that there was an increase in protein translation or decrease in protein degradation. In a separate study, NMDAR2A and 2B receptor subunit mRNA was found to be increased in cortex and in hippocampus (Follesa and Ticku, 1995).

Taken together, adaptation that occurs during ethanol exposure suggests that these neurotransmitter systems in particular may be important for modulating seizure and convulsion activity during withdrawal from ethanol, possibly in specific brain regions, most

notably the hippocampus, cortex, and occasionally, cerebellum. Alterations of gene and/or protein expression of receptor subunits appear to be regulated by ethanol treatments that also affect receptor pharmacology (receptor binding affinity, receptor density, functioning) and behavioral consequences, implicating potential causal relationships among these responses. One way to assess the relationship between the behavioral consequences of ethanol exposure/ withdrawal and alterations of gene expression is via genetic animal models such as WSP and WSR mice.

Correlated responses: Mechanisms underlying withdrawal severity in WSP and WSR mice

WSP and WSR selected lines have been invaluable for determining potential mechanisms underlying susceptibility and resistance to withdrawal. The selected genes may influence differences between the lines for nonselected traits (i.e., correlated responses to selection). In turn, these correlated responses can yield important information regarding the selected response to ethanol. Since WSP and WSR mice have been genetically selected for differences in withdrawal severity, it was hypothesized that there would be differential sensitivity to GABAergic and glutamatergic (NMDA-receptor specific) agents. By comparing responses of WSP and WSR mice to convulsant or anticonvulsant activity of agents of these receptor systems, correlated responses to the selected phenotype can be determined. For example, sensitivity to the convulsant properties of GABAergic and glutamatergic agents in WSP mice but not in WSR mice would suggest that these systems play a role in mediating ethanol withdrawal severity. For a more inclusive report of correlated responses to selection in WSP and WSR mice see Metten and Crabbe (1996).

$GABA_A$ receptor system

WSP mice had more severe withdrawal (measured by HIC) than WSR mice after chronic phenobarbital (Belknap, Danielson, Lame', Crabbe, 1988) and diazepam exposure (Belknap, Crabbe, and Laursen, 1989), both of which act through the $GABA_A$ receptor. WSP mice were more sensitive to the convulsant agents picrotoxin ($GABA_A$ receptor antagonist), CHEB (barbiturate), and 4-aminopyridine (potassium channel blocker) (Crabbe, Merrill, and Belknap, 1991). Pentylenetetrazole (PTZ) is a convulsant agent that binds to the picrotoxin site on the $GABA_A$ receptor complex. Tail-vein infusions of PTZ leading to myoclonus and writhing clonus indicated a greater sensitivity in WSP-1 versus WSR-1 mice but no difference in sensitivity between WSP-2 and WSR-2 mice. To investigate what functional aspects of this receptor system might be differentiated between the selected lines, binding affinity and receptor numbers were determined.

Binding of *t*-butylbicyclophosphothionate (TBPS) and flunitrazepam to the $GABA_A$ receptor was determined in ethanol-naive WSP and WSR mice (Feller, Harris, and Crabbe, 1988). TBPS binds to the picrotoxin site (in the ion channel) and flunitrazepam binds to the benzodiazepine site, which allosterically modulates GABA function. Differences in binding characteristics (receptor number or affinity) between the lines would suggest that genes for these systems were affected by selection. Flunitrazepam binding affinity and receptor density in whole brain and TBPS binding affinity in whole brain and in cortex, hippocampus, and cerebellum were the same for WSP and WSR mice. Hence, genetic differences in sensitivity to diazepam- or pictotoxin-induced convulsions could not be explained by changes in receptor number or affinity of $GABA_A$ receptors in those brains regions tested.

The effect of chronic ethanol treatment on $GABA_A$ receptor function has been investigated in WSP and WSR mice. When these mice were exposed to ethanol via a liquid

diet, they did not differ in stimulated Cl^--uptake in synaptoneurosomes from cerebral cortex; however, WSP mice were more sensitive to inhibition of Cl^--uptake by inverse agonists (Buck, McQuilkin, and Harris, 1991a). In the same study, flunitrazepam-enhancement of muscimol stimulated Cl^--uptake was attenuated by the ethanol treatment in WSR but not WSP mice. These results indicate that selection for ethanol-withdrawal susceptibility resulted in differential sensitivity to benzodiazepine inverse agonists. Taken together, these results suggest that $GABA_A$ receptor functions (e.g., Cl^- ion influx and certain binding characteristics) of the $GABA_A$ receptor may differ between WSP and WSR mice and contribute to ethanol withdrawal severity.

NMDA receptor system

If NMDA receptors are indeed involved in mediating withdrawal seizures, then WSP and WSR mice should show a difference in function or numbers of these receptors. Naive WSP were more sensitive to effects of NMDA on HIC than WSR mice (Crabbe, Merrill, and Belknap, 1993), suggesting that the behavioral differences observed between WSP and WSR mice may arise from inherent differences in the glutamate system. When infusions of NMDA were used to assess convulsions (myoclonus, tonic hindlimb extension) in ethanol-naive WSP and WSR mice (Kosobud and Crabbe, 1993), WSR mice were more sensitive to the NMDA-induced convulsions than WSP mice. Thus selection for resistance to ethanol withdrawal seizures appeared to correlate with NMDA-induced convulsion sensitivity. In addition, WSR mice were also found to be more sensitive to the anticonvulsant effect of MK-801, but electroconvulsive shock thresholds were elevated significantly more than WSP mice (Crabbe, Young, and Dorow, 1994).

Receptor binding function has assessed to ascertain if WSP and WSR mice differ in binding affinity or receptor denstiy. One group found the number of [^{3}H]MK-801 binding sites to be higher in hippocampus of naive WSP mice than WSR mice (Valverius et al., 1990); however, no differences in numbers of [^{3}H]MK-801 binding sites were detected by another group between naive WSP and WSR mice (Carter et al., 1995). Differences in route and duration of ethanol administration and assay conditions for each analysis may partially explain the discrepancy in results between the investigations. It is unclear from what level differences in glutamate and NMDA sensitivity between WSP and WSR stem.

The number of dihydropyridine-sensitive calcium channels (DHPSCC) on excitable membranes increases with prolonged exposure to ethanol, suggesting that these channels play an important role in the production of physical dependence (Guppy and Littleton, 1994). The number of DHPSCC binding sites in brain of WSP and WSR mice was investigated (Brennan, Crabbe, and Littleton, 1990). Mice were exposed to ethanol or air as per the selection protocol (Crabbe et al., 1985). Ethanol exposure led to a significantly greater up-regulation of DHPSCC binding sites from membrane preparations from WSP mouse brain than from WSR mouse brain.

Expression of $GABA_A$ receptor subunits in WSP and WSR mice

Despite the many demonstrable effects of ethanol on $GABA_A$ receptors and NMDA receptors during ethanol exposure and withdrawal, the mechanisms underlying these adaptive processes are unknown. It is apparent that multiple neurotransmitter and subcellular systems may be involved in mediating the severity of ethanol withdrawal convulsions, but how these differences are manifested in WSP and WSR mice to produce the observed behavioral differences is not clear. The effect of ethanol on the function of the

$GABA_A$ receptor suggested that the genetic differences may arise from changes in the expression of specific subunits (α, β, γ) comprising the GABA receptor.

Relative mRNA levels for the $GABA_A$ receptor in naive WSP and WSR have been examined to assess possible basal differences. Buck et al. (1991b) found that $\alpha 3$ and $\alpha 6$ subunit mRNAs were ~60% lower in WSP than WSR mice. However $\alpha 1$, γ_{2S}, and $\gamma 3$ subunit mRNA levels did not differ between naive WSP and WSR mice. Keir and Morrow (1994) found a greater difference in expression of the $\alpha 6$ in WSR versus WSP mice and slightly higher levels of $\alpha 1$ subunit mRNA in cerebellum of WSR versus WSP mice. No differences in expression levels between WSP and WSR mice was found for cortical $\alpha 1$ and $\beta 2$ subunits mRNA. In ethanol-treated mice, the cerebellar-specific $\alpha 6$ subunit mRNA was decreased by 60% WSR but not WSP mice, whereas $\alpha 1$ subunit mRNA was decreased by 40% in WSP mice but not in WSR mice (Buck et al., 1991b). Other subunit mRNAs did not differ in ethanol-induced expression between WSP and WSR mice. These results suggested a role for the GABA receptor in modulating withdrawal severity differences and may represent genotypic differences (i.e., non-ethanol induced). Therefore, it is apparent that there are genetic differences in the expression of some of the subunits of the $GABA_A$ receptor as well as an influence of ethanol on the expression of these subunits. These innate and ethanol-induced differences may modulate withdrawal severity, as defined by HIC scores. However, it is doubtful that these differences alone could account for the genetic difference in withdrawal between the selected lines.

When the results from the selected lines are taken together with results from the more generalized studies, it is apparent that chronic ethanol exposure produces adaptive effects on several levels of the $GABA_A$ receptor system (from gene expression to receptor function) and NMDA receptor system. Changes in mRNA and protein expression are

dependent on brain region, indicating that ethanol has selective effects that may contribute to the development of physical dependence and withdrawal seizures.

Effects of ethanol and withdrawal of ethanol on gene expression

The adaptive processes leading to physical dependence and a withdrawal syndrome most likely involve changes occurring on cellular and subcellular levels. The identification of specific targets for ethanol, especially at the level of the gene, is crucial for understanding how adaptation to chronic ethanol occurs. Ethanol is known to interact with several functional aspects of neurons, from neurotransmitter receptors to intracellular signaling mechanisms to regulation of gene expression (see Diamond and Gordon, 1997). In addition to effects of ethanol on gene expression of the constituents of $GABA_A$ and NMDA neurotransmitter systems, several other cellular components have been investigated for alterations in gene expression. What follows is a brief overview of effects of ethanol on expression of selected genes, which might be related to the development of physical dependence and ethanol withdrawal. Many of these genes tend to be ubiquitiously expressed in the brain; alterations of expression in selective cell populations or brain regions may indicate targets of action in the brain for ethanol.

Expression of immediate early genes (IEG), such as *c-fos* and *jun*D, is generally regarded as a marker of neuronal activity (Sager et al., 1988; Dragunow and Faull, 1989). Various stimuli can induce expression of *c-fos* in neurons, including electrical, physiological, and pharmacological stimuli (Morgan and Curran, 1989; Morgan et al., 1987; Dragunow and Robertson, 1982). Cell populations and brain regions involved in eliciting responses to particular stimuli can be mapped by *fos* and *jun* gene expression. For example, when ethanol is administered to cells or an animal, the expression of *c-fos*

indicates that there is active gene transcription occurring in cells. In the simplest model, expression of IEGs are assayed *in vitro*. Using neuroblastoma cells (SH-SY5Y cells), significant increases in mRNA levels of c-*jun* and *jun*D were observed after treatment with 100 mM ethanol continuously for 2-4 days; this treatment had no effect on *c-fos* and *fos*B mRNA levels (Ding et al. 1996). These results indicate that ethanol has effects on gene expression in these cells and support the hypothesis that ethanol produces adaptive changes in neurons in which IEGs may be involved.

Several studies have shown that ethanol withdrawal, but not ethanol intoxication, induced *c-fos* RNA or protein expression *in vivo* (Bouchenafa and Littleton, 1998; Morgan et al., 1992). The expression of *c-fos* in cerebral cortex, cerebellum, and hippocampus was examined in mice during ethanol withdrawal, and whether or not they were overtly expressing withdrawal signs was also examined (Dave, Tabakoff, and Hoffman,1990). *c-fos* expression was observed in all brain regions tested in mice that had a convulsion during withdrawal; the largest increases were in the hippocampus. No significant expression was detectable in any region from mice that did not have an overt seizure; however, increased levels of $\leq$ 2-fold were observed in hippocampus and cortex throughout the withdrawal period. The large increases in expression of *c-fos* were detected at the time of the seizure onset, suggesting that the seizure itself probably is not responsible for the change in expression. Morgan et al. (1992) showed that overt seizures were not necessary inducers of *c-fos* expression because expression of *c-fos* was detected in rats 6-8 hours after ethanol withdrawal independent of convulsive activity.

Using *c-fos* as a marker, the interactions of ethanol with NMDA and $GABA_A$ receptors have been investigated to elucidate pathways or mechanisms important in ethanol withdrawal. Indeed, both PTZ (a GABA antagonist) and NMDA induction of whole brain *c-*

fos was blocked by an acute preinjection of ethanol (Le et al., 1990, 1992; Wilce et al., 1993). FOS and c-JUN protein expression was used to determine specific brain pathways involved in mediating ethanol's inhibitory effects (Davidson et al.,1996). In cortex, ethanol decreased FOS immunoreactivity (FOS-IR) induced by PTZ and NMDA. Induction of FOS in the hippocampus by PTZ was decreased by 3.0 g/kg ethanol but this dose was ineffective in reducing NMDA-induced FOS-IR. In contrast, NMDA-induced c-JUN-IR in the hippocampus was decreased by ethanol. These results indicate that there is brain region and receptor specificity of the inhibitory actions of acute ethanol.

Taken together the results of experiments using *c-fos* or *jun* family members as molecular markers for neuronal activity indicate that specific populations of cells may be involved in the neuronal hyperexcitablility associated with ethanol withdrawal. The hippocampus, cortex, and to a lesser degree, the cerebellum all appear to be involved in the withdrawal syndrome.

Another class of proteins that is affected by ethanol are heat-shock protein, also known as chaperonins. This class of molecules can be induced by stress (such as heat or chemical) and function as protein chaperones during protein synthesis and trafficking (Ellis, 1994). Chronic ethanol exposure increased expression of the constitutively expressed Hsc70 *in vitro* (Miles et al., 1991,1992). Neuroblastoma cells treated for 2 or 6 days showed dose-dependent increase in expression. Hsc70 expression returned to control levels 48 hours after withdrawal from ethanol. Ethanol affects expression of members of another family of molecular chaperones, glucose-regulated protein (GRP) 78 and GRP94 (Miles et al., 1994). Neuroblastoma cells exposed to increasing concentrations of ethanol (25-100 mM) for 24 hours showed a dose-dependent increase expression of GRP78 and GRP94 mRNA above control levels. It is interesting that these proteins are localized to the

endoplasmic reticulum or otherwise involved in protein trafficking and sorting, suggesting that these represent a specific class of ethanol-regulated genes. Specific regions of the promoter region of Hsc70 appear to be important for conferring ethanol sensitivity (Hsieh et al., 1996).

Second messenger systems and constituents such as G-proteins (G_s, G_i), cyclic AMP (cAMP), and protein kinases (PKA and PKC) have been shown to be altered by chronic ethanol exposure (Diamond and Gordon, 1997). Chronic ethanol decreases cAMP production, in contrast to acute ethanol exposure, which increases cAMP production (Gordon, Mochly-Rosen, and Diamond, 1992). Functionally, the decrease in cAMP production probably arises from heterologous desensitization of the affected receptor (Diamond and Gordon, 1997) and has been correlated with decreases in mRNA for $G_s\alpha$ subunits (Mochly-Rosen et al., 1988; Rabin, 1993). The decrease of cAMP by chronic ethanol has been proposed as a model for dependence (Gordon, Collier, and Diamond, 1986).

In addition to the examples previously described, the list of ethanol-regulated genes includes, but is not limited to, most neurotransmitter receptors (Eravci, et al., 1997; Shen et al., 1997; Bruckner et al., 1997), components of the stress axis (Lee et al., 1997; Ogilvie et al., 1997); and metabolic enzymes (Chen, W. et al.; 1997; Chen,Y. et al., 1997; Tindberg et al., 1996; Simonyi et al., 1996). Molecular components of ethanol withdrawal *in vivo* have not been thoroughly investigated; indeed, the results of investigations of NMDA and $GABA_A$ receptor systems indicate that there are very complex interactions. It is likely that important components underlying withdrawal are in discrete populations of cells and associated with more than one neurotransmitter and/or cellular system.

WSP and WSR selected lines are good models for addressing the issue of identifying ethanol-induced alterations in gene expression related to withdrawal. Because of the underlying genetic differences between these selected lines, expression changes can be compared between the lines to identify likely candidates involved in withdrawal. It is unlikely that alterations in the above systems could completely account for the selection difference (i.e., genetic variance) observed in WSP and WSR mice. Although there are many candidate genes that have been studied for modulation by ethanol, it would be a formidable task to attempt to investigate expression differences between WSP and WSR mice for most of those genes.

Molecular approaches to identifying unrecognized ethanol-regulated genes

The genetics of WSP and WSR selected lines makes them a highly desirable model to attempt to identify ethanol-regulated gene products relating to withdrawal severity, without previous assumptions or knowledge of function of the genes. Molecular techniques that enable the direct detection of differentially expressed genes provide excellent alternatives for investigating genetic mechanisms involved in effects of ethanol. Techniques allowing for global assessment of gene expression have enabled investigators to identify genes that either would not be considered or that are unknown (not previously cloned or no function established). Among the techniques available for detecting differentially expressed genes from a large population of genes, the most widely used and best characterized are subtractive hybridization and PCR-based mRNA differential display. Subtractive cloning enables the detection of genes that are either increased or decreased in relative abundance. This technique and 2-D gel electrophoresis have been used to successfully identify ethanol-regulated genes in neuroblastoma cells (Miles et al., 1992). These investigators isolated

molecular chaperones Hsc70, GRP78 and GRP94, all of which showed increased expression after ethanol exposure when compared to untreated cells.

The development of mRNA differential display (mRNA-DD; Liang and Pardee, 1992) has enabled the simultaneous detection of many differentially expressed genes; both increased or decreased expression can be detected in the same population. mRNA-DD is a theoretically straightforward technique. Briefly, RNA is extracted from tissue and reverse transcribed with anchored oligo-dT primers. These primers direct reverse transcription of mRNA or expressed genes only and the anchor acts to divide the total mRNA population into smaller pools. A fraction of the reverse transcription reaction is amplified by PCR with the same oligo-dT primers and with arbitrary 5'-primers, resulting in a population of cDNA fragments that represent the 3' end of the expressed mRNAs. The resulting amplified products are separated on a DNA sequencing gel, resolving the PCR cDNA fragments by size as small as a single base-pair difference. From this, differentially expressed bands can be excised, and the DNA eluted and subsequently cloned and sequenced. This technique is highly amenable to the identification of ethanol-regulated genes in a complex behavioral model, such as withdrawal seizure susceptibility, where many genes influence a behavior.

Recently, Chen et al. (1997) used mRNA differential display to identify ethanol-regulated genes in rats chronically exposed to ethanol vapor. Mitochondrial NADH dehydrogenase showed a 2-fold increase in expression during exposure and remained elevated up to 48 hours following withdrawal from ethanol. Several other groups have used this technique to identify drug-induced changes in gene expression. For example, Douglass et al. (1995) have investigated effects of abused drugs on gene expression in cocaine or amphetamine dependent rats. Subsequent analysis of one of these products resulted in the identification of a novel brain specific peptide which is apparently

modulated transcriptionally by these psychomotor stimulant drugs. A psychostimulant - regulated G-protein β subunit gene ($rG\beta_1$) was identified in rat nucleus accumbens by subtractive differential display (Wang et al., 1997). This product was increased in drug-treated rats and appeared to be important for establishing cocaine behavioral sensitization, as determined by antisense treatment with $rG\beta_1$.

By applying this technique to WSP and WSR selected lines, ethanol-regulated genes can be identified and differential regulation by ethanol subsequently compared between the lines. To simply the process, one selected line (WSP-2) is chosen for the identification of differential regulation. Assuming that the displayed gene products showing alteration as a result of treatment are functionally relevant to the selected behavior, it was hypothesized that some of the differentially expressed genes identified from ethanol-exposed WSP-2 mice will have a role in susceptibility to ethanol-induced withdrawal convulsions.

RATIONALE

The objectives of this study were essentially two-fold. The first objective was to identify ethanol-regulated genes in brain. This was achieved using WSP-2 mice as a model. The whole brain was chosen to be investigated first; this way, ethanol-regulation of gene expression was assessed in a more global sense. Although several anatomical origins of ethanol-withdrawal seizures (convulsions) have been implicated (especially the hippocampus and cortex) distinct neural pathways have yet to be clearly established. It is probable that specific anatomical regions of the brain may be more or less responsive to ethanol, including ethanol's effects on gene expression, and this cannot be determined by a whole brain analysis. Region specific effects of ethanol on gene expression in the brain were examined in follow-up analyses.

The second objective was to characterize the regulation of a chosen product with respect to the severity of alcohol withdrawal and associated with the development of physical dependence. This was achieved by comparing ethanol-induced regulation in genetically susceptible lines (WSP-1 and WSP-2) with ethanol-induced regulation in genetically resistant lines (WSR-1 and WSR-2). Since WSP mice represent a distinct genetic population, it was hypothesized that ethanol-induced regulation of gene expression would not necessarily be observed in mice that do not express a withdrawal syndrome (WSR mice). For example, if ethanol induced an increase in expression in WSP-2 mice, it would be predicted that a similar effect would be seen in WSP-1 mice and no induction, or perhaps, a decrease, would be observed in both WSR lines. Selection pressure acts to change gene frequencies of trait-relevant gene therefore there are allelic differences in the reciprocally-selected lines (WSP vs. WSR), but not necessarily for the same genes. The assumption being made is that ethanol is acting on a particular set of alleles selected in WSP mice but not WSR mice. The expected result for this scenario would, for example, be a increase in gene expression in WSP mice but not in WSR mice. If alternate alleles at the same locus were important for resistance to withdrawal, then a change in expression in WSR mice, opposite to that observed in WSP mice, would be expected. It is important to note, however, that a gene identified as ethanol-regulated may not be a selected gene but downstream in a cascade. That is, it may be modulate transcription of the allele that is selected in WSP mice but not in WSR mice.

The alternative interpretation was that the ethanol-regulation observed in WSP-2 mice is not specific to withdrawal severity, but to a more generalized effect of ethanol on gene transcription and ethanol intoxication. In this case, a correlation between withdrawal severity and ethanol-regulation will not be expected. Either the same change in level of

expression will be seen in all selected lines (suggesting a generalized effect of ethanol intoxication) or there will be non-replicable (i.e., between replicate line) effects on gene expression. The latter case can arise due to chance fixation of alleles in one selected line. For example, if an increase in gene expression were observed in WSP-2 mice and were associated with withdrawal severity, then it would be expected that a similar increase would be observed in WSP-1 mice (since they were selected for the same ethanol response). Likewise, an increase in expression would not be expected in WSR mice. It was hypothesized that directional changes in gene expression (e.g., increased by ethanol in WSP-2 mice) may be specific to the selected phenotype (withdrawal convulsions) and consequently observed only in the WSP selected lines.

In addition, alternative genetic animal models were used for the characterization to independently assess the association of ethanol-induced regulation with these phenotypes. C57BL/6J and DBA/2J mice, two inbred strains, were tested. These particular inbred strains show marked differences in ethanol withdrawal severity. DBA/2J mice exhibit significantly more severe withdrawal convulsions than C57BL/6J mice (Goldstein and Kakihana, 1974; Crabbe et al., 1983). It was predicted that if there was an association between withdrawal severity and expression of the identified regulated gene, the ethanol-induced expression in DBA/2J mice would be similar to WSP mice and effects of ethanol on expression in C57BL/6J would be similar to WSR mice. The characterization of expression was examined in whole brain (as per the initial identification) and in selected brain regions to assess specific regional expression changes via chronic ethanol exposure. Interpretations of gene expression results from C57BL/6J and DBA/2J inbred strain studies must be regarded carefully. Since these strains differ on many of the pharmacological effects of ethanol,

differences may not necessarily reflect specificity of ethanol-induced gene expression with a single phenotype.

Experimental Design

The first experiment (Expt.1) was designed to identify ethanol-regulated genes in WSP-2 mice and confirm the regulation by Northern blot analysis in WSP-2 mice. WSP-2 mice were exposed to ethanol vapor for 72 hours at which time the whole brain was removed. Gene expression was compared between saline- and pyrazole-treated mice exposed to air and EtOH-treated mice exposed to ethanol vapor using mRNA differential display. The differential display method involves amplifying cDNA in the presence of one of eighty arbitrary (5′) primers and an anchored T-oligomer (3′) primer (the procedure will be described in more detail below). The goal was to identify and confirm at least one regulated gene. Ethanol-induced changes in gene expression were confirmed by Northern blot analysis in whole brain of WSP-2 mice. The mice were exposed to ethanol or air exactly as the mice used for differential display.

In Experiment 2, regional differences in ethanol-induced expression were examined. Since regulation of gene expression by ethanol could be different depending on brain region, specific brain regions (hippocampus, cerebellum, and cortex) were chosen for further confirmation analysis. These regions shows sensitivity to ethanol-induced effects on neurotransmitter function or gene expression as described in the sections above. In addition, the hippocampus and cortex appear to be involved in NMDA and/or GABA-induced seizure activity, which are inhibited by ethanol (Davidson et al., 1995, 1996) and have been implicated to be neuronally active as determined by *c-fos* expression during ethanol withdrawal (Bouchenafa and Little, 1998; Dave et al., 1990). The cortex, as was dissected from the brains of these mice, represents a heterogeneous mixture of cortical

tissues (frontal, occipital, temporal, and parietal). The cerebellum was also active during withdrawal (Dave et al., 1990).

For characterization of ethanol-induced changes in gene expression in specific brain regions, the hippocampus, cerebellum, and cortex were dissected from WSP-2 mice experimentally treated as described above. Individual samples were examined (i.e., there was no pooling of tissue). Ethanol regulation in these brain regions was characterized by Northern blots.

Experiments 3 and 4 were designed to test the hypothesis that the observed ethanol-regulation of gene expression was associated with ethanol withdrawal severity. In Experiment 3, WSP-1, WSR-1, and WSR-2 mice were exposed to ethanol or air as described. Initial characterization was performed with whole brain tissue to assess whether the observed changes from WSP-2 mice were observed in WSP-1 mice, WSR-1 and/or WSR-2 mice. Ethanol regulation of gene expression was characterized and compared in the same brain areas tested in WSP-2 mice: hippocampal, cerebellar, and cortical tissue. Northern blots were used to assess changes in gene expression after ethanol. Methodological limitations prevented the simultaneous analysis of all four genotypes (WSP-1, WSP-2, WSR-1, WSR-2). However, the relative change in expression can be compared (but not statistically correlated) between the selected lines.

In Experiment 4, the specificity of the confirmed ethanol-induced changes in gene expression was determined using C57BL/6J and DBA/2J inbred mice. These strains represent a different genetic animal model that can confirm an association between withdrawal severity and a directional change in gene expression following ethanol exposure. C57BL/6J and DBA/2J mice were exposed to ethanol as described. RNA from whole brain or hippocampus, cerebellum, or cortex was used for Northern analysis.

METHODS and RESULTS

EXPT. 1: mRNA differential display

The purpose of this experiment was to identify ethanol-regulated gene products. Total RNA from the WSP-2 mice was used as the template for the reactions (see Methods, below). This line shows a robust phenotypic withdrawal response, suggesting that there may be particularly important effects of ethanol on gene expression. In addition, the use of a single genotype simplified the procedure, was less time- and material-consuming, and allowed for all 3 pools of reactions (cDNA of A, T or G anchored oligo T-mers) for one upstream (arbitrary) primer to be assessed simultaneously on one gel.

1. Methods

Animals and Animal Husbandry. Male Withdrawal Seizure-Prone mice from replicate 2 (WSP-2; J. Crabbe, Portland, OR) were used for experiments. Mice were from selected generation 26 (filial generations 58 -68). Mice were 70-85 days old at the start of the experiments. All mice were housed in standard polypropylene containers (28 x 18 x 13 cm), 4 per cage. Mice were maintained on a 12:12 hr light cycle with *ad libitum* food (Purina Rodent Chow) and water. Daily room temperature was 21 ± 2°C. Cages and bedding were changed twice a week.

Drugs. Ethyl alcohol (200 proof, Pharmaco) was diluted to 20% (v:v) in 0.9% saline. Pyrazole hydrochloride (Sigma) was dissolved in 0.9% saline to a final concentration of 1.2 mmol/kg.

Ethanol exposure

Several methods have been employed to intoxicate a mouse sufficiently to produce a withdrawal syndrome. Common routes of administration include intragastric administration, liquid diets, and ethanol vapor inhalation. Each route of administration has its advantages and disadvantages; the decision to use a particular model depends on the ease of use of a particular method, and ultimately, what question is being addressed. Several factors determine the best methods; however, not all methods are as reliable as others in eliciting a robust withdrawal. Methods such as self-administration or simply the addition of ethanol to drinking water allow the animal to choose how much and often it drinks; therefore, stable and intoxicating BEC may not be maintained. For many investigations, liquid diets have been utilized; these are relatively simple to implement and can result in significant blood ethanol concentrations. However, it is nearly impossible to control how much a mouse drinks or how often; consequently, maintaining stable blood ethanol concentrations is not easy. Vapor inhalation, on the other hand, allows the investigator to control the amount of ethanol to which a mouse is exposed, duration of exposure, and stable blood ethanol concentrations are maintained throughout an exposure session.

The utility and reproducibility of ethanol vapor inhalation has been thoroughly explored. Since Goldstein and Pal (1971) first described the use of ethanol vapor to intoxicate mice and elicit a withdrawal syndrome, several facets of this paradigm, and of the withdrawal syndrome in mice, have been determined. A drawback to using ethanol vapor is the necessity to use pyrazole to inhibit ethanol metabolism. Pyrazole inhibits the function of alcohol dehydrogenase resulting in decreased ethanol metabolism and higher, stable blood ethanol concentrations than if pyrazole is not present.

Procedure. The ethanol chamber protocol and maintenance of ethanol vapor levels have been previously described (Terdal and Crabbe, 1994). Briefly, ethanol was pumped onto a filter paper wick within each ethanol chamber. Hospital air was pumped into the chambers at a rate of 55 liters/min. Mixing of ethanol and air was achieved by three small fans in each chamber. Chamber ethanol levels were 7.9 mg ethanol/liter air for 24 hours then reduced to 6-7.5 mg ethanol/liter air for the remaining 48 hours of treatment.

Mice were separated into 3 treatment groups: Saline (SAL), Pyrazole (PYR), or EtOH (ETOH). SAL and PYR represent control groups that were exposed to air only. Pyrazole was administered daily to inhibit metabolism of ethanol and help maintain steady blood ethanol levels. Without pyrazole present, ethanol is metabolized quickly leading to unstable blood levels during exposure and between animals (Goldstein, 1972). In addition, exclusion of pyrazole tends to result in higher mortality of subjects before the end of the exposure period (Goldstein, 1972). On Day 1, all mice were weighed and injected with the appropriate drug or vehicle prior to being placed into vapor chambers. ETOH mice were given 1.0 mmol/kg pyrazole mixed with 20% (v:v) ethanol (1.75 g/kg). PYR mice were given pyrazole in saline and SAL mice were given saline only. All injections were administered intraperitoneally according to body weight. Food and water were present at all times while the mice were in the chambers. On Day 2 and Day 3 (24 and 48 hours of exposure), all mice were briefly removed from vapor chambers for body weight measurement and injections of pyrazole (ETOH and PYR groups) or saline (SAL).

On Day 4, (72 hours of ethanol exposure), mice were removed from the chambers and a tail blood sample (20 μl) taken from the ETOH group or the tail snipped for PYR and SAL groups as a control. Immediately following tail blood sampling, mice were killed by cervical dislocation and the brain immediately removed and frozen in liquid nitrogen. All

brain samples were stored at -80°C until RNA isolation. All ethanol-treated samples used in later analyses were matched for blood ethanol concentration (BEC).

Differential Display Methods

RNA isolation. Total RNA from individual WSP-2 mouse whole brain was isolated using an acid-phenol extraction procedure (Chomczinsky and Sacchi, 1987) and quantitated with UV spectrophotometry. For differential display, 100 µg aliquots of selected samples were DNase treated with DNase I for 15 minutes at 37 °C (Gibco BRL; 1U/µg of RNA). The reaction was terminated by adding 5 µL DNase Stop solution (50 mM EDTA, 1.5 M sodium acetate, 1% SDS); samples were purified by extracting with one volume of Tris-buffered phenol:chloroform.

Reverse transcription. Two micrograms of DNase-treated total RNA from individual WSP-2 mouse whole brain tissue (n = 3 for SAL; n = 3 for PYR; n = 4 for ETOH) was reverse transcribed using a one-base anchored oligo dT primer, H-T_{11}M (GenHunter, Brookline, MA; H = *Hind* III restriction sequence, M = A, G, or C), with Superscript™ II RNase H^- reverse transcriptase (Gibco BRL), according to manufacturers directions. Briefly, 2 ug of RNA and one H-T_{11}M primer (0.1 µM) were heat denatured (70°, 10 min.). To each sample first strand buffer (BRL; 1X), DTT (0.01M), and 0.5 mM dNTPs (GenHunter) were added. Reactions were prewarmed to 42°C for 2 min before the addition of Superscript reverse transcriptase (200 U). Reactions proceeded for 50 min at 42°C and were heated to 70°C for 15 min to inactivate the enzyme. Reactions were performed in a volume of 20 µl and diluted to 200 µL final volume after heat inactivation of enzyme.

PCR amplification and cloning. Reverse-transcribed cDNA was amplified using the RNAimage™ kit (GenHunter). Two microliters of one reverse transcription reaction was used and amplified with the same anchored T-mer and an arbitrary primer (H-AP#, where H

= *Hind* III restriction sequence) as provided by the kit, [α-^{33}P]-dATP (DuPont NEN) and *Taq* polymerase (AmpliTaq, Perkin-Elmer). PCR reactions were performed in duplicate. Briefly, each reaction consisted of 2 μl of DNase-treated RNA, 2 μM dNTP, 0.2 μM H-AP primer, 0.2 μM H-T_{11}M primer, 2000 Ci/mmole [α-^{33}P]dATP, and 0.2 μL AmpliTaq. PCR was carried out as follows: denaturing step (94°C, 30 sec), annealing step (48°C, 1 min.), and elongation step (72°C, 30 sec.) for 40 cycles followed by final elongation step of 72°, 5 min for one cycle. Products were resolved on a 6% polyacrylamide/urea sequencing gel, vacuum-dried, and exposed to X-ray film (X-Omat, Kodak). Bands were assessed by visual inspection for changes in relative intensity between ETOH and SAL or PYR groups. Bands were excised for confirmation of regulation only if the increase or decrease was observed in all reactions of the ETOH group in comparison with the control groups (SAL and PYR). It was hypothesized that pyrazole would not have an effect on gene expression; products on the differential display gel were eliminated from further analysis if there was an effect of pyrazole.

Excised bands were boiled to elute the cDNA and briefly centrifuged to remove acrylamide and filter paper. The cDNAs were precipitated and reamplified with the appropriate primers (20 μM dNTP, 0.2 μM each H-AP and H-T_{11}M, 0.4μl AmpliTaq). Products were analyzed on a 1% agarose gel to determine size and amplification efficiency. Reamplified products were ligated directly into pCRII vector (Invitrogen) or pGEM T-Easy vector (Promega) using T/A cloning. Ligation reactions were used for transforming Electromax DH10B™ bacteria cells (Gibco BRL) by electroporation. Fifty to 100 μl of the transformation reactions were grown overnight on LB agar plates with ampicillin. Individual colonies were isolated and analyzed for insertion of cDNA product.

Sequencing. The cDNA product was sequenced using Sequenase 2.0 (USB) in the presence of [α-^{33}P]-dATP, according to manufacturer's recommendations or using automated DNA sequencing (ABI 373A sequencer using Taq dye terminator chemistry; VA Molecular Biology Core Facility, Portland, OR). Sequence information was used to search databases (GenBank, Bethesda, MD and EMBL, Hinxton, Cambridge) using the BLASTN program (Altschul et al., 1990). Sequence alignment was performed with Gapped BLAST.

2. Results

Total RNA from 4 ethanol-, 3 pyrazole- and 3 saline-treated WSP-2 mice was reverse transcribed to cDNA and used as templates for differential-display PCR amplification (Liang and Pardee, 1990). Several primer combinations (arbitrary primer plus the anchored T-mers) were tested, but further analysis was curtailed when confirmation of previous fragments commenced. Several cDNAs were identified as potential EtOH-regulated genes and subcloned. Efficiency of transformation varied greatly (from 30-100% of colonies contained inserts). Four to 12 colonies per differential display product were isolated from plates and analyzed for insert by restriction digest with H*ind* III. Upon cloning these fragments, it was found that 90% contained multiple transcripts, indicated by the pattern of restriction digestion with *Hae* III. One fragment appeared to contain a single transcript (2/4 colonies contained insert of the appropriate size). This fragment was preliminarily named 2-450A based on the combination of primers used (H-AP$^{\#}$2 and HT-A) and its relative size on the sequencing gel (~450 bp). The sequences of the primers that amplified this product are as follows: H-AP$^{\#}$2 = 5′-AAGCTTCGACTGT-3′ and HT_{11}-A = 5′-AAGCTTTTTTTTTTTA-3′. A scanned image of the differential display gel containing this cDNA product is shown in Figure 3.

The cDNA fragment was sequenced and this used to search databases for potential sequence homology. The fragment showed >85% nucleotide sequence homology to rat *rex* gene (Ninkina et al., 1997) and human neuroendocrine specific protein (NSP) gene (Roebroek et al., 1996). The sequence of the fragment is shown in Figure 4. Sequences for the *rex* gene and for NSP gene homology are also provided for comparison (Figure 5). Differential display preferentially targets the 3′ region of transcripts; therefore, the sequence homology is shown only for the 3′ region of hNSP. Exon 9 of NSP is common to both transcripts of NSP (NSP-A and NSP-C; Roebroek et al., 1996). This region contains two putative polyadenylation sites. The product isolated by mRNA differential display analysis has been provisionally named mouse NSP (mNSP).

Discussion

NSP protein was named from the fairly restricted patteren of expression in normal brain and endocrine tissues, including testis, ovaries, and parathyroid and in small-cell lung carcinoma-derived cell lines displaying a restricted neuroendocrine differentiation. There are two transcripts of human NSP expressed in normal brain tissue. Based on Northern blot analysis, the larger fragment is 3.4 kb and the smaller fragment is1.8 kb (Roebroek et al., 1993). The predicted sizes of the translated proteins were 776 amino acids and 208 amino acids, respectively. These proteins have been designated as NSP-A for the larger protein and NSP-C for the smaller one. A third transcript/protein product of intermediate size is limited to expression in small-cell lung carcinomas (SCLC) (Roebroek et al., 1993). This gene has been localized to the long arm human chromosome14

Figure 3. mRNA differential display analysis. Scanned image of an autoradiograph of the differential display product 2-450A (mNSP). Duplicate PCR reactions for individual saline (n = 3), pyrazole (n = 3), or ethanol (n = 4) mice were run and separated on a 6% polyacrlyamide/urea gel. The first lane contains a DNA molecular weight marker. Treatment groups are indicated above the lanes. Empty lanes under the "ethanol" label were failed reactions. The arrow indicates the potential ethanol-regulated product, 2-450A.

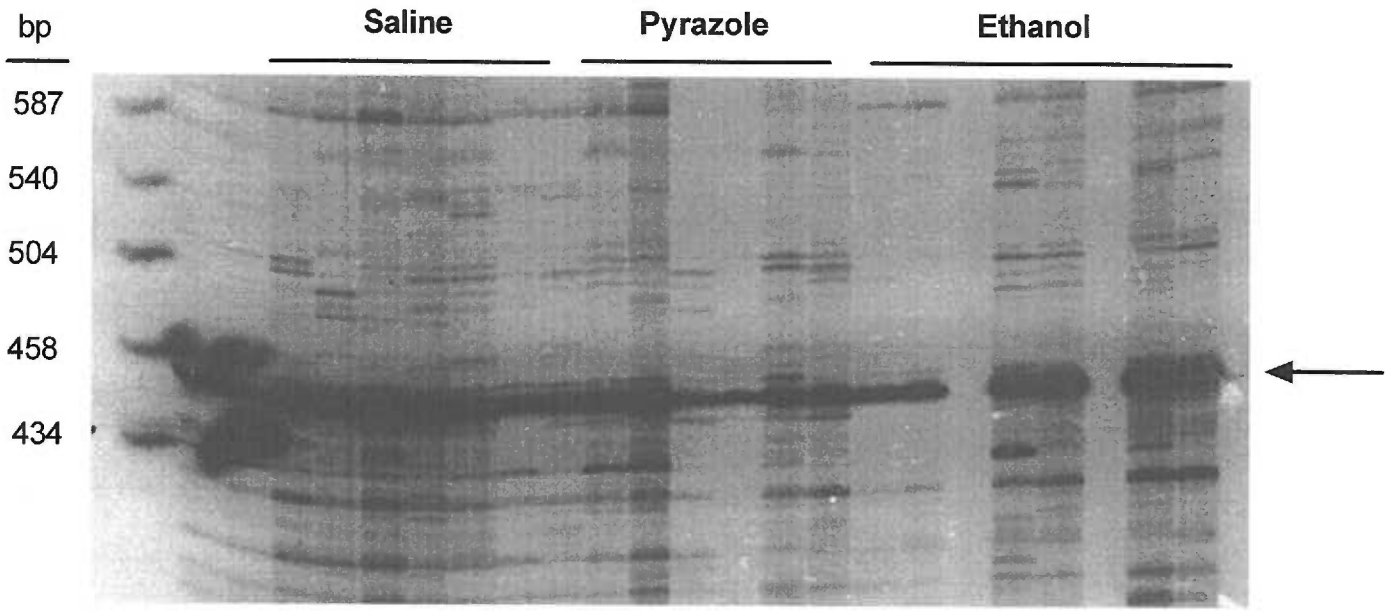

bp
Saline
Pyrazole
Ethanol
587
540
504
458
434

Figure 4. Sequence analysis of the differential display product, 2-450A. The differential display product, 2450A, was cloned into a T/A vector and sequenced as described in Methods. **Bold-face** nucleotides at the 5′ end represent vector sequence; **bold-face** and **underlined** nucleotides indicate the primers used for PCR amplification; poly-A adenylation sites are indicated by underlining only. Vector sequence was included because alignment to rat overlapped with a portion of this sequence.

5’

ctcggatccactagtaacggccgccagtgtgctggaattcggctt**aagcttcgactgt**cc 123

ctgtggctaggcctttcttgccaagtgcgctgacacatagtggatcgcttatcatgtcct 183

tgggttgctggatggattcatctctaataacactatatagaattgtaggccaatgtttta 243

gcatttccccacacacagagaacataaaaattaatataaaacagttgaccgtacatatgg 303

tgatccattttgtataacttagaatgatcgaattaaag**aataaa**tccaaaatgaacgcac 363

agtacttttctcctatgggatgcctgggctgatttacatgtatggtaactaaaggtgcca 423

gcatgttgactttattacaatttgtattacttcctctgtagttcctaatggactcaatca 483

cagactctggatatttgcacttatgtacttgatactgaatgcatc**aataaa**tgtgactaa 543

atg**taaaaaaaaaaagctt**aagccgaatt 572

Figure 5. Sequence homology of 2450A with hNSP and rS-rex$_b$. Nucleotide sequence of the differential display product and comparison with the 3′ regions of exon 9 of NSP (**A**), and rS-*rex*$_b$ (**B**). Conserved nucleotides are denoted by '|'; breaks in nucleotide (—) sequence indicate unaligned or deleted nucleotides.

A

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2450A:      121 tccctgtggctaggcctttcttgccaagtgcgctgacaca-tagtgga--tcgcttatca 177
                ||||  ||||||||||||||||  ||||  |||||  ||||   ||  |||||||  ||||||||  |
NSP  :      440 tccccgtggctaggcctttcctgccgagtgctctgatgcaatagtggaaatcgcttat-a 498

2450A:      178 tgtccttgggttgctggatggattcatctctaataacactatatagaattgtaggccaat 237
                ||||||||||||||||  ||||||  ||||  ||||||||  ||||||||||||||  |   ||
NSP  :      499 tgtccttgggttgctggttggattaatctttaataacaatatatagaattgtagactgat 558

2450A:      238 gttttagcatttccccacacacagagaacataaaaattaatataaaacagttgaccgtac 297
                ||||||||||||   |||  |||||  |  |||  |||||||  ||      |  ||||  |||||  ||
NSP  :      559 gttttagcatttttcca-acacacacaacgtaaaaataaa-----agcagtcgaccgcac 612

2450A:      298 atatggtgatccattttgtataacttagaatgatcgaattaaagaataaatccaaaatga 357
                ||||||  |||  ||||||||||||||  |||  ||  |||  ||  ||||||||||||||  |
NSP  :      613 ttatggtaatcagttttgtataacttaaaataattaaataaatgaataaatccaaaacaa 672

2450A:      358 acgcacagtacttttctcctatgggatgcctgggctgatttacatgtatggtaactaaag 417
                ||   ||||||||||  |  ||||||||   ||||||||||||||||||||||  ||||||
NSP  :      673 acatgcagtacttttgttgtatgggattggtgggctgatttacatgtatggttactaaaa 732

2450A:      418 -gtgccagcatgttgactttattacaatttgtattacttcctctgtagttcctaatggac 476
                 ||  ||||||||||  ||||||||||||||||||||||||  |||||||||||||||
NSP  :      733 agtaccagcatgttaactttattacaatttgtattactttctctgtagttcctaatggat 792

2450A:      477 tcaatcacagactctggatatttgcacttatgtacttgatactgaatgcatcaataaatg 536
                |||||  ||  ||||||||||||||||||||||||||||||||||||||||||  ||||||||
NSP  :      793 tcaattacggactctggatatttgcacttatgtacttgatactgaatgcataaataaatg 852

2450A:      537 tgactaaatgt 547
                |  |||||||||
NSP  :      853 ttactaaatgt 863
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B

```
2450A:      111  gcttcgactgtccctgtggctaggcctttcttgccaagtgcgctgacacatagtggatcg 170
                 ||||| |||||||||||||||||||| |||||||||||||||||||||||||||||| |||
rex-b:      3059 gcttctactgtccctgtggctaggcccttcttgccaagtgcgctgacacatagtgggtcg 3118

2450A:      171  cttatcatgtccttgggttgctggatggattcatctctaataacactatatagaattgta 230
                 |||||||||||||||||||||||| |||||||||||||||||||||||||||||| |||
rex-b:      3119 cttatcatgtccttgggttgctggttggattcatctctaataacactatatagaatcgta 3178

2450A:      231  ggccaatgttttagcattt---ccccacacacagagaacataaaaattaatataaaacag 287
                 |||||||||||||||||||   |||| |||||| ||||||||||||||||||||||||
rex-b:      3179 ggccaatgttttagcattttaccccccccacacacagaacataaaaattaatataaaacag 3238

2450A:      288  ttgaccgtacatatggtgatccattttgtataacttagaatgatcgaattaaagaataaa 347
                 |||||||| |||||||||||| |||||||||||| ||||||||||||||||||||||
rex-b:      3239 ttgaccgtgtatatggtgatccgttttgtataactaagaatgatcgaattaaagaataaa 3298

2450A:      348  tcc-aaaatgaacgcacagtacttttctcctatgggatgcctgggctgatttacatgtat 406
                 ||| |||||||||||||||||||||| |  ||||||||||||||||||||||||||||
rex-b:      3299 tccaaaaatgaacgcacagtacttttattgtatgggatgcctgggctgatttacatgtat 3358

2450A:      407  ggtaactaaaggtgccagcatgttgactttattacaatttgtattacttcctctgtagtt 466
                 |||||||||| |||||||||||||||||||||| ||||||||| ||||||||||||||
rex-b:      3359 ggtaactaaa-gtgccagcatgttgactttattacgatttgtatttcttcctctgtagtt 3417

2450A:      467  cctaatggactcaatcacagactctggatatttgcacttatgtacttgatactgaatgca 526
                 ||||| |||||||||||||||||||| ||||||||||||||||||||||||||||||
rex-b:      3418 cctaacggactcaatcacagactctgggtatttgcacttatgtacttgatactgaatgca 3477

2450A:      527  tcaataaatgtgactaaatgt 547
                 |||||||||||||| |||||
rex-b:      3478 tcaataaatgtgactcaatgt 3498
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(14q21→q22) by Southern blot analysis of human x mouse hybrid clones and fluorescent *in situ* hybridization analysis (Kools et al., 1994).

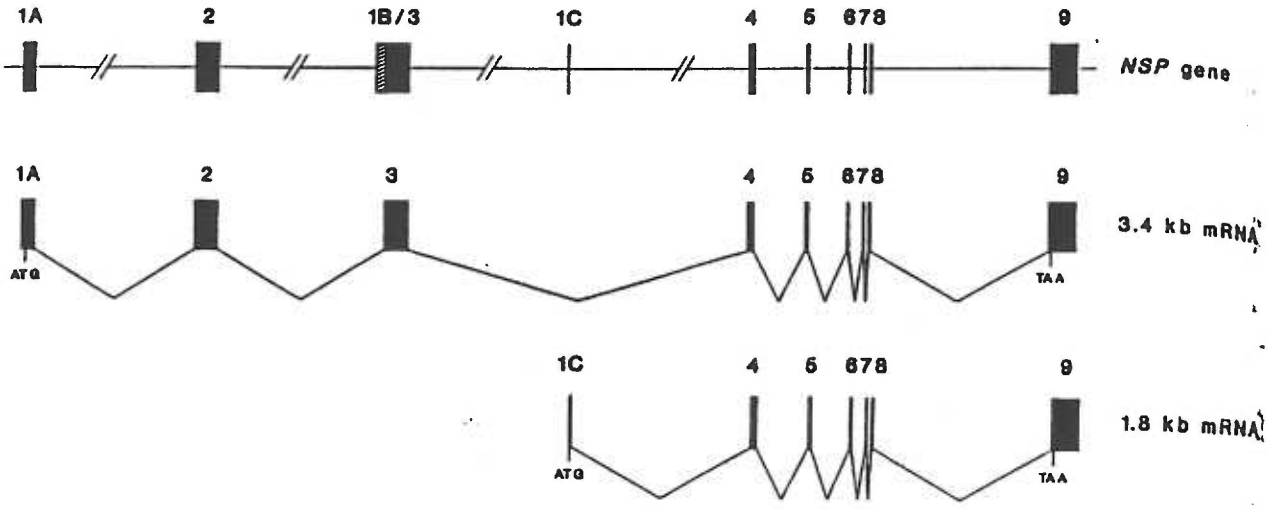
The genomic structure of the NSP gene revealed nine exons of variable size (47 bp - 774 bp) with interspersed introns, also of variable size (88 bp to ~ 120kbp). The cDNA sequence representing the mRNAs of NSP-A and NSP-C share common 3'-ends; however the 5'-end sequences are unique. The proposed gene structure is presented in Figure **6**. The exon composition was deduced for each transcript: exon 1A and exons 2 - 9 make up NSP-A (3.4 kb transcript) and exons 1C and exons 4 - 9 encode NSP-C (1.8 kb transcript) (Roebroek et al., 1996). Exon 1A and 1C refer to the first exon of each transcript. Exon 1C is probably located downstream of exon 3 upstream of exon 4, but is not transcribed in NSP-A (i.e., the 3.4 kb transcript). No exons upstream of exon 1C are transcribed in the 1.8 kb transcript. The rat homolog, *s-rex*, also has 2 transcripts of approximately the same size (Ninkina et al., 1997). Sequence preceding nucleotide 116 of the smaller *s-rex* transcript (*s-rex*$_s$) do not overlap with sequence from the larger transcript (*s-rex*$_b$). Together, these differences in exon composition of the transcripts suggested that these products were not generated simply by alternative splicing events but perhaps from different promoters (Roebroek et al., 1996; Ninkina et al., 1997).

Sequence analysis of the presumed promoter regions for each transcript did not reveal any TATA boxes upstream of exon 1A or 1C, however potential CCAAT boxes may be present (Roebroek et al., 1996). In addition, these putative promoter regions are G-C rich and contain SP1 elements that are common to "housekeeping" genes. Functional assays of promoter function provided more evidence for multiple promoter-directed expression of NSP transcripts (Roebroek et al., 1996). The deduced amino acid sequence of NSP-C is identical to NSP-A with exception of the first 20 amino acids Figure **6B**). The

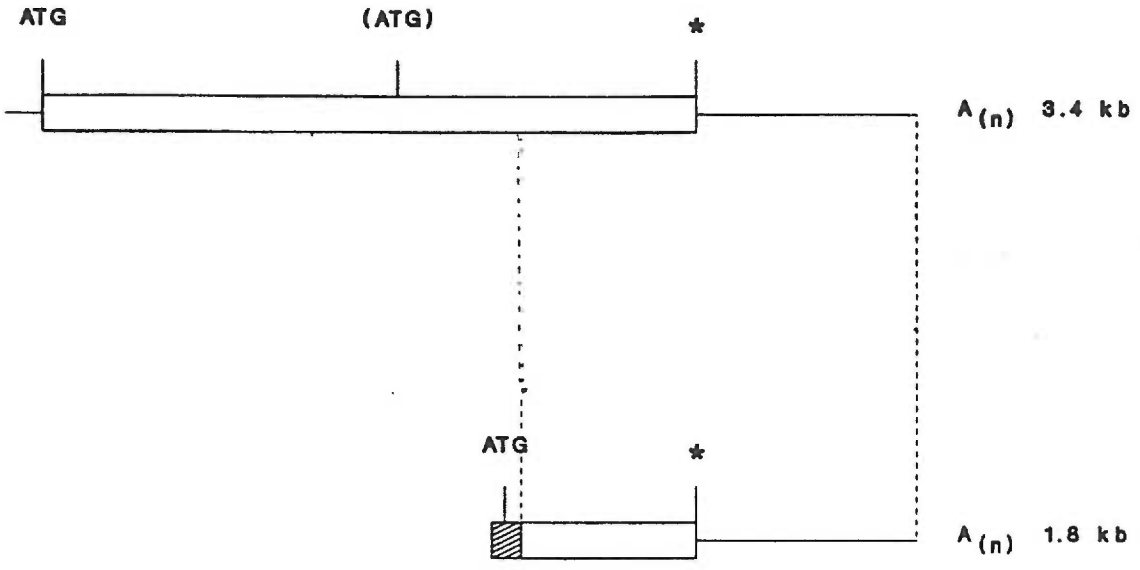
Figure 6. Predicted gene strucure of human Neuroendocrine Specific Protein. **A.** Predicted structure of exons and inrons and the two transcriptional products. Intronic regions, which are spliced out for each transcript, are indicated by the thin line. Exons are indicated by the boxes. Note that the 3′ regions are identical (exons 4-9). **B.** The 3.4 kb and 1.8 kb transcripts. The dotted line represents identical sequence downstream of that point. The * represents the addition of the poly A^+ tail [$A_{(n)}$].

(Adapted from Roebroek et al., 1996)

A


1A
2
1B/3
1C
4
5
678
9
NSP gene
1A
2
3
4
5
678
9
ATG
TAA
3.4 kb mRNA
1C
4
5
678
9
ATG
TAA
1.8 kb mRNA

B


ATG
(ATG)
*
A(n) 3.4 kb
ATG
*
A(n) 1.8 kb

predominant translational product for the 3.4 kb transcript is ~135 kDa whereas the 1.8 kb transcript encodes a much smaller protein of ~23 kDa, although in rat brain 2 minor proteins of ~16 kDa were detected (van de Velde et al., 1994).

Molecular characterization of NSP-A and NSP-C has revealed many similarities and a few differences in localization of protein expression. Immunohistochemical analysis of NSP-A revealed a fairly widespread expression of this protein throughout the rat brain. The most prominent immunoreactivity was detected in Purkinje cells of the cerebellum, superficial gray layer of the superior colliculus, piriform and entorhinal cortices, CA3 and dentate gyrus regions of the hippocampus, substantia nigra pars reticulata, caudate putamen, globus pallidus, subcommisural organ, median forebrain bundle and lateral habenula (van de Velde et al., 1994). Subcellular localization of NSP-A has indicated its association with the endoplasmic reticulum (van de Velde et al., 1994). The intense staining of Purkinje cells in the cerebellum was found in the dendritic trees, cell bodies, and axons. Expression of NSP-C was compared to NSP-A in human tissue (Senden et al., 1996). NSP-C protein was found in the granule and molecular cell layers of the cerebellum but not in Purkinje cells as observed for NSP-A. Within the molecular and granule cell layers, staining was light and diffuse for NSP-C. A small number of positive cells for NSP-A were found in the granular cell layer and in axons of the molecular cell layer. Staining for both proteins was detected in neurons of the cerebral cortex. No other brain regions were analyzed. The expression of these proteins in neuroendocrine tissue was compared; cells of the pituitary (both adenohypophysis and neurohypophysis) were strongly stained for both proteins. Staining was also observed for both proteins in the adrenal medulla. There were several tissues where NSP-A staining was observed but not NSP-C: islet cells of the pancreas, parathyroid, and neuroendocrine cells of the gastrointestinal tract (Senden et al.,

1996). It appears that there is overlapping, but not identical, expression of these proteins in some tissues. Thus, these results and the distinct protein sequence at the amino-terminus suggest that there may be functional differences between them.

Subcellular localization was accomplished by immunofluorescence labeling of human SCLC cell lines, COS-1 cells and immunoblotting microsomal fractions of rat pancreatic cells for NSP-C and NSP-A. A peripheral punctate pattern and strong perinuclear staining of cells was observed for NSP-A and NSP-C proteins; this staining highly resembled that of, and colocalized with, an established endoplasmic reticulum Ca^{2+}-ATPase (van de Velde et al., 1994). Mutations of the common carboxy-terminal hydrophobic region resulted in diffuse cytoplasmic staining, strongly suggesting that these proteins associate with the endoplasmic reticulum (ER) via the carboxy-terminus. In addition, NSP-A protein was found in microsomal fractions of cells and could only be solubilized by detergent, again highly suggestive of membrane-bound ER protein (van de Velde et al., 1994). The functional role of these proteins has not demonstrated. However, based on the expression patterns and association with the membrane of the ER, it has been suggested that NSP-C may play a role in transport of macromolecules involved in synaptic vesicle formation or packaging secretory products (Senden et al., 1996). It has also been suggested that these proteins may play a role in the regulation of intracellular Ca^{2+} levels (van de Velde et al., 1994).

Several other NSP-related transcripts or sequences have been identified. Two rat transcripts have been isolated that are enriched in synaptosomes as compared to cytoplasmic fractions (Ninkina et al., 1997). These transcripts, s-rex$_b$ and s-rex$_s$, correspond to the 3.4 kb and 1.8 kb NSP transcripts, respectively. A developmentally-regulated rat brain cDNA clone, Cl-13 (Wieczorek and Hughes, 1993), showed very high amino acid

sequence homology to NSP-C (van de Velde et al., 1994). No functions have been ascribed to any of these products.

EXPT. 2a: Confirmation of ethanol regulation in WSP-2 mouse whole brain

The amplification of the cDNA fragments used for differential display is a semi-quantitative; thus, it does not necessarily represent a reliable increase or decrease in expression of a particular message. To ensure that the increase observed after PCR amplification was a real effect, and not a false-positive, the PCR product was used as a probe to screen Northern blots containing RNA from additional ethanol-exposed WSP-2 mice treated as described and also used in the reverse transcription reactions.

2a.1 Methods

Northern analysis. 30 μg of total RNA from whole brains of individual ethanol-, pyrazole-, or saline-treated WSP-2 mice (n = 7-11/group) was fractionated on 1% agarose gels through 6% formaldehyde/ $NaPO_4$ buffer and transferred to nylon membranes overnight (Gene Screen, DuPont NEN). RNA molecular weight markers were run on every gel, excised fefore transfer and stained with ethidium bromide. Membranes were prehybridized in 50% formamide buffer [50% v:v formamide, 5X Denhardts solution (Ficoll, bovine serum albumin, and polyvinylpyrrolidone, 1:1:1), 5X saline sodium citrate (SSC) buffer, 50 mM $NaPO_4$ buffer, 1 mM sodium pyrophosphate, 100μg/ml Herring sperm DNA] containing 2% SDS, at 42°C for at least four hours, and hybridized overnight (42°, 16-20 hours) with a random primed probe from cloned differential display products. Radioactive probes were generated by random prime labeling (Amersham) 150-300 ng of differential display product with 50 μCi [α-^{32}P]-dCTP for 12-14 hrs. Blots probed with cDNA probes were washed at high stringency in SSC buffer (2X SSC at 25°C; 2X SSC/2% SDS at 65°C,

0.1X SSC at 25°C). All blots were stripped in boiling 2% SDS (three washes, 5 minutes each) and reprobed with a [γ-^{32}P]-ATP end-labeled oligo probe for 18S rRNA (5'-AGGGGGTCAGCGCCCGTCGGCATGTATTAG-3'; Operon) to control for loading. Autoradiographs hybridized with oligomer probes were washed at high stringency (2X SSC/0.1% SDS at 25°C; 2X SSC/0.1% SDS at 65°C; 2X SSC/0.1% SDS at 25°C; 2X SSC at 25°C). Blots were quantitated by scanning densitometry (Hoefer Instruments).

Northern blots contained total RNA from whole brain of WSP-2 mice that were treated identically to those used for the differential display reactions. Samples from all three treatment groups (saline, pyrazole, and ethanol) were used on a single gel for Northern analysis. Ethanol-treated samples were chosen based on BEC at time of withdrawal from the chambers. Mice were matched for BEC as closely as possible. The average BEC (mean ± sem) was 1.55 ± 0.11 mg/ml (Table 2).

Data analysis. Each sample was quantitated for relative expression levels for each transcript of the gene as well as for 18S rRNA. Inherent variability in the procedure (quantitation of RNA, loading of gel, transfer, length of film exposure) led to differences in the absolute density values from blot to blot. Therefore, it was necessary to adopt a strategy for indexing regulation that facilitated combining multiple blots. Each sample (i.e., transcript) was corrected for loading by dividing the integrated density value of the transcript by the sample's 18S rRNA value density value. For each transcript, the 18S-corrected values were normalized for each Northern blot by averaging the results of the whole blot (saline, pyrazole, and ethanol treatments) and then dividing each sample by the blot average ($X_i / \bar{X}_B$, where *i* stands for individual sample and *B* stands for the blot). The blot average therefore equaled 1.0 for each blot. Saline and pyrazole groups were compared by two-tailed Student's *t*-test. If there were no differences, then these groups

were combined to form a single control group. This way, the number of subjects for the control (saline and pyrazole air-exposed mice) and for ethanol groups were similar on each blot. The effect of ethanol versus control treatment on gene expression was then analyzed by two-tailed Student's t-test.

2a.2 Results: Whole brain

Northern analysis of gene expression was used to determine the effect of ethanol on steady-state mRNA levels for NSP. Figure 7 shows a representative Northern blot using the cloned differential display identified product, mNSP. A total of four Northern blots were quantitated. The average BEC for the 12 ethanol-treated mice was 1.55 ± 0.13 mg/ml (Table 2). Two transcripts were detected with this probe. One transcript was ~1.4 kb and the other ~3.0 kb in size. These sizes are similar to the sizes of the transcripts for NSP-A and NSP-C mRNA (Roebroek et al., 1993). Pyrazole had no effect on the expression of the 3.0 kb transcript ($t = 0.37$, $df = 14$, n.s.) or the 1.4 kb transcript ($t = 0.73$, $df = 15$, n.s.). Thus, data from saline- and pyrazole-treated mice were combined. The effect of ethanol on expression was then determined for each transcript independently.

There were modest increases in expression of these transcripts observed (Figure 8A and B). There was no significant effect of ethanol on the expression of the 1.4 kb transcript ($t = 0.86$, $df = 27$, n.s.). However, following ethanol treatment there was a significant increase in the steady-state level of the 3.0 kb transcript ($t = 2.59$, $df = 25$, $p < 0.02$).

2a.3 Discussion

The cDNA fragment isolated from the differential display gel detected two transcripts on Northern blots, one of 1.4 kb and the other of 3.0 kb. The estimated sizes

Figure 7. Northern blot analysis of mNSP in whole brain of WSP-2 mice. Representative Northern blot of whole brain RNA from WSP-2 mice exposed to ethanol-vapor (n = 3) or air (n = 2 saline, n = 2 pyrazole) for 72 hrs. The cDNA probe detected two transcripts of ~1.4 kb and ~3.0 kb, as indicated.

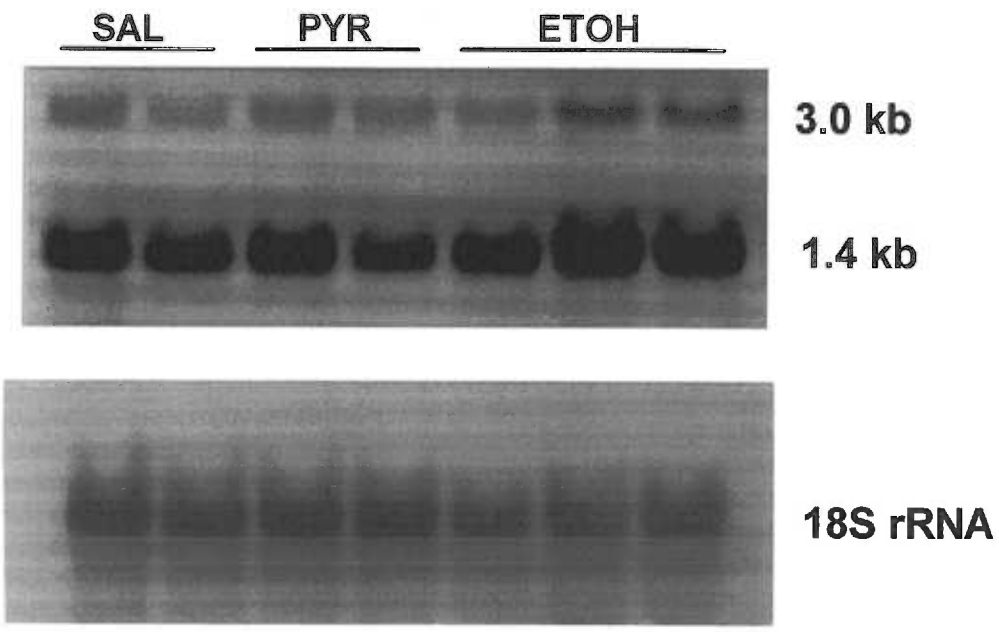

SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

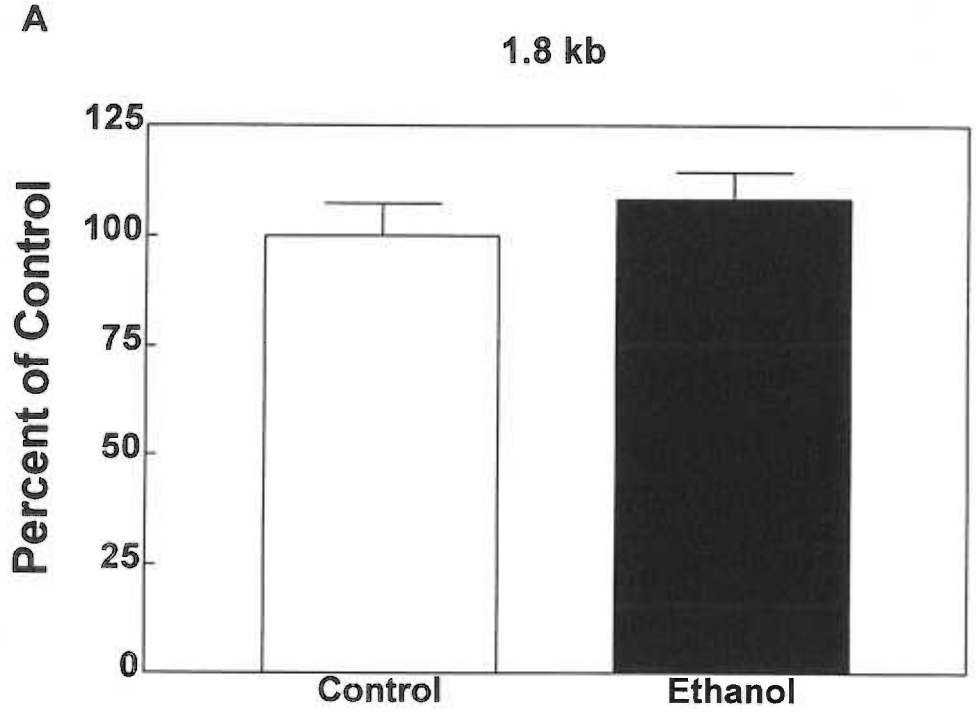
Table 2. Average blood ethanol concentration (mean ± sem) at time of removal from ethanol vapor chambers. BECs are presented for each replicate line for whole brain and brain region.

WHOLE BRAIN	LINE	BEC (mg/ml)
	WSP-1 (n = 9)	1.76 ± 0.13
	WSP-2 (n = 12)	1.55 ± 0.13
	WSR-1 (n = 6)	1.44 ± 0.12
	WSR-2 (n = 6)	1.45 ± 0.05

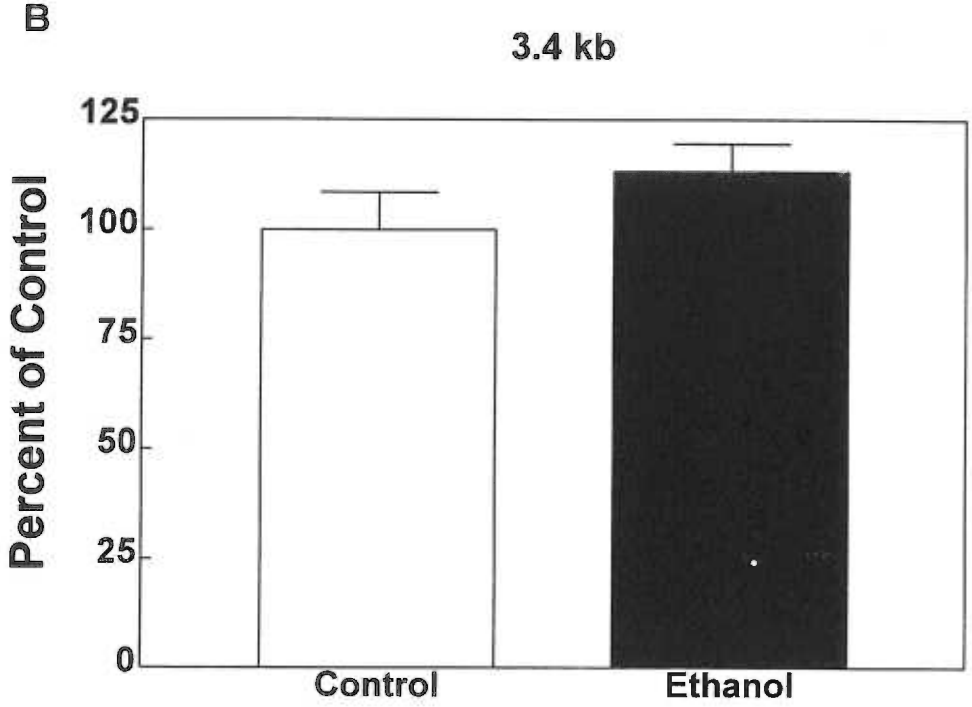
BRAIN REGION	LINE	BEC (mg/ml)
Hippocampus	WSP-1 (n = 8)	1.41 ± 0.15
	WSP-2 (n = 9)	1.75 ± 0.11
	WSR-1 (n = 9)	1.34 ± 0.06
	WSR-2 (n = 7)	1.49 ± 0.07
Cerebellum	WSP-1 (n = 7)	1.60 ± 0.11
	WSP-2 (n = 9)	1.75 ± 0.11
	WSR-1 (n = 11)	1.35 ± 0.06
	WSR-2 (n = 11)	1.47 ± 0.07
Cortex	WSP-1 (n = 6)	1.30 ± 0.15
	WSP-2 (n = 8)	1.92 ± 0.07
	WSR-1 (n = 7)	1.30 ± 0.07
	WSR-2 (n = 9)	1.56 ± 0.09

Figure 8. Ethanol-regulated expression of the transcripts of mNSP in whole brain tissue from WSP-2 mice. **A.** Ethanol-exposed versus air-exposed (control) samples for the 1.4 kb transcript. **B.** Ethanol-exposed versus air-exposed (control) samples for the 3.0 kb transcript. Scanning densitometry was used to quantitate autoradiographs. Each transcript was corrected for loading using its 18S rRNA integrated value. Samples on each blot were normalized to the blot average integrated value and are expressed as the percent of Control expression levels (mean ± sem). Saline- and pyrazole-groups were combined to form the Control group. See text for statistical analyses.

A


1.8 kb
Percent of Control
125
100
75
50
25
0
Control
Ethanol

B


3.4 kb
Percent of Control
125
100
75
50
25
0
Control
Ethanol

are slightly smaller than the transcripts of hNSP (Roebroek et al., 1993). The nucleotide sequence homology in addition to the correspondence in transcript size strongly implicates this cDNA to be the mouse homologue of neuroendocrine specific protein (NSP).

The Northern analysis of whole brain RNA did not detect any significant differences in expression following chronic ethanol exposure of the 1.4 kb transcript, but there was an increase in the relative abundance of the 3.0 kb transcript following chronic ethanol exposure. This increase was modest, 25% above control (i.e., saline and pyrazole-treated mice exposed to air only), but the observed magnitude of change following ethanol treatment was qualitatively similar to that estimated from the differential display gel. The differential regulation of the transcripts suggests that ethanol is interacting specifically with one transcript (e.g., via differences in promoter region). Another explanation for the differential effect of ethanol is the use of the whole brain to detect differences in ethanol-mediated changes in gene expression. The whole brain analyses gives a general indication as to whether ethanol is affecting the expression of genes at all. Whole brain analyses obviously do not allow region-specific effects to be detected. If there was a differential effect of ethanol on the expression of these transcripts, dependent on brain region, modest effects would have been masked among regions that do not show ethanol regulation. The selected phenotype (withdrawal severity) is a complex behavioral trait and there is not much evidence implicating specific brain regions that might be targets of ethanol with regard to modulating convulsion susceptibility. In order to more fully characterize ethanol regulation, hippocampal, cerebellar, and cortical tissue were also analyzed by Northern analysis.

EXPT. 2b: Characterization of brain regional expression and regulation in WSP-2 mice: Hippocampus, cerebellum, and cortex

2b.1 Methods

WSP-2 mice were exposed to ethanol as described above, removed from the vapor chambers, and the hippocampus, cerebellum, and cortex were immediately removed and frozen. Tissues was extracted from individual mice using RNA STAT-60 (Tel-Test, Inc.). Twenty micrograms (20 μg) of hippocampal, cerebellar, or cortical issue RNA was fractionated on 1% agarose/ formaldehyde gels and transferred to nylon membranes (Gene Screen) overnight. Four saline, 4-5 pyrazole, and 7-9 ethanol-treated mice were analyzed per brain region. The average BEC for the ethanol-groups were as follows (see Table 2): hippocampus,1.75 ± 0.11 mg/ml (n = 9); cerebellum, 1.75 ± 0.11 mg/ml (n = 9); cortex, WSP-2 1.92 ± 0.07 mg/ml (n = 8). All other procedures and data analyses were identical to the whole brain analysis described above.

Saline and pyrazole results were compared first, for each transcript from each brain region. There were no significant differences found for either transcript (all t's < -2.0 to 2, $df = 7$, n.s.). Therefore, saline- and pyrazole-treated mice were combined to form a single control group against which ethanol treatment was compared by Student's t-test.

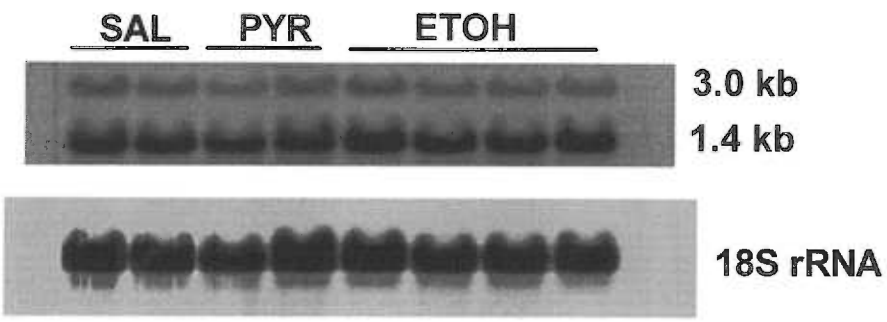
2b.2 Results

2b.2.1. Hippocampus

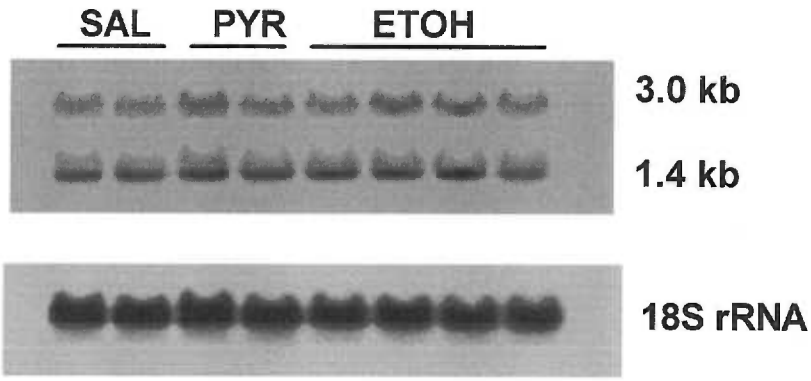
A representative Northern blot of hippocampal tissue is shown in Figure 9A. The Northern blots looked identical to those from whole brain: two transcripts (1.4 kb and 3.0 kb) were detected by the probe. The relative abundance of the 3.0 kb transcript was 5-fold less than the 1.4 kb transcript.

Figure 9. Northern blot analysis of mNSP in specific brain regions of WSP-2 mice. Representative Northern blots from hippocampus (**A**), cerebellum (**B**), and cortex (**C**) are shown. Mice were exposed to ethanol or air for 72 hrs. Treatment groups are designated as SAL for saline-treated, PYR for pyrazole -treated, and ETOH for ethanol-treated. Blots were probed with a cDNA fragment corresponding to mNSP as described in Methods. Transcripts of 1.4 kb and 3.0 kb were detected.

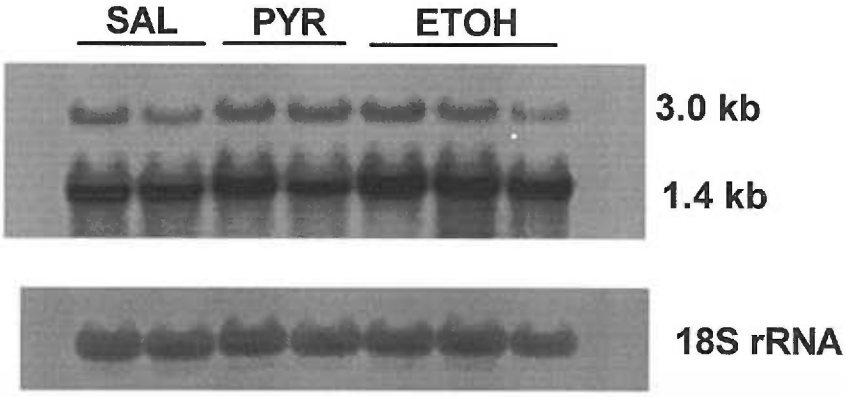
A


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

B


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

C


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

1.4 kb transcript

Results from scanning densitometry analysis of the Northern blots are depicted in Figure 10A. Ethanol treatment increased the expression of this transcript in the hippocampus ($t=2.95$, $df=16$, $p<0.01$). This increase was 41%, substantially greater than what was observed in whole brain.

3.0 kb transcript

In contrast to the 1.4 kb transcript, chronic ethanol treatment did not significantly alter the expression of the 3.0 kb transcript (Figure 10B) ($t=1.62$, $df=16$, n.s.). Although the absolute magnitude of the change was about the same as the 1.4 kb transcript, there was much greater variance in the ethanol group for the 3.0 kb transcript.

2b.2.2 Cerebellum

A representative Northern blot for cerebellar tissue is shown in Figure 9B. The 3.0 kb transcript is more abundant in cerebellum than hippocamus; the ratio of relative abundance of the 3.0 kb transcript to the 1.4 kb transcript in the cerebellum was 1:2 (see saline groups).

1.4 kb transcript

In the cerebellum, chronic ethanol treatment did not affect the relative abundance of the 1.4 kb transcript ($t=0.33$, $df=16$, n.s.; Figure 10C).

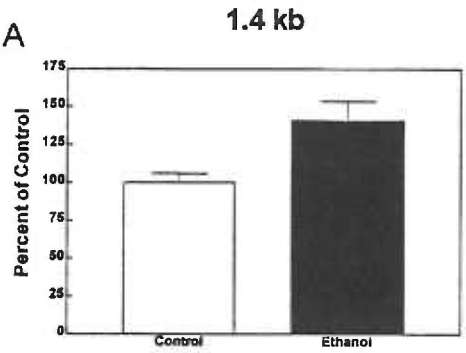
3.0 kb transcript

In contrast to whole brain and hippocampus, chronic ethanol decreased the expression of this transcript 25% below control (air-exposed) mice in cerebellum (Figure 10D); however, this apparent decrease did not reach statistical significance ($t=-1.8$, $df=16$, n.s.).

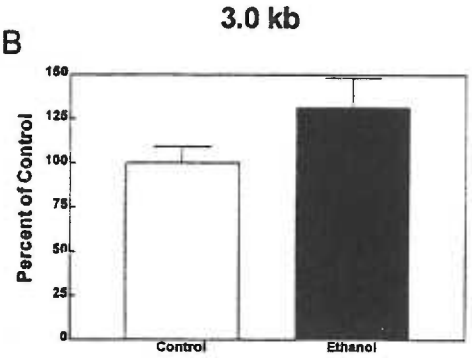
Figure 10. Expression of the 1.4 kb and 3.0 kb transcript of mNSP in hippocampal, cerebellar, and cortical tissue from WSP-2 mice. **A.** Ethanol-exposed (n = 9) versus air-exposed (control; n = 9) samples for the 1.4 kb transcript in hippocampus. **B.** Ethanol-exposed (n = 9) versus air-exposed (control; n = 9) samples for the 3.0 kb transcript in hippocampus. **C.** Ethanol-exposed (n = 9) versus air-exposed (control; n = 9) samples for the 1.4 kb transcript in cerebellum. **D.** Ethanol-exposed (n = 9) versus air-exposed (control; n = 9) samples for the 3.0 kb transcript in cerebellum. **E.** Ethanol-exposed (n = 8) versus air-exposed (control; n = 9) samples for the 1.4 kb transcript in cortex. **F.** Ethanol-exposed (n = 8) versus air-exposed (control; n = 9) samples for the 3.0 kb transcript in cortex. Scanning densitometry was used to quantitate autoradiographs. Each transcript was corrected for loading using its18S rRNA integrated value. Samples on each blot were normalized to the blot average integrated value and are expressed as the percent of Control expression levels (mean ± sem). Saline- and pyrazole-groups were combined to form the Control group. See text for data analyses.

HIPPOCAMPUS

A

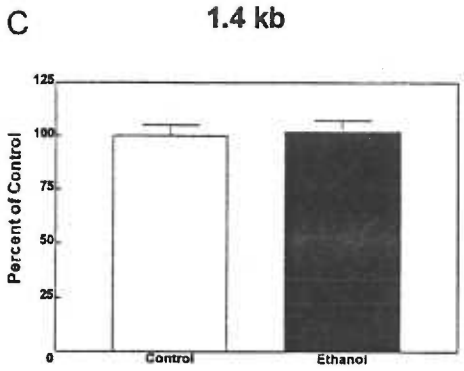


B

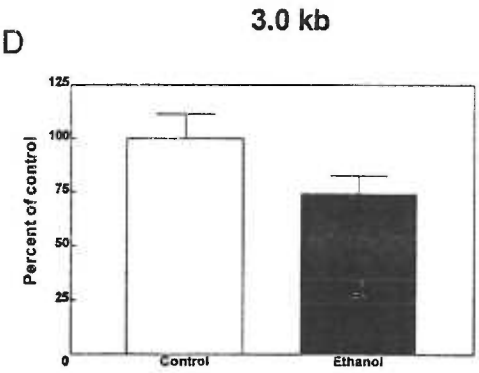


CEREBELLUM

C

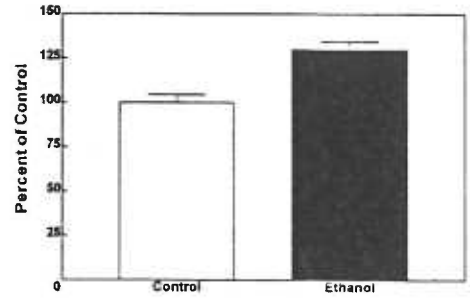


D

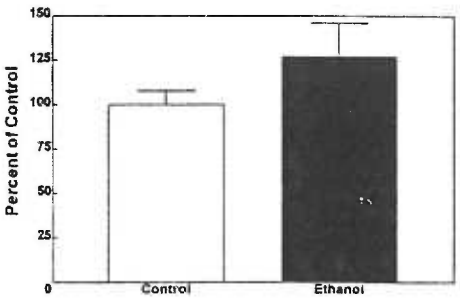


CORTEX

E



F



2b.2.3 Cortex

A representative Northern blot is shown in Figure 9C; the two transcripts are detected in this tissue as well. The relative abundances of the 3.0 kb and 1.4 kb transcripts appeared to be similar to that observed in the hippocampus.

1.4 kb transcript

The effect of ethanol on the expression of the 1.4 kb transcript is shown in Figure 10E. Ethanol significantly increased the expression of this transcript versus air-exposed mice ($t = 4.42$, $df = 15$, $p < 0.01$). This increase was 30% above the control level, similar to the effect observed in hippocampus.

3.0 kb transcript

There was a 27% increase in expression in mice treated with ethanol (Figure 10F). This increase was equivalent to the effect on the1.4 kb. However, this increase was not statistically significance ($t = 1.37$, $df = 15$, n.s.), probably due to large variability in the ethanol group.

2b.3 Summary

Several features of ethanol-mediated regulation are apparent from these analyses. It is clear from the results of the brain region analyses the expression observed from the differential display gel and from whole brain are "averages" of regional expression. Although the magnitude of ethanol-induced regulation from whole brain were in agreement with that observed from the differential display gel, the results for the selected brain regions indicate that the nature of ethanol-mediated regulation of mNSP is complex.

Results from hippocampal and cortical tissues showed fairly modest effects of chronic ethanol exposure on the steady-state levels of mNSP. Chronic ethanol exposure

increased the expression of both transcripts to some degree in both brain regions. The increase was statistically significant for the 1.4 kb transcript in the hippocampus.

The most interesting and surprising results were gleaned from the cerebellum. In the cerebellum, the transcripts were regulated quite differently from each other: the 1.4 kb transcript was not affected by ethanol at all whereas the 3.0 kb transcript was decreased. The decrease in expression was surprising and differed from the effect of ethanol on the expression of either transcript in any other brain region or the whole brain. Although the decrease was not statistically significant, the fact that there was an apparent decrease by ethanol suggests that this is a relevant and specific effect of ethanol. Since the pyrazole group did not differ from the saline group, the results of ethanol treatment on the expression of these transcripts in cerebellum cannot be attributed to an effect of pyrazole on gene expression.

EXPT. 3: Characterization of expression and ethanol-regulation in WSP-1, WSR-1, and WSR-2 selected lines of mice

3.1 Methods and Data analysis

Animals. Male Withdrawal Seizure-Prone mice of replicate 1 (WSP-1) and Withdrawal Seizure-Resistant mice from both replicates (WSR-1, WSR-2) were used. Mice were from selected generation 26 (filial generations 58 -68). Mice were 70-85 days old at the start of the experiments. Mice were exposed to ethanol as described under "Expt. 1 Methods" with the following exceptions. All ethanol-treated mice received a loading dose of 1.5 g/kg ethanol with 1.0 mmol/kg pyrazole. Pyrazole-treated mice were given 1.0 mmol/kg pyrazole in 0.9%saline. Due to differences between the selected lines in tissue concentrations of ethanol achieved at a given chamber vapor concentration, WSP-1 and

WSP-2 mice were placed in a separate ethanol chamber from each other and from WSR mice. This allowed different concentrations of ethanol vapor to be maintained in each chamber and has proven to be the optimal method for maintaining equivalent blood ethanol concentrations in all lines over the 3-day period (Terdal and Crabbe, 1994). Equivalent BEC levels at time of withdrawal are necessary for controlling for pharmacokinetic effects on behavioral and also molecular differences (see Terdal and Crabbe, 1994). Chamber ethanol levels were maintained as follows: both WSR lines= 7-8 mg ethanol/liter air for all 3 days; WSP-1 chamber levels were 6.5 -7.5 mg ethanol/liter air for the first 24 hrs, then 6-7 mg /liter for 48-72 hours.

After data were normalized, data from blots of the same genotype could be combined. Each replicate line was analyzed separately. This method was chosen because 1) WSP-2 were previously analyzed independently, and 2) WSP-1 and WSR-1 and -2 mice were not all exposed simultaneously or run on the same gels.

The relative steady-state expression was compared first between the Air groups (saline and pyrazole) for each selected line. When no difference was found between these groups, the saline and pyrazole groups were combined to form a single control group. If there were significant differences between the treatment groups exposed to air only, then these groups remained separate for the analyses with the ethanol treatment group except where noted and discussed. The effect of ethanol on the relative abundance of each transcript was analyzed by independent Student's *t*-test for each replicate line when saline and pyrazole groups could be combined or one-way ANOVA when all three treatment groups compared. Data are graphically presented as percent change from the Control (or Saline) group.

3.2 Results

Blood ethanol concentration

The choice of samples to be used for ethanol groups was based on BEC levels. Animals with extreme BEC values (above 2 mg/ml or below 1.0 mg/ml) were not analyzed. Blood ethanol concentrations were averaged for each line (x replicate) for whole brain, hippocampus, cerebellum, and cortex (Table 2). BECs for the three brain regions are not identical due to either deficiency in amount of RNA extracted (limiting further use on Northern blots), integrity of RNA, or lost tissue.

A two-way ANOVA was used to determine if there were any line differences in BEC for any of the brain areas. Although the average BEC for WSP mice was slightly higher, it was not significantly different between the Lines [$F(1, 29) = 2.57$, n.s.]. There was no effect of replicate or interaction of Replicate with Line [both $F's(1,29) < 1.0$, n.s.]. For the hippocampus, there were no significant differences in BEC between the Lines [$F(1, 30) = 3.28$, $p = 0.08$] and no effect of Replicate or interaction of Line x Replicate [both $F's\ (1,30) < 3.0$, n.s.]. The Line effect was close to significance; WSP mice had slightly higher BECs in general.

For cerebellum samples, WSP mice had higher BECs than WSR. There was a significant Line effect [$F(1,34) = 10.7$, $p < 0.01$], but no effect of Replicate [$F(1,34) = 2.03$, n.s.] or interaction [$F(1,34) = 0$, n.s.], indicating that both WSP lines had greater BECs than WSR (see Table 2). The difference between WSP and WSR in BEC was 0.29 mg/ml and within a similar range as for whole brain and hippocampus and is unlikely to contribute to the effect of ethanol on gene expression in cerebellum.

In cortex, there was no effect of selected line on BEC [$F(1,26) = 2.01$, n.s.] but there was a main effect of Replicate [$F(1,26) = 16.02$, $p < 0.001$]. Replicate 2 lines (WSP-2 and

WSR-2 lines) had higher BECs than Replicate 1 lines. There was no interaction of Replicate with Line [$F(1,26) = 2.09$, n.s.].

3.2.1a Whole Brain

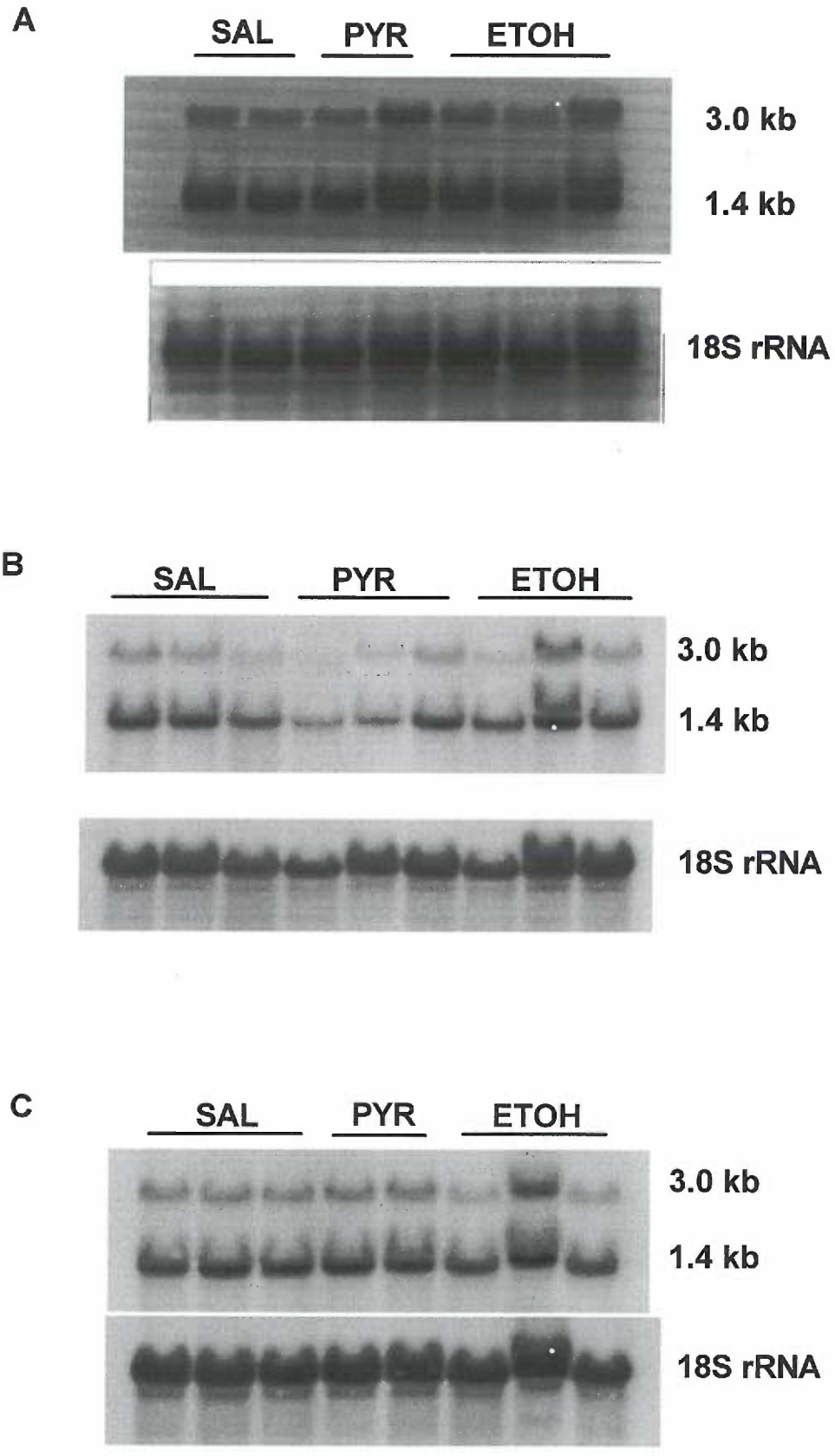
Six to seven mice from each genotype made up the saline groups, 4-6 mice per genotype were used for the pyrazole group and 4-9 mice per genotype were used for ethanol groups. Data for WSP-2 mice were already presented and analyzed; they are included in graphs for side-by-side comparison of ethanol effects on gene expression. The average BECs for each genotype were as follows: WSP-1: 1.76 ± 0.13 mg/ml (n = 9); WSP-2: 1.55 ± 0.13 mg/ml (n = 12); WSR-1; 1.44 ± 0.12 mg/ml (n = 6); WSR-2: 1.45 ± 0.05 mg/ml (n = 6).

Northern analysis was performed for WSP-1, WSR-1, and WSR-2 selected lines. The average BEC (mg/ml ± sem) at time of withdrawal from the chambers is presented in Table 2. Representative Northern blots are shown in Figure 11. These blots resemble the blots obtained from WSP-2 mice (see Figure 7). Results from Northern analyses are summarized in Tables 3 and 4, located at the end of the Results.

Effect of Pyrazole

Saline and pyrazole treated mice exposed to air only were initially compared for effects of pyrazole on gene expression. Pyrazole did not have significant effects on the expression of these transcripts in WSP-1 mice [1.4 kb transcript: $t = -0.83$, $df = 11$, n.s.; 3.0 kb transcript: $t = -0.50$, $df = 10$, n.s.], or WSR-1 mice [1.4 kb transcript: $t = 1.41$, $df = 9$,n.s. ; 3.0 kb transcript: $t = 0.83$, df = 8, n.s.]. For WSR-2 mice, there was no effect of pyrazole on the expression of the 1.4 kb transcript ($t = 1.54$, $df = 9$, n.s.); however, pyrazole alone had a slight effect on expression of the 3.0 kb transcript in WSR-2 mice which was marginally

Figure 11. Representative Northern blot of mNSP expression in whole brain of WSP-1, WSR-1 and WSR-2 mice. Scanned image of a northern blot autoradiograph from whole brain of mice exposed to ethanol-vapor or air for 72 hrs. Treatment groups are designated as SAL for saline-treated, PYR for pyrazole -treated, and ETOH for ethanol-treated. The cDNA probe detected two transcripts of 1.4 kb and 3.0 kb.


A
SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA
B
SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA
C
SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

significant (t=-2.28, df=9, p=0.05). The effect of pyrazole on the expression of the 3.0 kb transcript was to increase expression ~23% above the expression of this transcript in mice injected with saline only and exposed to air. However, because the expression changes observed in whole brain represent changes from various discrete brain regions and there is region-specific effects of ethanol on expression, this effect was ignored for the whole brain (i.e., saline and pyrazole combined) and explored when necessary in regional brain tissue.

1.4 kb transcript

Ethanol treatment did not alter the expression of the 1.4 kb transcript in WSP-1 (t=0.65, df=20, n.s.). This result is depicted in Figure 12A. Data from WSP-2 mice are shown in Figure 12C.

There was no effect of ethanol on the expression of this transcript in WSR-1 mice (t=0.55, df=15, n.s.) or WSR-2 mice (t=-0.85, df=15, n.s.). These results are shown in Figures 12B and D, respectively.

3.0 kb transcript

Following ethanol exposure, there was a 27% increase in expression of the 3.0 kb transcript in WSP-1 mice (Figure 13A). This observation was not supported by the statistical analysis (t=1.73, df=20, n.s.). Data from WSP-2 mice are shown in Figure 13C.

In WSR-1 mice, ethanol had no effect on the expression of the 3.0 kb transcript (t=-0.35, df=13, n.s.; Figure 13B). In WSR-2 mice, there was no effect of ethanol on expression when compared with the combined air group (saline- and pyrazole-treated mice) (t=-0.66, df=13, n.s.). However as can be seen from the graph (Figure 13D), there was a slight decrease from control in the ethanol-treated mice for the 3.0 kb transcript. Since there was an increase in expression by pyrazole alone detected (see analysis above,

Figure 12. Expression of the 1.4 kb transcript of mNSP in whole brain from WSP-1, WSR-1, WSP-2, and WSR-2 mice. Mice were exposed to ethanol vapor or air (control) for 72 hrs. **A.** Ethanol-exposed (n = 8) versus control (n = 13) samples for WSP-1 mice. **B.** Ethanol-exposed (n = 6) versus air-exposed control (n = 17) samples for WSR-1 mice. **C.** Ethanol-exposed versus air-exposed (control) samples for WSP-2 mice (included for comparison). **D.** Ethanol-exposed (n = 6) versus control (n = 11) samples for WSR-2 mice. Scanning densitometry was used to quantitate autoradiographs. Data are from 2-3 Northern blots per genotype. Each transcript was corrected for loading using the sample 18S rRNA integrated value. Samples on each blot were normalized to the blot average integrated value and are expressed as the percent of Control expression levels (mean ± sem). Saline- and pyrazole-groups were combined to form the Control group. See text for data analyses.

Whole Brain
1.4 kb Transcript

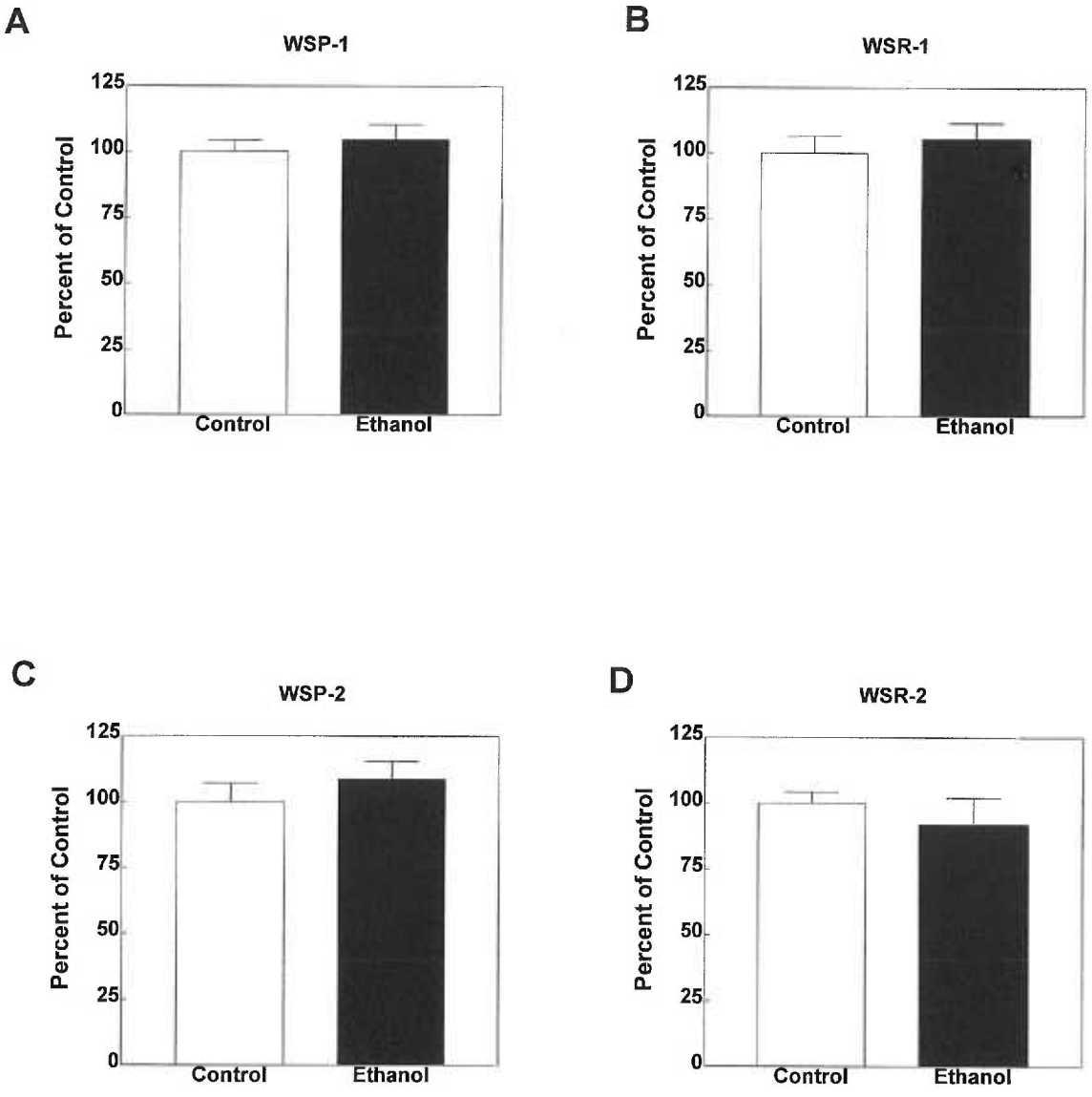
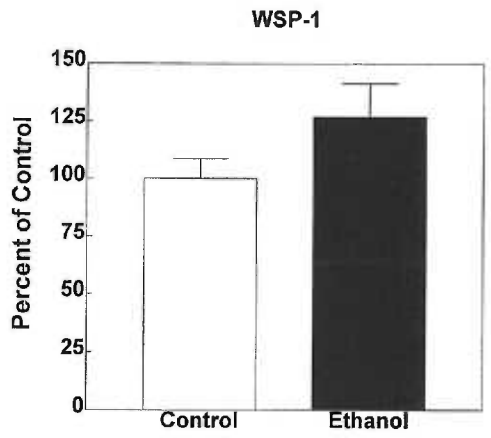


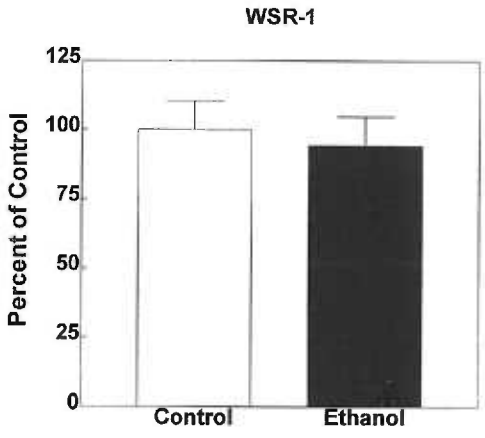
Figure 13. Expression of the 3.0 kb transcript of mNSP in whole brain from WSP-1, WSR-1, WSP-2, and WSR-2 mice. Mice were exposed to ethanol vapor or air (control) for 72 hrs. **A.** Ethanol-exposed (n = 8) versus control (n = 13) samples for WSP-1 mice. **B.** Ethanol-exposed (n = 6) versus control (n = 17) samples for WSR-1 mice. **C.** Ethanol-exposed versus control samples for WSP-2 mice. **D.** Ethanol-exposed (n = 6) versus control (n = 11) samples for WSR-2 mice. Scanning densitometry was used to quantitate autoradiographs. Data are from 2-3 Northern blots per genotype. Each transcript was corrected for loading using the sample 18S rRNA integrated value. Samples on each blot were normalized to the blot average integrated value and are expressed as the percent of Control expression levels (mean ± sem). Saline- and pyrazole-groups were combined to form the Control group. See text for data analyses.

Whole Brain
3.0 kb Transcript

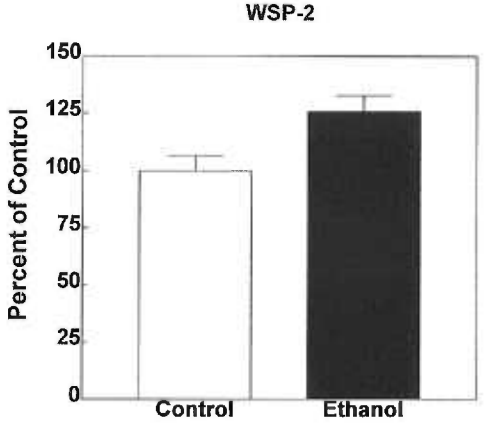
A



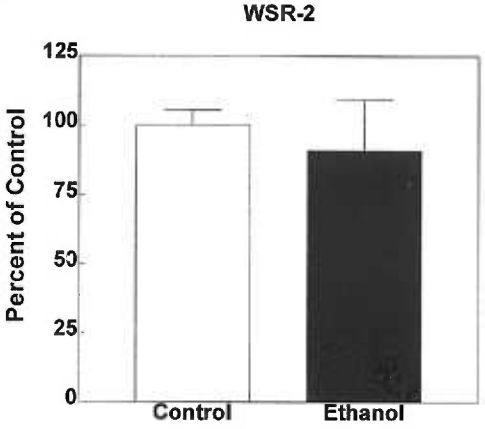
B



C



D



"**3.2.1a** ...Pyrazole effects..."), it is possible that this represents a fairly significant decrease in expression by ethanol if compared to the pyrazole group only. As discussed previously, the whole brain does not represent region-specific effects of ethanol on expression. The results from WSP-2 mice (discussed in Section 2.3) clearly illustrated the point that the direction of effect of ethanol in specific brain regions cannot be predicted by the results of the whole brain.

3.2.1b Discussion: Whole brain

In general, these results from the selected lines indicate that ethanol was not effective in altering the expression of the 1.4 kb transcript. Although there was a fair amount of variability in the data from the 3.0 kb transcript in WSP-1 mice, these results otherwise suggested that ethanol increased the expression of this transcript in both WSP lines. In WSR-2 mice ethanol may have decreased expression of the 3.0 kb transcript, if compared to the increase observed from pyrazole administration. However, the effects observed in WSR-2 mice were not seen in WSR-1 mice. Despite the fact that the data represent effects from the entire brain, it would suggest that there is a different effect of action of ethanol in these selected lines.

3.2.2a Results: Hippocampus

The average BEC at withdrawal from vapor chambers are shown in Table 2. WSP-2 mice had slightly higher BECs than the other lines mice however there were no significant differences found, as reported above.

The effect of pyrazole on gene expression was analyzed in each line separately. There was no effect of pyrazole on the expression of the 1.4 kb transcript [all t's = -0.16 to

1.18, *df*=8-15, n.s.] or the 3.0 kb transcript [all *t*'s =-0.09 to 1.2, *df*=8-16, n.s.]. Representative Northern blot are shown in Figure 14. In hippocampal tissue, the 3.0 kb transcript is clearly not as abundant as the 1.4 kb transcript, as observed in WSP-2 mice.

1.4 kb transcript

Exposure to ethanol produced a small increase of 10% of this transcript in WSP-1 mice (Figure 15A). This increase did not reach statistical significance [*t*=0.74, *df*=16, n.s.]. Data from WSP-2 mice are shown in Figure 15C.

There was a modest increase in expression of this transcript in WSR-1 mice, 16% above control (Figure 15B). This increase did not quite reach statistical significance [t= 1.88, df=26, *p*=.07], however it is suggestive of an ethanol effect. A similar increase (17% above control) was observed in WSR-2 mice (Figure 15D), but did not reach statistical significance [*t*=1.08, *df*=18, n.s.].

3.0 kb transcript

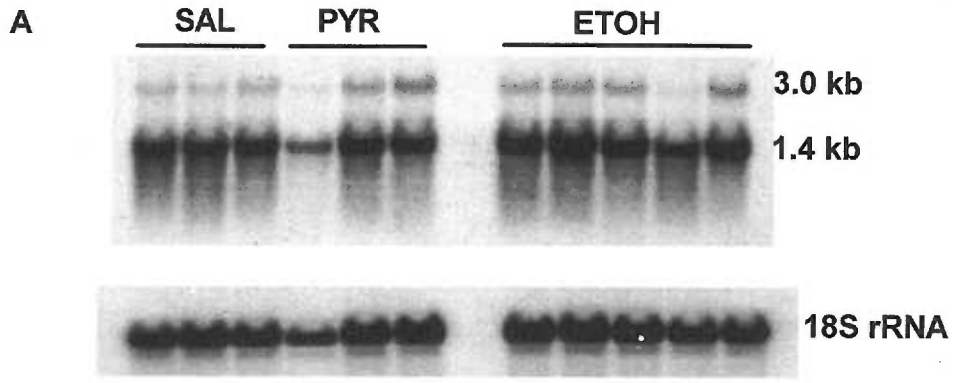
In WSP-1 mice, there was no observable effect by ethanol on expression of this transcript [*t*=0.24, *df*=16, n.s; (Figure 16A)]. Data for WSP-2 mice are shown in Figure 16C.

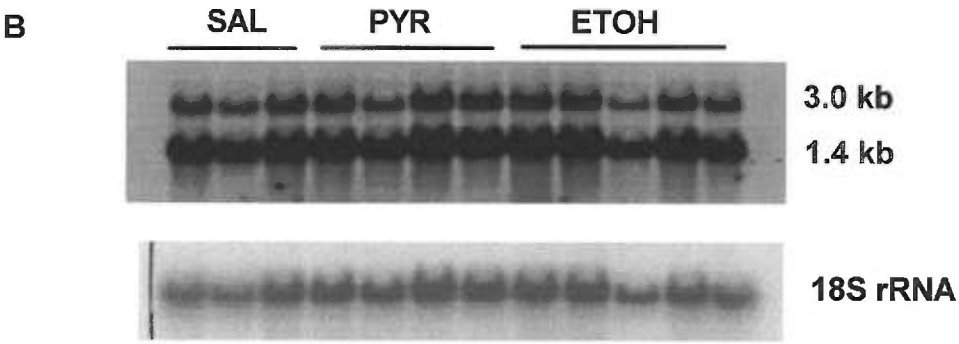
Very little ethanol-regulation was apparent in WSR mice (Figure 16B and D). No significant effects of ethanol-treatment were detected for WSR-1 mice [*t*= 0.34, *df*=27, n.s.] or for WSR-2 mice [*t*= 0.03, *df*=18, n.s.].

3.2.2b Discussion: Hippocampus

There were no significant differences in the relative percent change from control levels between WSP-1, WSR-1, or WSR-2 for either transcript. However, there were

Figure 14. Northern blot analysis of mNSP expression in hippocampal tissue of WSP-1, WSR-1, and WSR-2 mice. **A.** WSP-1 **B.** WSR-1 **C.** WSR-2. Scanned image of representative Northern blots of RNA from WSP-1 mice exposed to ethanol-vapor or air for 72 hrs. Treatment groups are designated as SAL for saline-treated, PYR for pyrazole -treated, and ETOH for ethanol-treated. The cDNA probe detected two transcripts of ~1.4 kb and 3.0 kb.


A
SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA


B
SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

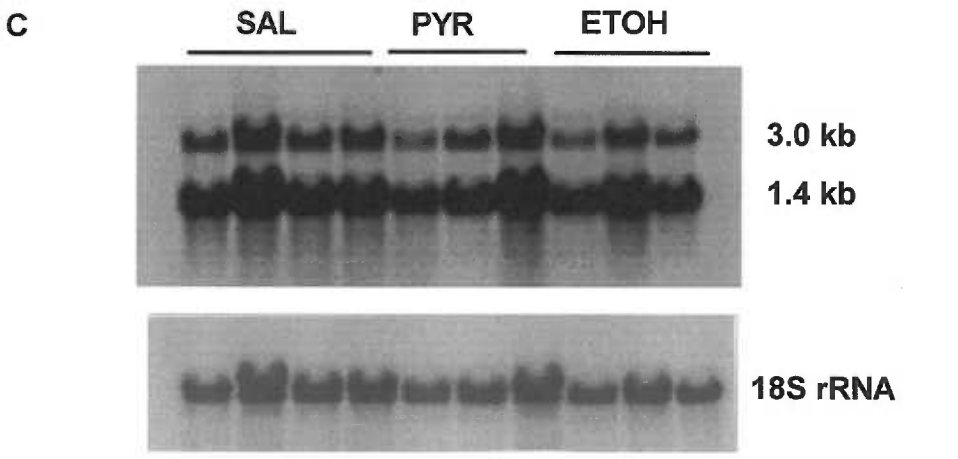

C
SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

Figure 15. Expression of the 1.4 kb transcript of mNSP in hippocampal tissue from WSP-1, WSR-1, WSP-2, and WSR-2 mice. Mice were exposed to ethanol vapor or air (control) for 72 hrs. **A**. Ethanol-exposed (n = 11) versus air-exposed (control; n = 7) samples in WSP-1 mice. **B**. Ethanol-exposed (n = 11) versus control (n = 17) samples from WSR-1 mice. **C**. Ethanol-exposed versus air-exposed (control) samples from WSP-2 mice. **D**. Ethanol-exposed (n = 7) versus control (n = 13) samples from WSR-2 mice. Scanning densitometry was used to quantitate autoradiographs. Data are from 2-3 Northern blots per genotype. Each transcript was corrected for loading using the sample 18S rRNA integrated value. Samples on each blot were normalized to the blot average integrated value and are expressed as the percent of Control expression levels (mean ± sem). Saline- and pyrazole-groups were combined to form the Control group. See text for data analyses.

Hippocampus
1.4 kb Transcript

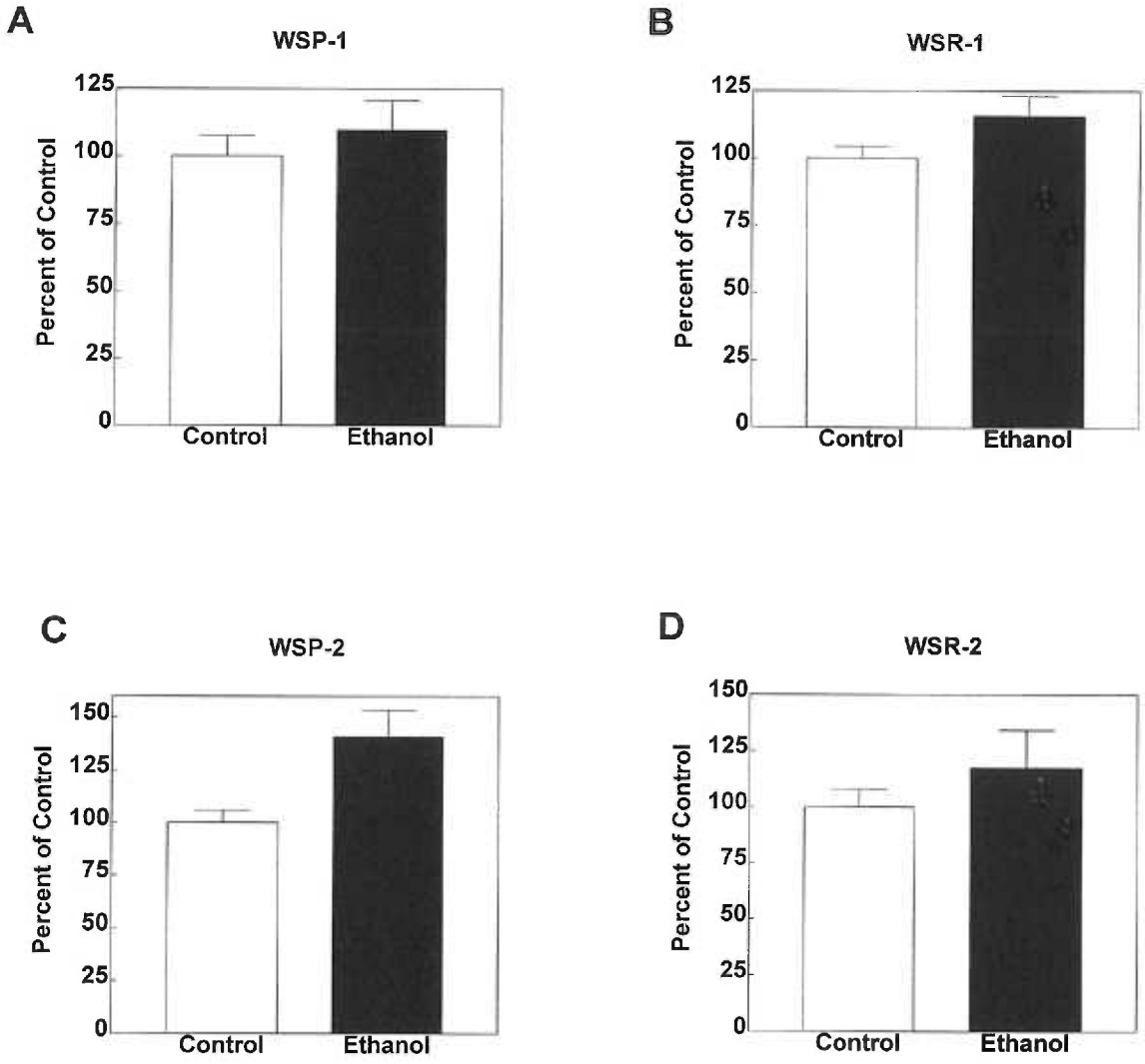
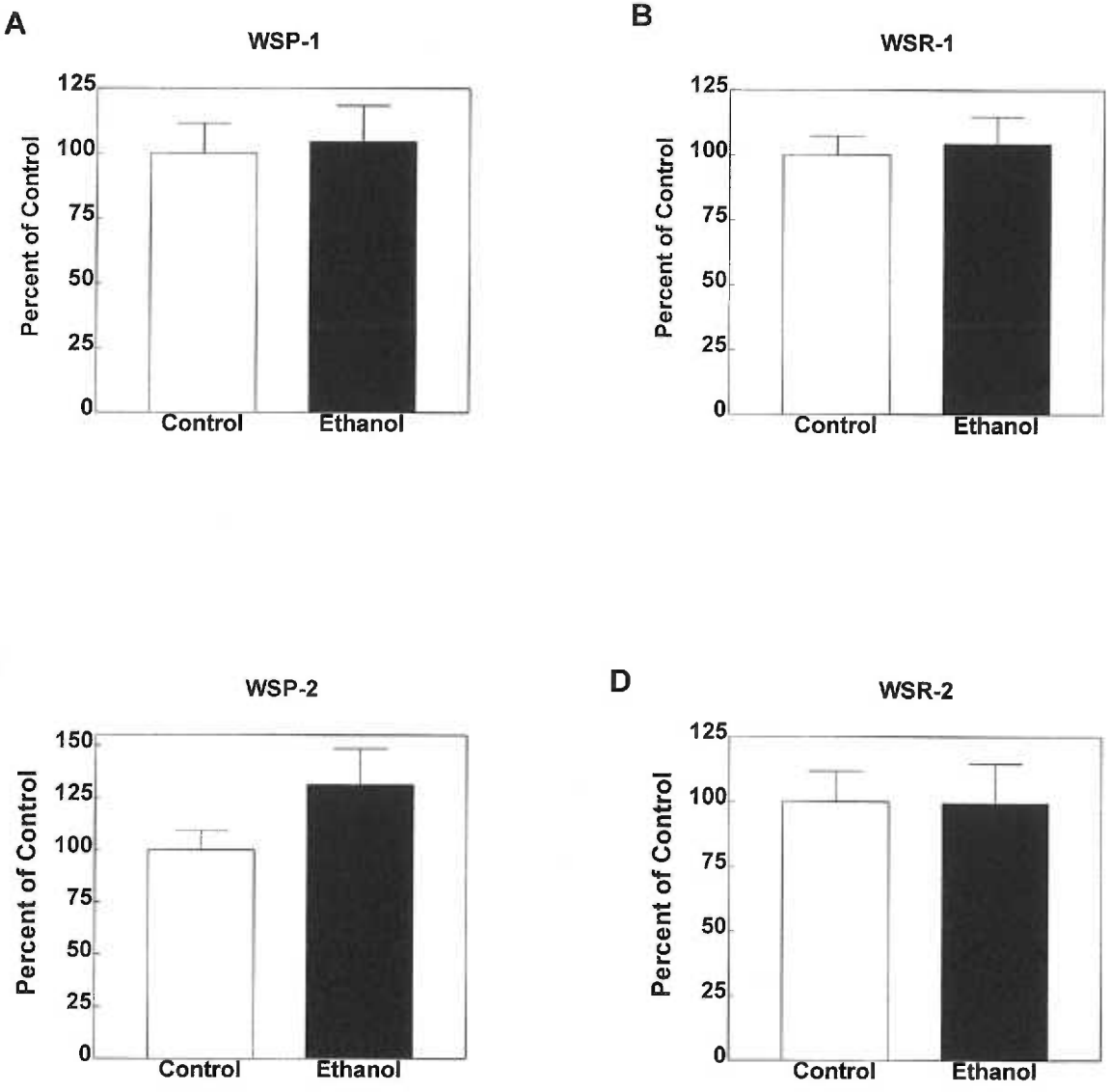


Figure 16. Expression of the 3.0 kb transcript of mNSP in hippocampal tissue from WSP-1, WSR-1, WSP-2, and WSR-2 mice. Mice were exposed to ethanol vapor or air (control) for 72 hrs. **A.** Ethanol-exposed (n = 11) versus air-exposed (control; n = 7) samples in WSP-1 mice. **B.** Ethanol-exposed (n = 11) versus control (n = 17) samples from WSR-1 mice. **C.** Ethanol-exposed versus air-exposed (control) samples from WSP-2 mice. **D.** Ethanol-exposed (n = 7) versus control (n = 13) samples from WSR-2 mice. Scanning densitometry was used to quantitate autoradiographs. Data are from 2-3 Northern blots per genotype. Each transcript was corrected for loading using the sample 18S rRNA integrated value. Samples on each blot were normalized to the blot average integrated value and are expressed as the percent of Control expression levels (mean ± sem). Saline- and pyrazole-groups were combined to form the Control group. See text for data analyses.

Hippocampus
3.0 kb Transcript



modest effects on expression of the 1.4 kb transcript in all selected lines, which were much greater for WSP-2 mice than any of the other lines. Essentially no effect of ethanol exposure on expression levels of the 3.0 kb was found for WSP-1 or either WSR line. The results for expression of these transcripts in WSP-1 hippocampus were different from those observed for WSP-2 mice. In WSP-2 mice, modest increases in the expression of both transcripts were observed. However in the replicate line, WSP-1 mice, there were no effects of ethanol on the expression of either transcript. The relative difference in regulation in expression of the 1.4 kb transcript between WSP-1 and WSP-2 mice suggest that increased in expression may not be necessary for enhanced withdrawal severity in WSP-1 mice but may contribute to the phenotype of WSP-2 mice.

Overall, these results suggest that although there was a modest effect of ethanol on expression of these transcripts in the hippocampus, the effect probably does not contribute significantly to phenotypic differences between WSP and WSP lines.

3.2.3a Cerebellum

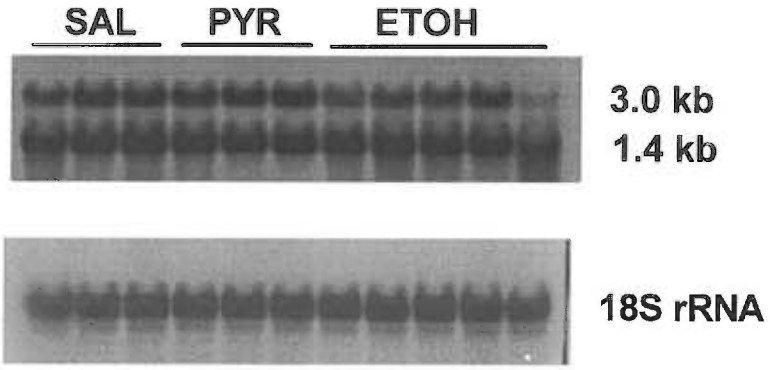
Representative Northern blots are shown in Figure 17. Qualitatively, there appeared to be greater expression of the 3.0 kb transcript than in hippocampus. Due to technical problems with either RNA integrity or yield, not all mice used for the hippocampus analyses could be used for cerebellum analyses. Thus, the average BEC values are slightly different for hippocampus versus cerebellum. The average BEC at 72 hrs. are shown in Table 2.

Effect of Pyrazole

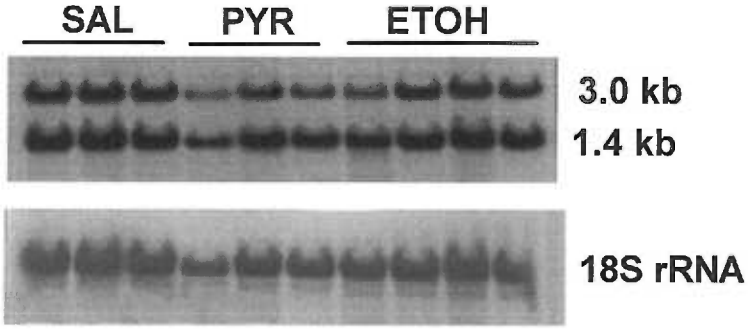
Saline and pyrazole-treated mice exposed to air were compared in independent *t*-tests for each replicate line (WSP-1, WSR-1, WSR-2). There were no significant differences

Figure 17. Northern blot analysis of mNSP in cerebellar tissue of WSP-1, WSR-1, and WSR-2 mice. **A.** WSP-1 **B.** WSR-1 **C.** WSR-2. Scanned image of representative Northern blots of RNA from mice exposed to ethanol-vapor or air for 72 hrs. Treatment groups are designated as SAL for saline-treated, PYR for pyrazole-treated, and ETOH for ethanol-treated. The cDNA probe detected two transcripts of 1.4 kb and 3.0 kb.

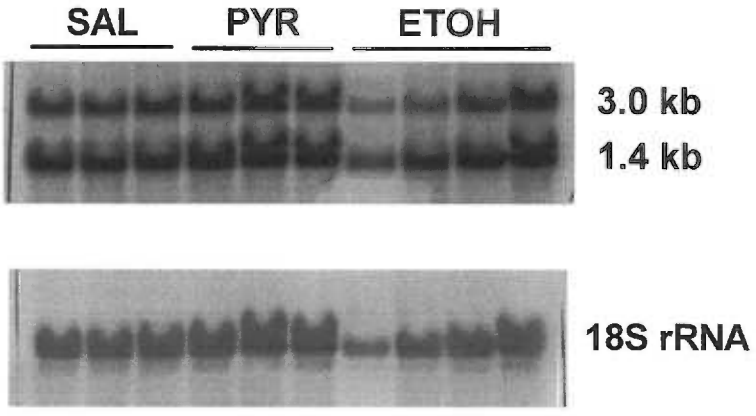
A


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

B


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

C


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

for the 3.0 kb transcript between saline and pyrazole-treated WSP-1 mice ($t=0.89$, $df=10$, n.s.). There was a marginal effect of pyrazole on the expression of the 3.0 kb transcript in WSR mice ($t=1.87$, $df=14$, n.s.). In WSR-2 mice, there was no effect at all ($t=0.122$, $df=16$, n.s.).

For the 1.4 kb transcript, there was a highly significant effect of pyrazole in WSP-1 mice ($t=3.38$, $df=10$, $p<0.01$), with pyrazole leading to a 16% decrease in expression. These data were therefore analyzed by independent one-way ANOVA for treatment group. In WSR-1 mice, there was no effect of pyrazole on relative abundance of the 1.4 kb transcript ($t=-1.07$, $df=15$, n.s.). There was no significant effect of pyrazole on the expression of this transcript in WSR-2 mice ($t=1.28$, $df=16$, n.s.).

1.4 kb transcript

The data for the 1.4 kb transcript analysis for WSP-1 mice are shown in Figure 18A. Although the ethanol-treated group showed a decrease in expression compared to the saline group, it did not appear to be different from the pyrazole-treated mice. A one-way ANOVA indicated that there was a significant effect of treatment [$F(2,16)=6.42$, $p<0.01$]. Post-hoc analysis by Tukey's test indicated that pyrazole treatment produced a significant decrease in expression compared to saline-treated, air-exposed mice, but not to ethanol-treated mice. The decrease observed in the ethanol group, therefore, can be attributed to pyrazole's effect. Data from WSP-2 mice are shown in Figure 18C.

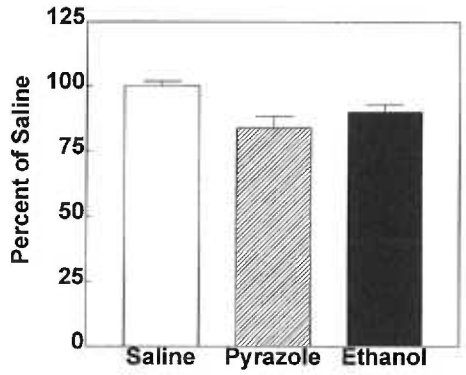
There was no effect of pyrazole on gene expression in WSR-1 or WSR-2 mice cerebellum, therefore these data were analyzed by a *t*-test for control (air) versus ethanol treatment. In WSR-1 mice, there was no observable difference in expression of the 1.4 kb transcript following ethanol treatment (Figure 18B) ($t=0.91$, $df=27$, n.s.).

It is clear from Figure 18D, that ethanol did not have any effect on the expression of the 1.4 kb transcript in WSR-2 mice ($t=0.38$, $df=27$, n.s).

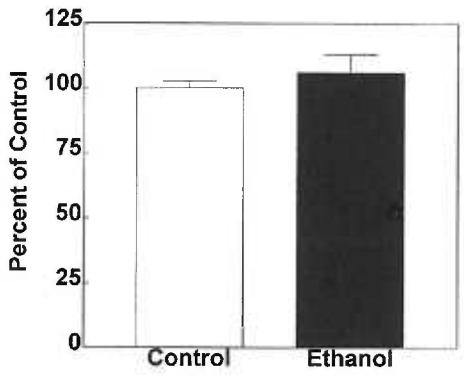
Figure 18. Expression of the 1.4 kb transcript of mNSP in cerebellum from WSP-1, WSR-1, WSP-2, and WSR-2 mice. Mice were exposed to ethanol vapor or air (control) for 72 hrs. Data are presented as percent (mean ± sem) of Saline group levels (WSP-1) or Control group levels (WSP-2, WSR-1, WSR-2). **A.** Percent of saline (n = 6) levels for pyrazole-treated (n = 6) and ethanol-treated (n = 7) WSP-1 mice. **B.** Ethanol-exposed (n = 12) versus air-exposed (control; n = 17) samples for WSR-1 mice. **C.** Ethanol-exposed versus control samples for WSP-2 mice. **D.** Ethanol-exposed (n = 11) versus control (n = 18) samples for WSR-2 mice. Data are from 2-3 Northern blots per genotype. Scanning densitometry was used to quantitate autoradiograms. Each transcript was corrected for loading using the sample 18S rRNA integrated value. Samples on each blot were normalized to the blot average. Saline- and pyrazole-groups were combined to form the Control group (WSP-2, WSR-1, and WSR-2). See text for data analyses.

Cerebellum
1.4 kb Transcript

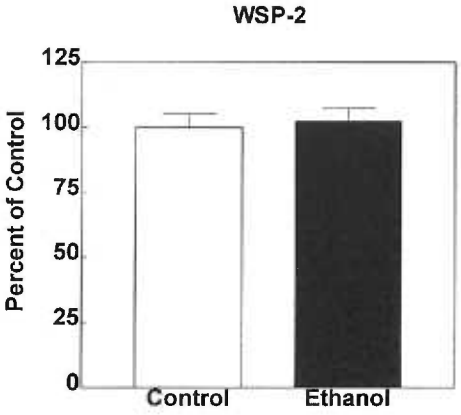
A



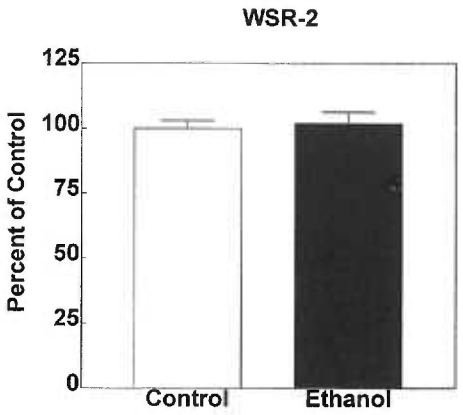
B



C



D



3.0 kb transcript

In WSP-1 mice there appeared to be a decrease in the expression of the 3.0 kb transcript following pyrazole and ethanol exposure (Figure 19A). However, no statistically significant effect was detected (t=-1.57, df=17, n.s.). Data from WSP-2 mice are shown in Figure 19C.

There was no effect of ethanol on the expression of the 3.0 kb transcript in WSR-1 mice (Figure 19B) or WSR-2 mice (Figure 19D). The lack of ethanol effect in WSR-1 mice was indicated by the t-test (t=0.20, df=25, n.s.). The statistical analysis did not yield any significant results in WSR-2 mice (t=0.79, df=27, n.s.; Figure 19D).

The observed decrease in expression of the 3.0 kb transcript by ethanol in WSP mice appears to be specific to the selected phenotype, although the results were not statistically significant in WSP-1 mice. Thus, ethanol appears to decrease the expression of this transcript preferentially in mice susceptible to withdrawal seizures.

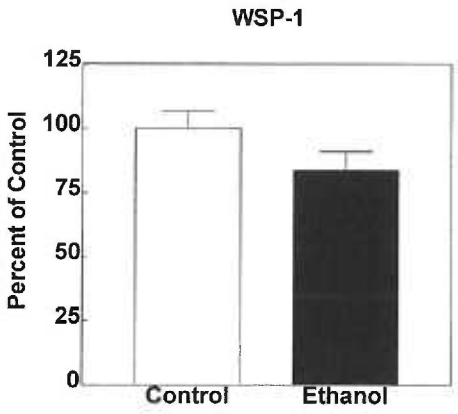
3.2.3b Discussion: Cerebellum

The expression of these transcripts in cerebellum of WSP and WSR mice was markedly different, depending both on the genotype of the mice and on the transcript. It appeared that there was a selective effect of ethanol on the expression of the 3.0 kb transcript in WSP-1 and WSP-2 mice, which led to a large decrease in expression following ethanol treatment. This may be additive with the decrease in expression by pyrazole observed in these mice. This decrease in expression by ethanol was not observed for the 1.4 kb transcript in WSP mice.

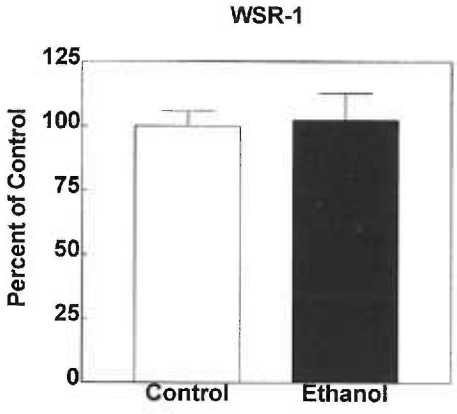
Figure 19. Expression of the 3.0 kb transcript of mNSP in cerebellum from WSP-1, WSR-1, WSP-2, and WSR-2 mice. Mice were exposed to ethanol vapor or air (control) for 72 hrs. Data are presented as percent (mean ± sem) of Control group levels. **A.** Ethanol-treated (n = 7) versus control (n = 12) WSP-1 mice. **B.** Ethanol-exposed (n = 12) versus air-exposed (control; n = 17) samples for WSR-1 mice. **C.** Ethanol-exposed versus control samples for WSP-2 mice. **D.** Ethanol-exposed (n = 11) versus control (n = 18) samples for WSR-2 mice. Data are from 2-3 Northern blots per genotype. Scanning densitometry was used to quantitate autoradiograms. Each transcript was corrected for loading using the sample 18S rRNA integrated value. Samples on each blot were normalized to the blot average. Saline- and pyrazole-groups were combined to form the Control group (WSR-1 and WSR-2). See text for data analyses.

Cerebellum
3.0 kb Transcript

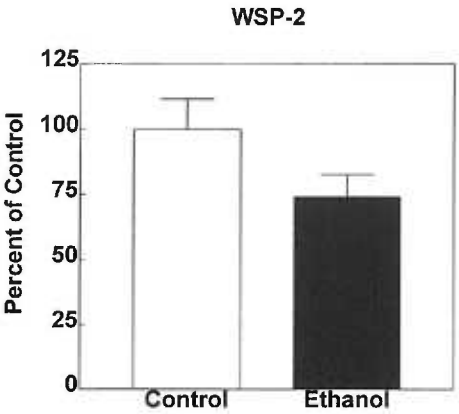
A



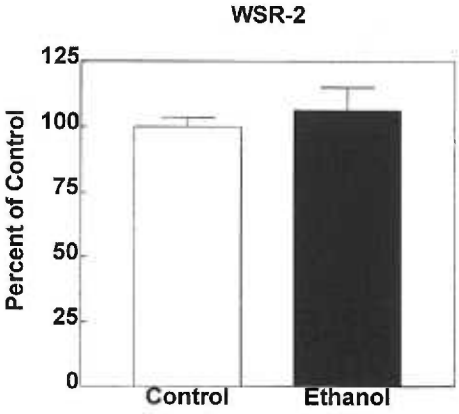
B



C



D



In WSR mice there were no substantial effects of ethanol on the expression of either transcript. There was a suggestion of a decrease in expression by pyrazole for the 3.0 kb transcript that was reversed by ethanol. However this effect was not observed for the 1.4 kb and was in opposition to the effects observed in WSP mice for the 3.0 kb transcript.

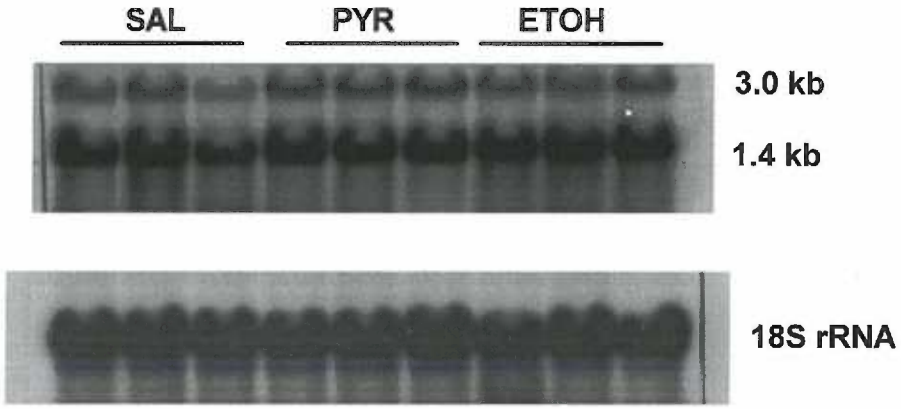
3.2.4a Cortex

Representative Northern blots are shown in Figure 20. The relative abundance of the two transcripts with respect to one another was similar to that observed in the hippocampus, with the 3.0 kb transcript being expressed at much lower levels than the 1.4 kb transcript. As with cerebellar tissue, technical difficulties made it impossible to use exactly the same mice for all three tissue analyses.

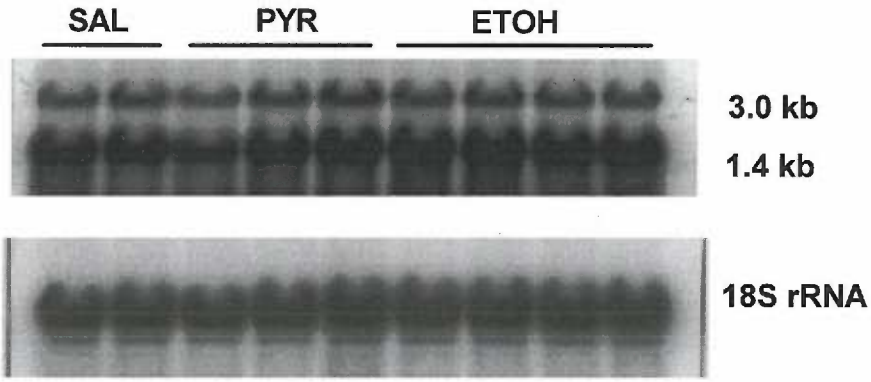
There was no effect of pyrazole treatment in WSP-1 on the expression of the 1.4 kb transcript ($t=0.2$, $df=9$, n.s.) or the 3.0 kb transcript mice ($t=-0.35$, $df=9$, n.s.). For WSR-1 mice, there was a marginal effect of pyrazole compared to saline on the expression on the 1.4 kb transcript ($t=2.35$, $df=7$, $p=0.05$); the effect of pyrazole was in the direction of suppressing expression. No effect on the expression of the 3.0 kb transcript was detected in WSR-1 mice ($t=-0.69$, $df=7$, n.s.). No effects of pyrazole were detected in WSR-2 mice for either the 1.4 kb transcript ($t=1.60$, $df=11$, n.s.) or the 3.0 kb transcript ($t=-0.14$, $df=11$, n.s.). Since there was only a single incidence of pyrazole having a (marginal) effect on expression of these transcripts, it was decided to combine the saline and pyrazole groups into a single control group. The data were then analyzed by Students *t*-test for each replicate line.

Figure 20. Representative Northern blot from cortex. WSP-1 (**A**), WSR-1 (**B**), and WSR-2 (**C**) mice were treated with saline (SAL), pyrazole (PYR), or ethanol (ETOH) for 72 hours. RNA from cortical tissue was used for northern blots; mNSP was used as the probe. Representative autoradiographs from Northern blot analysis were scanned.

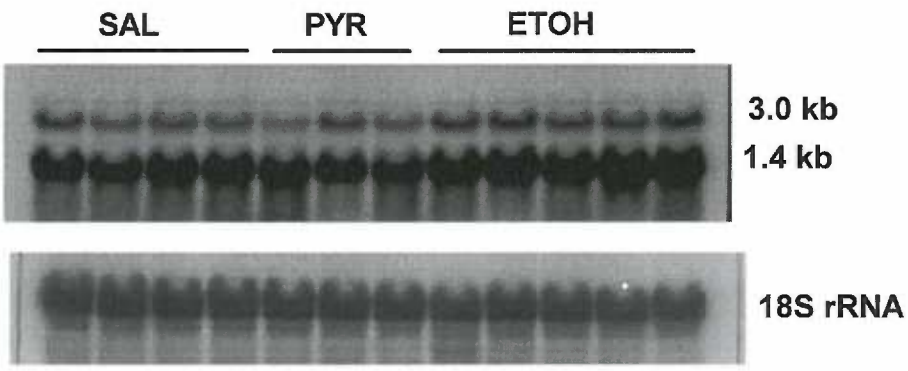
A


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

B


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

C


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

1.4 kb transcript

In WSP-1 mice, a modest increase in expression of the 1.4 kb transcript was observed (Figure 21A). This result was supported by the Students *t*-test ($t = 2.49$, $df = 15$, $p < .05$). The magnitude of the increase in expression by ethanol was 13% above control (air-exposed) mice. Data from WSP-2 mice are shown in Figure 21C.

In WSR-1 mice, there was no effect of ethanol on the expression of the 1.4 kb transcript when compared to mice exposed to air only ($t = 0.49$, $df = 14$, n.s.). This result can be seen in Figure 21B.

There was a slight effect of ethanol to increase the expression of the 1.4 kb transcript in WSR-2 mice (Figure 21D). This small effect of ethanol was not statistically significant ($t = 1.77$, $df = 20$, n.s.).

3.0 kb transcript

The expression of the 3.0 kb transcript was unaltered by ethanol in WSP-1 mice (Figure 22A). A Students *t*-test, comparing relative abundance of control levels versus ethanol-treatment detected no significant differences ($t = -0.09$, $df = 15$, n.s.). Data from WSP-2 mice are shown in Figure 22C.

Results for WSR-1 mice are presented in Figure 22B. There was no significant difference in expression of this transcript by ethanol for WSR-1 mice ($t = 0.99$, $df = 14$, n.s.). In WSR-2 mice no effect of ethanol expression of the 3.0 kb transcript was observed ($t = 1.08$, $df = 20$, n.s.; Figure 22D).

Overall, ethanol did not have a significant effect on the expression of the 3.0 kb transcript in the cortex of any of these selected lines.

Figure 21. Expression of the 1.4 kb transcript of mNSP in cortex from WSP-1, WSR-1, and WSR-2 mice. Mice were exposed to ethanol vapor or air (control) for 72 hrs. Data are presented as percent (mean ± sem) of Control (air) levels. **A.** Ethanol-treated (n = 6) versus control (n = 11) WSP-1 mice. **B.** Ethanol-treated (n = 7) versus control (n = 9) WSR-1 mice. **C.** WSP-2 mice. **D.** Ethanol-treated (n = 9) versus control (n = 13) WSR-2 mice. Data are from 2-3 Northern blots per genotype. Scanning densitometry was used to quantitate autoradiograms. Each transcript was corrected for loading using the sample18S rRNA integrated value. Samples on each blot were normalized to the blot average integrated value. Saline- and pyrazole-groups were combined to form the Control group. See text for data analyses.

CORTEX
1.4 kb Transcript

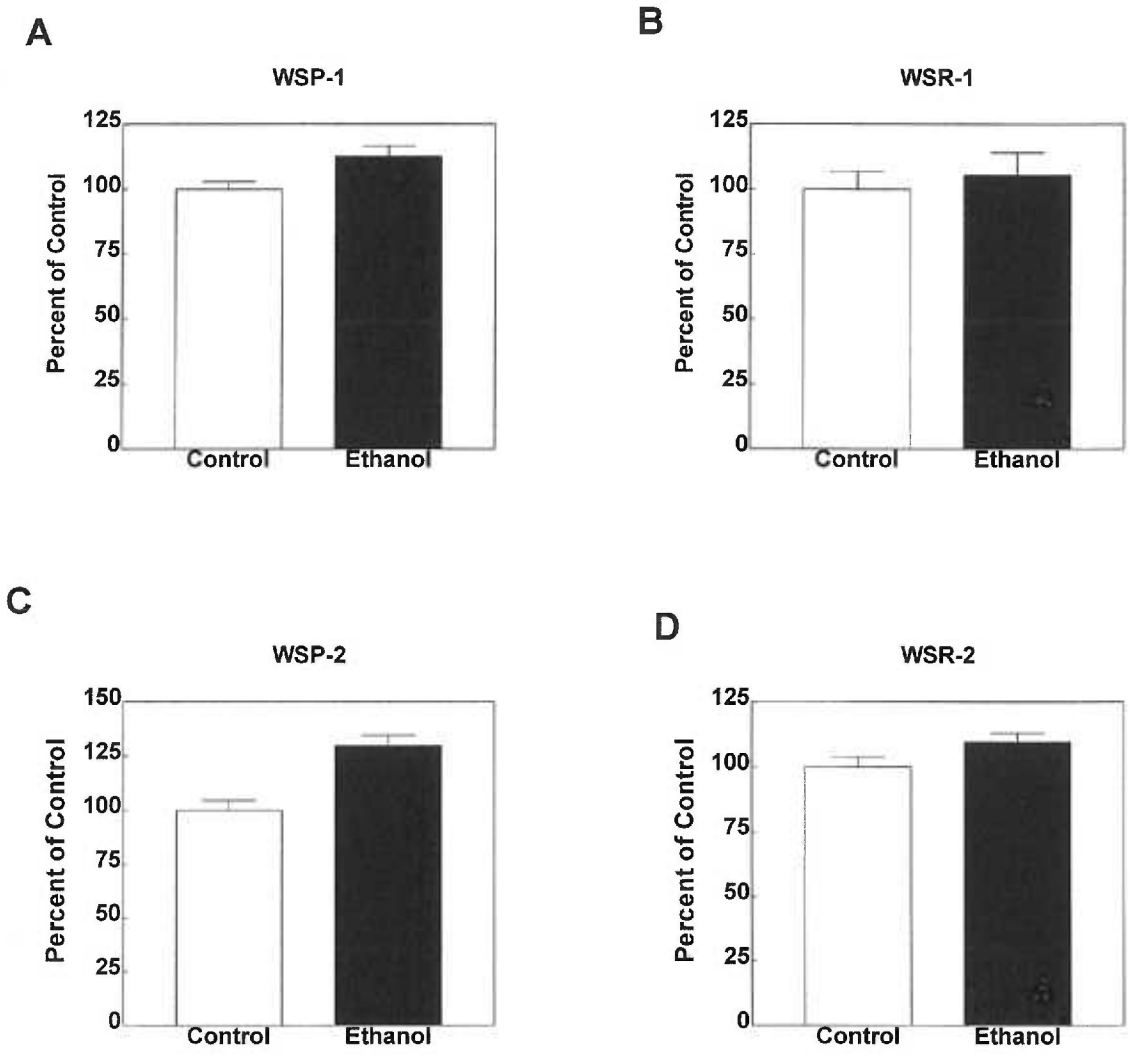
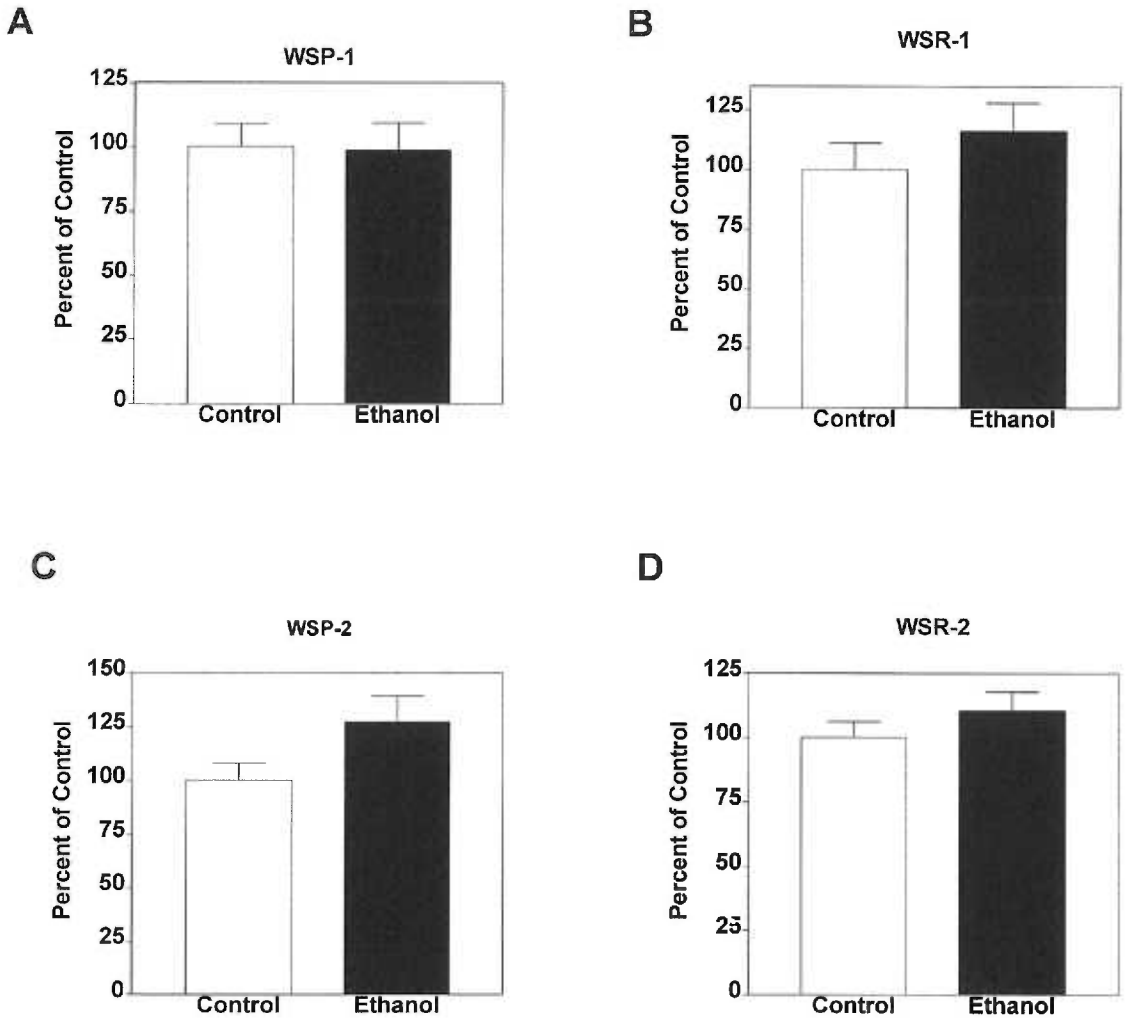


Figure 22. Expression of the 3.0 kb transcript of mNSP in cortex from WSP-1, WSR-1, WSP-2, and WSR-2 mice. Mice were exposed to ethanol vapor or air (control) for 72 hrs. Data are expressed as percent (mean ± sem) of Control expression levels. **A.** Ethanol-treated (n = 6) versus control (n = 11) WSP-1 mice. **B.** Ethanol-treated (n = 7) versus control (n = 9) WSR-1 mice. **C.** WSP-2 mice. **D.** Ethanol-treated (n = 9) versus control (n = 13) WSR-2 mice. Data are from 2-3 Northern blots per genotype. Scanning densitometry was used to quantitate autoradiograms. Each transcript was corrected for loading using the sample 18S rRNA integrated value. Samples on each blot were normalized to the blot average integrated value. Saline- and pyrazole-groups were combined to form the Control group (WSR-1 and WSR-2). See text for data analyses.

CORTEX
3.0 kb Transcript



3.2.4b Discussion: Cortex

There was a small, yet significant, effect of ethanol on the expression of the 1.4 kb transcript in WSP-1 mice, generally supporting the effect of ethanol observed in WSP-2 mice. Ethanol exposure did not effect the expression of either transcript in WSR-1 or WSR-2 mice. These results suggests a positive correlation between an increase in expression of the 1.4 kb transcript and withdrawal severity. However, the effect in WSP-1 mice was smaller than that observed in WSP-2 mice and there was a suggestion that there could be an effect of ethanol on expression of this transcript in WSR-1 mice.

3.3 Summary

The results for the expression of the 3.0 kb transcript and 1.4 kb transcript in WSP and WSR mice are summarized in Tables 3 and 4, respectively. For each brain region analyzed, the percent change from control treatment is presented. The modest increase in expression by ethanol observed from the differential display gel was confirmed on whole brain RNA-containing Northern blots from WSP mice. Northern blot analysis revealed that the gene fragment identified from differential display consisted of two transcripts. Sequence analysis strongly suggest this product is the mouse homologue to human neuroendocrine specific protein. Since expression of this gene is fairly widespread in the brain (van de Velde et al., 1994), the results from whole brain analysis did not reflect specific regional effects of ethanol on expression of these gene products. In order to more adequately assess this, the effect of ethanol on expression in hippocampus, cerebellum, and cortex was examined.

In general, ethanol increased expression of mNSP in hippocampus and cortex, although there was both transcript and genotype specificity. A surprising effect of ethanol

Table 3. Summary of results for the 1.4 kb transcript in WSP and WSR selected lines and C57BL/6J and DBA/2J inbred strains of mice. The effect of ethanol versus air-exposed mice for each transcript and statistical significance are presented (↑= increase; ↓= decrease).

Brain region	Ethanol effect (percent control)	Statistical Significance
Hippocampus		
WSP-1	↑11%	n.s.
WSP-2	↑ 41%	$p<0.01$
WSR-1	↑ 16%	n.s.
WSR-2	↑ 18%	n.s.
C57BL/6J	↑ 28%	$p< 0.001$
DBA/2J	↑ 17%	$p< 0.02$
Cerebellum		
WSP-1	↑ 7% vs. pyrazole	n.s.
WSP-2	↓ 2%	n.s.
WSR-1	↑ 6%	n.s.
WSR-2	0%	n.s.
C57BL/6J	↑ 14%	n.s.
DBA/2J	↓ 10%	$p<0.05$
Cortex		
WSP-1	↑ 13%	$p<0.05$
WSP-2	↑ 30%	$p <0.01$
WSR-1	↑ 5%	n.s.
WSR-2	↑10%	n.s.
C57BL/6J	↑10%	n.s.
DBA/2J	↑15%	$p<0.05$

Table 4. Summary of results from Northern analysis of the 3.0 kb transcript in WSP and WSR selected lines and C57BL/6J and DBA/2J mice. The effect of ethanol versus air-exposed mice (percent change form control) for each transcript and statistical significance are presented (↑= increase; ↓= decrease).

Brain region	Ethanol effect (percent control)	Statistical Significance
Hippocampus		
WSP-1	↑ 4%	n.s.
WSP-2	↑ 31%	n.s.
WSR-1	↑4%	n.s.
WSR-2	0 %	n.s.
C57BL/6J	↑ 12%,	n.s.
DBA/2J	↑ 24%	n.s.
Cerebellum		
WSP-1	↓ 16%	n.s.
WSP-2	↓ 25%	n.s.
WSR-1	0 %	n.s.
WSR-2	↑ 6%	n.s.
C57BL/6J	↑ 27%	$p=0.06$
DBA/2J	↓ 23%	$p<0.01$
Cortex		
WSP-1	0 %	n.s.
WSP-2	↑27%	n.s.
WSR-1	↑16%	n.s.
WSR-2	↑10%	n.s.
C57BL/6J	0%	n.s.
DBA/2J	0%	n.s.

was to decrease expression of the 3.0 kb transcript in cerebellum of the mice that selected for susceptibility to ethanol withdrawal convulsions. In general, there was a complex interaction between the effect of ethanol on the expression of these transcripts, brain region, and the genotype of the mice.

3.4. Expression of mNSP in saline-treated groups.

In aorder to assess if there were differences in expression of the 1.4 kb or 3.0 kb transcript of mNSP under non-ethanol conditions, the relative steady-state levels of expression in saline-treated mice was analyzed. Ten micrograms of total RNA was analyzed using Northern blots as previously described. RNA from hippocampus, cerebellum, and cortex was analyzed on a single gel. Two sets of 2-3 individual mouse samples from each selected line were pooled together; therefore, two lanes were loaded on the gel for each genotype. A scanned image of the autoradiograph of the Northern blot is shown in Figure 22 A-C, separated into each brain region. A semi-quantitative analysis was performed; relative abundance of each transcript was determined and reported at percent of WSP-2 mice (Figure 23 A-F). In general there weren't any differences in relative abundance, although there was a fair amount of variability between the genotypes. In cortex, only one WSR-1 sample was loaded, due to space constraints on the gel, and one WSP-2 sample was degraded.

Figure 23. Baseline expression of mNSP. Representative Northern blot of hippocampus (**A**), cerebellum (**B**), and cortex (**C**) from saline-treated WSP-1, WSP-2, WSR-1, and WSR-2 mice. Lanes are labeled for each genotype. Ten micrograms of total RNA was loaded in each lane. Autoradiographs were scanned.

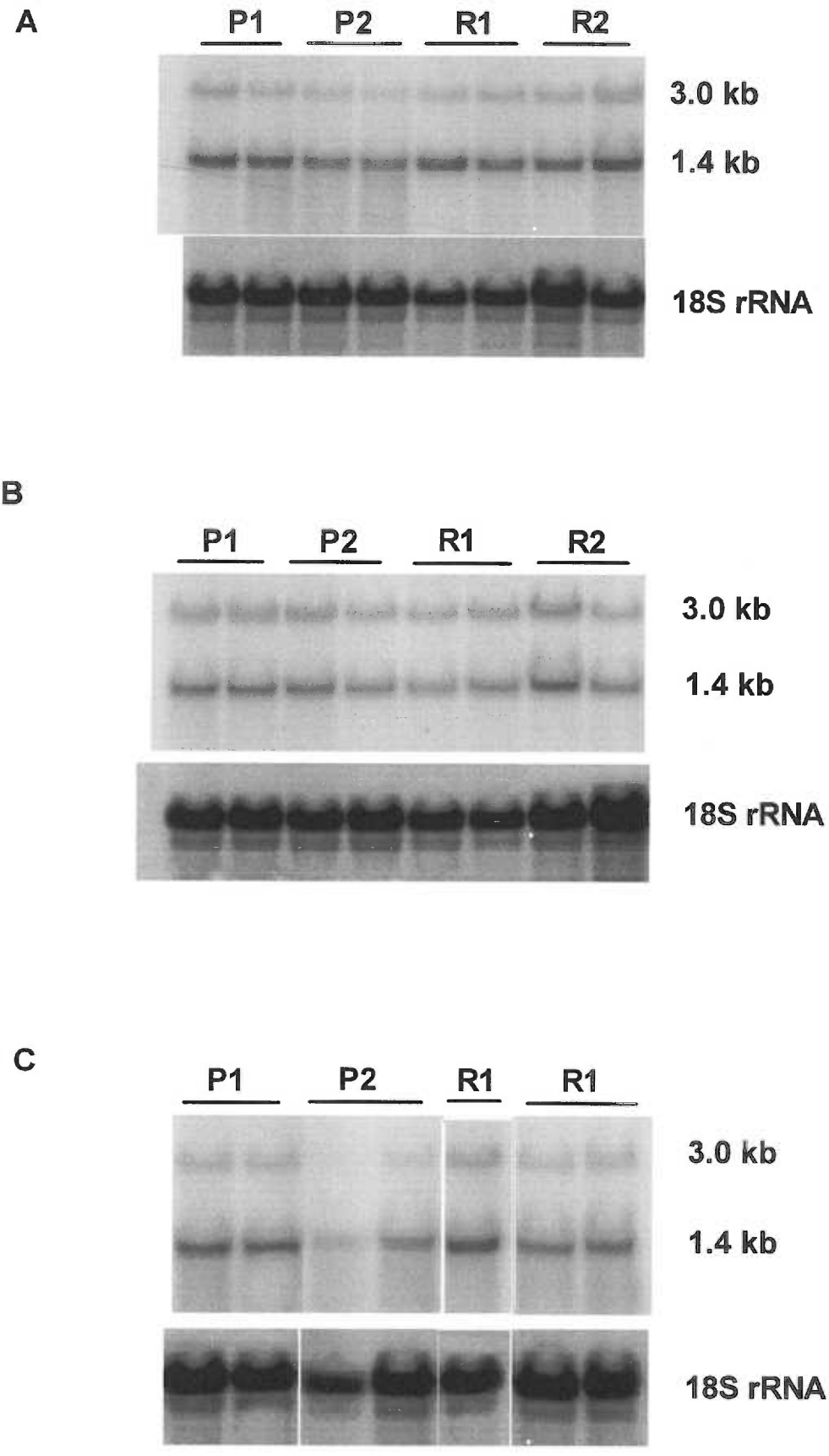
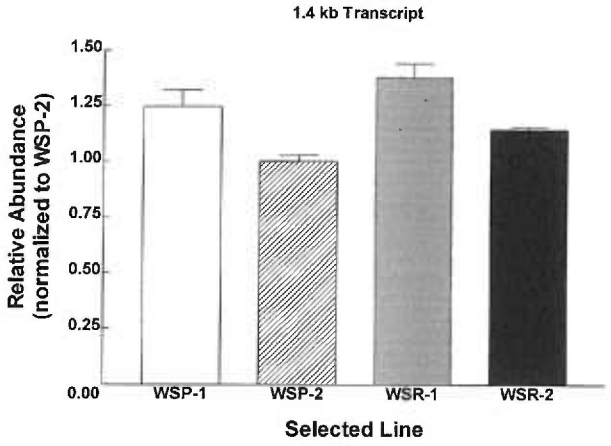
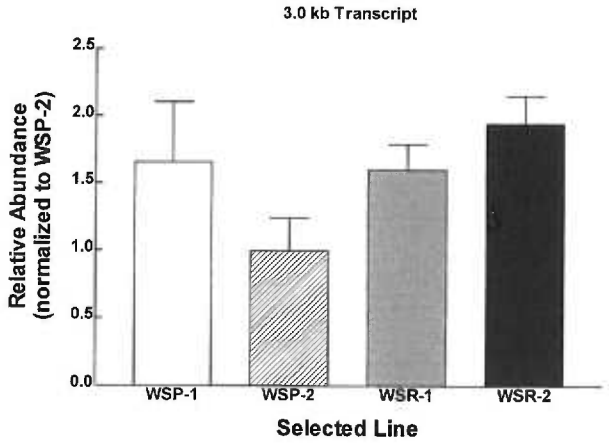

A
P1
P2
R1
R2
3.0 kb
1.4 kb
18S rRNA
B
P1
P2
R1
R2
3.0 kb
1.4 kb
18S rRNA
C
P1
P2
R1
R1
3.0 kb
1.4 kb
18S rRNA

Figure 24. Relative abundance of mNSP in saline-treated mice. **A.** Hippocampus, 1.4 kb transcript (n = 2). **B.** Hippocampus, 3.0 kb transcript (n = 2). **C.** Cerebellum, 1.4 kb transcript (n = 2). **D.** Cerebellum, 3.0 kb transcript (n = 2). **E.** Cortex, 1.4 kb transcript (n = 2 for WSP-1, WSR-2; n = 1 for WSP-2, WSR-1). **F.** Cortex, 3.0 kb transcript (n = 2 for WSP-1, WSR-2; n = 1 for WSP-2, WSR-1). Data from the autoradiograph in Figure 22 were quantitated using scanning densitometry and presented normalized to WSP-2 levels (mean± sem).

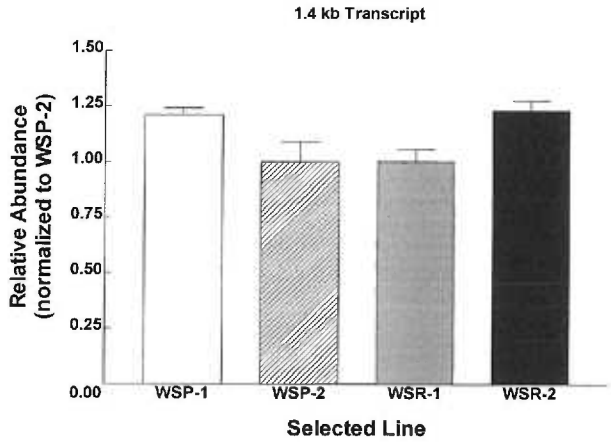
A


1.4 kb Transcript
Relative Abundance (normalized to WSP-2)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
WSP-1
WSP-2
WSR-1
WSR-2
Selected Line

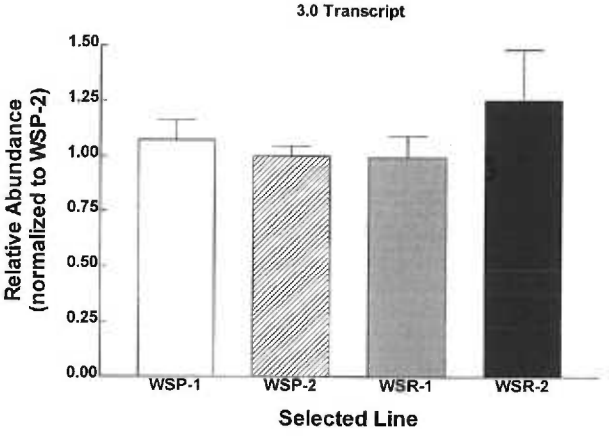
B


3.0 kb Transcript
Relative Abundance (normalized to WSP-2)
2.5
2.0
1.5
1.0
0.5
0.0
WSP-1
WSP-2
WSR-1
WSR-2
Selected Line

C


1.4 kb Transcript
Relative Abundance (normalized to WSP-2)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
WSP-1
WSP-2
WSR-1
WSR-2
Selected Line

D


3.0 Transcript
Relative Abundance (normalized to WSP-2)
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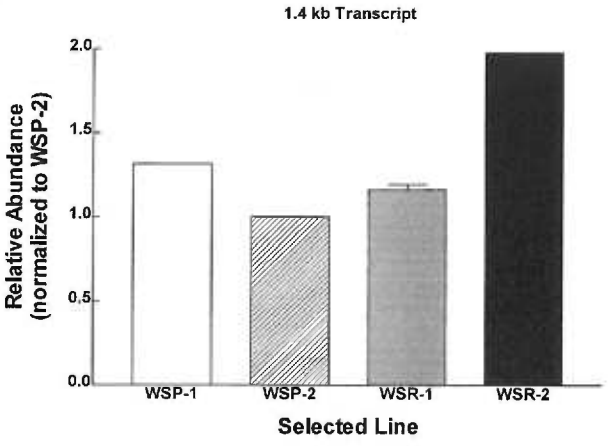
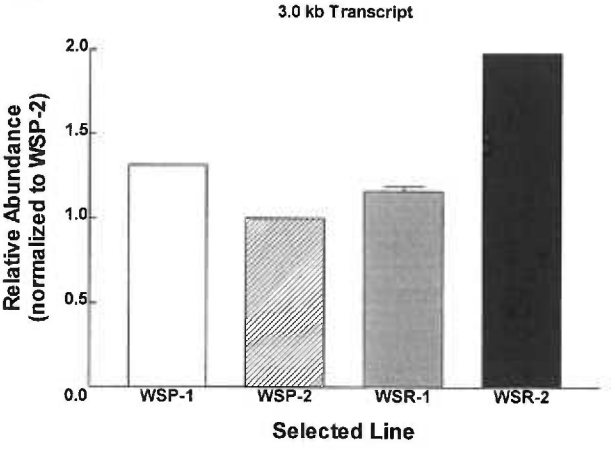
1.25
1.00
0.75
0.50
0.25
0.00
WSP-1
WSP-2
WSR-1
WSR-2
Selected Line

E


1.4 kb Transcript
Relative Abundance (normalized to WSP-2)
2.0
1.5
1.0
0.5
0.0
WSP-1
WSP-2
WSR-1
WSR-2
Selected Line

F


3.0 kb Transcript
Relative Abundance (normalized to WSP-2)
2.0
1.5
1.0
0.5
0.0
WSP-1
WSP-2
WSR-1
WSR-2
Selected Line

Expt. 4: Inbred strain studies: C57BL/6J and DBA/2J mice

It was hypothesized that ethanol's effect on gene expression could be a generalized effect of ethanol, i.e., reflecting an adaptation of cells that does not correspond to a specific ethanol-induced behavioral change (e.g., dependence, tolerance).

Alternatively, changes in gene expression could be correlated with a specific set of behavioral alterations such as susceptibility to withdrawal seizures. Selected lines of mice are an ideal model to investigate these correlations. However, the validity of the correlation is increased if the results from genetically selected lines of mice are substantiated in other animal models, such as independently selected lines (for the same phenotype) or inbred strains that differ in ethanol responses. C57BL/6J and DBA/2J mice differ in their withdrawal severity (Goldstein and Kakihana, 1974; Crabbe et al., 1983). To strengthen the hypothesis that a changes in the relative abundance of mNSP in brains of ethanol-treated mice are specific to withdrawal seizures, in particular a decrease in expression in the cerebellum, these inbred strains were used. It was predicted that DBA/2J mice exposed to ethanol vapor would show a decreased expression of the 3.0 kb transcript but not the C57BL/6J mice. Additionally, increased expression in hippocapmus and cortex would be expected, especially in the C57BL/6J mice but also in DBA/2J mice. These mice were exposed to ethanol as described in Methods. RNA was used for Northern analyses to assess changes in gene expression of the differential display product, mNSP.

4.1 METHODS

Animals and Animal husbandry. Male C57BL/6J and DBA/2J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and, after arrival, allowed to habituate for 2 weeks prior to ethanol exposure. Mice were 65-75 days old at the start of experiments. All mice were housed in standard polypropylene containers (28 x18 x13 cm), 4 per cage. Mice

were maintained on a 12:12 hr light cycle with *ad libitum* food (Purina Rodent Chow) and water. Daily room temperature was 21 ± 2°C. Cages and bedding were changed twice a week.

Drugs. Ethyl alcohol (200 proof, Pharmaco) was diluted to 20% (v:v) in 0.9% saline. Pyrazole hydrochloride (Sigma) was dissolved in 0.9% saline and injected at a concentration of 1 mmol/kg for both strains.

Ethanol exposure.

Whole brain studies. 40 male C57BL/6J and DBA/2J mice were divided into three treatment groups (EtOH, PYR, SAL) and exposed to ethanol vapor or air as described above. On Day 1, mice were weighed, injected with 1.5 g/kg 20% ethanol/1.0 mmol/kg pyrazole (EtOH group), 1.0 mmol/kg pyrazole (PYR group), or saline (SAL group). On Days 2 and 3 (24 and 48 hrs), mice were briefly removed from the chambers, weighed and injected with 1 mmol/kg pyrazole (EtOH and PYR groups) or saline (SAL group), and returned to the chambers. Seventy-two hours after being placed in the chambers, mice were removed. Mice in the EtOH group had a 20 µl blood sample taken from the tail while PYR and SAL groups of mice only received a tail snip. A subset of each of the groups of mice (4 saline-, 4 pyrazole-, and 8 ethanol-treated mice from each strain) was set aside for withdrawal severity scoring for handling-induced convulsions. These mice were scored for HIC for the 13 hours (t = 0 to t = 12) plus hours 24 and 25 after withdrawal and excluded form the Northern analysis. The remaining mice were used for the gene expression study. These mice were immediately killed by cervical dislocation, the brain removed, and rapidly frozen in liquid nitrogen. Samples were stored at -80°C until processing.

Regional expression. Thirty-six male C57BL/6J and DBA/2J mice were exposed to ethanol as described above. Immediately after removal from the vapor chambers, a tail blood

sample (ethanol-groups) or a tail snip (air-groups). Mice were immediately killed by cervical dislocation, and hippocampus, cerebellum, and cortex rapidly removed, and rapidly frozen in liquid nitrogen. Samples were stored at -80°C until processing.

Chamber levels for C57BL/6J mice were 7.5-8 mg ethanol/liter air for the first 24 hrs, 6-9 mg ethanol/liter air for 48-72 hrs. DBA/2J mice were maintained at 5-5.5 mg ethanol/liter air for the first 24 hrs, and 4-7 mg ethanol/liter air for the next 48 hours.

RNA isolation, Northern blot analyses, and quantitation were identical as described above.

Data analysis

Behavioral data. The area under the withdrawal curve (AUC) was calculated for each mouse. Statistical differences were analyzed using a two-factor ANOVA for Strain x Treatment, where treatment included pyrazole and ethanol only. All saline mice had scores of 0, except one DBA/2J mouse, and because there was no variance associated with the C57BL/6J saline group, both saline groups were eliminated from the ANOVA.

Northern analysis. Integrated densitometry values were normalized by the procedure described in section "**2a.1 Methods**". C57BL/6J and DBA/2J mice data were analyzed separately. Data were analyzed first for changes in expression by pyrazole for each strain (saline versus pyrazole), followed by analyses for ethanol effects. All data were analyzed by Student's *t*-test.

4.2 RESULTS and DISCUSSION

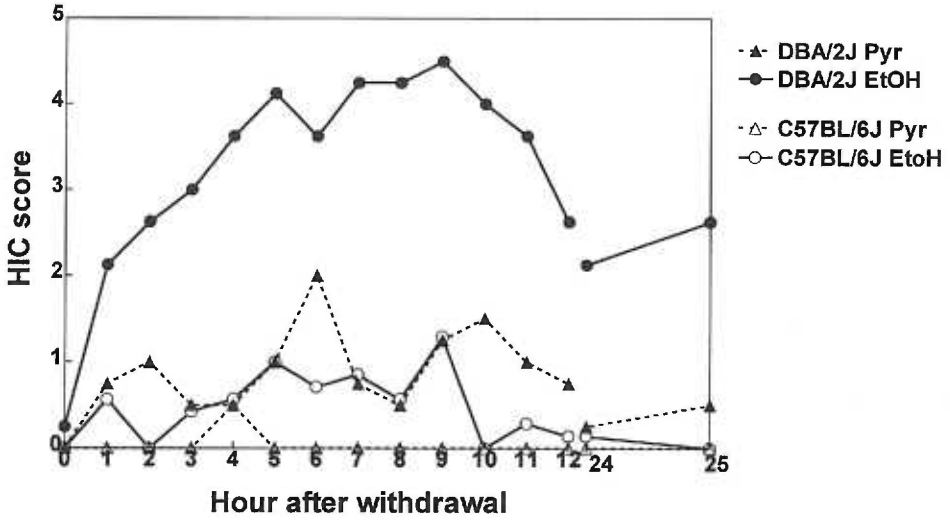
4.2.1 Withdrawal severity

C57BL/6J and DBA/2J mice were scored for HIC every hour for 12 consecutive hours and at hour 24 and 25 from withdrawal from chambers. One C57BL/6J mouse had to be euthanized after the second hour of withdrawal due to extremely poor health. A graph of the HIC response over time is shown in Fig 25A. As can be seen the from the graph, DBA/2J mice exhibited a robust withdrawal syndrome, as indexed by HIC scores, whereas C57BL/6J mice showed very little response. The area under the withdrawal curve (AUC) was calculated for each strain and treatment group and is shown in Figure 25B.

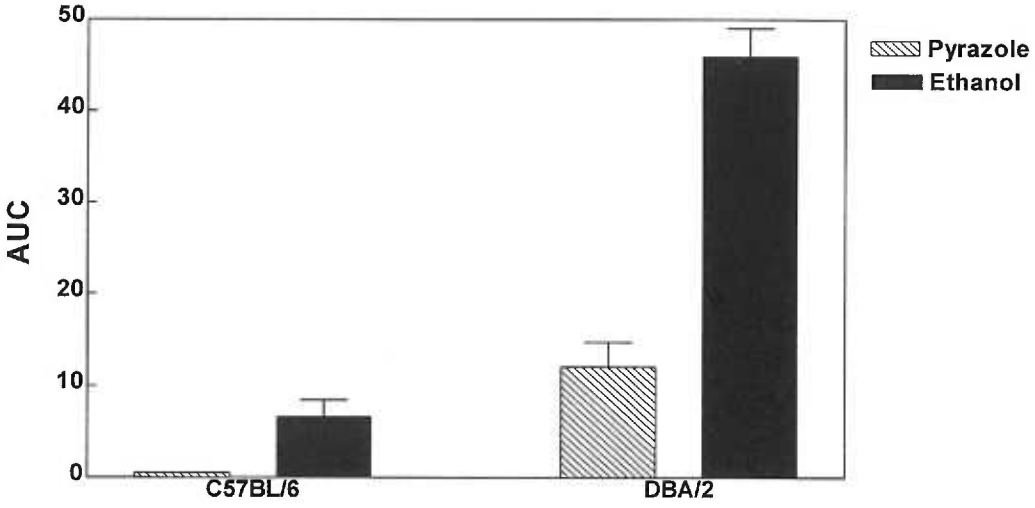
Ethanol-treatment significantly enhanced HICs in DBA/2J mice compared to C57BL/6J mice. A two-factor ANOVA was used to analyze AUC for pyrazole and ethanol-treated C57BL/6J and DBA/2J mice. There were significant main effects of Strain [$F(1,19)=80.95$, $p<0.0001$], Treatment [$F(1,19)=50.08$, $p<0.0001$), and the interaction of Strain x Treatment [$F(1,19)=24.29$, $p<0.001$]. Simple main effects analysis of the interaction revealed significant difference of strain (DBA>C57) in AUC for ethanol-treatment ($p<0.0001$) and for pyrazole treatment ($p<0.05$). Despite the greater effect of pyrazole on eliciting HICs in DBA/2J mice than on C57BL/6J mice, the effect of ethanol withdrawal was much more profound. For the group of mice used for HIC scoring, the BEC values (mean ± sem) for the ethanol-treated C57BL/6J and DBA/2J mice were 1.26 and 1.68 mg/ml, respectively.

Figure 25. Ethanol withdrawal in C57BL/6J and DBA/2J mice. **A.** HIC scores during the withdrawal period. Mice were scored hourly for 12 consecutive hours after removal from chamber, then at 24 and 25 hours. HIC are mean ± sem for n = 4 mice per strain for pyrazole groups and n = 7 mice (C57BL/6J) and n = 8 (DBA/2J) for ethanol groups. **B.** AUC (mean ± sem) were calculated for HIC during the withdrawal period (12 hours plus hours 24 and 25 following withdrawal). Saline scores were 0. AUC for C57BL/6J mice were plotted on the same scale as the DBA/2J mice for direct comparison of the difference in severity between the strains.

A


HIC score
Hour after withdrawal
DBA/2J Pyr
DBA/2J EtOH
C57BL/6J Pyr
C57BL/6J EtoH

B


AUC
C57BL/6
DBA/2
Pyrazole
Ethanol

4.2.2 Expression of mNSP

4.2.2a Whole brain

The data for C57BL/6J and DBA/2J mice were analyzed for the global effect of ethanol on entire brain expression of mNSP. Mice used in the EtOH groups for C57BL/6J and DBA/2J mice were matched for BEC. BEC at time of withdrawal from the chambers is presented in Table 5. There were no significant differences between C57BL/6J mice and DBA/2J mice in BEC ($t = 1.05$, $df = 22$, n.s.).

A representative Northern blot is shown in Figure 26. The effect of pyrazole treatment on expression of the 1.4 kb transcript was not signficant for either strain (C57BL/6J: $t = 0.04$, $df = 5.8$, n.s; DBA/2J: $t = 1.74$, $df = 4.2$, n.s.). Expression of the 3.0 kb transcript was also unaffected by pyrazole treatment (C57BL/6J: $t = -0.07$, $df = 10$, n.s.; DBA/2J: $t = -0.62$, $df = 9$, n.s.). Therefore, saline and pyrazole-treated mice exposed to air only were combined for the analysis of ethanol versus air exposure.

1.4 kb transcript

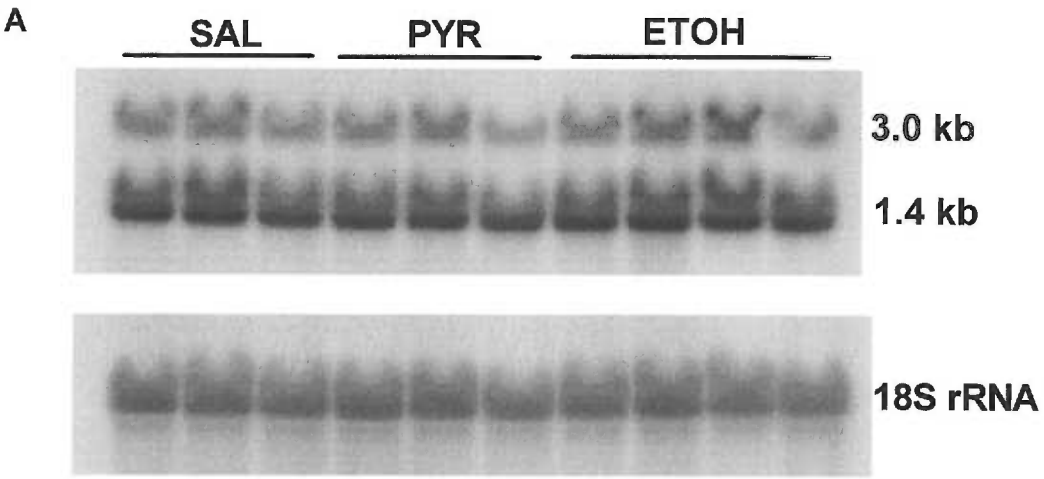
Results from the Northern blot analysis of C57BL/6J mice are presented in Figure 27A. In whole brain of C57BL/6J mice exposed to ethanol vapor for 3 days, there was no effect of ethanol on steady-state levels of the 1.4 kb transcript ($t = 0.66$, $df = 18$, n.s.).

Three days of chronic exposure to ethanol vapor did not alter the steady-state mRNA levels of the 1.4 kb transcript in DBA/2J mice (Figure 27B). No statistically significant differences between ethanol- or air-exposed mice were detected ($t = 1.29$, $df = 17$, n.s.).

Table 5. Blood ethanol concentration for C57BL/6J and DBA/2J mice. The average BEC (mean ± sem) at time of withdrawal from chambers for mice used for each brain region analysis is shown.

STRAIN	BRAIN REGION	BEC (mg/ml)
C57BL/6J	Hippocampus	1.60± 0.10
	Cerebellum	1.43 ± 0.16
	Cortex	1.43 ± 0.16
DBA/2J	Hippocampus	1.42± 0.06
	Cerebellum	1.46± 0.06
	Cortex	1.46± 0.06

Figure 26. Representative Northern blots for whole brain from C57BL/6J and DBA/2J mice. **A.** C57BL/6J mice. **B.** DBA/2J mice. Autoradiographs were scanned. The two transcripts of mNSP are labeled as 1.4 kb and 3.0 kb; 18S rRNA was used as the loading control.


A
SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

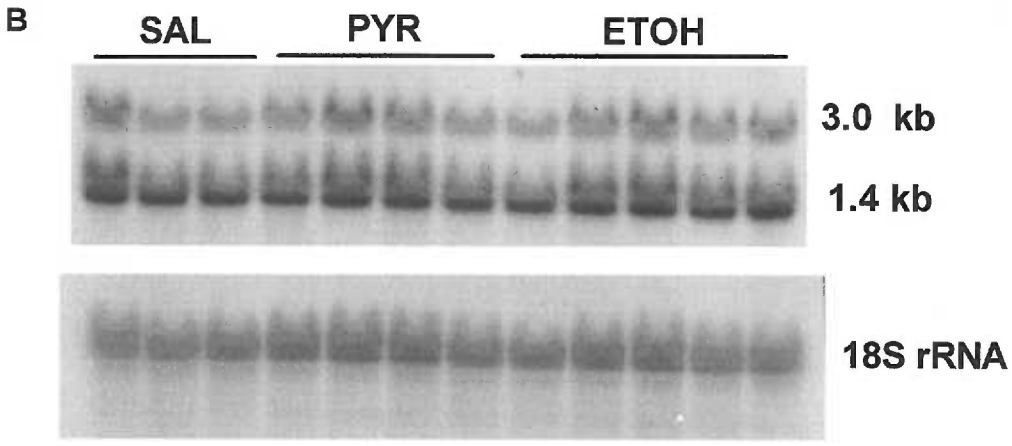
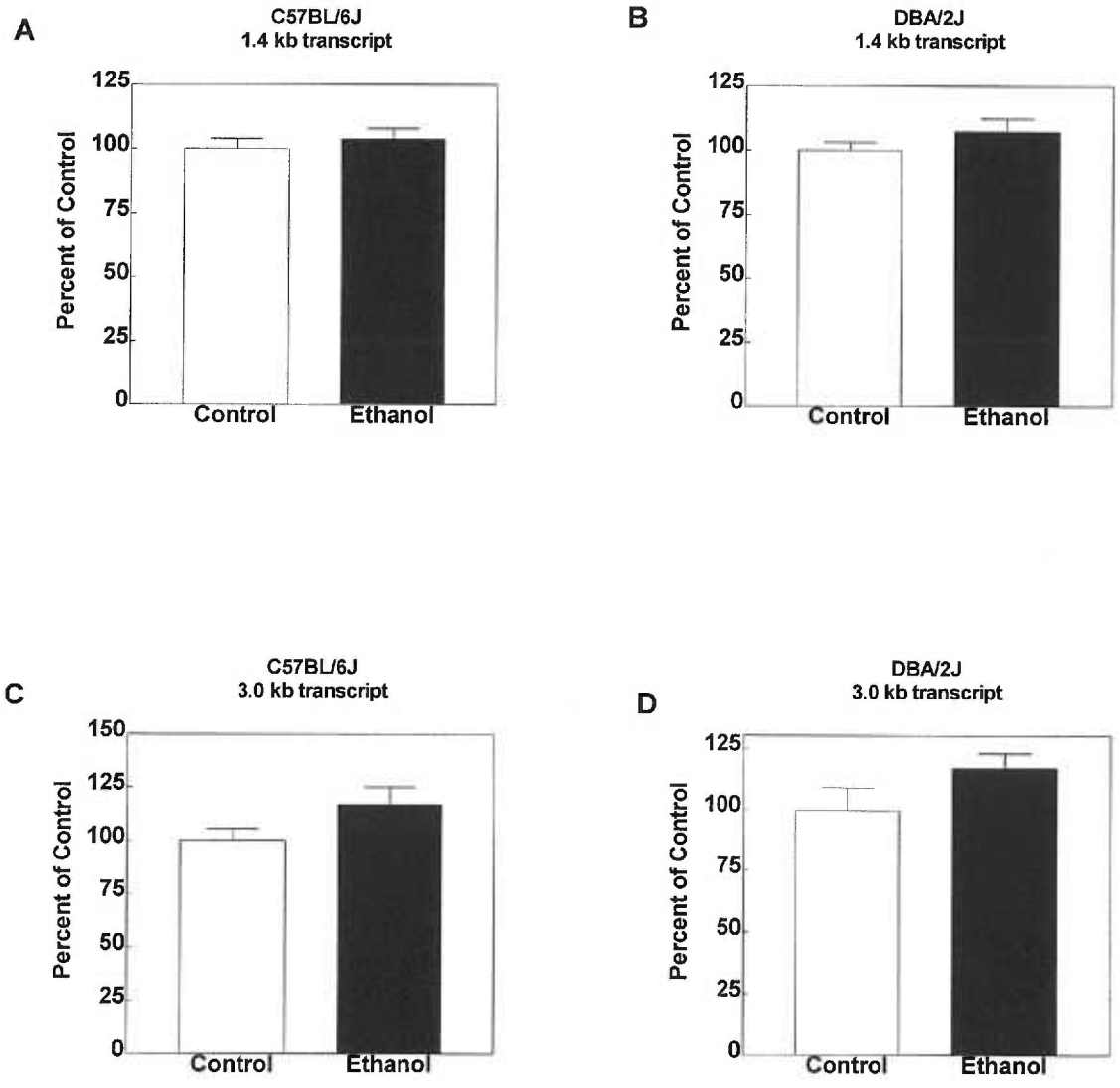

B
SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

Figure 27. Quantitative analysis of Northern blots for whole brain of C57BL/6J and DBA/2J mice. Data are presented for each transcript. **A.** 1.4 kb transcript for C57BL/6J mice (control: n = 12; ethanol: n = 8). **B.** 1.4 kb transcript for DBA/2J mice (control: n = 11; ethanol: n = 8). **C.** 3.0 kb transcript for C57BL/6J mice (control: n = 12; ethanol: n = 8). **D.** 3.0 kb transcript for DBA/2J mice (control: n = 11; ethanol: n = 8). Data represent mean ± sem for 2 Northern blots for each strain.

Whole Brain


A
C57BL/6J
1.4 kb transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol
B
DBA/2J
1.4 kb transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol
C
C57BL/6J
3.0 kb transcript
Percent of Control
150
125
100
75
50
25
0
Control
Ethanol
D
DBA/2J
3.0 kb transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol

3.0 kb transcript

In C57BL/6J mice exposed to ethanol vapor, expression of the 3.0 kb transcript was increased 17% above air-exposed mice (Figure 27C). However, this increase was not statistically significant ($t = 1.80$, $df = 18$, $p = 0.09$).

The expression of the 3.0 kb transcript was increased 17.5% in DBA/2J mice exposed to ethanol vapor for 3 days (Figure 27D). This modest increase in expression was not significantly different from air-exposed mice ($t = 1.4$, $df = 17$, n.s.).

Summary

Ethanol had a modest effect to increase steady-state levels of the 3.0 kb transcript for both strains, and no effect on the expression of the 1.4 kb transcript. There was essentially no difference in the effect of ethanol on expression levels of the 3.0 kb transcript, which could indicate that this transcript of mNSP is not involved the behavioral difference in withdrawal severity between these strains. However, since it was apparent from the WSP and WSR results that whole brain analysis does not represent changes in gene expression by ethanol in specific brain regions, analysis of effects of ethanol specific brain regions may yield differences between C57BL/6J and DBA/2J mice.

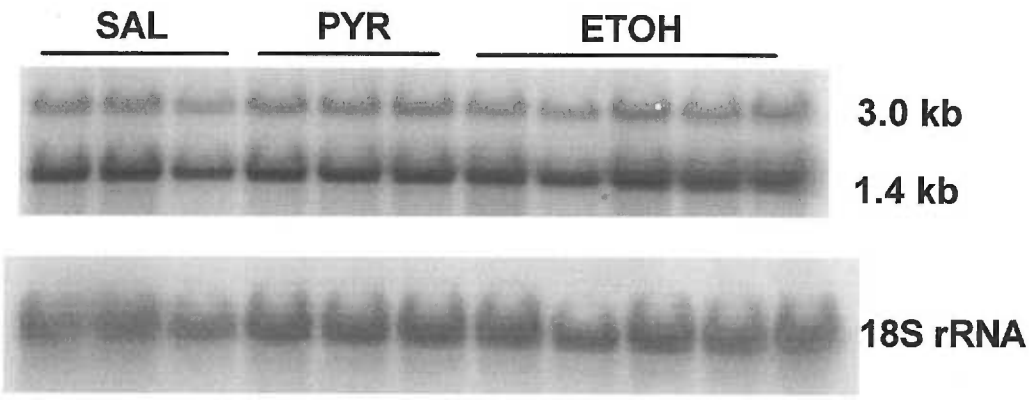
The same brain regions were analyzed for regulation of the transcripts of mNSP. If the expression of this gene in the cerebellum has an important role in mediating withdrawal severity, as indicated by results from WSP and WSR, then it was hypothesized that a similar decrease in expression of the 3.0 kb transcript would be observed in DBA/2J mice only.

4.2.2b Hippocampus

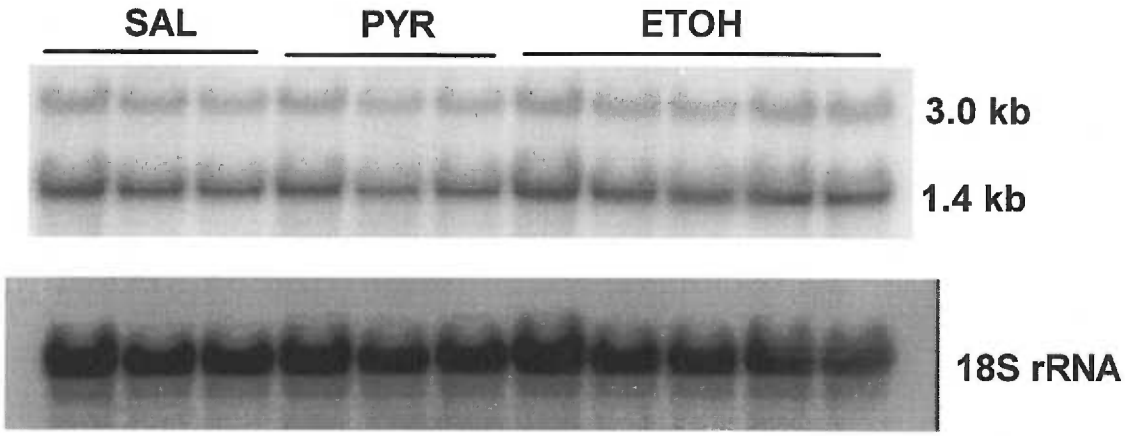
A representative Northern blot is shown in Figure 28. In general, the hybridization pattern was identical to that observed in WSP and WSR mice.

Figure 28. Representative Northern blots for C57BL/6J and DBA/2J mice hippocampus. **A**. C57BL/6J mice. **B**. DBA/2J mice. Northern blots are shown as scanned images of autoradiographs.

A


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

B


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

1.4 kb transcript

There was no effect of pyrazole on expression of the 1.4 kb transcript in hippocampal tissue of C57BL/6J mice ($t=0.016$, $df=10$, n.s.). However, ethanol significantly increased the expression of the 1.4 kb transcript ($t = 5.29$, $df=19$, $p< 0.0001$). The observed increase was 28%. These results can be observed in Figure 29A.

Results for DBA/2J mice are shown in Figure 29B. In DBA/2J mice, pyrazole had no effect on expression of the 1.4 kb transcript ($t= -1.34$, $df=9$, n.s.). There was a significant effect of ethanol to increase the expression of this transcript ~17% ($t= 2.56$, $df =18$, $p <0.02$).

3.0 kb transcript

In C57BL/6 mice, there was no effect of pyrazole on the expression of the 3.0 kb transcript ($t =0.025$, $df=10$, n.s.). Ethanol had no significant effect on the expression of this transcript ($t= 0.093$, $df=19$, n.s.); however the change from control was observed as an increase of 12% (Figure 29C).

In DBA/2J mice, there was no effect of pyrazole ($t=0.4$, $df=9$, n.s.). In cortex of these mice ethanol had no significant effects on transcription ($t=1.51$, $df=18$, n.s.). There was a qualitative increase in expression of 24% (Figure 29D).

Ethanol increased the expression of both transcripts fairly modestly in hippocampus of both strains of mice. The differences were only statistically significant for the 1.4 kb transcript. The magnitude of the increase was different between strains depending on the transcript.

4.2.2c Cerebellum

Figure 30 shows the results from Northern blots containing cerebellar tissue from C57BL/6J mice (Figure 30A) and DBA/2J mice (Figure 30B). Similar to the WSP and WSR

Figure 29. Expression of the 1.4 kb and 3.0 kb transcripts of mNSP in hippocampal tissue from C57BL/6J or DBA/2J mice following ethanol exposure. **A.** Control levels (n = 12) versus ethanol (n = 9) for the 1.4 kb transcript in C57BL/6J mice. **B.** Control (n = 11) versus ethanol-treated (n = 9) levels for the 1.4 kb transcript in DBA/2J mice. **C.** Control levels (n = 12) versus ethanol (n = 9) for the 3.0 kb transcript in C57BL/6J mice. **D.** Control (n = 11) versus ethanol-treated (n = 9) levels for the 3.0 kb transcript in DBA/2J mice. Autoradiographs were quantitated by scanning densitometry. Each transcript was corrected for loading using its 18S rRNA integrated value. Samples on each blot were normalized to the blot average and are expressed as the percent change in expression from control (mean ± sem). Saline- and pyrazole-groups were combined to form the Control group. Two Northern blots for each strain were quantitated. See text for statistical analyses.

Hippocampus

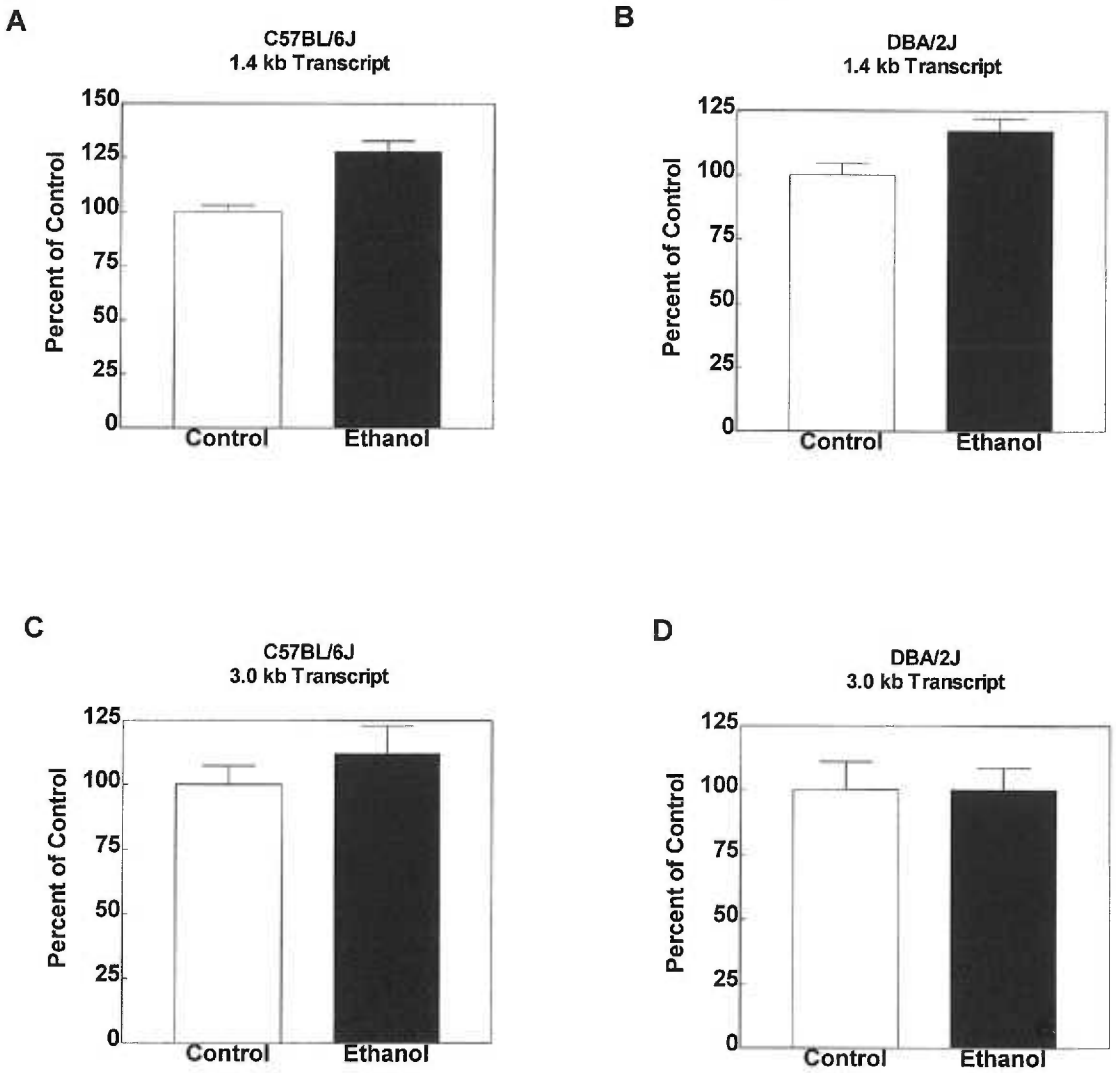
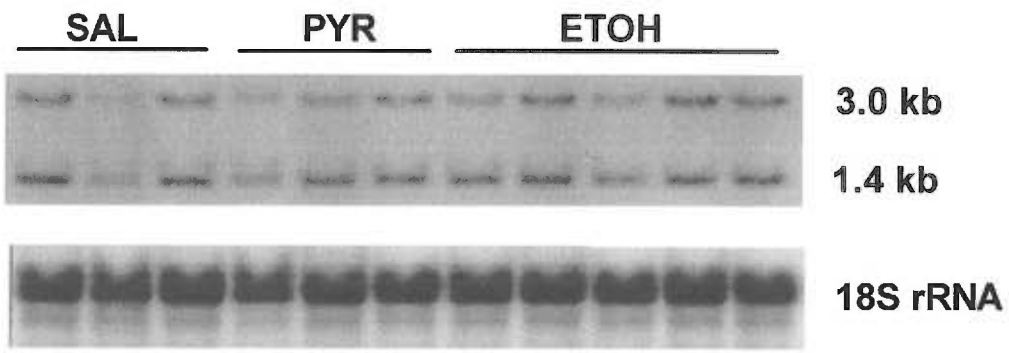
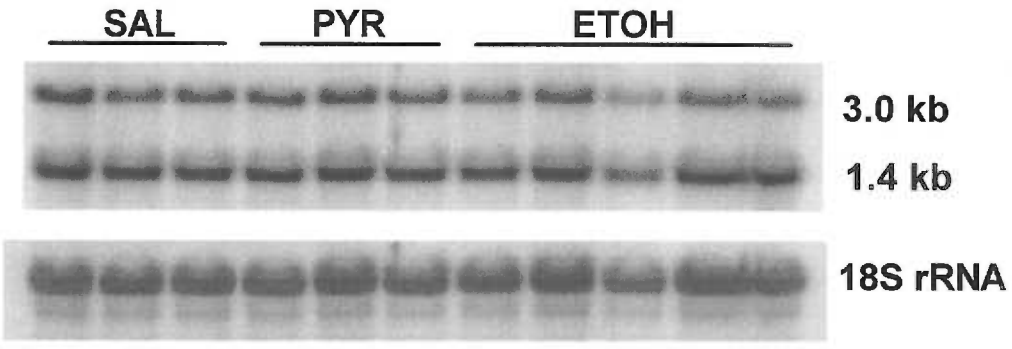


Figure 30. Representative Northern blot from cerebellum of C57BL/6J or DBA/2J mice. **A.** C57Bl/6J mice **B.** DBA/2J mice. Autoradiographs were scanned.

A


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

B


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

results, there is a greater abundance of the 3.0 kb transcript in cerebellum in comparison to hippocampal tissue.

1.4 kb transcript

Figure 31A shows the results of ethanol exposure on the expression of the 1.4 kb transcript in C57BL/6J mice. In C57BL/6J mice, there was no effect of pyrazole on expression of the 1.4 kb transcript (t=-0.58, df=10, n.s.). Ethanol increased the expression 14%, although this did not reach statistical significance (t= -1.74, df=20, n.s.).

In DBA/2J mice, pyrazole did not affect the expression of this transcript (t=1.43, df=10, n.s.). Ethanol decreased expression 10% below control levels. This was a significant decrease in expression of this transcript (t=2.41, df=20, $p<0.05$; Figure 31B).

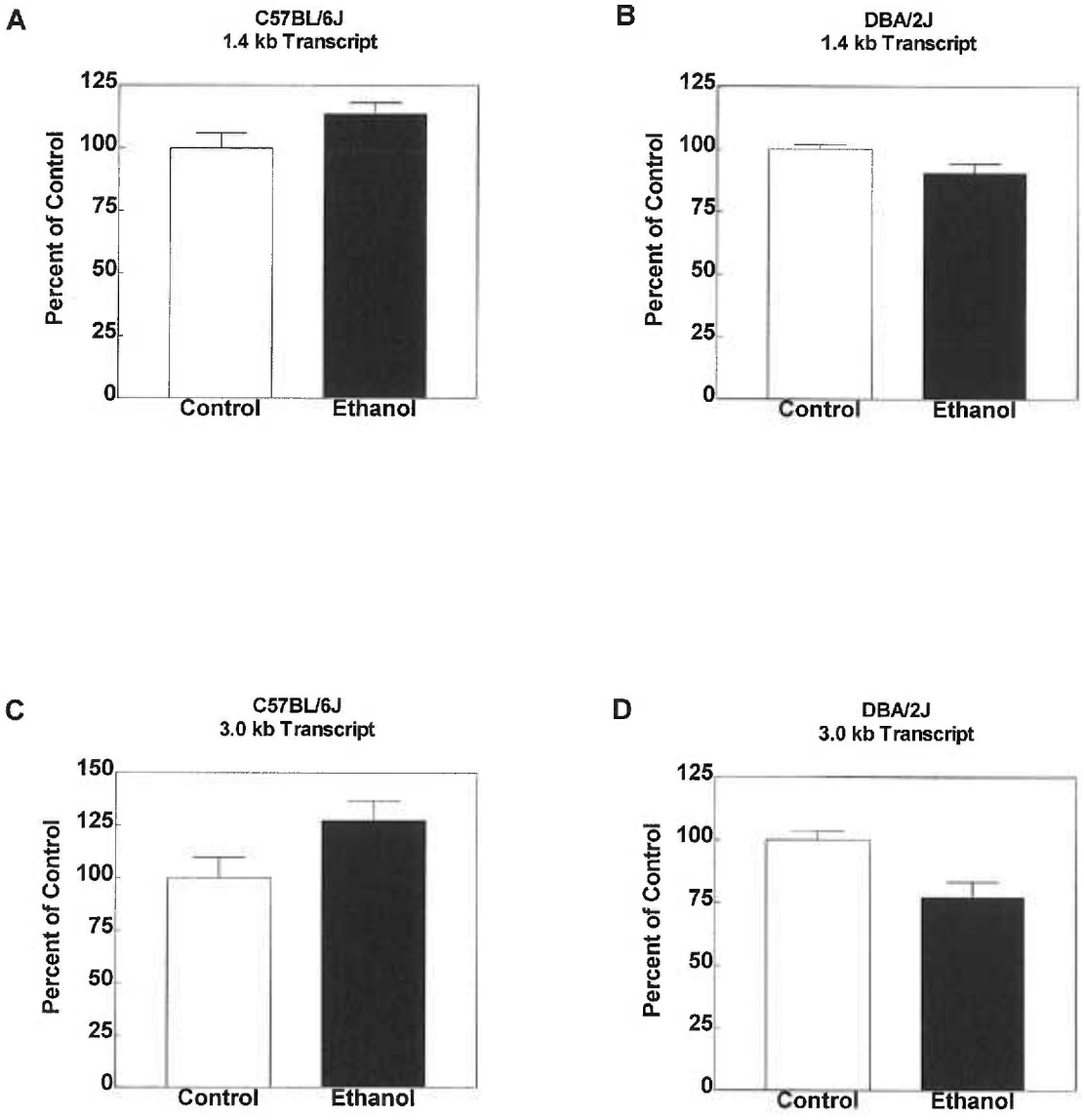
3.0 kb transcript

In C57BL/6J mice, there was no effect of pyrazole on expression of the 3.0 kb transcript in cerebellum (t = -0.755, df= 10, n.s.); therefore, the saline and pyrazole groups have been combined as the control group. Exposure to ethanol for 72 hours increased the expression of the 3.0 kb transcript 27% above control levels (Figure 31C). This increase in expression by ethanol was marginally significant (t=2.013, df=20, p=0.06).

Since pyrazole did not have an effect on gene expression in DBA/2J mice (t=1.03, df=10, n.s.), the saline and pyrazole groups were combined as the control group. In contrast to C57BL/6J mice, ethanol decreased expression 23% lower than control (Figure 31D). The effect of ethanol was significantly different from air-exposed mice (t= -3.42, df=20, $p<0.01$).

Figure 31. Expression of the 1.4 kb and 3.0 kb transcripts of mNSP in cerebellar tissue from C57BL/6J or DBA/2J mice following ethanol exposure. **A.** Control (n = 12) versus ethanol (n = 10) levels for the 1.4 kb transcript in C57BL/6J mice. **B.** Control (n = 12) versus ethanol (n = 10) levels for the 1.4 kb transcript in DBA/2J mice. **C.** Control (n = 12) versus ethanol (n = 10) levels for the 3.0 kb transcript in C57BL/6J mice. **D.** Control (n = 12) versus ethanol (n = 10) levels for the 3.0 kb transcript in DBA/2J mice. Autoradiographs were quantitated by scanning densitometry. Each transcript was corrected for loading using its 18S rRNA integrated value. Samples on each blot were normalized to the blot average and are expressed as the percent change in expression from control (mean ±sem). Saline- and pyrazole-groups were combined to form the Control group. Two Northern blots for each strain were quantitated. See text for statistical analyses.

Cerebellum


A
C57BL/6J
1.4 kb Transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol
B
DBA/2J
1.4 kb Transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol
C
C57BL/6J
3.0 kb Transcript
Percent of Control
150
125
100
75
50
25
0
Control
Ethanol
D
DBA/2J
3.0 kb Transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol

The results for the 1.4 kb transcript were very similar to the 3.0 kb transcript, that ethanol decreased expression of the transcripts in DBA/2J mice and increased the expression of the transcripts (modestly) in the C57BL/6J mice. These results are similar to the results from the 3.0 kb transcript in cerebellum of WSP and WSR mice in that the mice susceptible to expressing withdrawal convulsions (WSP and DBA/2J) show a decrease in the expression of this transcript after ethanol treatment and the mice that exhibit very minor withdrawal convulsions (WSR and C57BL/6J) had no effect on expression of this transcript after ethanol exposure.

4.2.2d Cortex

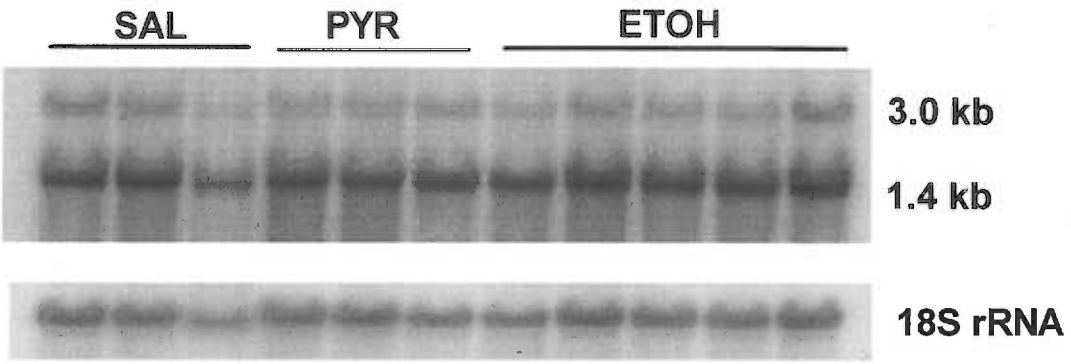
A representative Northern blot is shown in Figure 32. The two transcripts, which were observed for all other tissues, were present. Pyrazole effects on transcription were compared to saline-treated mice. No differences between saline or pyrazole treatment were observed for C57BL/6J mice (1.4 kb transcript: $t=1.01$, $df=10$, n.s.; 3.0 kb transcript: $t=0.36$, $df=10$, n.s.) or for DBA/2J mice (1.4 kb transcript: $t=1.58$, $df=6$, n.s.; 3.0 kb transcript: $t=0.82$, $df=6$, n.s.). Therefore, saline- and pyrazole-treated mice were combined to form the control group against which ethanol-treatment was compared.

1.4 kb transcript

Chronic ethanol exposure had a modest, but nonsignificant effect on the expression of the 1.4 kb transcript in C57BL/6J mice. The increase in expression by ethanol was less than 10% of air-exposed mice (Figure 33A). A Students *t*-test comparing ethanol versus control marginally significant results ($t=1.98$, $df=20$, $p=0.06$).

Figure 32. Representative Northern blots for cortex of C57BL/6J and DBA/2 J mice. C57BL/6J (**A**) and DBA/2J (**B**) mice were treated for 72 hours with saline (SAL), pyrazole (PYR), or ethanol (ETOH). Autoradiographs from representative blots were scanned.

A


SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

B

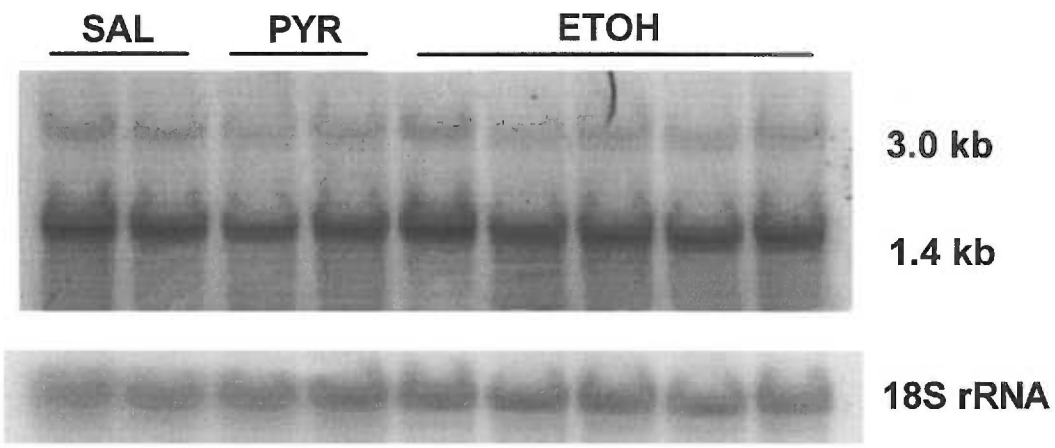
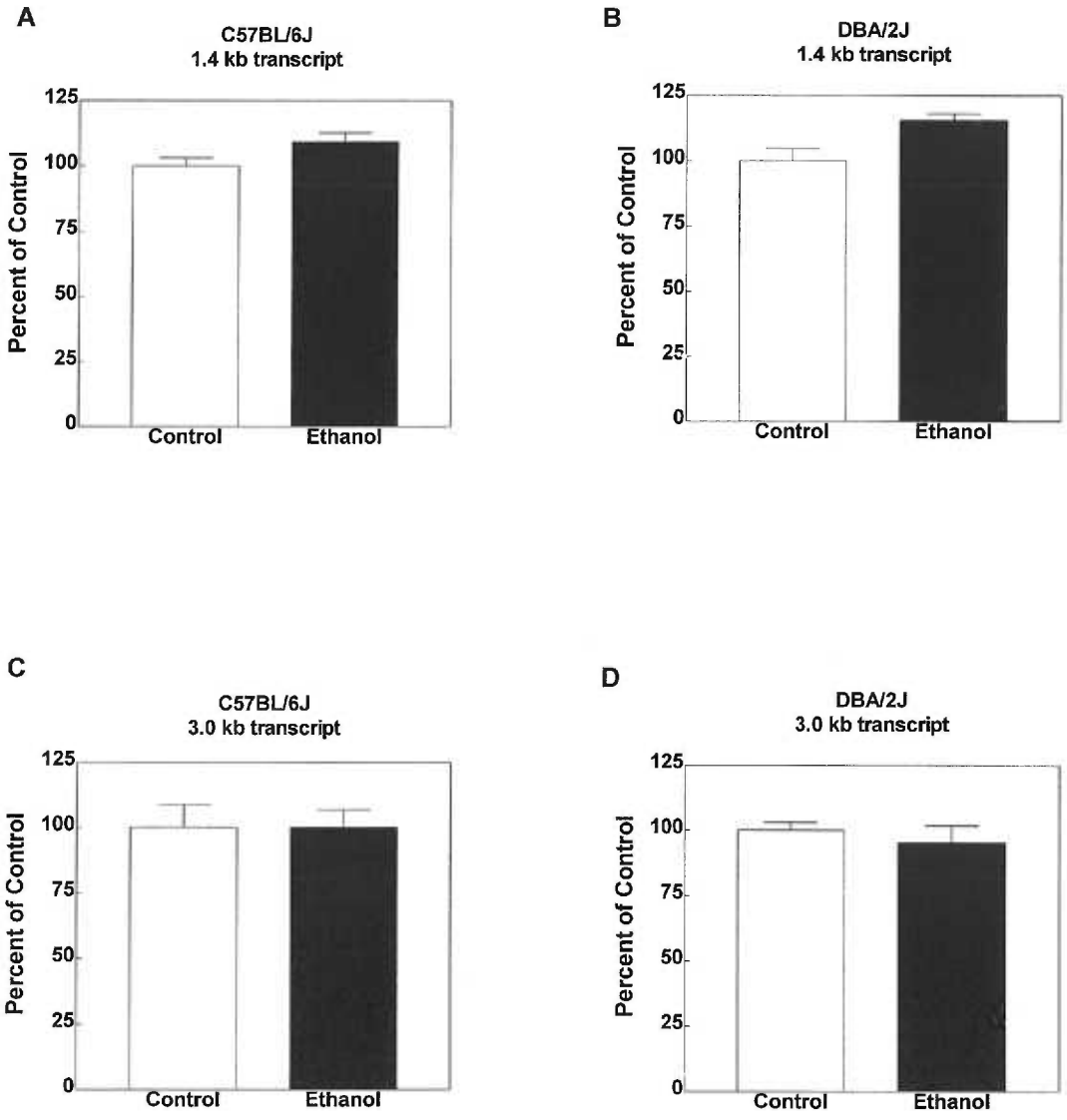

SAL
PYR
ETOH
3.0 kb
1.4 kb
18S rRNA

Figure 33. Expression of the 1.4 kb and 3.0 kb transcripts of mNSP in cortical tissue from C57BL/6J or DBA/2J mice following ethanol exposure. **A.** Control (n = 12) versus ethanol (n = 10) levels for the 1.4 kb transcript in C57BL/6J mice. **B.** Control (n = 8) versus ethanol (n = 10) levels for the 1.4 kb transcript in DBA/2J mice. **C.** Control (n = 12) versus ethanol (n = 10) levels for the 3.0 kb transcript in C57BL/6J mice. **D.** Control (n = 12) versus ethanol (n = 10) levels for the 3.0 kb transcript in DBA/2J mice. Autoradiographs were quantitated by scanning densitometry. Each transcript was corrected for loading using its 18S rRNA integrated value. Samples on each blot were normalized to the blot average and are expressed as the percent change in expression from control (mean ± sem). Saline- and pyrazole-groups were combined to form the Control group. Two Northern blots for each strain were quantitated. See text for statistical analyses.

Cortex


A
C57BL/6J
1.4 kb transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol
B
DBA/2J
1.4 kb transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol
C
C57BL/6J
3.0 kb transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol
D
DBA/2J
3.0 kb transcript
Percent of Control
125
100
75
50
25
0
Control
Ethanol

In DBA/2J mice, ethanol increased the expression of the 1.4 kb transcript 15% above control levels (Figure 33B). A Students *t*-test confirmed a significant increase by ethanol as compared to air-exposed mice ($t = 3.14$, $df = 16$, $p < 0.01$).

3.0 kb transcript

Ethanol had no effect on the expression levels of the 3.0 kb transcript in C57BL/6J mice ($t = 0.17$, $df = 20$, n.s.; Figure 33C).

In contrast to the ethanol-induced increase in expression of the 1.4 kb transcript, ethanol had no effect on the expression of the 3.0 kb transcript in DBA/2J mice (Figure 33D). No significant results were revealed from the statistical comparison of ethanol- versus air-exposed mice ($t = 0.61$, $df = 16$, n.s.).

Ethanol modestly modified the expression of the 1.4 kb transcript in the cortex, but appeared to have no effect on the expression of the 3.0 kb transcript. The increase in expression of the 1.4 kb transcript was greater in the DBA/2J mice than in the C57BL/6J mice. However, the small effect of ethanol in C57BL/6J mice nearly reached statistical significant, suggesting that the effect of ethanol was probably not due to chance. Since the cortex dissection was not isolated to a specific cortical region, this could represent a mixture of effects of ethanol in discrete cortical regions.

DISCUSSION

Using mRNA differential display, several ethanol-regulated products were identified in WSP-2 mice. One product was confirmed using Northern blot analyses and detected two transcripts; one was 1.4 kb and the other was 3.0 kb. Sequence analysis of this cDNA showed >85% nucleotide sequence homology with human neuroendocrine specific protein. Homologous transcriptional products have been cloned and the expression has been partially characterized from chicken and rat (Ninkina et al.,1997) but there is limited information regarding expression of NSP in mouse (see Roebroek et al., 1993; van de Velde et al., 1994). The effect of ethanol on expression of these transcripts was dependent on the brain region (hippocampus, cerebellum, or cortex), genotype, and transcript of mNSP.

Effect of brain region and genotype on ethanol-regulation

Whole brain. The Northern analysis of whole brain RNA did not detect any significant differences in expression of the 1.4 kb transcript following chronic ethanol exposure for any genotype. However, the relative abundance of the 3.0 kb transcript was increased by ethanol 25% above air-exposed mice in WSP-1 & -2 mice, very little, if at all, in WSR mice and increased 17% in both C57BL/6J and DBA/2J mice. The differential regulation of the transcripts suggests that actions of ethanol are specific for one transcript (see discussion below). Although the effect of ethanol differed between WSP and WSR selected lines, a similar magnitude of effect was observed between DBA/2J and C57BL/6J mice. The magnitude of ethanol-induced regulation in whole brain was not dramatic, and only significant in WSP-2 mice (3.0 kb transcript), but necessary as a first step in confirming ethanol regulation. In addition, it demonstrated that ethanol regulation appeared to be slightly different in magnitude between WSP and WSR selected lines.

Cerebellum. The expression of these transcripts in cerebellum of WSP and DBA/2J mice was markedly different from C57BL/6J and WSR mice. Ethanol decreased expression of the 3.0 kb transcript in WSP-1, WSP-2, and DBA/2J mice, whereas no effect or an increase in expression of this transcript was observed in WSR-1, WSR-2, and C57BL/6J mice. The direction of change in expression by ethanol exposure was associated with the selected phenotype (decrease observed in both replicates of WSP mice; no effect observed in both replicates of WSR mice). In addition, the decrease in expression by ethanol observed in DBA/2J mice is also consistent with their withdrawal phenotype, similar to WSP mice. Thus, in cerebellum, the effect of ethanol on expression of the 3.0 kb transcript was associated with the genotype of mice that display a relatively severe withdrawal. There were no consistent patterns of expression alterations by ethanol for the 1.4 kb transcript in WSP and WSR mice, although in C57BL/6J and DBA/2J ethanol had opposite effects on expression. The decrease in expression observed in cerebellum of WSP selected lines and DBA/2J mice was surprising, considering that the original finding from differential display was an increase in expression. This stresses the importance of investigating brain regional differences in expression; it cannot be assumed that effects of ethanol in one brain region (or whole brain) represents effects in other brain regions.

Hippocampus and cortex. In the hippocampus and cortex, the effect of ethanol was not associated with the ethanol withdrawal. Chronic ethanol exposure increased the expression of the 1.4 kb transcript in each genotype, although the magnitude of the increase varied with strain and selected line. The increase of the 1.4 kb transcript in hippocampus was largest in WSP-2 mice and C57BL/6J mice who exhibit quite different magnitude of convulsion severity during ethanol withdrawal, while the remaining genotypes (WSP-1, WSR-1 & -2, DBA/2J) had lower, fairly equivalent increases. Ethanol increased the

expression of the 3.0 kb transcript in hippocampus of WSP-2 mice, C57BL/6J and DBA/2J mice only; essentially, no effect of ethanol on expression of the 3.0 kb transcript was observed in WSP-1 or either WSR lines. In the cortex, there was no effect of ethanol on expression of the 3.0 kb transcript in either C57BL/6J or DBA/2J mice.

Implications of ethanol's effects on expression of mNSP transcripts

Many genes contribute to the expression of the WSP and WSR selected phenotypes. Although it is likely that alleles with major effects (for either susceptibility or resistance to withdrawal convulsions) will be present in multiple mouse strains that express the same phenotype, not all alleles contributing to the phenotype in a selected line would be expected to be present in all such selected lines. Since C57BL/6J and DBA/2J mice were not selected for any specific phenotype, it is not unexpected that the results from these strains were not always consistent with WSP or WSR mice. However, to find differential effects of ethanol on gene expression from inbred strains that is consistent with the differential effects on expression in cerebellum of WSP and WSR mice strengthens the hypothesis that decreased expression of this gene is associated with more severe withdrawal.

In hippocampus or cortex, the lack of replicated effect in WSP selected lines indicates that increased expression of this gene may not be highly important for determining increased susceptibility to ethanol withdrawal seizures. It is possible that selection pressure led to changes in gene frequency affecting expression of mNSP in WSP-2 mice and, although not present in WSP-1 mice, these alleles may contribute to the phenotype of WSP-2 mice. This would suggest that this gene is not necessary for determining withdrawal severity; there may be alternative genetic pathways that lead to the same phenotype.

Alternatively, alleles may have been randomly fixed that are more (or less) responsive to ethanol. Thus, an effect of ethanol on transcription of mNSP would not be the result of selected genes.

Although the function of NSP has not been elucidated, it has been hypothesized that these proteins may play a role in protein trafficking and possibly vesicular transport (Senden et al., 1996). NSP-A (translational product of the human 3.4 kb transcript) was localized to Purkinje cells in the cerebellum, but NSP-C was not (Senden et al., 1996), suggesting that there may be subregional translational regulation (determination of RNA was not determined at this level for NSP-C). The differential ethanol-regulation of the 3.0 kb transcript, which would correspond to NSP-A, compared to the 1.4 kb transcript could indicate that Purkinje cells specific targets of ethanol. Differential ethanol regulation between genotypeswith differing seizure-susceptiblity has implications for compromised cellular function within the cerebellum. Purkinje cells receive numerous inputs from parallel fibers (axons of granule cells), basket cells, and climbing cells. The Purkinje cells are the only cell type of the cerebellum that projects from the cerebellar cortex; altered synaptic function would have diverse actions. A decrease in the mRNA levels of metabotropic glutamate receptors and inositol 1,4,5-triphosphate receptor (both located in Purkinje cells) was observed in mice following long-term ethanol exposure (Simonyi et al., 1996). One could speculate that changes in the signal transduction system could result in alterations of subsequent cellular functions such as protein trafficking.

The increases in expression of mNSP transcripts in hippocampus and cortex of several of the genotypes suggests that these brain regions may be particularly sensitive to ethanol. Although no consistent genotypic differences in degree of expression increase of NSP were observed in these regions, it cannot be ruled out that these regions may also

participate in seizure-susceptibility. More detailed analyses of the temporal and spatial (anatomical) ethanol-regulated expression pattern must be conducted to assess adequately the role of NSP products in modulating convulsions.

Mechanisms underlying the differential effect of ethanol on mNSP gene expression

There appears to be a fairly high level of constitutive expression of mNSP in all tissues examined, particularly of the 1.4 kb transcript, suggesting that this protein product (corresponding to human NSP-C) may play integral roles in cellular functioning. Basal levels (i.e., from saline-control mice) were not greatly different between the brain regions or the selected lines, although there was a higher level of expression of the 3.0 kb transcript (corresponding to NSP-A) in cerebellum as compared to hippocampus or cortex. In the presence of ethanol, there was a difference in expression of one or both of the transcripts. To complicate the issue, this regulation of expression was not only brain region-specific but genotype-specific as well.

Differences in ethanol-regulated expression between the selected lines suggest that there are allelic differences between the WSP and WSR lines that modulate transcriptional regulation of this gene. It can be speculated that the decrease in expression of the 3.0 kb transcript of mNSP (corresponding to hNSP-A) in cerebellum of WSP-1, WSP-2, and DBA/2J mice represents a common effect of ethanol on transcriptional regulation. Selection may have led to changes in gene frequency of genes affecting transcriptional machinery that results in a decrease in expression of mNSP following chronic exposure to ethanol. These alleles may be present in DBA/2J mice, but not in C57BL/6J mice. Since WSP-1 &-2 mice are replicated lines selected for severe ethanol withdrawal and DBA/2J mice have a similar phenotype, it is hypothesized that some of the genes selected for withdrawal severity,

contribute to the expression of mNSP and are present on DBA/2J mice. Expression of selected genes appears to be tissue-specific, as the decrease was limited to the cerebellum in this investigation.

Since there is no information on transcriptional regulation of NSP, mechanisms of ethanol-regulation of transcription can only be speculated. Sequences of the promoter regions of mNSP may differ among genotypes. The promoter region is typically characterized by several important regions (sequences) that can bind various transcription factors, activators, enhancers, or suppressors. Variation at this level, which is reflected as transcription ability, is mostly what differentiates cell/tissue phenotype. Miles et al. (1992) have suggested that there are specific ethanol-responsive genes, those to which there is regulation of gene transcription by ethanol. Although it is not known by what mechanisms transcriptional regulation by ethanol occurs, it is hypothesized that inducible binding factors or activator proteins acting on the promoter regions are responsible (Miles, 1995). Promoter regions of ethanol-responsive genes have been investigated to find sequences that are important for imparting ethanol sensitivity (Miles, 1995). Indeed, removal or mutation of specific sequences of Hsc70 promoter attenuate ethanol-induced expression of Hsc70 (Miles at al., 1991). Mutations of the promoter region of GRP78 have identified distal sequences that are important for ethanol responsiveness (Hsieh et al., 1996). Deletion of the nucleotides -85 to -104 results in a loss of ethanol induction. Thus, it is apparent that there are "ethanol-responsive" promoter regions of genes. It is not known whether the same or similar sequences are represented in a variety of ethanol-responsive genes. There may specific inducible transcription factors that bind NSP in the presence of ethanol. In the case of a decrease in expression by ethanol, the function of this kind of factor would presumably suppress transcription to some degree.

Ethanol regulation of gene expression could occur through several mechanisms. Evidence from IEG expression studies (e.g., *c-fos*, *jun*D) indicates one pathway upon which ethanol can act (Morgan et al., 1992; Ding et al., 1996). As transcription factors, these IEGs combine to form dimers that bind to specific DNA sequences. Activation of FOS and JUN tends to be transient, however, and therefore changes in gene expression of these genes after chronic or continuous drug exposure would not be expected to be observed. However, effects of IEGs on subsequent gene expression may in fact be important for adaptation to chronic drug exposure. It is conceivable that ethanol also affects other classes of IEGs, termed "effectors", that have been described for other drugs of abuse (Fosnaugh et al., 1995). These are immediate early genes that directly modulate cellular function versus indirectly through the classical action on gene expression. One such putative effector, *arc* (for activity-related, cytoskeleton-associated) has been proposed in mediating acute responses to cocaine. These molecules also appear to be effective for identifying relevant brain structures important to mediate acute versus chronic drug effects.

There is also evidence indicating ethanol's effects on signal transduction systems, such as PKA and PKC cascades (see Diamond and Gordon, 1997). Chronic drug exposure can lead to long-lasting changes in signal transduction systems such as up-regulation of the cAMP pathway that ultimately leads to persistent changes in target gene transcription and phosphorylation of target proteins, including ion channels (Nestler and Aghajanian, 1997). These effects have best been described for cocaine and morphine administration. Chronic opiate administration increases specific subtypes of adenylyl cyclase in locus coeruleus neurons, mediated by cAMP response element-binding protein (CREB). Chronic ethanol administration lead to a decrease in cAMP production in NG108-15 cells (Mochly-Rosen et al., 1988; Rabin, 1993), resulting in a reduction of PKA function (Coe et al., 1995) but up-

regulated the cAMP pathway in nucleus accumbens (Nestler and Aghajanian, 1997), indicating that there may be distinct cellular and anatomical pathways leading to increased or decreased function. In return, changes in gene expression can led to the changes in receptor subunit expression and constituents of signal transduction systems (Miles et al., 1993). Together, alterations at these initial stages of gene transcription can lead to changes in later gene expression that are representative of cellular adaptation and that could contribute to enhanced hyperexcitability characteristic of a withdrawal syndrome.

Ethanol itself may not directly interact with the NSP gene to modulate transcription. For example, allelic variations in receptor subunit constituents or protein kinase isozymes may be partly responsible for genetic variability in gene expression. Alternatively, ethanol may increase mRNA degradation. Since Northern blot analysis measures steady-state levels of mRNA, a decrease in expression could result from a decrease in the initiation of transcription and/or increases in degradation of transcripts. Increases in transcription can arise from an increased stability of RNA molecules or from increases in the rate of transcription.

Ethanol-regulated gene expression of other ER proteins

Several other resident ER proteins have been identified as ethanol-responsive. The glucose-regulated proteins, GRP78 and GRP94, show increased expression following chronic ethanol exposure (Miles et al., 1994). These proteins are molecular chaperones and involved in protein folding and trafficking. Signal peptidase is another ER protein that was increased in rat brain following chronic ethanol exposure (Signs and Jacquet, 1994). The function of signal peptidase is to cleave the N-terminal amino acid signal sequence needed for appropriate membrane translocation. It has been hypothesized that the increases in ER proteins reflect a vulnerability of the ER to ethanol. Changes in protein

expression and location may be necessary for adaptation to chronic ethanol. Alternatively, as discussed in Signs and Jacquet (1994), ethanol may increase proliferation of the ER membrane, thereby increasing the amount of membrane proteins such as signal peptidase. These explanations do not necessarily fit the results for mNSP. There were increases in mRNA expression of mNSP, which would fit with the hypothesis of increased in biosynthesis of ER membrane; however, decreases in expression of mNSP would not follow this hypothesis. That is, ethanol may not have a generalized effect on ER membranes. However, neither signal peptidase, GRP78, nor GRP94 have been investigated for ethanol alterations in gene expression in specific brain regions; these proteins may also show region-specific directional changes. Although it is not a resident of the ER, increased expression of Hsc70 mRNA has been observed (Miles et al., 1992). Hsc70 is a molecular chaperone, assisting in protein folding. Increases in theses molecular chaperones suggest there may a need for increased proteins (e.g., structural or metabolic proteins) as the cells adapt to the persistence of ethanol.

Although specific protein targets of Hsc70, GRPs, or NSP are not known, it would be interesting to determine if any of these ethanol regulated products interact with the candidate genes implicated in ethanol dependence and withdrawal (e.g., $GABA_A$ or NMDA receptors, calcium channels, G-proteins).

Magnitude of changes in expression levels

Another interesting characteristic of the ethanol-mediated effect on NSP expression was the magnitude of expression. Indeed, the significant increases and decreases were generally about 15-30% above or below air-exposed mice (with the exception of WSP-2 mice). In some respects, this may not appear to be very impressive; however, it is not known how protein levels are affected. Since these transcripts are constitutively expressed

at a fairly high level (based on intensity of hybridization), the decrease in expression may have a profound effect on protein levels. Since the function of NSP proteins is not known it is not possible to predict what effect decreasing the final product would have on cell function.

The effect of ethanol on the magnitude of gene expression is variable. The ethanol effect on the mNSP transcript levels were near the range observed for some other ethanol-regulated genes. Hsc70 was increased about 30% -50% above control, depending on length of ethanol exposure (Miles et al., 1991). $GABA_A$ receptor subunit expression in WSP and WSR mice was altered 40-60% by ethanol (Buck et al., 1991). Some products show quite large increases in expression; GRP78 and GRP94 have 150-200% increases over control (non-ethanol treated cells) (Miles et al., 1994). Mitochondrial NADH dehydrogenase mRNA levels were increased 2.4 fold over control in hippocampus of rats following chronic ethanol treatment; in cortex, an increase of ~1.2 fold was observed and considered to be unaffected by ethanol treatment (Chen et al., 1997). Ding et al. (1996) observed an increase of 125-150% in c-jun and jun D in SH-SY5Y cells exposed to ethanol for 4 days. NMDA receptor subunit NR2A mRNA was decresed 25% in cerebellum of ethanol-treated mice; however there was not a cncomitant change in protein levelsof this subunit in cerebellum (Snell et al., 1996).

In general, it appears that the observed effect of ethanol on relative mRNA levels of mNSP are not dissimilar to some other ethanol-regulated products. Several factors can influence the magnitude of expression. There may be temporal changes in expression; measuring the relative abundance at a specific timepoint may not reflect the greatest changes from control. Differences in the route of administration of ethanol may also effect mRNA levels. Methods such as liquid diets do not necessarily maintain animals at a

constant BEC level; fluctuations in BEC levels over the course of ethanol administration may affect the transcription processes.

Role of BEC

In general, the effect of ethanol in all brain regions was greatest in WSP-2. It could be argued that there may be a dose-dependent effect of ethanol on the expression of these transcripts since the BEC values for WSP-2 tended to be higher than in the other lines. This is plausible but seems unlikely since similar effects of ethanol on expression of all transcripts was not observed.

In particular, the expression difference observed in cerebellum, both between the transcripts and between genotype is not consistent with this explanation. DBA/2J and C57BL/6J mice (used for cerebellum analysis) had very similar BECs yet had very different expression patterns following ethanol. The BEC differences between WSP and WSR mice was very similar to the difference between WSP and DBA/2J mice, although different effects of ethanol on transcription were observed between these combinations. Taken together, it seems that the differential effect of ethanol on the expression of the transcripts and between brain regions and genotype are not effects of the difference in total ethanol exposure but derive from regulatory mechanisms associated with ethanol exposure.

Summary

The modest increase in expression by ethanol observed from the differential display gel was confirmed on whole brain RNA-containing Northern blots from WSP mice. Brain region, genotype, and transcript specific ethanol-regulated expression was found. Increased expression was observed predominantly in hippocampus and cortex; however, expression

changes were not consistent with genotype or withdrawal phenotype in these regions. In the cerebellum a decrease in expression was observed in mice that exhibit a robust withdrawal syndrome, characterized by severe HICs; no change or increase in expression was observed in mice that do not exhibit severe HICs. These results suggest that a decrease in expression of the 3.0 kb transcript of mNSP may play a role in determining susceptibility severe ethanol withdrawal. The mechanism(s) by which ethanol is exerting this effect is not known. Mechanisms of transcriptional regulation of mNSP have not been identified.

In general, the results indicate that mNSP is an ethanol-regulated gene, which may contribute to the development and/or expression of physical dependence. This gene joins the list of ethanol-regulated genes expressed in the brain including neurotransmitter receptors, IEGs, metabolic enzymes, and molecular chaperones. It is likely that a combination of these effects is involved in mediating withdrawal severity.

Future directions

Studying the time course of ethanol regulation would give insight as to whether regulation of these products occurs soon after ethanol exposure or as a result of the continued exposure, as well as whether the changes persist into the withdrawal period. Preliminary evidence from whole brain of WSP and WSR mice that have been withdrawn from ethanol, without experiencing overt convulsions, suggests that the expression of NSP in WSP mice decreased at 6 hours after withdrawal but did not return to control levels. It is not known whether there are changes in the specific brain regions examined here. An important feature of expression analysis is determining changes in protein levels in parallel with alterations in mRNA levels. Thus, it will be important to assess changes in NSP-A and NSP-C protein in these mouse lines to determine if they reflect the observed alterations in

gene expression. It is assumed that these changes in mRNA would be reflected as changes in protein levels but this may not be the case if protein stability is altered or if the mRNA translation is affected.

In addition to determining the temporal sequence of expression regulation by ethanol, a greater spatial resolution would be beneficial. *In situ* hybridization would allow the simultaneous assessment of many brain regions. However, because the cDNA probe used for the current analyses detected two transcripts, full length cDNA sequence would need to be performed to determine regions that would detect only one of the transcripts.

The differential results in terms of transcript regulation suggests that there may be independent promoter regions upstream from the start of transcription for these two transcripts. These promoter regions may differ in ethanol responsiveness within a brain region. Between brain regions, a more complex scenario was revealed. However, because these studies were performed in selected lines of mice, this may directly reflect the results of changes in gene frequency. Sequencing this gene in WSP and WSR mice would assist in identifying allelic variation. If the promoter regions were different, functional assays could be performed to identify regions that were ethanol-responsive and had the ability to confer the response.

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