The Kappa-Opioid Receptor System: A Role in Ethanol Withdrawal Severity?

by

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List of Abbreviations

AA	alcohol preferring
Acb	nucleus accumbens
AcbSh	nucleus accumbens, shell
AcbC	nucleus accumbens, core
AMPA	amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANA	alcohol avoiding
ANOVA	analysis of variance
AR	autoradiography
AUC	area under the curve
B6	C57BL/6J
BECs	blood ethanol concentrations
bp	base pair
Ca^{2+}	calcium
cDNA	complementary deoxyribonucleic acid
Cg	cingulate cortex
Cl_	claustrum
Cl^-	chloride
CNS	central nervous system
CPP	conditioned place preference
CPu	caudate putamen
CTA	conditioned taste aversion
δ	delta
D2	DBA/2J
DEn	dorsal endopiriform nucleus
DEPC	diethylpyrocarbonate
DLU/mm^2	digital light units per millimeter squared
$DOP\text{-}R^{aa}$	delta opioid receptor
dpm	decays per minute
d-NTP	deoxy-nucleotidetriphosphate
DSM-IV	Diagnostic and Statistical Manual, Fourth Edition
DZ	dizygotic
e-EPSP	epileptiform excitatory post synaptic potential
EL	epilepsy like
EtOH	ethanol, alcohol
EtOH-6	ethanol withdrawn
EtOH-0	ethanol intoxicated
FF	face and forelimb clonus
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCRs	G-protein coupled receptors

[a] Opioid receptor nomenclature based on recommendations of International Union of Pharmacology Nomenclature for Opioid Receptors (http://www.iuphar.org).

HCl	hydrochloride
HIC	handling induced convulsion
HS	heterogeneous stock
ICV	intracerebroventricular
ip	intraperitoneal
κ	kappa
KA	kainate
KOP-R	kappa opioid receptor
KPBS	potassium phosphate buffered saline
K^+	potassium
*l*AcbSh	lateral nucleus accumbens, shell
L-DOPA	levodopa
μ	mu
*m*AcbSh	medial nucleus accumbens shell
MC	myoclonic twitch
min	minute
MOP-R	mu opioid receptor
MR	maximum resolution
MZ	monozygotic
*Nco*I	*Nocardia corallina*
NMDA	N-methyl-D-aspartate
nor-BNI	nor-binaltorphimine
Pdyn	prodynorphin mRNA
PEAK HIC	peak handling induced convulsion
Penk	proenkephalin
Pir	piriform cortex
PCR	polymerase chain reaction
Pomc	proopiomelanocortin
DTT	dithiothreitol
PTZ	pentylenetetrazole
Pyr	pyrazole
RB	running bouncing
RNase	ribonuclease
rRNA	ribosomal ribonucleic aicid
RT	room temperature
*Sal*I	*Streptomyces albus*
sc	subcutaneous
SD	Sprague-Dawley
SN	substantia nigra
SNP	Sardinian non-preferring
SP	Sardinian preferring
SDS	sodium dodecyl sulfate
SR	super resolution
SS	seizure sensitive
SSC	saline sodium citrate
TBPS	[35S]t-butylbicyclophosphorothionate

TE	Tris-EDTA
TEA	triethanolamine
THE	tonic hindlimb extension
Tu	olfactory tubercle
U50	U-50,488H
VTA	ventral tegmental area
WSP	Withdrawal Seizure-Prone
WSR	Withdrawal Seizure-Resistant

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Abstract

Alcohol (ethanol, EtOH) dependence (alcoholism) is a significant and costly social problem. A great deal of emphasis has been placed on understanding the mechanisms that contribute to EtOH dependence, but far less is known about the neurochemical and genetic alterations contributing to the severity of EtOH withdrawal. This dissertation presents a molecular pharmacological and behavioral characterization of the effects of EtOH exposure and withdrawal on the kappa opioid receptor (KOP-R) system in Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) mice. These selectively bred mouse lines were derived from a genetically heterogeneous founder population, which was selectively bred over multiple generations to create lines that exhibit mild and severe EtOH withdrawal handling induced convulsions (HICs) upon removal from chronic EtOH vapor exposure. The kappa opioid receptor system was selected based on its proposed role in the modulation of other seizure types, where it has been observed that increased kappa opioid receptor activity can be either pro- or anti-convulsant. We hypothesized that the KOP-R system is activated more robustly in WSP mice compared to their WSR counterparts, and this activity reflects a compensatory response to the increased seizure sensitivity of these mice. At the molecular pharmacological level we characterized two components of this system: prodynorphin (*Pdyn*) mRNA: the precursor for the endogenous KOP-R peptide ligand dynorphin, and the KOP-R.

Whole brain Northern blot analysis and *in situ* hybridization of Pdyn mRNA, along with KOP-R autoradiography, examined overall and brain-region specific locations in which differential expression of this mRNA and/or receptor occurred. At the whole

brain level, a significant increase in Pdyn mRNA abundance was observed in WSP mice, while non-significant increases in Pdyn mRNA were observed in WSR mice. In situ hybridization in the caudate-putamen, nucleus accumbens core and shell, olfactory tubercle, and piriform cortex confirmed our findings in whole brain, with significant increases in Pdyn again occurring in nearly every brain region examined. In contrast to the whole brain findings, however, significant effects of withdrawal were present primarily in WSP-1 mice. Smaller increases in Pdyn mRNA were observed in WSP-2 mice and non-significant decreases in *Pdyn* mRNA were often observed in WSR mice. Theses findings suggest that the KOP-R system was activated to a greater extent in WSP mice during EtOH withdrawal. Increased Pdyn mRNA in brain regions of seizure-susceptible WSP mice was likely a response to depletion of endogeneous dynorphin peptide stores. However, it is unclear why this response did not result in seizure suppression in these animals. One possible explanation was that there were differences in KOP-R density among the lines, thus, we characterized the KOP-R in these lines as well.

Examination of the KOP-R system in these lines revealed more complex EtOH effects. Significant increases in KOP-R abundance were observed during EtOH withdrawal in the caudate-putamen, lateral nucleus accumbens shell and olfactory tubercle of WSP-1 mice, and EtOH exposure increased KOP-R density in the caudate-putamen of WSP-1 mice as well. EtOH exposure also increased the KOP-R density in the cingulate cortex of WSR mice. Finally, basal levels of KOP-R were significantly different among these lines; most WSR-2 brain regions tended to contain greater KOP-R densities than both WSP lines and the WSR-1 line. Specifically, WSR-2 mice had the highest levels of KOP-R in the lateral nucleus accumbens shell, olfactory tubercle, and

dorsal endopiriform nucleus. There was also a trend for this effect in the caudate-putamen. In the cingulate cortex opposite patterns of expression were observed between the replicates, with WSP-1<WSR-1, while WSP-2>WSR-2. In the medial nucleus accumbens shell, WSP-2 mice displayed significantly less KOP-R than the remaining three lines, while in the claustrum and dorsal endopiriform nucleus WSP-1 mice displayed significantly lower KOP-R densities. The levels of basal KOP-R in the dorsal endopiriform nucleus were particularly interesting because a gradient of expression was observed, increasing from least to most with WSP-1<WSP-2<WSR-1=WSR-2. This corresponds to a similar graded HIC response among the lines when the withdrawal scores are corrected for baseline HIC differences. These findings suggested that KOP-R activation in the dorsal endopiriform nucleus might be an important mechanism for HIC seizure suppression, and that the lower density of KOP-Rs in WSP mice might predispose them to more severe EtOH withdrawal HICs.

Finally, we examined the effects of KOP-R agonist and antagonist administration in these lines. Similar to our molecular characterizations, effects were observed in replicate one animals. Administration of the KOP-R antagonist nor-BNI 5-6 days prior to the onset of withdrawal resulted in a significant increase in HIC severity in WSR-1 mice during EtOH withdrawal. In contrast, no effects of nor-BNI were observed in WSR-2 mice, and both WSP lines were unaffected by pretreatment with this KOP-R antagonist. Importantly, the effects of nor-BNI were only observed in animals that had been exposed to EtOH, thus the nor-BNI induced increases in HIC severity in WSR-1 mice appear to have been an EtOH-specific response, rather than a global change in HIC susceptibility in WSR mice following nor-BNI administration. KOP-R agonist administration (U50) in

WSP and WSR mice altered HICs in WSP mice, rather than in WSR mice. Thus, the observed effects of the agonist were complementary to those of the KOP-R antagonist. That is, while antagonist administration potentiated HICs in WSR mice, agonist administration attenuated HICs in WSP mice. In contrast to an EtOH-specific effect of nor-BNI, U50 administration resulted in a non-specific reduction in seizure severity. That is, both EtOH naïve and EtOH exposed WSP mice showed decreased HIC severity during withdrawal following administration of U50 consistent with an anti-convulsant effect of this drug. The results of these studies further support a modulatory role of the KOP-R system in EtOH withdrawal seizures. Based on the suppressive effect of the KOP-R agonist on basal HICs in the WSP mice, these results also suggest that this system may participate in general seizure activity as well. A better understanding of the role the KOP-R system plays in EtOH withdrawal severity may eventually aid in the develop of novel therapeutic agents for the treatment of alcohol dependence and withdrawal.

Chapter I
Introduction

In every saloon, there could be a costs jar. For every dollar you push across the bar, two bucks would go into the jar to pay for the damages. That would cover the cost of the city cops arresting and jailing a drunken driver — or repairing the damages in a DUI accident."

Excerpted from: Alcohol: Cradle to Grave
Eric Newhouse, Great Falls Tribune

Alcohol dependence is a significant social problem

Alcohol (ethanol; EtOH) dependence is a disease characterized by compulsive drug seeking even in the presence of negative consequences (First, 2000). As highlighted by the above quote, the expenses of this problem are enormous. Every year Americans spend nearly 185 billion dollars dealing with the effects of this disease, and this dollar value doesn't include hidden costs such as profits lost by employers, policing, and counseling and therapy for the addict and their family members (Newhouse, 1999; Harwood, 2000; NIAAA, 2001). Clinically, complications related to alcohol dependence result in substantial costs for society. It was estimated that in 1999, 18.9 billion dollars were attributable to expenditures made to treat "the adverse medical consequences of alcohol consumption" (Harwood, 2000; NIAAA, 2001). In addition, the National Institute on Alcohol Abuse and Alcoholism estimates that each year more than 100,000 people die as either a direct or indirect consequence of alcohol abuse.

Physiological aspects of dependence and withdrawal

During withdrawal from alcohol after chronic exposure, both physiological and psychological symptoms occur. These range from mild tremor and anxiety to severe physical symptoms that include alcohol withdrawal seizures and delirium tremens (Table

1). Severe alcohol withdrawal often requires medical treatment, and withdrawal symptoms are included among the criteria for a diagnosis of alcohol dependence in the DSM-IV (First, 2000, Table 2). Unlike withdrawal from some drugs of abuse, the physical manifestations of severe EtOH withdrawal can be life-threatening. Thus, it is likely that alcoholics who are physically dependent upon alcohol contribute significantly to the costs already noted. Additionally, it has been observed that "a past history of tolerance or withdrawal is associated with a more severe clinical course (i.e., an earlier onset of dependence, higher levels of substance intake, and a greater number of substance-related problems)," (First, 2000). The presence of physiological withdrawal signs also identifies alcohol dependent patients that may require more specialized medical care, and these symptoms can be indicative of greater relapse liability (Langenbucher et al., 1997; Langenbucher et al., 2000; Schuckit et al., 2003). These observations highlight the significant need for a better mechanistic understanding of the neurochemical alterations contributing to alcohol withdrawal severity and alcoholism in order to develop better methods for treating this potentially life threatening event and reducing relapse.

Table 1. DSM-IV Criteria for Alcohol Withdrawal

(A) Cessation of (or reduction in) alcohol use that has been heavy and prolonged.

(B) Two (or more) of the following, developing within several hours to a few days after Criterion A:

(1) autonomic hyperactivity (e.g., sweating or pulse rate greater than 100)
(2) increased hand tremor
(3) insomnia
(4) nausea or vomiting
(5) transient visual, tactile, or auditory hallucinations or illusions
(6) psychomotor agitation
(7) anxiety
(8) grand mal seizures

(C) The symptoms in Criterion B cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

(D) The symptoms are not due to a general medical condition and are not better accounted for by another mental disorder.

Adapted from (First, 2000).

Table 2. DSM-IV Criteria for a diagnosis of alcohol dependence

A maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by three (or more) of the following, occurring at any time in the same 12-month period:
(1) tolerance, as defined by either of the following: **(a)** a need for markedly increased amounts of the substance to achieve intoxication or desired effect **(b)** markedly diminished effect with continued use of the same amount of the substance
(2) withdrawal, as manifested by either of the following: **(a)** the characteristic withdrawal syndrome for the substance **(b)** the same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms
(3) the substance is often taken in larger amounts or over a longer period than was intended
(4) there is a persistent desire or unsuccessful efforts to cut down or control substance use
(5) a great deal of time is spent in activities necessary to obtain the substance (e.g., visiting multiple doctors or driving long distances), use the substance (e.g., chain-smoking), or recover from its effects
(6) important social, occupational, or recreational activities are given up or reduced because of substance use
(7) the substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance (e.g., continued drinking despite recognition that an ulcer was made worse by alcohol consumption)

Adapted from (First, 2000).

Dopaminergic reward pathways and psychological aspects of EtOH dependence and withdrawal

While the presence or absence of physical withdrawal symptoms during withdrawal is dependent upon the particular drug being abused, the production of negative affective states such as dysphoria can be observed during withdrawal from all drugs of abuse including EtOH (Koob, 2000). This effect is due to alterations in the function of the mesolimbic dopamine system, a reward pathway activated by all drugs of abuse (Nestler, 2001).

Although the mechanisms by which an abused drug alters function of the mesolimbic reward system is not identical for all drugs, the neurochemical endpoint is the same: there is increased release of dopamine within the nucleus accumbens (Acb) (Koob et al., 1998a). This neurochemical effect contributes to positive affective states, and contributes to the rewarding effects of all drugs of abuse. In contrast, during a subsequent withdrawal period, dopamine release is decreased. This reduced dopaminergic tone can result in a negative affective state, which in turn promotes drug seeking behavior and relapse (Nestler, 2001).

Reward dysregulation and withdrawal

One theory that attempts to explain the psychological component of addiction suggests that in the presence of chronic drug exposure, a neuroadaptive process occurs that leads to the establishment of a new hedonic homeostatic setpoint (Koob and Le Moal, 1997; Koob, 2003). Upon withdrawal of abused drugs, an opposing negative affective state is observed that is believed to be a major underlying contributor to relapse.

This opponent-process theory was first suggested by Solomon and colleagues (Solomon and Corbit, 1974; Solomon, 1977; reviewed in Robinson and Berridge, 2003). Briefly, this theory suggested that the use of abused drugs produced a rewarding affective state that the authors termed the "A" state, which is opposed by the activation of a negative "B" state, which serves to return the system to its normal homeostatic state. The sum of these two opponent processes determines the affective state experienced by the individual. During the early phases of drug abuse, the "A" process predominates, resulting in the production of reward, but in addicted individuals the "B" processes prevail, and the net result is the production of a negative affective state that promotes further drug use. Koob and colleagues expanded upon this theory to include the concept of allostasis, which he defines as "maintenance of stability outside of the normal homeostatic range, where an organism must vary all the parameters of its physiological systems to match them appropriately to chronic demands" (Koob and Le Moal, 2001). Thus, Koob posited that a new (negative) pathological set point is achieved that, upon removal of the drug, produces dysphoria. This negative affective state contributes to the development of alcohol and drug dependence and is likely to play a significant role in alcoholic relapse. Thus, a further understanding of the neurochemical mechanisms that contribute to the affective states produced during withdrawal should also help to identify potential therapeutic targets in the prevention of alcoholic relapse.

There is a genetic contribution to alcoholism

It has been well documented in the literature that alcoholism is a complex disease influenced by both genetic and environmental factors. Studies of adult children of alcoholics have shown a 3-4 fold increase in risk to develop alcoholism in this population

when compared to the general public (Goodwin et al., 1973; Cotton, 1979; Schuckit, 1999). Much of the early data suggesting a genetic contribution to alcoholism was gathered from adoption and twin studies (Goodwin et al., 1973; Cotton, 1979). For example, early studies examined the risk for developing alcoholism in adopted away offspring of alcoholics that were raised in a home with a non-biological relative. When compared to adopted children who were not born to alcoholic parents, a significantly increased risk to become alcoholic was revealed in the child born to the alcoholic parent (Goodwin et al., 1973; Cloninger et al., 1981). This finding suggests the genetic makeup of the child contributed to their increased risk for alcoholism since these children were more likely to become alcoholic even when raised apart from an environment that might contribute to a propensity to develop alcoholism.

Twin studies also support the presence of a genetic contribution to alcoholism. Twin studies involve comparing monozygotic (MZ) twins, dizygotic (DZ) twins, and the general population. MZ twins share identical genetics, while DZ twins generally share half of their alleles, and are thus similar to any other sibling with the exception of having shared to same pre-natal environment. A tendency for greater concordance between MZ twins than between DZ twins and the general population indicates a genetic contribution to the trait being studied (Heath, 1995). An early study performed in a Swedish population revealed that the MZ twin of an alcoholic parent had a risk ratio of 9.1, and DZ twins had a risk ratio of 6.2 (Kaij, 1960). These ratios provide an index of the increased risk to develop alcoholism when compared to the general population, and again support the presence of a genetic contribution to alcoholism.

Animal models used to study alcohol related traits

More recent approaches have harnessed the power of animal models to explore this complex genetic disorder (Crabbe, 1989; Crabbe and Belknap, 1992; Browman and Crabbe, 1999). For example, a large number of inbred (and therefore genetically identical) mouse strains exist, that show great variability in their response to EtOH and EtOH withdrawal. Another important genetic animal model for studying EtOH related traits is the selected line. Both inbred and selected lines will be described in further detail later. Because animal models can be examined under tightly controlled conditions, and because large populations of genetically identical animals can be produced, animal models make it possible to examine specific genetic questions at the neurochemical level that are not possible in human subjects. In this way, animal models generate data that are often complementary to observations made in humans. The convergence of similar findings across multiple animal models can provide strong evidence that a particular neurotransmitter system or biochemical pathway participates in the production of an EtOH related response, and suggest potential targets for examination in human populations. While no single line is a perfect model of a complex disease such as alcoholism, they provide important insight into the genetic puzzle that is alcoholism, and continue to contribute significantly to our understanding of the multiple molecular alterations that can contribute to this disorder.

Inbred strains and alcohol related responses

Individuals within an inbred strain are, by definition, genetically identical. Inbreeding is usually achieved by carrying out brother-sister mating over multiple generations. Following application of this breeding scheme for twenty or more

generations, a genetically identical population of animals is produced that are homozygous at all genetic loci (Crabbe and Belknap, 1992). When a particular characteristic is examined in these animals under identical environmental conditions, differences within an inbred strain reflect the contribution of environmental factors, while differences between inbred strains represent a genetic contribution to the character under investigation (Crabbe, 1989).

C57BL/6J and DBA/2J mice are examples of inbred strains

Two well-characterized inbred mouse strains used in studies that examine genetic and molecular contributions to EtOH-related traits are the C57BL/6J (B6) and the DBA/2J (D2) strains. The B6 inbred strain shows a preference for EtOH in a two-bottle choice paradigm; in contrast, the D2 inbred strain avoids consuming EtOH when given such a choice (Fuller, 1964; McClearn, 1968; Rodgers, 1972; Belknap et al., 1993). With respect to EtOH withdrawal, it has been observed that B6 mice undergo mild EtOH-withdrawal handling induced convulsions (HICs), which are a measure of physical dependence upon EtOH (Goldstein and Pal, 1971). In contrast, D2 animals exhibit significant HICs during withdrawal from EtOH. Thus, B6 mice that exhibit preference for EtOH exhibit minimal HICs, while D2 mice avoid EtOH consumption, and exhibit severe HICs during EtOH withdrawal (Crabbe et al., 1980; Crabbe et al., 1994). This finding suggests an inverse genetic relationship between EtOH preference and withdrawal severity that is supported by a rapidly growing body of evidence (Crabbe et al., 1983c; Metten and Crabbe, 1994; Chester et al., 1998; Metten et al., 1998; Chester et al., 2002; Chester et al., 2003).

Selected lines and alcohol related responses

Selected lines represent another powerful tool created for studying the genetic contribution to particular phenotypes such as alcohol-related traits. These lines are generally rat or mouse populations that have been selectively bred to study discrete components of the alcohol response. These lines are bred under selection pressure over multiple generations to create two highly divergent groups displaying the extremes of a characteristic of interest. The general approach to selection involves measuring a particular trait of interest in a genetically heterogeneous founder population. Those animals that score at the two extremes of a normally distributed trait are bred with equivalently scoring animals. Thus, for example, animals that exhibit a "high" response are bred with other high responders, while those that exhibit a "low" response are bred with low responders. The measurement of the trait of interest and selective breeding of animals from the two population extremes is continued for multiple generations, and over time, a divergence from the mean of the founder population is observed, such that the mean of each group approaches the extreme ends of the original score distribution. A diagram depicting the hypothetical response to selection is presented in Figure 1. The ability to select for a trait is proof of principle that the trait of interest is under at least partial genetic control (Crabbe et al., 1990c). Because there is the possibility that alleles unrelated to the trait of interest will become fixed by chance during the selection process, it is important that selected lines be independently replicated (Crabbe et al., 1990c). The probability that the same gene will become fixed by chance in the second replicate is very low, thus the second line can be used to confirm initial findings.

In addition to providing a model that allows one to dissect the genetic contributions to the selected trait, selected lines are also useful models for identifying correlated responses to selection (Crabbe, 1989). This refers to the presence of an additional unselected trait that differs between the lines due to the influence of common genetic mechanisms for both traits. Thus, the identification of additional disparate responses to EtOH or other drugs in selected lines suggests that some of the genes contributing to the selected trait also contribute to the correlated response to selection (Crabbe and Belknap, 1992).

Withdrawal Seizure-Prone and Withdrawal Seizure-Resistant selected lines represent one example of selectively bred mouse lines

Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) mice are an example of selectively bred lines. Beginning with genetically heterogeneous stock (HS), the product of an eight way cross of inbred strains that included A, AKR, BALB/c, C3H/2, C57BL, DBA/2, Is/Bi, and RIII strains (McClearn et al., 1970), WSR and WSP mice were selected for mild and severe EtOH withdrawal seizure severity, respectively. HICs were used as the index of withdrawal severity following 72 hours of chronic EtOH vapor inhalation. HICs are discrete behaviors that vary in severity based on the intensity of withdrawal, and thus provide a quantifiable response for the characterization of physical dependence (Goldstein and Pal, 1971). The scale used for quantification of this response and subsequent selection was based on a four-point scale published by Goldstein (Goldstein and Pal, 1971; Goldstein, 1972b), later modified to a seven-point scale as shown in Table 3. Following chronic EtOH exposure, withdrawal severity was measured in the offspring from the first cross by scoring HICs (Crabbe et al., 1985).

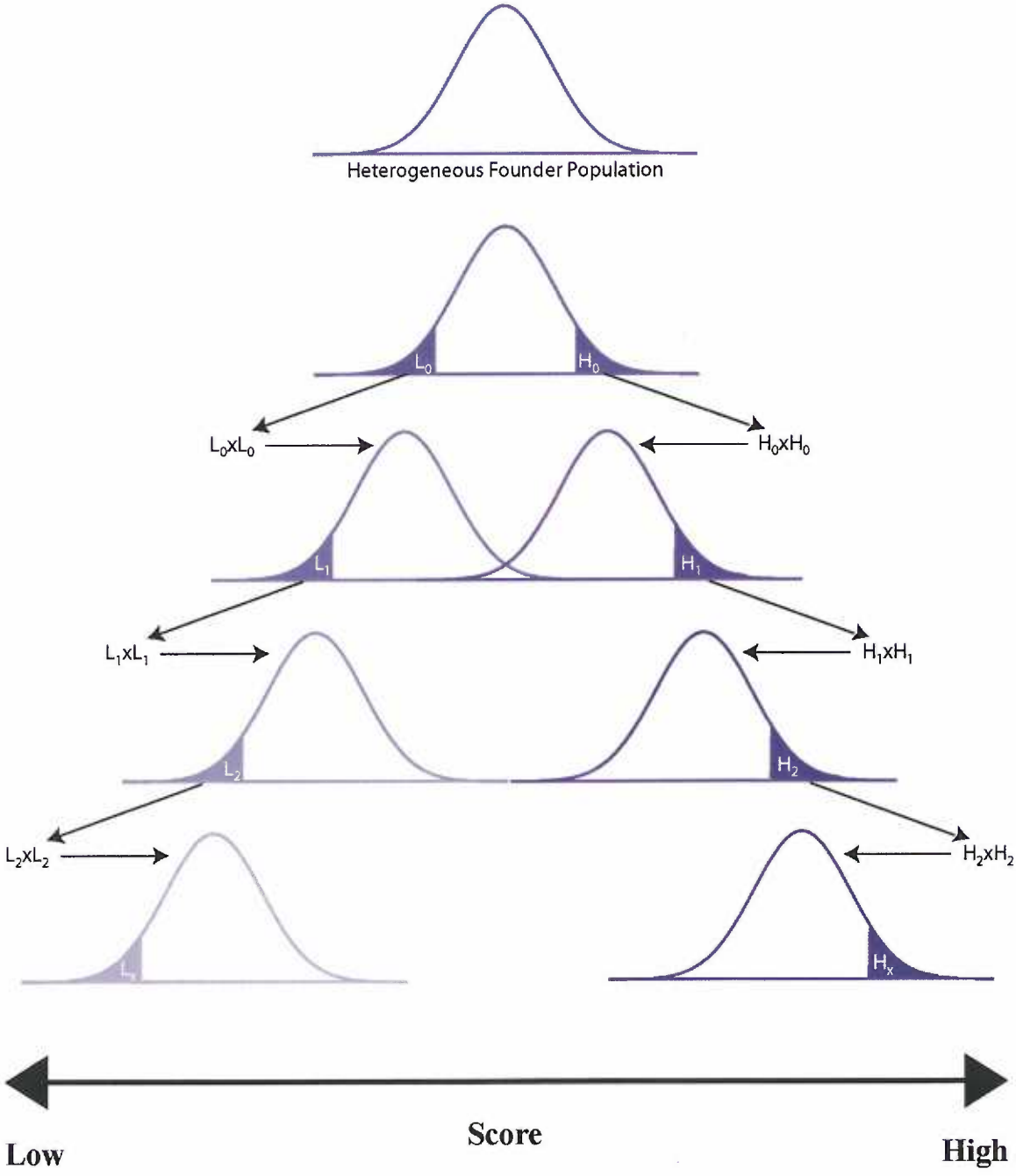

Figure 1. Selective Breeding Scheme. Selective breeding begins with a genetically heterogeneous founder population that is approximately normally distributed. From that population, low (L) and high (H) scoring animals are selected, and bred with like animals (L0xL0; H0xH0). From the resulting offspring (L1 and H1), animals exhibiting low scores are again selected from the L population to be crossed, and animals exhibiting high scores from the H population are again selected to be crossed. This process is repeated over multiple generations to produce two populations divergent on a particular trait. Drawing adapted from (Crabbe and Belknap, 1992).

Table 3. The ethanol withdrawal seizure scores

HIC Score	Behavioral Observations
0	No convulsion after gentle 180° spin
1	Only facial grimace after gentle 180° spin
2	Tonic convulsion elicited by gentle 180° spin
3	Tonic-clonic convulsion after 180° spin
4	Tonic convulsion when lifted by tail, no spin
5	Tonic-clonic convulsion when lifted by tail, no spin
6	Severe tonic-clonic convulsion when lifted by tail, no spin
7	Severe tonic-clonic convulsion elicited before lifting by the tail

Adapted from (Crabbe et al., 1991).

Those that achieved high HIC scores were bred with other offspring that had scored similarly, while those that exhibited low scores, and thus had mild withdrawal, were bred with other low scoring animals. This process was carried out over multiple generations, and the result was the production of two populations that expressed a 10-fold difference in their HIC scores during withdrawal from EtOH by the 11th selected generation (Crabbe and Phillips, 1993). Selection continued to the 26th generation, at which point selection was relaxed with no loss of the phenotype. This indicated that the selection pressure placed on these selected lines had fixed those genes important for EtOH withdrawal seizure severity and resistance in a homozygous state, making WSP and WSR mice a rich resource for examining the role of candidate genes in EtOH withdrawal convulsions and correlated responses to selection.

What is currently known about EtOH withdrawal mechanisms in WSP and WSR mice?

One of the earliest descriptions of the link between drug dependence and withdrawal was made by Himmelsbach in reference to morphine (Himmelsbach, 1941), when he noted that the physiological mechanisms that produce dependence alter homeostatic processes within the organism, eventually leading to tolerance. Upon withdrawal of drug, a characteristic withdrawal syndrome is produced. Thus, physiological dependence is inferred if tolerance and/or a characteristic withdrawal response is observed in an organism. It is not surprising then, that much of our knowledge regarding mechanisms of withdrawal stems from the examination of animal models of dependence. Neurotransmitter systems that have been extensively

characterized for their role in drug dependence and withdrawal include the glutamate, γ-aminobutyric acid (GABA), and dopamine receptor systems. Based on the observations of Himmelsbach, these systems are also likely to contribute to the alcohol withdrawal syndrome. A brief summary of the role these systems have been proposed to play in withdrawal is presented here, with a focus on the findings observed in WSP and WSR mice.

Glutamate

Ionotropic glutamate receptors are an important EtOH target (Davis and Wu, 2001). Three ionotropic glutamate receptor subtypes exist: N-methyl-D-aspartate (NMDA), kainate (KA), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Davis and Wu, 2001). Following acute exposure to EtOH, NMDA receptor function is inhibited, which contributes to the acute central nervous system depressant effects of EtOH. In contrast, chronic exposure to EtOH results in an increase in NMDA receptor expression and increased neuronal hyperexcitability (reviewed in Samson and Harris, 1992; Davis and Wu, 2001; Allgaier, 2002). Upon withdrawal, this neuroadaptive response persists, resulting in a hyperexcitable state. Glutamate receptors are coupled to voltage sensing ion channels. Binding of glutamate to the receptor, along with a concomitant release of a voltage dependent magnesium block, results in the opening of a channel that is highly permeable to calcium. Thus, up-regulation of NMDA receptors results in increased calcium permeability. Excessive calcium has been shown to be excitotoxic, and it has been suggested this may be a mechanism that contributes to the generation of EtOH withdrawal seizures and the neurodegeneration that can be observed in alcoholics (Samson and Harris, 1992; Davis and Wu, 2001).

Multiple investigations on NMDA receptors have been performed in WSP and WSR mice. At the cellular and molecular levels, conflicting results have emerged regarding the density of NMDA receptor binding sites in the lines, with early data suggesting that naïve WSP mice have a greater number of hippocampal NMDA binding sites than WSR mice (Valverius et al., 1990). In addition, the same authors observed significant increases in the density of MK-801 binding sites (i.e. NMDA sites) in the same brain region in both lines following six days of EtOH exposure via a liquid diet. However, similar to the observations in naïve animals, the NMDA receptor density in EtOH-treated WSR mice was significantly lower than that of WSP mice. In fact, the increases in receptor density within EtOH treated WSR mice did not exceed the density observed in EtOH naïve WSP mice (Valverius et al., 1990). Thus, the authors concluded that altered NMDA receptor density may contribute to the differences in EtOH withdrawal seizures observed between the lines. In contrast to these findings, under different binding conditions, no difference in MK-801 binding to NMDA receptors was observed in naïve WSP and WSR mice (Carter et al., 1995). Additionally, under conditions that produced HICs in these lines, EtOH exposure again failed to alter MK-801 binding. The authors suggested that the disparate findings were due to the absence of equilibrium binding conditions in the earlier investigations, and suggest that alterations in NMDA receptor binding in WSP and WSR mice do not contribute to the divergent EtOH withdrawal response of the lines (Carter et al., 1995).

Immunocytochemical analysis of glutamate levels in the CA1 region of the hippocampus of WSP and WSR mice revealed that in animals that had been carefully handled in order to avoid seizures (i.e., seizure naïve), there was increased basal density

of glutamate immunoreactivity in WSP mice when compared to WSR mice (Buckman and Meshul, 1997). The authors concluded that these observations reflected an inherent genetic difference in glutamate levels between the two lines, and that this difference might contribute to the divergent HIC severity observed in both naïve and EtOH-withdrawing WSP and WSR mice. *In situ* hybridization analysis of NMDA receptor subunits in the hippocampus and cortex of these lines revealed differences in regional distribution of the various subunits, but no differences in mRNA abundance for the subunits between the WSP and WSR lines (Mason et al., 2001). In addition, analysis of subunit protein expression using Western blot analysis and immunocytochemistry again revealed no difference in the expression of various subunits between the lines. The authors concluded that there was no correlation between subunit mRNA and protein expression and the divergent convulsions observed in WSP and WSR lines. Thus, based on the findings, it is difficult to definitively determine whether or not there is a correlation between EtOH withdrawal severity and the NMDA receptor system.

It has been demonstrated behaviorally that NMDA administered to naïve WSP and WSR mice resulted in minimal changes in HICs severity. However, during withdrawal from an acute 4 g/kg ip injection of EtOH, NMDA significantly and selectively increased HIC scores in WSP mice (Crabbe et al., 1990b; Crabbe et al., 1991a; Crabbe et al., 1993). In addition, treatment with the NMDA receptor antagonist dizocilpine decreased HIC severity during withdrawal from an acute, high-dose (4 g/kg) of EtOH in withdrawing WSP animals (Crabbe et al., 1993). Finally, during withdrawal from chronic EtOH vapor inhalation, differential sensitivities of NMDA and KA receptors were observed between the WSP and WSR mice, with WSR mice exhibiting

decreased sensitivity to NMDA, while WSP mice did not show alterations in sensitivity to NMDA from controls. Kainic acid administration to withdrawing WSP and WSR mice revealed that while both lines exhibited an increased sensitivity to the drug, WSP mice exhibited a trend toward a greater response to this drug during withdrawal than WSR mice (Finn and Crabbe, 1999). Taken together, the behavioral data suggests that activity at NMDA receptors may indeed contribute to EtOH withdrawal HICs.

GABA

Similar to the characterization of the NMDA receptor, thorough investigations on GABA have been performed in WSP and WSR mice. GABA is the major inhibitory neurotransmitter in the nervous system. Alcohol withdrawal is often treated with drugs active at $GABA_A$ receptors, such as benzodiazepenes, suggesting that this system participates in the manifestation of withdrawal. Acute exposure to alcohol potentiates the effects of GABA at $GABA_A$ receptors. This occurs via interactions of EtOH with the receptor, and subsequent potentiation of Cl^- flux. The net result is membrane hyperpolarization and inhibition of neuronal firing (Grobin et al., 1998; Davis and Wu, 2001). Following chronic exposure to EtOH, tolerance develops to the effects of EtOH at GABA receptors, resulting in reduced sensitivity of the receptor to this drug. The exact mechanisms that produce this response are not clear, but the result is a net decrease in inhibitory neurotransmission that persists upon the removal of EtOH. This disinhibition may in turn contribute to EtOH withdrawal hyperexcitability.

In WSP and WSR mice, a role for the GABA system in the expression of EtOH withdrawal seizures has been examined using a variety of approaches. Behaviorally, the effects of multiple $GABA_A$ antagonists have been examined in naïve animals.

Pentylenetetrazol (PTZ), bicuculline, and picrotoxin produce convulsions in both the WSP and WSR lines. However, WSP mice were more sensitive to these drugs than WSR mice as measured by HICs (Feller et al., 1988; Crabbe et al., 1991a). Interestingly, this finding was not accompanied by changes in the density or affinity of $GABA_A$ receptor binding sites, as measured by [^{35}S]t-butylbicyclophosphorothionate (TBPS) or [^{3}H]flunitrazepam binding (Feller et al., 1988), suggesting alterations in receptor abundance are not the mechanism contributing to the differing sensitivity of $GABA_A$ receptors in naïve WSP and WSR mice. In addition, immunocytochemical analysis of GABA immunoreactivity in the CA1 region of the hippocampus and the somatosensory cortex revealed no differences between the lines (Buckman and Meshul, 1997). At the mRNA level, differences in $GABA_A$ receptor subunits have been observed between both naïve and EtOH-treated WSP and WSR mice. While differences were observed between the two lines, the line exhibiting greater levels of subunit expression was dependent upon the brain region and subunit being examined (Buck et al., 1991b; Keir and Morrow, 1994). Administration of two $GABA_A$ receptor antagonists pentylenetetrazole (PTZ) or (+)bicuculline during EtOH withdrawal significantly decreased the threshold dose for onset of myoclonic twitch (MC) and face and forelimb clonus (FF), two convulsive measures thought to originate in forebrain circuits. This indicates an increased sensitivity to $GABA_A$ receptor antagonism in both of these lines. In addition, a significant decrease in the threshold to onset of PTZ- and (+)bicuculline-induced running bouncing (RB) clonus and tonic hindlimb extension (THE), convulsive measures originating in the hindbrain, was observed in both lines (Finn and Crabbe, 1999). The presence of an effect of these GABA antagonists in both lines during EtOH withdrawal suggest that

GABAergic mechanisms do not participate in the divergent EtOH withdrawal HICs measured in these animals. Thus, differences in the sensitivity of GABA receptors may contribute to the differing HIC severity observed in naïve WSP and WSR mice, however a role for GABA in EtOH withdrawal-related convulsions is more equivocal.

Dopamine

As discussed earlier, the dopaminergic reward system is a common target for all drugs of abuse, and activation of this system is likely to contribute to the addictive potential of abused drugs, including EtOH (Nestler, 2001). EtOH administration has been demonstrated to increase the firing of dopaminergic neurons of the ventral tegmental area (VTA) (Gessa et al., 1985; Brodie et al., 1990; Diana et al., 1993a), and EtOH exposure elicits an increase in the release of dopamine in the Acb, a key brain region in reward circuitry (Imperato and Di Chiara, 1986; Di Chiara and Imperato, 1988b). This increased Acb dopamine release has been hypothesized to contribute to the rewarding effects of EtOH. The converse appears to occur upon withdrawal from chronic EtOH; decreased activity of dopaminergic neurons of the VTA (Diana et al., 1992; Diana et al., 1993b; Shen, 2003), and a concurrent decrease in DA release in the Acb have both been observed in withdrawn rats (Imperato and Di Chiara, 1986; Rossetti et al., 1992; Diana et al., 1993b; Weiss et al., 1996). This decreased dopamine release has been hypothesized to contribute to relapse to EtOH drinking (Weiss and Porrino, 2002).

Thus, a role for the dopaminergic reward system in EtOH reward and relapse is likely, but is it possible that alterations in dopaminergic systems also contribute to physical withdrawal severity, specifically HICs, as well? Some early studies suggest that altered dopamine receptor function may indeed contribute to EtOH withdrawal related

HICs. Administration of dopamine or its precursor L-Dopa reduced HICs in mice undergoing withdrawal from EtOH inhalation, while administration of a dopamine antagonist, haloperidol increased HIC severity (Blum et al., 1976). More recent examinations demonstrated increases in striatal dopamine levels in rats that experienced audiogenic seizures during withdrawal from EtOH administered in a liquid diet compared to controls that received water (Mirovsky et al., 1995). A second study examined the effects of the dopamine D2 antagonist cyamemazine on HICs in mice withdrawn from EtOH liquid diet. The data also revealed a significant decrease in HIC severity in animals that received cyamemazine versus controls (Naassila et al., 1998). Thus, it appears that manipulations of dopamine signaling may alter both the affective and physical components of EtOH withdrawal.

To our knowledge, there have been no direct biochemical examinations of the dopamine receptor system and its contributions to withdrawal in WSP and WSR mice. However there is behavioral evidence suggesting that WSP and WSR exhibit different EtOH reward and aversion related responses as measured by conditioned place preference (CPP) paradigms, as well as conditioned taste aversion (CTA) studies (Crabbe et al., 1992; Chester et al., 1998). Given the probable contribution of the dopaminergic system to EtOH reward states, it is possible there are differences between WSP and WSR mice in dopaminergic function.

Are there other neurotransmitter candidates that may participate in EtOH withdrawal?

Based on the data described above, it appears that both GABAergic and glutamatergic, and possibly dopaminergic, mechanisms participate in the generation of EtOH withdrawal seizures. However, the lack of effective therapeutic interventions and the complex polygenic nature of alcohol related traits suggest that alcohol-related phenotypes are likely not attributable to activity only within these systems. Thus, it is of interest to identify other potential participants in EtOH withdrawal responses.

Alcohol drinking is one of the many correlated responses to selection that have been investigated in WSP and WSR mice. WSR mice drink more EtOH than WSP mice in a two-bottle choice paradigm (Kosobud et al., 1988). Thus, like the observations made in B6 and D2 mice, a similar inverse relationship between EtOH preference and EtOH withdrawal severity appears to be present in WSP and WSR mice, and it is possible that these traits are influenced by a common set of genes. Thus, genes that have been suggested to contribute to EtOH drinking behaviors may also contribute to EtOH withdrawal severity and serve as interesting targets for characterization.

The opioid receptor system: an additional contributor to EtOH withdrawal?

A particularly interesting candidate for investigation in WSP and WSR mice is the kappa-opioid receptor (KOP-R) system, which consists of prodynorphin mRNA (*Pdyn*), dynorphin peptide, and the KOP-R. The KOP-R is a member of the opioid receptor system, which includes at least three receptor subtypes known as the mu- (μ-, MOP-R), delta- (δ-, DOP-R), and kappa- (κ-, KOP-R) opioid receptors (reviewed in Narita et al.,

2001). Each of these receptors, in turn, has a preferred endogenous peptide ligand(s): beta-endorphin, enkephalins and dynorphins, respectively, which are derived from specific mRNA precursor molecules. Proopiomelanocortin (*Pomc*) is the precursor for beta-endorphins, proenkephalin (*Penk*) for the enkephalins, and prodynorphin (*Pdyn*) is the precursor molecule for dynorphin peptides (Table 4). It is important to note that the ligand-receptor relationship is not 1:1. Rather, these ligands binds preferentially to one receptor and exhibit much lower affinities to one or more of the remaining receptors (Table 4). Opioid receptors are widely distributed throughout the brain, with particularly high densities in limbic brain regions as well as thalamic nuclei and regions important for visceral function (Mansour et al., 1988). The distribution of the peptide ligands for these receptors is equally extensive (Khachaturian et al., 1985).

A variety of investigations have generated data supporting a link between EtOH dependence and the endogenous opioid system. Particularly interesting are data gathered in B6 and D2 mice. Specifically, analysis of the KOP-R system in select brain regions of naïve alcohol-avoiding D2 mice and naïve alcohol-preferring B6 mice revealed significantly higher levels of KOP-R mRNA, prodynorphin mRNA, dynorphin peptides and KOP-R in the Acb of D2 animals (Jamensky and Gianoulakis, 1997). It was proposed that this difference might contribute to the differing alcohol consumption observed between the two lines. However, given the apparent inverse correlation between EtOH preference and withdrawal severity, it is also possible that these alterations contribute to the differences in EtOH withdrawal severity observed between these inbred strains.

Table 4. Opioid receptor family

Receptor Type	Endogeneous Ligand	Large molecular weight peptide precursor (mRNA)	Relative Affinity of Peptide Ligand for Receptors
μ- (mu-, MOR, MOP)	β-endorphin	Proopiomelanocortin, (*Pomc*)	μ,δ ($\mu=\delta$)
δ- (delta-, DOR, DOP)	[Met]enkephalin		δ,μ ($\delta>>\mu$)
	[Leu]enkephalin	Proenkephalin, (*Penk*)	δ,μ ($\delta>>\mu$)
κ- (kappa-, KOR, KOP)	Dynorphin A(1-17)		κ
	Dynorphin A(1-8)		κ
	Dynorphin B		
	α-neoendorphin		
	β-neoendorphin	Prodynorphin, (*Pdyn*)	

Adapted from (Gianoulakis, 2004; Janecka et al., 2004).

Changes in components of the KOP-R system have also been implicated in seizure severity in other genetic seizure models. For example, a mouse model of epilepsy known as the EL (epilepsy-like) mouse has been compared to its parent strain, the ddY mouse, in an attempt to correlate differences in KOP-R abundance with seizure susceptibility. In adult animals that exhibit generalized seizures, greater levels of KOP-R were present in the cerebral cortex, hippocampus, striatum and amygdala of EL mice when compared to their seizure resistant ddY counterparts (Kai et al., 1998). The authors suggested that the increases in KOP-R were responsible for the predisposition toward developing seizures in EL mice, as opposed to the seizures causing an increase in the KOP-R levels. The authors also suggested that the increase in KOP-R levels might be due to decreased dynorphin abundance in EL mice. Increases in opioid receptor numbers have also been observed in the seizure-sensitive (SS) Mongolian gerbil, another genetic seizure model, when compared to their seizure-resistant counterparts. This increase was observed in both pre- and post-seizure SS animals. Again, it was suggested that this increase might be due to a deficit in endogenous ligand for the receptor, and that this deficit might contribute to the seizure sensitivity of this model (Lee et al., 1986).

Examinations in other seizure models have suggested a seizure-protective role for KOP-R ligands. Pre-administration of U-50488H, a KOP-R agonist, dose-dependently increased the seizure latency and decreased seizure duration in rats treated with systemic pilocarpine. Administration of the KOP-R antagonist nor-binaltorphimine increased the incidence of these seizures (Bausch et al., 1998). The same effect has also been observed in mice using two other KOP-R agonists: U69,593 and PD117,302 (Przewlocka et al., 1994b). Finally, the KOP-R system has been demonstrated to participate in a variety of

other chemically induced and electrical seizure models (reviewed in Simonato and Romualdi, 1996). However the role of the KOP-R system in EtOH withdrawal seizures remains relatively uncharacterized.

Interestingly, a limited characterization of the opioid receptor system has already occurred in WSP and WSR mice. However, this work focused on Met-enkephalin, an endogenous ligand for the DOP-R. Examination of Met-enkephalin levels in these lines revealed differences in the levels of this peptide in naïve animals; both WSP-1 and WSP-2 lines had significantly higher whole-brain levels of this peptide than their respective WSR counterparts (Plotkin et al., 2001). This difference persisted in EtOH-dependent replicate one animals, but not in withdrawing WSP-1 and WSR-1 lines, where levels of Met-enkephalin were increased in WSR-1 animals compared to that of the WSP-2 line. In contrast, this difference was not present in the EtOH-dependent WSP-2 and WSR-2 groups, but did re-emerge in withdrawing WSP-2 and WSR-2 mice. These findings suggest that basal differences in Met-enkephalin levels may contribute to withdrawal seizure susceptibility in these lines, and support the hypothesis that the opioid receptor system may participate in this phenotype. Thus, we sought to extend studies investigating the role of the opioid receptor system in the HIC severity of WSP and WSR mice to include the KOP-R system.

Another effect of the KOP-R system that has been well documented is its effect on affective states. The opioid system interacts with dopaminergic systems to modulate the rewarding effects of EtOH consumption and the dysphoria produced with drug withdrawal (reviewed in Herz, 1997, 1998). Activation of MOP and DOP receptors results in reward, while KOP-R activation produces dysphoria (Nestler, 2002). For

example, systemic administration of KOP-R agonists produces conditioned taste and place aversions in Sprague-Dawley (SD) rats (Mucha and Herz, 1985). Site-specific microinjection of KOP-R agonists, dynorphin or the dynorphin analog E-2078 into the Acb also produces conditioned place aversion in SD rats (Bals-Kubik et al., 1993). Introduction of KOP-R agonists into the Acb and caudate-putamen (CPu) of Sprague Dawley rats via microdialysis results in decreased dopamine in both of these areas. This suggests that the aversive effects observed in these paradigms are the result of KOP-R mediated alterations within the dopaminergic systems that decrease reward (Di Chiara and Imperato, 1988a).

It has been hypothesized that an aversive state is produced during EtOH withdrawal, suggesting a possible role for the KOP-R system in this state. There is some evidence to support this hypothesis. *In situ* hybridization analysis performed in male Wistar rats demonstrated increases in *Pdyn* mRNA in the nucleus accumbens (Acb) following withdrawal from chronic EtOH administered in the drinking water (Przewlocka et al., 1997). Thus, increased dynorphin abundance in the Acb during EtOH withdrawal may result in a KOP-R mediated inhibition of dopaminergic Acb neurons. The decreased levels of dopamine would then contribute to the negative affect produced during EtOH withdrawal (Spanagel et al., 1992). Manipulations of the opioid receptor system have also been observed to affect EtOH-drinking behavior (reviewed in Gianoulakis, 1993). In addition, genetically selected lines of rats and mice that show differences in their preference for EtOH also show differential expression of opioid receptors, endogenous opioid receptor ligands, and mRNA precursors for ligand and receptor (de Waele et al., 1995; Jamensky and Gianoulakis, 1997; Marinelli et al., 2000).

Based on the extensive data suggesting a role for the KOP-R in seizure generation and potential participation in reward states, and the presence of disparate seizure severity and EtOH consumption in WSP and WSR mice, we have chosen to examine the KOP-R system in this animal model of EtOH withdrawal severity.

Rationale and Specific Aims:

The data presented in this thesis are the result of a multi-disciplinary approach consisting of both molecular pharmacological and behavioral neuroscience techniques. Using this combined approach, we examined the role of the KOP-R system in a genetic animal model of mild and severe EtOH withdrawal. Based on the data presented in the introduction, we hypothesized that KOP-R activation *is anti-convulsant, and that the endogenous KOP-R system is activated during withdrawal from chronic EtOH exposure to a greater extent in WSP mice versus WSR mice, reflecting a compensatory adaptive response occurring in an attempt to decrease HIC severity in WSP mice.* Three sets of studies were designed to test this hypothesis; their specific aims and a brief description of the approach taken to address each aim are briefly outlined below.

Specific aim one: Characterize Pdyn mRNA abundance in WSP and WSR mice during EtOH exposure and withdrawal in whole brain using Northern Blot analysis.

WSP and WSR mice were utilized as a mouse model of severe and mild EtOH withdrawal severity respectively, and were first examined for differences in *Pdyn* expression in whole brain. WSP and WSR mouse lines were exposed to 72 hours of chronic EtOH vapor exposure followed by brain harvest at distinct behavioral time points. Intoxicated animals were euthanized immediately upon removal from EtOH; an additional group of withdrawn animals were killed following six hours of withdrawal. This is a timepoint at which the WSP line begins to exhibit peak withdrawal symptoms (Finn and Crabbe, 1999). In this way we were able to distinguish between alterations in *Pdyn* mRNA that were due to EtOH exposure from those that were the result of EtOH

withdrawal. An alteration in the abundance of *Pdyn* mRNA in both replicates of one line, with no alterations or changes in the opposite direction within the other line would suggest a correlated response to selection. Such data would be consistent with a genetic contribution of the KOP-R system to EtOH withdrawal related phenotypes.

Specific aim two: Examine the effects of EtOH exposure and withdrawal on Pdyn and KOP-R expression in specific frontal forebrain regions believed to participate in seizure generation, withdrawal induced aversion and dysphoria, or both, using in situ hybridization and receptor autoradiography.

Because Northern blot analysis does not identify specific brain regions displaying altered *Pdyn* mRNA abundance, we examined the frontal forebrain using *in situ* hybridization and receptor autoradiography. These approaches allowed us to identify the effects of both EtOH exposure and withdrawal on *Pdyn* and KOP-R abundance in specific brain regions, and to test the hypothesis that alterations in the KOP-R system were genetically correlated with EtOH withdrawal-related phenotypes. By examining KOP-R system expression in specific brain regions, we would be able to suggest a potential role for this system in physical or affective components of EtOH withdrawal based on known biological function of the region. *In situ* hybridization and KOP-R autoradiography were utilized to examine levels of *Pdyn* and KOP-R respectively, in the Acb and the CPu. These brain regions are components of the mesolimbic dopaminergic reward pathway, and EtOH exposure- or withdrawal-induced alterations in these brain regions might modulate affective states in these lines via changes in KOP-R signaling. Furthermore, differences between the two lines in *Pdyn* abundance in these regions

would suggest that these states are differentially modulated and that affective responses to EtOH might have been selected in addition to seizure severity in these lines. The CPu also participates in motor control, thus activation of the KOP-R system in this brain region might also contribute to the divergent seizure susceptibility of these lines. In addition, the olfactory tubercle (Tu) was also examined for differences in both *Pdyn* and KOP-R abundance. This brain region participates in dopaminergic reward, but has also been implicated in the generation of seizure activity. Differences in *Pdyn* or KOP-R abundance between the lines in these brain regions would identify potential participants in EtOH-withdrawal seizure circuitry, and would suggest that the KOP-R system might contribute to the withdrawal severity of these lines. A few brain regions were only characterized using one of the two approaches, because either *Pdyn* or KOP-R expression was absent in these regions. Sites characterized using KOP-R autoradiography only included the cingulate cortex (Cg), claustrum (Cl) and the dorsal endopiriform nucleus (DEn), brain regions involved in seizure circuitry. An additional site that was characterized using only *in situ* hybridization for *Pdyn* was the piriform cortex (Pir) another brain region implicated in seizure circuitry.

Specific aim three: Explore the functional involvement of the KOP-R system in the generation and severity of EtOH withdrawal seizures by administering a KOP-R agonist and antagonist at various timepoints during EtOH exposure and withdrawal, followed by scoring of EtOH HICs to evaluate seizure severity.

In this series of studies we further explored the role of the KOP-R system in EtOH withdrawal seizures and their severity. The work described thus far has been

molecular in nature; in addition the animals of interest exhibit a distinct, observable, quantifiable behavioral phenotype in the HIC. As already stated, one of the goals of this dissertation work was to utilize molecular pharmacological and behavioral approaches to examine the role of the KOP-R system in EtOH withdrawal. Thus, WSP and WSR mice were exposed to 72 hours of chronic EtOH vapor inhalation and the effects of pharmacological intervention with the KOP-R agonist U-50,488H (U50) on HICs was examined. In addition, the effects of administration of a KOP-R antagonist nor-binaltorphimine (nor-BNI), on HICs were also examined. Because KOP-R agonist administration tends to be anti-convulsant, we hypothesized that agonist administration would be anti-convulsant in WSP mice, thus decreasing the HICs observed during EtOH-withdrawal. Blockade of KOP-R using the antagonist should exacerbate the HIC response of WSP mice, and may also result in the expression of HICs in WSR mice. Thus, both WSP and WSR lines were utilized in these studies to examine whether or not the HIC behavior of the lines could be manipulated to more closely resemble the HICs of their counterpart line.

Chapter II
Elevated Prodynorphin Expression Associated With Ethanol Withdrawal Convulsions

Preface

The following chapter contains data that been previously published. The data presentation and introduction and conclusion have been slightly modified to reflect suggestions made by thesis committee members, however the data previously published remains identical to that presented here. The reference for this article is Beadles-Bohling AS, Crabbe JC, Wiren KM (2000) Elevated prodynorphin expression associated with ethanol withdrawal convulsions. Neurochem Int 37:463-472.

Abstract

The hypothesis that KOP-R system activity may in part mediate convulsions exhibited during EtOH withdrawal was tested by exposing WSP and WSR mice to chronic EtOH. Whole brain was harvested for RNA isolation and prodynorphin mRNA steady-state levels in whole brain were examined using Northern blot analysis. The data revealed significantly increased levels of prodynorphin expression during withdrawal in mice susceptible to EtOH withdrawal convulsions, with non-significant increases in prodynorphin steady-state levels in mice resistant to EtOH withdrawal convulsions. These findings were not due to basal differences in prodynorphin expression between the WSP and WSR mice. To verify that the differences observed were not due to an EtOH-induced global alteration in gene transcription, mRNA levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase were measured. Glyceraldehyde-3-phosphate dehydrogenase expression was unchanged following both chronic exposure to EtOH and chronic exposure accompanied by withdrawal. These results extend our understanding of prodynorphin's role in generalized seizure activity to include EtOH withdrawal induced convulsions. Our findings suggest that prodynorphin expression is modulated during EtOH withdrawal convulsions, or alternatively, prodynorphin may mediate the severity of EtOH withdrawal convulsions.

Introduction

EtOH withdrawal is a physiological response to the absence of EtOH that is exhibited after an organism has become physically dependent upon alcohol. This response is believed to be a physical adaptation that the nervous system undergoes in the presence of EtOH (Himmelsbach, 1942) and is included in the National Institute on Alcohol Abuse and Alcoholism's definition of alcoholism. Withdrawal can be displayed in various forms, and in humans can include symptoms such as nausea, sweating, shakiness and anxiety (Victor and Adams, 1953; Victor, 1990), (http://silk.nih.gov/silks/niaaa1/publication/booklet.htm). In addition, estimates suggest that 4-7% of alcoholics (Hauser, 1990) suffer from seizures that can be life threatening and even fatal; therefore, it is important that we understand the mechanisms underlying EtOH dependence and withdrawal. The purpose of this study was to begin to elucidate the genes contributing to EtOH withdrawal convulsions. We hypothesized that EtOH withdrawal convulsions might be mediated by the endogenous KOP-R system and therefore we chose to characterize mRNA expression of the endogenous ligand, prodynorphin.

We utilized a model of EtOH dependence and withdrawal in combination with selectively bred mouse lines. Selective breeding was accomplished by starting with a genetically heterogeneous stock of mice that showed a normal distribution of the trait under selection. High and low scoring animals were selectively mated, such that high scoring males were mated with high scoring females, and low scoring males were mated with low scoring females. By exerting this pressure over multiple generations a divergent pair of lines was created. Ideally, the genes contributing to the trait of interest, and only

the genes contributing to this trait, would become fixed during this process, while all other alleles would be selected for randomly. Of course, it is possible that a set of genes might become fixed by chance early on in this selection process, thus it is very important that the selection be replicated. Thus, this approach results in two lines of animals divergent on a particular trait and a phenotypically identical replicate pair of lines. In this case, HS/Ibg mice were selectively bred for their sensitivity or resistance to handling induced convulsions (HICs) when withdrawn from chronic EtOH exposure. This produced the Withdrawal Seizure-Prone (WSP) and the Withdrawal Seizure-Resistant (WSR) mice (Crabbe et al., 1983a; Crabbe et al., 1985). Thus, when withdrawn, WSP mice show severe HICs whereas WSR mice show negligible HICs. The first replicate pair of lines is known as the WSP-1 and WSR-1 mice, replicate two lines are identified as the WSP-2 and WSR-2 mice (Crabbe et al., 1983a; Crabbe et al., 1985; Crabbe, 1987; Crabbe et al., 1990b; Crabbe and Phillips, 1993).

The WSP and WSR mouse lines serve as powerful tools for genetic and molecularly based research on alcoholism. From a genetic perspective, the ability to selectively breed animals and observe divergence on a trait suggests that selection pressure has been exerted on genes that contribute to the trait of interest. Because the WSP mice have been specifically selected for severe EtOH withdrawal convulsions, these animals are likely to produce an enhanced response of the systems involved with withdrawal. Furthermore, because of the genetic power of these selectively bred animals, an observation of alterations in both replicate WSP-1 and WSP-2 lines of mice, and an absence of these alterations in WSR mice, would strongly suggest a role for the KOP-R system in this selected withdrawal phenotype.

Prodynorphin was the gene chosen for study based on current evidence suggesting a role for the KOP-R system, and opioids in general, in seizures (Tortella et al., 1985; Caldecott-Hazard and Engel, 1987; Gale, 1988; Hong et al., 1993; Harrison et al., 1995; Lothman, 1996; Simonato and Romualdi, 1996; Hosford et al., 1997). This peptide is the endogenous ligand for the KOP-R, a member of the opioid receptor family. This family of receptors includes at least three receptor subtypes known as the KOP, MOP, and DOP receptors (for review, see Dhawan et al., 1996). Each receptor has its own endogenous peptide ligands: dynorphins, beta-endorphins, and enkephalins respectively. Studies of the KOP-R system have revealed that KOP-Rs are widely distributed throughout the brain, with particularly high densities in limbic brain regions as well as thalamic nuclei and regions important for visceral function (Mansour et al., 1988). The distribution of the peptide ligand for this receptor is equally extensive but not always co-localized (Khachaturian et al., 1985).

While little work has focused on the role of the KOP-R system in the convulsions exhibited during EtOH withdrawal, alterations in this system have been observed in other models of seizure disorders. For example, the hippocampus and amygdala of both seizure-naïve and seizure-experienced epilepsy-like (EL) mice contain significantly more KOP-Rs compared to ddY mice, their seizure-nonsusceptible parent strain (Kai et al., 1998). While these authors postulated that this was due to a decrease in dynorphin peptide abundance in these animals, which in turn might contribute to their seizure-susceptibility, they have not measured dynorphin levels. On the other hand, radioimmunoassay studies performed in another model, the seizure sensitive Mongolian gerbils, revealed significantly *higher* levels of dynorphin A peptide in the hippocampus

of both naïve and post-seizure animals compared to their corresponding seizure resistant counterparts (Lee et al., 1987). In Swiss Webster mice, intracerebroventricular injections of KOP-R agonists produced convulsions (Bansinath et al., 1991). Thus, depending upon the species and convulsive model used, KOP-R agonists can be either seizure-protective or seizure promoting (Frey, 1988; Fischer et al., 1993; Przewlocka et al., 1994b; Bausch et al., 1998).

Several studies have focused on the effects of kindled seizures on prodynorphin expression mRNA levels. Sixty minutes following a seizure in amygdala kindled rats, increased levels of prodynorphin mRNA were observed in hippocampus, striatum and hypothalamus (Romualdi et al., 1995). Prepiriform cortex kindling also altered prodynorphin levels. However, prodynorphin levels were decreased in the hippocampus with an increase or no change in prodynorphin observed in the striatum of these animals (Xie et al., 1989). In rats injected with kainic acid to produce seizures, an increase in hippocampal prodynorphin mRNA was observed 3 hours following the injection (Douglass et al., 1991). Taken together, these data support a role for the KOP-R system in seizure activity, but again suggest that the species and seizure model employed are important factors when examining alterations in the KOP-R system.

In terms of alcohol exposure, work performed in B6 mice using a liquid diet paradigm suggested that prodynorphin abundance was specifically increased in a variety of brain regions following chronic exposure to EtOH (Gulya et al., 1993). These inbred mice are known to consume high levels of alcohol when given a choice between EtOH and water (McClearn and Rodgers, 1959; Belknap et al., 1993). The findings of Gulya et al. (1993), along with findings that prodynorphin expression (Przewlocka et al., 1992;

Przewlocka et al., 1997) and dynorphin peptide abundance (Przewlocka et al., 1994a) were altered by EtOH exposure in rats support a role for the KOP-R system in animal models of EtOH exposure. In addition, previous work found that the concentration of met-enkephalin, an endogenous DOP-R ligand, was significantly greater in WSP mice than in WSR mice, which also supports a role for the opioid system in animal models that show altered responses to EtOH (Plotkin et al., 1998). Plotkin suggested there might be a link between increased met-enkephalin levels and susceptibility to EtOH withdrawal convulsions. However, this data was gathered from naïve animals, thus, the effects of EtOH withdrawal on met-enkephalin are uncharacterized.

Based on models of seizure activity and mouse models of EtOH consumption and EtOH withdrawal convulsions, we hypothesized that alterations in the KOP-R system expression might occur in animals susceptible to EtOH withdrawal convulsions. In the experiments presented here, the effects of both EtOH exposure and withdrawal on the KOP-R ligand, prodynorphin, were examined in WSP and WSR mice using Northern blot analysis. Using chronic EtOH vapor exposure, the phenomenon of EtOH withdrawal was separated from that of EtOH exposure to more clearly identify whether EtOH exposure, withdrawal, or both alter the KOP-R system in a mouse model of EtOH withdrawal convulsions.

Materials and Methods

All animal procedures and animal care were reviewed and approved by the Portland Oregon VA Medical Center Institutional Animal Care and Use Committee and met NIH guidelines for appropriate care and use of animals in research. All efforts were made to minimize animal suffering during these experiments and the minimum numbers of animals necessary to answer our questions were used.

Animal Subjects.

WSP and WSR mice were bred in the laboratory of J. C. Crabbe in Portland, OR. Drug-naïve adult male mice (age range 56-97 days) from selected generation 26 (filial generations G_{58}-G_{71}) were used. The WSP and WSR selective breeding protocol was replicated, thus there are two independently derived replicate WSP and WSR lines (Crabbe et al., 1983a; Crabbe et al., 1985; Crabbe, 1987; Crabbe et al., 1990b; Crabbe and Phillips, 1993). Mice of both replicates of the WSP (WSP-1 and WSP-2) and WSR (WSR-1 and WSR-2) lines were tested in these studies. Average ages of the animals at the onset of the experiments ranged from 72.8-79.7 days; mean body weights ranged from 25.9-29.6 g. Mice were maintained under a light/dark cycle of 0600-1800 light with water and Purina Lab Diet chow available *ad libitum*. Room temperatures were maintained at 22±1°C. EtOH exposure was initiated between 0800-0930 hr. Animals were removed from the chambers on the final day at 0830 and subgroups of the EtOH-exposed mice were placed in a quiet room for six hours until euthanasia at 1430.

Chronic EtOH exposure and withdrawal.

Mice were made dependent upon EtOH using a method described by Goldstein (Goldstein, 1972a) with modifications previously published (Terdal and Crabbe, 1994). On day one, male WSP-1, WSP-2, WSR-1 and WSR-2 mice were weighed, injected ip with EtOH (1.5 g/kg, 20% v/v in 0.9% saline for WSP-1, WSR-1 and WSR-2 and 1.75 g/kg, 20% v/v in 0.9% saline for WSP-2 animals) and 1.0 mmol/kg pyrazole HCl. Two separate control groups received either pyrazole (1.0 mmol/kg in 0.9% saline) or saline (0.9%). Pyrazole is an EtOH dehydrogenase inhibitor that was administered to maintain consistent blood EtOH concentrations (BECs). The mice were then placed into wire mesh cages in a large plastic chamber into which EtOH vapor and air were introduced. EtOH vapor concentrations within the chamber were monitored with a gas chromatograph. At 24 and 48 hr, the mice were removed from the chamber, weighed and injected with pyrazole or saline. If an animal lost greater than 20% of its body weight at the final injection timepoint (48 hr), it was excluded from the study. A subgroup of animals had 20 µl of blood drawn from the tip of the tail daily for gas chromatography determination of average BECs. The mice were then replaced in the chamber and the vapor concentration of EtOH was adjusted up or down to achieve BECs at or near 2.0 mg/ml. At the 72-hr chronic exposure timepoint, which was 24 hr after the final pyrazole injection, the mice were removed from the chambers, weighed, and all animals had blood samples drawn for BEC determinations. A subgroup of the EtOH exposed animals were euthanized immediately to harvest brain from intoxicated animals (EtOH-0) and the remaining animals were allowed to withdraw for six hours, (6-8 hours corresponds to peak withdrawal in WSP mice), and then sacrificed for brain harvest (EtOH-6). The

animals chosen for this study had either a 72-hour BEC greater than 1.5mg/ml or had an average exposure greater than 1.5 mg/ml over the 72 hours. BECs ranged from 1.01 to 3.17 mg/ml over the 72-hour exposure.

Determination of BEC.

For analysis of BECs, the method described by Kosobud and Crabbe (1986), was utilized. Briefly, 20 μl of blood was drawn from the tip of the tail and added to 50 μl of 3M $ZnSO_4$, mixed, and stored on ice until all blood samples were collected. Fifty μl of $Ba(OH)_2$ (5%) and 300 μl of ice-cold distilled water were then added to each sample, and the samples were vortexed and centrifuged at 4°C for 5 minutes at 3000 rpm. The supernatant was analyzed for EtOH concentration using gas chromatography.

Drug sources and preparation.

Pyrazole HCl was purchased from Sigma Chemical Co. (St. Louis, MO). EtOH (ethyl alcohol, absolute, 200 proof) was purchased from AAPER Alcohol and Chemical (Shelbyville, KY). Reagent chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The random primer complementary DNA (cDNA) kit RPN.1601Z was obtained from Amersham Corp. (Arlington Heights, IL). Radioisotopes [α-^{32}P]-dCTP and [γ-^{32}P]-ATP, and GeneScreen nylon membrane filters were purchased from New England Nuclear Corp. (Boston, MA). Pyrazole HCl was dissolved in saline (0.9%) and administered ip at an ambient temperature of 22±1°C. EtOH was mixed with 0.9% saline and injected ip or introduced without mixing as a vapor into the chambers.

Tissue harvest.

Animals were killed via cervical dislocation and whole brain was isolated immediately. Brains were transferred to sterile centrifuge tubes, submerged in liquid nitrogen, and held on dry ice until transfer to an -80°C freezer for storage until RNA isolation.

RNA isolation and Northern blot analysis.

RNA was isolated by the single step acid phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). RNA was quantitated by spectrophotometric determination at 260 nm. Total cellular RNA (20μg) was denatured at 65°C for 15 minutes with 2.2M formaldehyde and fractionated by electrophoresis through a 1% agarose-2.2M formaldehyde gel. RNA species were transferred to GeneScreen nylon membrane filters overnight using standard capillary blotting techniques in 1.5 M sodium chloride-0.15 M sodium citrate (10X SSC). The bound material was fixed by UV cross-linking (Stratalinker 1800, Stratagene, LA Jolla, CA). Blots were prehybridized at 42°C in Ullrich's solution containing 50% deionized formamide, 5X SSC, 5X Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% gelatin), 100 μg/ml sonicated salmon sperm DNA, and 2% sodium dodecyl sulfate (SDS).

All cDNA hybridization probes were isolated as gel-purified cDNA sequences. Hybridization occurred at 42°C with a ^{32}P-labelled random primed prodynorphin cDNA probe generated using [α-^{32}P]d-CTP (Feinberg and Vogelstein, 1983). The prodynorphin cDNA was generously provided by J. Douglas. The prodynorphin probe was a 1700 base pair fragment excised with PstI and EcoRI from pSP64D1.7 (Civelli et al., 1985). Post-

hybridization washes included two five-minute washes at room temperature in 2X SSC; two 30-minute washes at 65°C in 2xSSC/2% SDS and a final rinse in 0.1X SSC at room temperature. Bound probe was visualized by autoradiography using Kodak AR film and NEN Reflection Intensifying Screens at -80°C. Membranes were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) using a ^{32}P-labelled random primed cDNA probe generated using [α-^{32}P]d-CTP. The GAPDH probe was an 800 base pair fragment excised with PstI from pRGAPDH-13 provided by Gary McKnight (Fort et al., 1985). All blots were also probed for 18S ribosomal RNA (rRNA) which served as a loading control. The 18S rRNA probe was an antisense 30-mer oligonucleotide (AGGGGGTCAGCGCCCGTCGGCATGTATTAG) synthesized by Operon (Alameda, CA) and end-labeled with [γ-^{32}P]ATP. Size markers were RNA standards run in adjacent lanes, excised, and stained with ethidium bromide.

Quantitation and statistical analysis.

Quantitation of films was performed via densitometry from non-saturated films in the linear response range utilizing Gel Expert, Version 3.1 (Nucleotech Corp., San Carlos, CA). Statistical Analysis was performed using Students' t-test. Multiple groups were tested using one-way ANOVA with significance set at $p<0.05$. Significant findings were further analyzed post-hoc using Bonferroni's multiple comparisons test.

Results

Chronic EtOH vapor exposure.

WSP and WSR mice exhibiting average blood EtOH concentrations (BECs) ranging from 1.50-2.24 mg/ml at the completion of their 72-hour exposure were used for this analysis. The mean BEC for the WSP-1 mice was 1.71±0.06 mg/ml. WSP-2 mice had an average BEC of 1.50±0.09 mg/ml. BECs of the WSR mice were 2.24±0.11 mg/ml for the WSR-1 and 1.84±0.05 mg/ml for the WSR-2 animals. Two-way ANOVA revealed a significant main effect of line ($F[1,46]=23.73$, $p<0.0001$) (WSR>WSP) and a significant main effect of replicate ($F[1,46]=11.27$, $p<0.001$) (replicate-1>replicate-2) but no significant line by replicate interaction.

Prodynorphin expression in WSP mice.

Expression of steady-state prodynorphin mRNA levels from whole brain was examined by Northern analysis (Figure 2) in four groups of WSP mice: saline control, pyrazole control, the EtOH exposed group (EtOH-0) and a final group that was EtOH exposed and withdrawn for six hours (EtOH-6); this timepoint is peak withdrawal for these animals. There was no significant difference between the saline and pyrazole control groups (Table 5); thus the data for these animals were collapsed and expressed as a single control group. One-way ANOVA revealed significant differences among treatments ($F[2,20]=11.15$, $p<0.001$, Figure 2A). EtOH-0 WSP-1 mice did not display significant alterations in whole brain steady-state prodynorphin mRNA versus control as examined by Northern blot analysis. In contrast, whole brain prodynorphin mRNA was

Table 5. Comparison of Pdyn abundance in saline and pyrazole controls

Group	Saline		Pyrazole		*p* value
	Mean ± SEM	N	Mean ± SEM	N	
WSP-1	0.63 ± 0.12	6	0.74 ± 0.17	6	0.60
WSP-2	0.49 ± 0.15	7	0.56 ± 0.10	9	0.72
WSR-1	1.26 ± 0.13	4	1.18 ± 0.10	4	0.67
WSR-1	2.31 ± 0.27	4	2.52 ± 0.06	6	0.39

Values are expressed as ratio of Pdyn abundance to 18S abundance, arbitrary units. The values do not represent the relative abundance of *Pdyn* among the four lines. Comparisons can only be made between Saline and Pyrazole values within a replicate line.

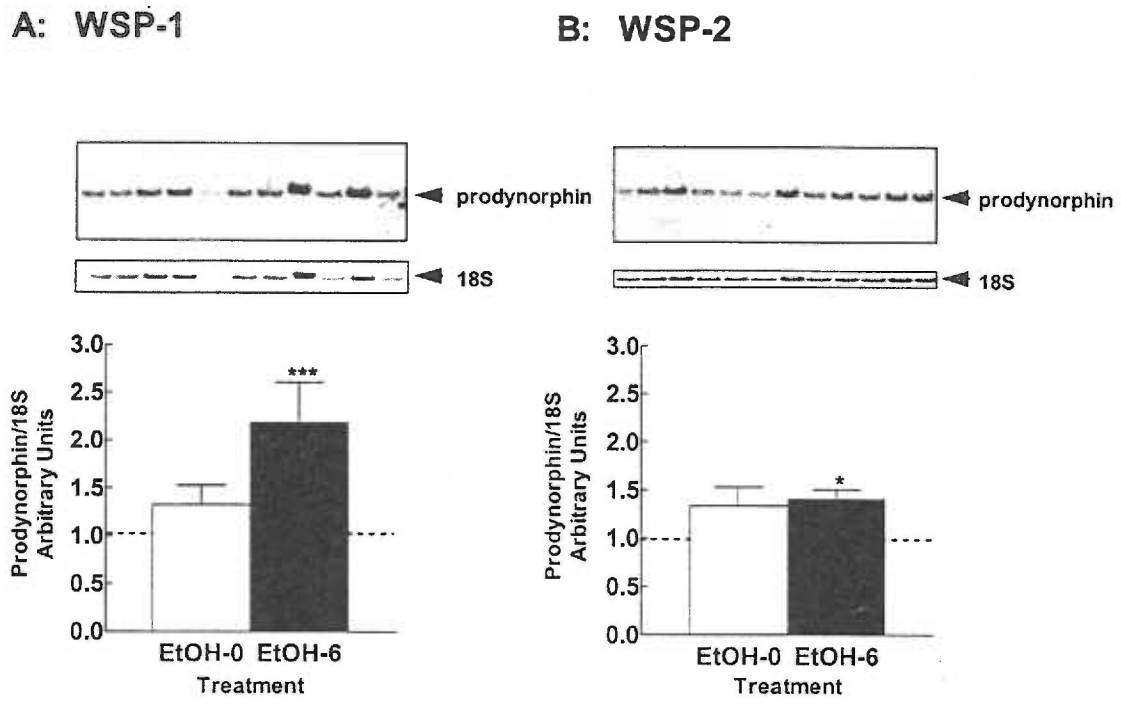

Figure 2. Prodynorphin expression in (A) WSP-1 and (B) WSP-2 mice. The top panels are representative Northern blots of prodynorphin mRNA steady-state levels and 18S rRNA steady-state levels for the WSP-1 and WSP-2 lines. The 18S blot serves as a loading control. Northern blot loading in From left to right, lanes 1-3, Saline; lanes 4-6, pyrazole; lanes 7-9, EtOH-0; lanes 10-11 (A) or 10-12 (B), EtOH-6. The saline and pyrazole treatment groups were not significantly different when examined by Student's t-test, thus these values have been collapsed and are referred to as control. The bar graphs in (A) and (B) represent the mean±SEM of each treatment group, normalized to 18S and then to control; control values are reflected by the dotted line. (A) Analysis following one-way ANOVA ($F[2,20]=11.15$, $p<.001$) using Bonferroni's multiple comparison's test revealed prodynorphin mRNA steady-state levels were significantly increased in withdrawn (EtOH-6) WSP-1 mice (***$p<0.001$) compared to control. No significant increases in prodynorphin steady-state levels were observed in the WSP-1 exposed (EtOH-0) mice compared to control. (B) Similar results were observed in the WSP-2 mice. One-way ANOVA revealed significant differences between treatments ($F[2,26]=5.26$, $p=0.0128$). Bonferroni's multiple comparison's test revealed that the withdrawn (EtOH-6) mice had significantly elevated prodynorphin mRNA compared to controls, with no significant alterations observed in the exposed (EtOH-0) mice compared to control.

significantly increased in EtOH-6 WSP-1 mice compared to control prodynorphin steady-state levels ($p<0.001$). This increase was approximately 2.2-fold. ANOVA on the WSP-2 line yielded similar results ($F[2,26]=5.26$, $p<0.02$). No significant alterations in prodynorphin steady-state levels were present in EtOH-0 animals, but EtOH withdrawal significantly increased prodynorphin steady-state levels in the EtOH-6 mice compared to controls ($p<0.05$). In these mice the increase in prodynorphin steady-state levels was 1.4-fold (Figure 2B). Thus EtOH withdrawal significantly increased prodynorphin expression in both replicate WSP lines.

Prodynorphin expression in WSR mice.

Neither EtOH intoxication nor EtOH withdrawal altered whole brain prodynorphin mRNA levels in the WSR mice (Figure 3). These results were observed in both the WSR-1 (Figure 3A) and WSR-2 (Figure 3B) replicate selected lines of mice.

GAPDH expression in WSP and WSR mice.

GAPDH expression was analyzed in each strain to verify that alterations in prodynorphin expression were not due to global transcriptional alterations induced by chronic EtOH exposure. There were no significant alterations in GAPDH expression in the WSP lines (Figure 4) or in the WSR lines of mice (data not shown) following EtOH exposure or withdrawal.

Basal prodynorphin expression in WSP and WSR mice.

Basal prodynorphin steady-state mRNA abundance was examined in saline treated animals to determine whether the selection process had produced a divergence in

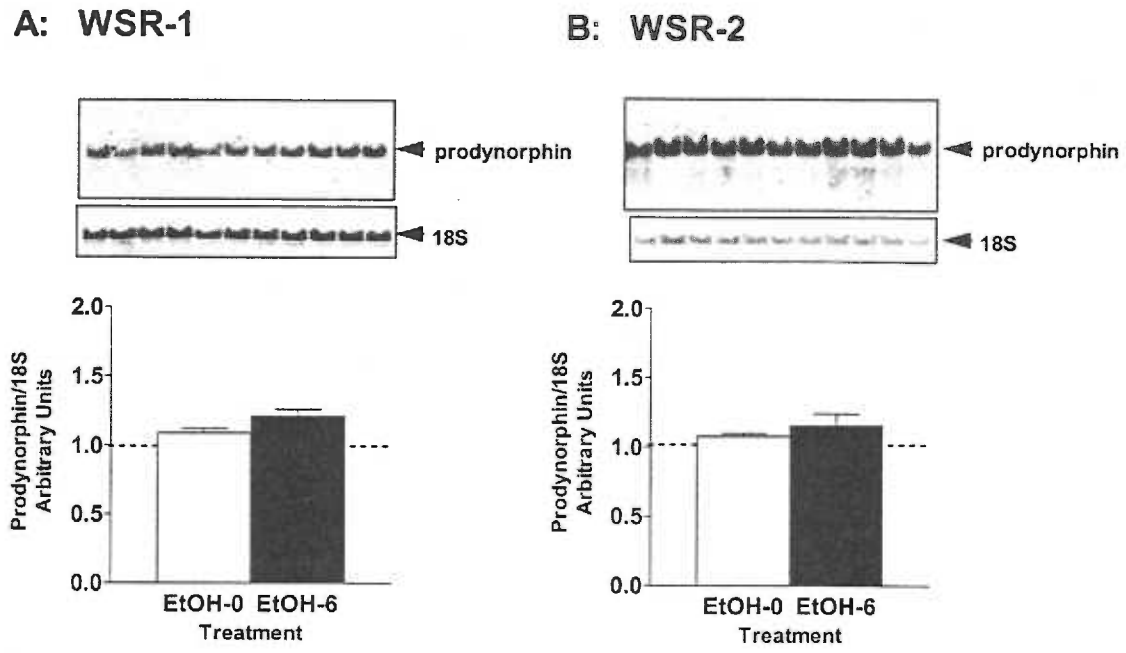

Figure 3. Prodynorphin expression in (A) WSR-1 and (B) WSR-2 lines of mice. The top panels of (A) and (B) are representative Northern blots of prodynorphin mRNA steady-state levels and 18S rRNA steady-state levels. 18S served as a loading control. Northern blot loading from left to right: lanes 1-2, Saline; lanes 3-5, Pyrazole; lanes 6-8, EtOH-0; lanes 9-11, EtOH-6. Student's t-test revealed no significant differences between the saline and pyrazole control groups, thus they have been collapsed and are referred to as control. The bar graphs in (A) and (B) represent the mean±SEM of each treatment group, normalized to control; control values are reflected by the dotted line. (A) Prodynorphin steady-state levels were not significantly altered in the exposed (EtOH-0) or the withdrawn (EtOH-6) WSR-1 animals compared to control as revealed by one-way ANOVA. B. Prodynorphin mRNA steady-state levels were also not significantly altered in the exposed (EtOH-0) or the withdrawn (EtOH-6) WSR-2 mice compared to control.

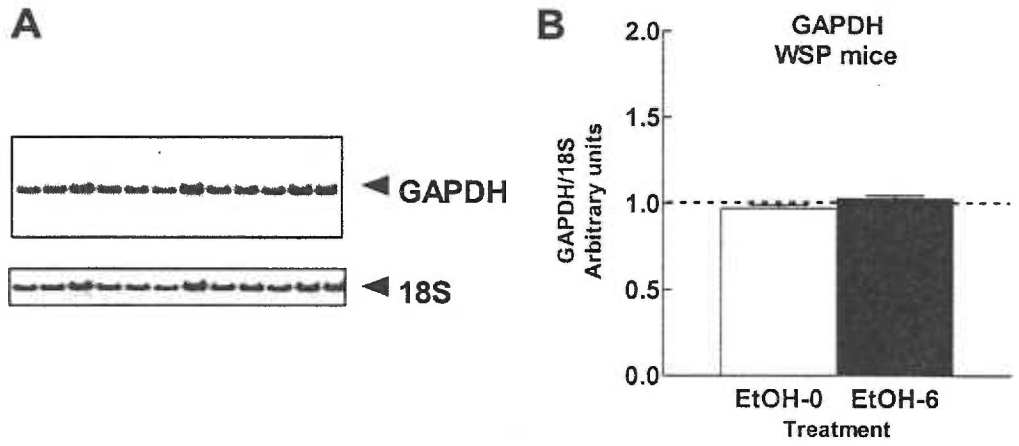

Figure 4. **GAPDH expression in WSP mice. (A)** Representative Northern blot of GAPDH expression in WSP-2 mice. The top panel is GAPDH expression and the bottom panel is 18s rRNA, a loading control. Loading from left to right: lanes 1-3, saline; lanes 4-6, pyrazole; lanes 7-9, exposed (EtOH-0); and lanes 10-12 withdrawn (EtOH-6). The values of the saline and pyrazole groups in each line were not found to be significantly different using Student's t-test, thus they are collapsed and referred to as control, which is depicted as a dotted line. **(B)** The mean±SEM of each treatment normalized to control values is presented; the data are collapsed across the WSP-1 and WSP-2 lines. One-way ANOVA revealed GAPDH expression was not altered in the WSP lines. One-way ANOVA also revealed no alterations in GAPDH expression in the WSR lines (data not shown).

the levels of prodynorphin expressed basally in the WSP and WSR lines. ANOVA revealed no significant differences in prodynorphin mRNA steady-state levels among saline treated WSP-1, WSP-2, WSR-1 or WSR-2 lines of mice (Figure 5).

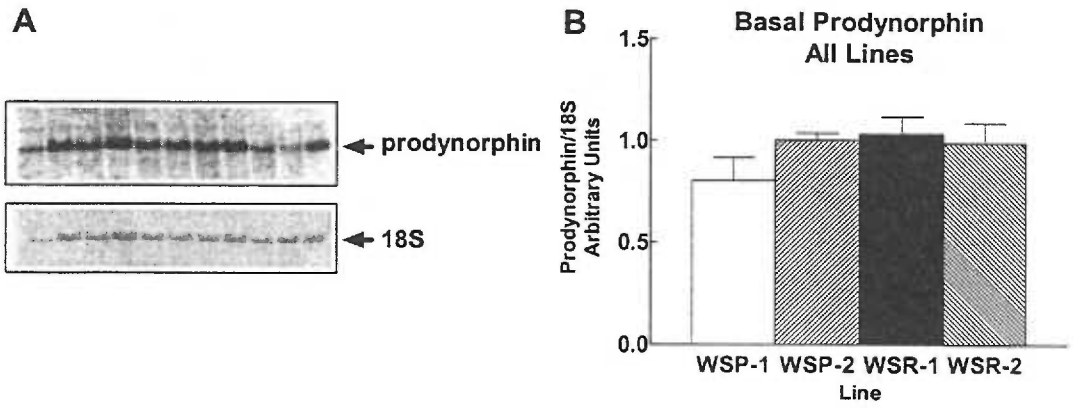

Figure 5. **Northern blot analysis of basal prodynorphin mRNA steady-state levels in WSP-1, WSP-2, WSR-1 and WSR-2 lines. (A)** The top panel is a representative Northern blot of prodynorphin mRNA and 18S rRNA. The 18S rRNA served as a loading control. Loading for the blot from left to right: lanes 1-2, WSP-1; lanes 3-5, WSP-2; lanes 6-8, WSR-1, lanes 9-11, WSR-2. **(B)** The bar graph represents the mean±SEM of basal prodynorphin expression in each line normalized to WSP-2 animals. One-way ANOVA revealed no significant differences in prodynorphin mRNA steady-state levels among the four lines.

Discussion

The data presented represent the first examination of prodynorphin mRNA steady-state levels in WSP and WSR mice, a mouse model of severe EtOH withdrawal that was selected for their differences in handling-induced convulsion sensitivity (Crabbe et al., 1990b). We have shown that prodynorphin steady-state mRNA was significantly increased in whole brain of both replicate lines of WSP mice following withdrawal from chronic EtOH exposure. In contrast, non-significant increases in prodynorphin steady-state levels were observed in the WSR mice. Therefore, these results suggest that the endogenous ligand for the KOP-R may play a role in the severity of EtOH withdrawal convulsions.

The system we chose to use for our studies has some advantages over liquid diet administration, which was utilized by Guyla et al. in their analysis of EtOH regulation of dynorphin levels (Gulya et al., 1993). Gulya et al. showed that prodynorphin expression in B6 mice was altered by chronic exposure to EtOH (Gulya et al., 1993). However, the liquid diet paradigm used did not prevent the animals from withdrawing from EtOH while sleeping. In the vapor exposure paradigm the animals do not go through periods of abstinence (and thus possibly withdrawal) during sleep as they might in a liquid diet or a chronic injection paradigm. By utilizing EtOH vapor inhalation chambers, we have been able to separate exposure to EtOH and EtOH withdrawal into two distinct events. This has allowed us to suggest that the alterations in mRNA expression observed were associated with the animal's withdrawal from EtOH rather than exposure. Our results therefore help clarify the issue of prodynorphin regulation by withdrawal rather than

exposure, and suggest that the route of administration for EtOH should be considered when determining the effect of EtOH on gene expression.

There is conflicting evidence for the role of prodynorphin's peptide product in seizure activity. Multiple animal models have been used, and the timepoints in which the peptide or its mRNA have been examined vary. In some animal models, increased prodynorphin suggested increased seizure susceptibility when examined in naïve subjects (Lee et al., 1987). Other models have studied prodynorphin levels only after seizure generation, and the results have varied. In rat models of amygdala and systemic kainic acid kindling, prodynorphin levels were increased (Douglass et al., 1991; Romualdi et al., 1995). Amygdaloid kindling produced by injecting kainic acid directly into the amygdala produced significant decreases in prodynorphin abundance (Lason et al., 1983), and in prepiriform cortex kindling models a decrease was also observed (Xie et al., 1989). Nevertheless, our results are consistent with a role for prodynorphin expression in EtOH withdrawal convulsions. Future studies will examine the effects of withdrawal on the KOP-R system in additional mouse models in order to extend these finding to other genetically unique populations of mice and strengthen the generalizability of our findings.

It is important to note that the observed alterations in prodynorphin steady-state levels were not due to a global alteration in gene expression due to chronic exposure to EtOH, as was shown by the analysis of GAPDH expression. Additionally, the differences observed between the two lines of mice were not due to an inherent difference in basal prodynorphin steady-state levels generated between the two lines during the

selective breeding process. Thus, the alterations we have observed were seen only in the presence of severe withdrawal following chronic EtOH exposure.

Our data do not allow us to make conclusions regarding a causal role for prodynorphin in EtOH withdrawal convulsions. Several alternatives are possible; for example, prodynorphin may be modulating the expression of convulsions or the severity of these convulsions, or prodynorphin may be modulated by convulsive activity. We believe the latter is unlikely because the handling protocol greatly minimizes the observed convulsive activity these animals exhibit. However, this option cannot be ruled out, as a small subset of the animals do convulse prior to sacrifice. Future studies using behavioral pharmacological approaches and time course studies will focus on distinguishing between these two alternative roles for prodynorphin in EtOH withdrawal.

Of course it is possible that prodynorphin may also participate in additional aspects of withdrawal. For example, KOP-R activation has been suggested to play a role in mediating aversion, specifically via the mesolimbic dopaminergic pathways (reviewed in Herz, 1997). This alternative explanation is currently being examined in our laboratory via behavioral and molecular approaches.

Because our analysis did not investigate specific anatomic regions, we do not know if the brain regions thought to be involved in the generation of seizures correspond to the regions showing prodynorphin alterations. A more comprehensive analysis of regional alterations of prodynorphin abundance in WSP and WSR mouse brain using *in situ* hybridization analysis is currently underway in our laboratory. These findings should provide insight into the potential role(s) of prodynorphin in EtOH withdrawal.

Furthermore, time course studies and confirmation of changes in peptide levels will need to be pursued.

To conclude, this work has demonstrated that steady-state prodynorphin mRNA is increased in whole brain of WSP mice, a mouse model of severe EtOH withdrawal convulsions, following withdrawal from 72 hours of chronic EtOH exposure. This is in contrast to the absence of changes in prodynorphin levels in the WSR mice, which are resistant to EtOH withdrawal convulsions. These findings advance the work of Guyla et al., who found that prodynorphin expression was increased by EtOH exposure via a liquid diet (Gulya et al., 1993). In addition, our inability to detect changes in prodynorphin mRNA steady-state levels in the EtOH-0 animals suggests that the method of exposure must be considered when examining EtOH's effects on gene expression. Finally, our data suggest that changes in the expression of the ligand for the KOP-R may be involved with EtOH withdrawal seizure severity, although the exact role for this molecule will need to be further characterized. These findings also extend the current data suggesting a role for opioid receptor ligands in seizure generation to convulsions produced during EtOH withdrawal, and suggest that alterations in the KOP-R system should be examined in further mouse models of EtOH withdrawal and other withdrawing species to determine the generalizability to humans. A better understanding of the role of the KOP-R system in EtOH withdrawal may eventually allow identification of potential therapeutic targets for the treatment of withdrawing alcoholics.

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Chapter III
Ethanol Withdrawal Elicits Prodynorphin and Kappa-Opioid Receptor Expression In Distinct Brain Regions in a Genetic Model of Enhanced Withdrawal Severity

Preface

The following chapter represents a manuscript in preparation. The data presented here has been presented in part at Annual Meetings of the Research Society on Alcoholism.

Abstract

Abrupt withdrawal from chronic EtOH can produce convulsions that are likely due to EtOH neuroadaptations. In the absence of EtOH, a state of neuronal hyperexcitability is unmasked that contributes to the generation of seizure activity. While significant efforts have focused on elucidating dependence mechanisms, the alterations that participate in EtOH withdrawal severity are less well characterized. The present studies characterized the KOP-R system in Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) mice, selected lines that display severe and mild (respectively) handling induced convulsions (HICs) upon removal from chronic EtOH exposure. Previous data have demonstrated significant increases in whole brain prodynorphin mRNA in WSP mice during EtOH withdrawal, while no significant effects of EtOH exposure or withdrawal were observed in WSR mice. These data suggest altered prodynorphin contributes to the divergent phenotypes exhibited by these lines, but they do not identify specific anatomical targets. Thus, we have further characterized two components of the KOP-R system: prodynorphin mRNA and the KOP-R, in WSP and WSR mice using *in situ* hybridization and receptor autoradiography, respectively. The analyses occurred in the nucleus accumbens, caudate-putamen, olfactory tubercle, piriform cortex, claustrum, dorsal endopiriform nucleus, and cingulate cortex, forebrain regions that may contribute to EtOH withdrawal seizures, affective states, or both. *In situ* hybridization analyses confirmed previous findings; EtOH withdrawal significantly increased prodynorphin in WSP mice in multiple brain regions, with no effects observed in the WSR lines. Differences in basal KOP-R abundance were also observed across brain regions; WSR mice tended to exhibit higher basal KOP-R binding than WSP mice.

Finally, significant alterations in KOP-R density were observed during EtOH withdrawal in WSP mice. We propose that alterations in the KOP-R system of these lines mediate withdrawal severity and that the KOP-R system is a common neurochemical target for withdrawal induced aversive states.

Introduction

It is known that chronic EtOH exposure can result in physical dependence. This likely occurs as a result of central nervous system (CNS) adaptations that enable an organism to function in the presence of this CNS depressant and thus re-establish internal homeostasis (Himmelsbach, 1942, 1943). A great deal of effort has focused on elucidating the neuroadaptive alterations that occur to produce EtOH dependence (Koob et al., 1998b). As a result, it has been demonstrated that during chronic EtOH exposure, the function of multiple neurotransmitter systems is altered. For example, down-regulation of GABA receptors occurs along with a concomitant up-regulation of NMDA receptor function (reviewed in Crews et al., 1996; Davis and Wu, 2001). The net effect of these responses is increased neuronal excitability, the development of tolerance to the effects of EtOH, and, following long-term exposure, physical dependence. Removal of EtOH (i.e., EtOH withdrawal), eliminates the central nervous system depressant effects of the drug, and unmasks a state of neuronal hyperexcitability. EtOH withdrawal seizures are one manifestation of this hyperexcitable state that are observable in nearly every species studied. Interestingly, while a great deal of effort has been focused on understanding how dependence develops, we know much less about the mechanisms that contribute to EtOH withdrawal.

Unlike withdrawal from some drugs of abuse, severe EtOH withdrawal can be a life-threatening event. Symptoms can range from mild tremor or anxiety, to more serious physical manifestations including alcohol withdrawal seizures and delirium tremens (First, 2000). This observation highlights the importance of understanding the neurochemical alterations contributing to the physical and affective components of EtOH

withdrawal to develop better methods for treating this potentially life threatening event and to help prevent alcoholic relapse.

The complex polygenic nature of alcoholism makes it a challenge to dissect the genetic contributions to this disease. However, animal models that exhibit differences in one or more alcohol-related phenotypes exist. Selective breeding is one way that these models are developed, and the ability to select for a phenotype is proof of principle that the trait of interest is at least partially controlled by genetic factors (Crabbe and Belknap, 1992). In addition to the selected phenotype, these lines often display differences in the expression of non-selected traits, which is termed a correlated response to selection and reflects the selection of a gene (or genes) that the phenotypes share in common (Crabbe et al., 1990c). Thus, selected lines can be powerful tools for examining genetically influenced, alcohol-related traits.

Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) mice are lines that have been selected in replicate for mild and severe EtOH withdrawal seizure severity (Crabbe et al., 1983a; Crabbe et al., 1985). Handling induced convulsions (HICs) are characterized as an index of withdrawal severity following 72 hours of EtOH vapor inhalation. In addition to the selected phenotype, a large number of correlated responses to selection have been examined in WSP and WSR mice (for recent review see Metten and Crabbe, 1996). For example, WSP and WSR mice showed different EtOH consumption patterns and differences in their response to conditioned taste aversion (CTA) and conditioned place preference (CPP) paradigms. Although neither line showed a profound preference for EtOH, WSR mice consumed larger quantities of EtOH than WSP mice (Kosobud et al., 1988). This is in contrast to the observation that WSP mice

exhibit preference for an EtOH paired floor in CPP experiments, while WSR mice do not (Crabbe et al., 1992). Finally, WSP mice showed a smaller initial CTA than WSR animals (Chester et al., 1998). It has been suggested that some of the genes contributing to EtOH withdrawal severity also participate in alcohol drinking behavior, and presumably, the rewarding and aversive aspects of EtOH consumption. This hypothesis is supported by a growing body of evidence that demonstrates an inverse genetic correlation between withdrawal severity and EtOH consumption (Metten et al., 1998; Chester et al., 2002; Chester et al., 2003). Selection pressure has fixed many of the genes important for EtOH withdrawal seizure severity or resistance in a homozygous state in WSP and WSR mice, and it appears that some of the same genes that contribute to EtOH withdrawal seizures might also participate in rewarding and aversive states associated with EtOH consumption and withdrawal. Thus, these selected lines represent a rich resource for identifying candidate genes that may participate in EtOH withdrawal seizures and withdrawal-induced aversion.

Because little is known about the mechanisms contributing to EtOH withdrawal HICs, one approach to examining their origin might be to identify those systems participating in other seizure types. An interesting candidate for investigation in WSP and WSR mice is the KOP-R system. This system consists of prodynorphin (*Pdyn*), dynorphin, and the KOP-R. *Pdyn* is the mRNA precursor for dynorphin peptide, which in turn, is the endogenous ligand for the KOP-R. Differences in dynorphin and KOP-R receptor abundance between seizure sensitive and seizure resistant animals models have been observed, as well as alterations in the KOP-R system pre- and post-seizure induction (De Sarro et al., 1992; Fischer et al., 1993; Przewlocka et al., 1994b; Przewlocka et al.,

positive and negative reward states contribute to relapse, and relapse is a significant factor in addictive processes that is not well understood (Self and Nestler, 1995, 1998).

Because our previous analyses of *Pdyn* mRNA abundance occurred at the whole brain level, we could not identify brain region specific changes that might help elucidate the specific role of the KOP-R system in EtOH withdrawal or other correlated responses to selection in these lines. Thus, we sought to further characterize differences in the response of the KOP-R system to EtOH exposure and withdrawal in WSP and WSR mice. Using *in situ* hybridization analysis and KOP-R autoradiography (AR), we examined the effects of chronic EtOH exposure and subsequent withdrawal on *Pdyn* and the KOP-R. We focused on brain regions that have been identified as participants in seizure activity, affective states, or both: the nucleus accumbens, caudate-putamen, olfactory tubercle, piriform cortex, claustrum, dorsal endopiriform nucleus, and cingulate cortex. Changes in prodynorphin message and KOP-R receptor abundance were correlated with the differences in withdrawal severity and drinking behavior between the two lines.

In this report, we show that EtOH withdrawal significantly increased prodynorphin in WSP mice in multiple brain regions, with no effects observed in the WSR lines, a finding that confirms our previous observations. In addition, differences in basal KOP-R abundance were also observed across brain regions; WSR mice tended to exhibit higher KOP-R binding than WSP mice. Finally, significant alterations in KOP-R density were observed during EtOH withdrawal in WSP mice. We propose that alterations in the KOP-R system of these lines is involved in mediating their selected

differences in withdrawal HIC severity and that the KOP-R system is a common neurochemical target for withdrawal induced aversive states.

Materials and methods

All animal procedures and animal care were reviewed and approved by the Portland Oregon VA Medical Center Institutional Animal Care and Use Committee and met NIH guidelines for appropriate care and use of animals in research.

Animal subjects

WSP and WSR mice were bred and generously provided by the laboratory of Dr. John Crabbe in Portland, OR. Drug-naïve adult male mice from selected generation 26 (filial generations G_{77}-G_{90}) were used. The WSP and WSR selective breeding protocol was replicated, thus there are two independently derived replicate WSP and WSR lines (Crabbe et al., 1983a; Crabbe et al., 1985; Crabbe, 1987; Crabbe et al., 1990b; Crabbe and Phillips, 1993). Male mice of both replicates of the WSP (WSP-1 and WSP-2) and WSR (WSR-1 and WSR-2) lines were tested in these studies. Ages of the animals at the onset of the experiments ranged from 55-93 days, mean age 77 days; body weights ranged from 21.6-34.6g, with a mean body weight of 27.6g. Mice were maintained under a light/dark cycle of 0600-1800 light with water and Purina Lab Diet chow available *ad libitum*. Room temperatures were maintained at 22±1°C. EtOH exposure was initiated between 0730-0930 hr.

Drug sources, reagents and preparation

Pyrazole HCl (Pyr) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). EtOH (ethyl alcohol, absolute, 200 proof) for use in chemical assays was purchased from AAPER Alcohol and Chemical (Shelbyville, KY), and Pharmco

Products, Inc. (Brookfield, CT) for use in the EtOH vapor chambers and injections. Pyr was dissolved in saline (0.9%) and administered via intraperitoneal (ip) injection. EtOH (20% v/v) was mixed with 0.9% saline and injected ip or introduced without mixing as a vapor into the chambers. Radioisotope [α-^{35}S]-UTP was purchased from PerkinElmer Life Sciences Inc. (Boston, MA). [^{3}H]-CI-977 was purchased from Amersham Biosciences, (Piscataway, NJ). Additional reagent chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified in the text.

Chronic EtOH exposure and withdrawal

Mice were made dependent upon EtOH using a method described by Goldstein (Goldstein, 1972a) with modifications previously published (Terdal and Crabbe, 1994; Beadles-Bohling et al., 2000). On day one control or EtOH treated male WSP-1, WSP-2, WSR-1 and WSR-2 mice were weighed, injected ip with EtOH (1.5 g/kg for WSP-1, WSR-1 and WSR-2 and 1.75 g/kg for WSP-2 animals) and 1.0 mmol/kg Pyr (an alcohol dehydrogenase inhibitor). Controls were placed into air chambers and received Pyr only; a saline control was not included because previous data has shown there was no difference between Pyr and saline treated animals with respect to prodynorphin gene expression (Beadles-Bohling et al., 2000). At 24 and 48 hrs, the animals were briefly removed from the chambers, weighed, and blood samples were collected from each animal for BEC determination. Following 72-hr of chronic exposure, all mice were removed from the chambers, weighed, and again had blood samples drawn for BEC determinations. Following EtOH exposure, the animals were euthanized for tissue processing as outlined below.

Determination of BEC.

For analysis of BECs, the method described by Kosobud, et al (1986) was utilized. Briefly, 20 µl of blood was drawn from the tip of the tail and added to 50 µl of 3M $ZnSO_4$. Fifty µl of $Ba(OH)_2$ (5%) and 300 µl of ice-cold distilled water were then added to each sample, and the samples were vortexed and centrifuged at 4°C for 5 minutes at 3000 rpm. The supernatant was analyzed for EtOH concentration using gas chromatography.

Brain collection and sectioning.

Brain tissue was harvested from a subgroup of the EtOH exposed (i.e., intoxicated) WSP and WSR animals immediately after removal from the chambers at time 0 (EtOH-0). The remaining animals were allowed to withdraw for six hours (a time point within peak withdrawal, Finn and Crabbe, 1999) before tissue harvest (EtOH-6). The animals selected for examination of RNA or receptor abundance were chosen using the following criteria: the subject had either a 72-hour BEC greater than 1.5mg/ml or the subject received an average exposure greater than 1.5 mg/ml over the 72 hour vapor exposure paradigm. BECs ranged from 0.82 to 3.32 mg/ml over the 72-hour exposure. Brains were rapidly removed and frozen in isopentane cooled in a dry ice-isopropyl alcohol slurry, and were stored at -80°C until sectioning. Prior to sectioning, the tissue was embedded in O.C.T. Compound (Sakura Finetech USA, Inc.; Torrance, CA). Fourteen µm coronal sections were cut between –17 and -22°C using a Leica CM1850 cryostat (Leica Microsystems, Bannockburn, IL) with a disposable blade. *In situ* hybridization and receptor autoradiography slides were collected from the brains of the

same animals. Coronal sections were collected from the forebrain. Examination of *in situ* hybridization for *Pdyn* occurred within the nucleus accumbens core (AcbC), shell (AcbSh), piriform cortex (Pir), olfactory tubercle (Tu) and caudate-putamen (CPu). The regions analyzed by *in situ* hybridization fell between 1.10 and 1.42mm anterior to Bregma as defined in the atlas "The Mouse Brain in Stereotaxic Coordinates, second edition" (Paxinos and Franklin, 2001). Receptor autoradiography was performed between 0.62 and 1.34 mm anterior to Bregma, in similar regions to those analyzed by *in situ* hybridization: the CPu and Tu. Additional regions analyzed by KOP-R AR included the medial portion of the accumbens shell (*m*AcbSh), cingulate cortex (Cg), claustrum (Cl), dorsal endopiriform nucleus (DEn), and a region encompassing the lateral AcbSh (*l*AcbSh). Slices from the same identified anatomical locations were included in a single experimental run.

Riboprobe template production.

Forward ($_{5'}$CAA ATA CCC CAA GAG CTC$_{3'}$) and reverse ($_{5'}$AGT CAC CAC CTT GAA CTG ACG$_{3'}$) primers based on published rat *Pdyn* sequence (Civelli et al., 1985) were designed using Oligo Software (Molecular Biology Insights, Inc.; Cascade, CO). These primers were predicted to produce a 187 bp portion of the mouse *Pdyn* gene corresponding to bps 716-903 of published mouse sequence (accession number AF026537). D2 female midbrain RNA was subjected to reverse transcription and the resulting product was amplified by PCR. Appropriate size was confirmed by agarose gel electrophoresis. The amplicon was then T/A cloned using pGEM-T Easy (Promega Corp., Madison, WI) overnight at 4°C, electroporated into DH10B electro-competent bacteria and grown overnight under ampicillin selection using blue/white screening. Four

clones were linearized using either *SalI* or *NcoI* and subjected to automated DNA sequence analysis with the Dye Terminator Cycle Sequencing System (PerkinElmer Life Sciences, Boston, MA) on an ABI 377 DNA sequencer. All sequences were subjected to BLAST analysis to verify the mouse *Pdyn* sequence. One plasmid with the desired orientation for production of an anti-sense strand riboprobe using the T7 promoter site in pGEM-T Easy was designated *Pydn*-187 and used for all riboprobe synthesis and labeling.

Riboprobe synthesis and labeling.

Pdyn-187 was linearized using *SalI*, and the resulting linear product produced a template for the synthesis of a ^{35}S-UTP (PerkinElmer Life Sciences, Boston, MA) labeled *Pdyn*-187 anti-sense cRNA probe using T7 RNA Polymerase. Digestion of the same plasmid by *NcoI* generated a linear template that produced a sense strand of the *Pdyn* riboprobe utilizing the SP6 RNA polymerase promoter site in pGEM-T Easy. Synthesis was carried out using RiboProbe In Vitro Transcription System-SP6/T7 (Promega Corp., Madison, WI). Unincorporated nucleotide was removed from the reaction using a Sephadex G-50 NICK Spin Column (Amersham Biosciences, Piscataway, NJ). Four 100-μl fractions were collected via gravity flow from the column, counted on a Quick-Count (Bioscan, Inc, Washington, DC), and the highest activity fraction was used to prepare the probe solution. The labeled cRNA probe was denatured at 65°C for 5 minutes in DEPC H_2O containing 500µg/ml of yeast tRNA and 50 µM dithiothreitol (DTT) and diluted to 5-10x10^6 dpm/ml in hybridization buffer containing 50% Formamide, 0.25M sodium chloride, 1X Denhardt's Solution and 10% Dextran Sulfate

(Amersham Biosciences, Piscataway, NJ). Probe was stored in 1 ml aliquots for no more than 1 month at -20°C until hybridization.

In situ hybridization

Following slicing and mounting on Superfrost Plus slides (FisherBrand, Pittsburgh, PA), the slides were stored at -80°C in slide boxes containing desiccant until 1-2 weeks prior to use, when they were transferred to storage at -20°C. Slides were transferred from –20°C storage directly into 4% paraformaldehyde:sodium borate fixative for 1 hour at room temperature. Slides were then washed 2X for 10 minutes in sterile 0.02M potassium PBS (KPBS) at room temperature (RT), followed by incubation for 15 minutes at 37°C with 0.0005% Proteinase K in TE Buffer. Slides were rinsed briefly in Tris-EDTA (TE) buffer without agitation, transferred to 0.1 M triethanolamine (TEA) buffer at pH 8.0 for 3 minutes, then acetylated in 0.1 M TEA-acetic anhydride (0.25%) for 10 minutes at room temperature before rinsing gently in 2X Saline-Sodium Citrate (SSC) buffer, 2x for 2 minutes. Finally, the slides were dehydrated quickly in ascending alcohol concentrations: 50% EtOH, 2 min; 70% EtOH, 2 min; 95% EtOH, 2 min; 100% EtOH, 2x for 2 min. Slides were drained and air-dried for 10 minutes, followed by storage with desiccation under vacuum at RT for a minimum of 2 hours before hybridization.

One ml aliquots of ^{35}S-UTP labeled probe hybridization solution were heated to 65°C for 5 minutes, and 90 µl of solution was pipetted onto a 22x60 mm cover glass. The slides were incubated in a humidified Slide Moat (Boekel Scientific, Feasterville, PA), for 16-20 hours at 55°C. Following the hybridization, the slides were soaked in 4X

SSC for 30-60 minutes to remove the cover slips. Slides were subsequently rinsed 4 times, 5 min each in 4X SSC using gentle agitation followed by RNase digestion (10 μg/ml) for 25 min at 37°C. The slides were rinsed and gradually desalted in descending concentrations of SSC (2X, 1X, 0.5X) with 1 mM DTT for ten minutes each. A final 30-minute wash in 0.1X SSC at 65°C was carried out followed by a rinse in 0.1X SSC for 5 min at RT. The slides were quickly dehydrated in ascending EtOH concentrations, and dried at RT under vacuum with desiccation for 30 minutes. Non-specific hybridization controls consisted of similarly prepared sense riboprobes. In addition, some tissue sections were processed for RNase digestion, in which tissue sections were treated with RNase (50 μg/ml) after paraformaldehyde fixation and acetylation, and then hybridized with antisense riboprobe as described.

Kappa opioid receptor autoradiography.

Sections adjacent to those used for *in situ* hybridization analysis were used in the KOP-R AR experiments. Sections were allowed to come to RT, rapidly transferred to a slide rack, and immediately subjected to the autoradiography protocol using [^{3}H]CI-977 as the KOP-R AR ligand as adapted from Kitchen et al. (Kitchen et al., 1997). Briefly, the slides were preincubated in 50 mM Tris-HCl buffer, pH 7.4, containing 0.9% NaCl for 30 minutes at RT to remove endogenous opioids. The slides were then transferred to NaCl-free, 50 mM Tris-HCl, containing approximately 2.5 nM [^{3}H]CI-977, and incubated for 60 minutes at room temperature. Non-specific binding was defined by incubating sequential slides in the parallel buffer with the addition of 1 μM naloxone (an opioid receptor antagonist). The slides were washed 3 times for 5 min each in fresh ice-

cold Tris-HCl buffer, briefly air dried and stored under vacuum with desiccation for a minimum of 2 hours.

Quantitation of in situ hybridization, receptor autoradiography and statistical analyses of data.

Brain regions from a minimum of three adjacent sections per subject were measured bilaterally from anatomically matched sections; group sizes are noted in the figure legends. Brain areas from comparable brain sections were initially identified using thionin stained adjacent sections and by visual comparison of landmarks within the radiolabeled images. Further verification was obtained by lightly counterstaining the slides used for the assay (*Pdyn in situ* hybridization or KOP-R AR). Regions selected for measurement did not noticeably differ between sections or subjects.

In situ hybridization autoradiographs were generated for quantitative analysis by apposition of dried slides containing equivalent brain regions from animals of each group to Cyclone Super Resolution (SR) phosphor screens (PerkinElmer Life Science, Boston, MA) for 8-14 days in the presence of ^{14}C autoradiographic microscale standards (American Radiolabeled Chemicals, Arlington Heights, IL). Following phosphor screen exposure, the images were digitized at 600 dpi using the Cyclone Storage Phosphor System (PerkinElmer Life Sciences, Boston, MA). Once acceptable 16-bit .tif images had been obtained, the slides were lightly counterstained with thionin and coverslipped for visualization of anatomical structures and confirmation of anatomical location.

Dried slides were apposed to Kodak MR Film (Kodak Co.; Rochester, NY) for 8 weeks to generate KOP-R autoradiographs. [^{3}H]methacrylate polymer standards

(Amersham Biosciences; Piscataway, NJ) were also apposed to each film to aid in signal quantification. Receptor autoradiographs were digitized using a ScanMaker 9800XL Scanner with transparency adaptor (Microtek, Carson, CA) at 1200 dpi using 16-bit resolution. Both methods produced high-resolution .tif images for analysis using Optiquant Software (Version 4.0, PerkinElmer Life Science, Boston, MA). The data for both the *in situ* hybridization analysis and receptor autoradiography were expressed as digital light units per mm^2 (DLU/mm^2), an expression of density defined by the Optiquant Software that shows a linear relationship with DPM/mm^2. Brain regions of interest were outlined manually with the aid of an Intuos2 graphics tablet (Wacom Technology Corporation; Vancouver, WA). For the *in situ* hybridization data, DLU/mm^2 values for the 14-C standard were plotted against known dpm/mm^2 values to generate a standard curve. DLU/mm^2 values were then determined for each identified brain region, background was subtracted, and the values were converted to dpm/mm^2 using linear regression. All values were within the linear portion of the standard curve. Finally, values were multiplied by a decay factor to account for differences in probe activity at the time of use. In order to combine data from multiple *in situ* hybridization passes in the analyses, all data were normalized within pass to their respective Pyr control.

Constraints on the number of slides that could be simultaneously run in a single *in situ* hybridization pass limit the comparisons made within and between the WSP and WSR replicate lines. Multiple passes of *in situ* hybridization assays were required due to the large number of experimental groups (4 lines x three treatments = 12 groups) and to obtain a large enough sample size for statistical analysis. As a result, the approach we employed was intended to allow for comparisons between the replicate lines, i.e. WSP-1

and WSR-1 animals were always run concurrently, and the same approach was used for the WSP-2 and WSR-2 groups. Thus, each replicate from the selected lines was exposed to identical hybridization conditions within a single pass. In order to combine all the passes for each replicate, the DPM/mm2 values for control animals were arbitrarily set to one and experimental values were normalized to their respective control group. The WSP-1 and WSP-2 lines were compared by 2-way ANOVA, as were the WSR-1 and WSR-2 lines. If no main effect of replicate was present, the data were collapsed across replicate, and reanalyzed for treatment effects.

A priori assumptions were made about the effects of EtOH on *Pdyn* abundance based on previous studies performed in our laboratory. Specifically, we reported significant alterations in *Pdyn* mRNA abundance in whole brain of WSP-1 and WSP-2 mouse lines during withdrawal from EtOH, but not in either WSR replicate (Beadles-Bohling et al., 2000). In addition, the effect of alcohol withdrawal was more robust in the WSP-1 line compared to effects observed in the WSP-2 line (Beadles-Bohling et al., 2000). Based on these findings, we predicted that *Pdyn* mRNA would be selectively altered by EtOH treatment and withdrawal in brain regions of WSP mice with no observable alterations in the WSR lines, and that the effects on *Pdyn* expression would be more robust in the WSP-1 line than the WSP-2 line. Thus, even if the two-way ANOVA on replicate and treatment effects in WSP mice revealed no main effects or interactions, the lines were still each analyzed individually for treatment effects.

The receptor autoradiography data were initially analyzed using a three-way ANOVA with line, replicate and treatment as factors, followed by simple main effects and post-hoc analyses where indicated. Data were considered significant at $p \leq 0.05$. In

contrast to the *in situ* hybridization analyses, the receptor autoradiography data were obtained using identical radiolabeled ligand, and the slides were apposed to film simultaneously. As a result, data were not normalized to control and it was possible to compare absolute abundance of the receptor across multiple brain regions and between the selected lines.

We have observed significant differences in basal HIC severity between the seizure-prone lines; WSP-2 mice display greater HICs than WSP-1 mice, while WSP-1 animals display greater HICs than WSP-2 mice during EtOH withdrawal. In contrast, the seizure-resistant lines show no baseline HICs and are therefore not different in their drug-naïve response. Thus, there is a continuum within the lines with respect to basal seizure severity. WSR-1 and WSR-2 lines showing negligible activity and WSP-1 mice show baseline HICs, but the severity of their seizures is less than the baseline HICs observed in WSP-2 mice (WSR-1=WSR-2<WSP-1<WSP-2) (A.S. Beadles-Bohling unpublished observations). Given these observations, we applied an approach similar to that outlined in Crabbe, 1990 (Crabbe et al., 1990c) to analyze potential differences in basal KOP-R abundance across the replicate lines. If a three-way ANOVA revealed no main effect of treatment and no significant interactions of treatment with line and/or replicate, and if there was no effect of treatment observed when each line and replicate was analyzed individually, the data were collapsed over treatment group to increase the sample size for the analysis of inherent differences between the lines and replicates. The results were interpreted as discussed by Crabbe et al., (1990). If both replicates showed significant line differences that were in the same direction, there was strong evidence for a correlated response to selection, while a difference between lines within only one replicate was

considered moderate evidence for a genetically correlated response to selection. An absence of significant differences between either replicate suggested weak evidence for a genetically correlated response.

Because inherent differences in KOP-R abundance were observed across the lines, analyses of EtOH-specific effects on KOP-R abundance were performed using data that were normalized to their respective pyrazole controls. We analyzed the WSP lines for replicate differences and in a separate analysis examined the WSR lines for replicate differences. When no replicate differences were observed in the WSP lines, the data were reanalyzed for treatment effects collapsed across replicate. However, to determine whether replicates one and two exhibited different levels of sensitivity to KOP-R system alterations, each replicate was also analyzed independently. WSR lines were only analyzed independently for treatment effects if there was a significant interaction between replicate and treatment in the two-way ANOVA.

Results

EtOH exposure

The KOP-R system may play a role in mediating withdrawal severity. To test this prediction, WSP and WSR animals were chronically exposed to EtOH and withdrawn, followed by characterization of *Pdyn* by *in situ* hybridization and KOP-R by receptor autoradiography. EtOH exposure was carefully monitored, and BECs of the animals were maintained as closely as possible to the desired range of 1.8-2.0 mg/ml. Upon removal from EtOH (at the 72-hour time point), BECs were determined for all EtOH-exposed animals, and are as follows (expressed as mg EtOH/ml blood ± standard error of the mean; SEM): WSP-1, 1.97±0.08; WSP-2, 1.89±0.10, WSR-1, 1.73±0.06, and WSR-2, 1.77±0.11. Prior to the EtOH vapor exposure paradigm, the EtOH exposed animals were pseudo-randomly divided into two experimental groups: EtOH exposed animals that were to be immediately euthanized (EtOH-0), and EtOH withdrawn animals that were transferred to a quiet room and allowed to withdraw for six hours (EtOH-6). In order to verify that there were no differences between the BECs of these two subgroups, the 72 hour BECs of the EtOH treated mice were analyzed by ANOVA with the factors line, replicate and experimental group. The analysis revealed no significant main effect of group (EtOH-0 vs. EtOH-6) on the BECs, and no interactions of this factor with line or replicate, indicating that BECs were comparable across groups. Thus the BEC data is presented collapsed across these two experimental groups for each line and replicate. Analysis of the 72-hour BECs by two-way ANOVA with the factors line and replicate

revealed a significant main effect of line [$F(1,95)=4.89$, $p<0.05$] (WSP>WSR). No main effect of replicate or interactions between line and replicate were observed.

Prodynorphin in situ hybridization analysis

Brain regions that may play a role in seizure circuitry (Pir, Tu, Acb and CPu) (Gale, 1992; Loscher and Ebert, 1996; Mraovitch and Calando, 1999; Deransart and Depaulis, 2002; Ma and Leung, 2002; Slaght et al., 2002; Malas et al., 2003), and EtOH drinking and affective states (Acb and CPu) (Samson et al., 1992; Jamensky and Gianoulakis, 1997; Koob, 2003), were examined for alterations in *Pdyn* abundance. Digestion with RNase prior to hybridization with labeled *Pdyn* cRNA resulted in no detectable signal, as did the utilization of sense strand RNA for hybridization (data not shown). The brain regions analyzed for *Pdyn* mRNA abundance are depicted in figure 6. Patchy binding was also observed in the cortex, however, due to the difficulty in defining clear boundaries for the binding, it was not included in the analysis. The brain regions in which we observed *Pdyn* mRNA binding were consistent with previous characterizations of *Pdyn* mRNA expression in mouse brain (Gulya et al., 1993; Jamensky and Gianoulakis, 1997; Turchan et al., 1997; Jamensky and Gianoulakis, 1999). Representative *in situ* hybridization images are depicted in Figure 6B.

Piriform Cortex. Analysis of *Pdyn* mRNA abundance in Pir was performed only in replicate one of the WSP and WSR lines due to the presence of tissue artifacts that prohibited analysis of this brain region in replicate-2 animals. A decrease of 32% in *Pdyn* abundance compared to controls was observed in EtOH exposed WSP-1 mice, while EtOH withdrawn animals exhibited an 8% decrease. A 3% decrease in *Pdyn* mRNA was present in EtOH intoxicated WSR-1 mice, while withdrawing WSR-1 mice

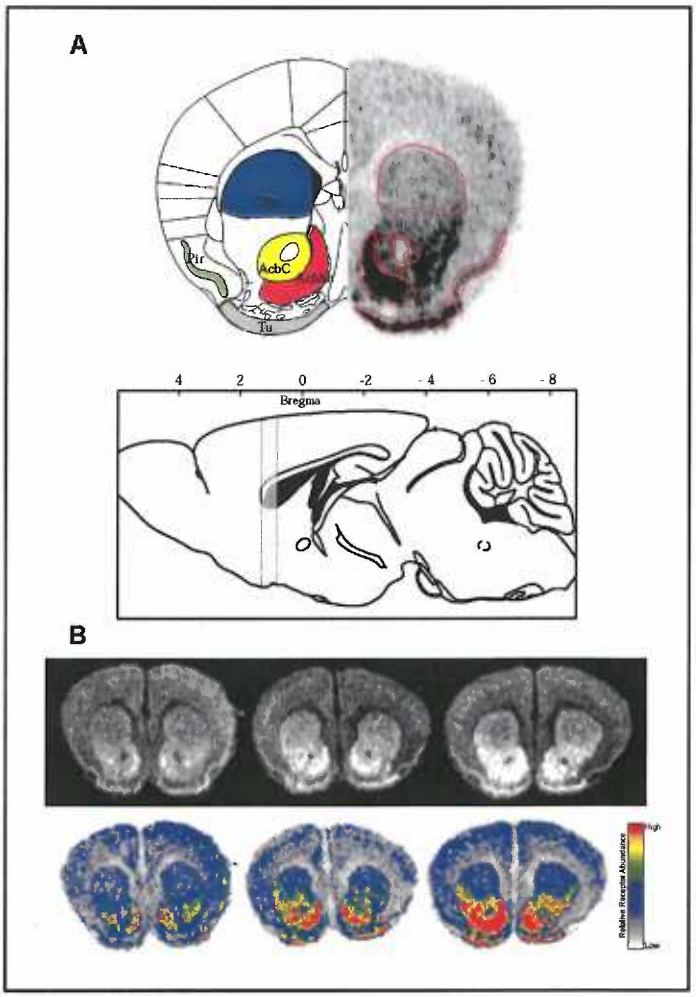


Figure 6. *In situ* hybridization analysis of prodynorphin mRNA. (A) Line drawing and representative autoradiogram indicating the regions analyzed using prodynorphin mRNA *in situ* hybridization. Upper panel is a coronal slice located 1.18 mm from Bregma. The left side of the image has been adapted from *The Mouse Brain In Stereotaxic Coordinates*, with permission from the publisher (Paxinos and Franklin, 2001). The brain regions analyzed by *in situ* hybridization analysis are highlighted and include the caudate-putamen (CPu, blue), piriform cortex (Pir, green), olfactory tubercle (Tu, gray), nucleus accumbens core (AcbC, yellow), and nucleus accumbens shell (AcbSh, red). The right side of the image is a representative autoradiogram showing the brain regions as outlined for quantitation. Lower panel: Sagittal image from the same atlas showing the rostral to caudal range from which the slices analyzed were taken. **(B)** Representative *in situ* hybridization images in WSP-1 mice. Both panels from left to right: WSP-1 pyrazole control, WSP-1 ethanol intoxicated, WSP-1 ethanol withdrawn. Upper panel: darkfield representations of prodynorphin autoradiograms in WSP-1 animals highlighting brain regions expressing prodynorphin mRNA. Note expression of *Pdyn* in CPu, AcbSh, AcbC, Tu and Pir. Bottom panel is the same data displayed using a pseudo-color image to highlight the increasing abundance of prodynorphin expression in nucleus accumbens shell across the three treatment groups. Additional abbreviation in figure: aca, anterior commissure, anterior part.

exhibited a 3% increase in *Pdyn* abundance. However, analysis by ANOVA was non-significant, indicating treatment did not significantly alter *Pdyn* in the piriform cortex of WSP-1 or WSR-1 mice (Figure 7A, Piriform Cortex).

Olfactory Tubercle. *Pdyn* mRNA abundance in the Tu also was also examined only in the replicate-1 line due to the presence of tissue artifacts as described for the Pir data. EtOH withdrawal significantly increased *Pdyn* mRNA abundance in this line by 56% ($p<0.001$), while a non-significant 11% increase in *Pdyn* was observed in EtOH intoxicated WSP-1 mice, (Figure 7B, Olfactory Tubercle). An 8% decrease in *Pdyn* abundance was observed in the EtOH intoxicated WSR-1 mice, and a 25% decrease in *Pdyn* abundance was present in the withdrawing WSR-1 group. However, these effects did not reach significance in WSR-1 mice. Thus, the WSR-1 line was not significantly affected by EtOH exposure or withdrawal based on the results of a one-way ANOVA for treatment effects.

Nucleus Accumbens Core. Non-significant increases in *Pdyn* expression of 8% and 2% were present in the AcbC of EtOH intoxicated WSP-1 and WSP-2 mice (respectively) (Figure 8A, Accumbens Core). In contrast, EtOH withdrawal significantly increased *Pdyn* mRNA abundance by 23% in the WSP-1 line, and by 11% in WSP-2 mice. EtOH treated WSR-1 mice showed a 20% decrease in *Pdyn* abundance and a decrease of 13% percent was observed in the WSR-2 groups. The effect of treatment was significant in WSP-1 mice [$F(2,54)=6.07$, $p<0.05$], but no main effect of replicate was present and there was no replicate by treatment interaction. Thus, the data was collapsed on replicate, and reanalyzed for main effects of treatment. Collapsed across replicate the main effect of treatment in WSP mice was significant [$F(2,46)=5.85$, $p<0.01$]. Post hoc

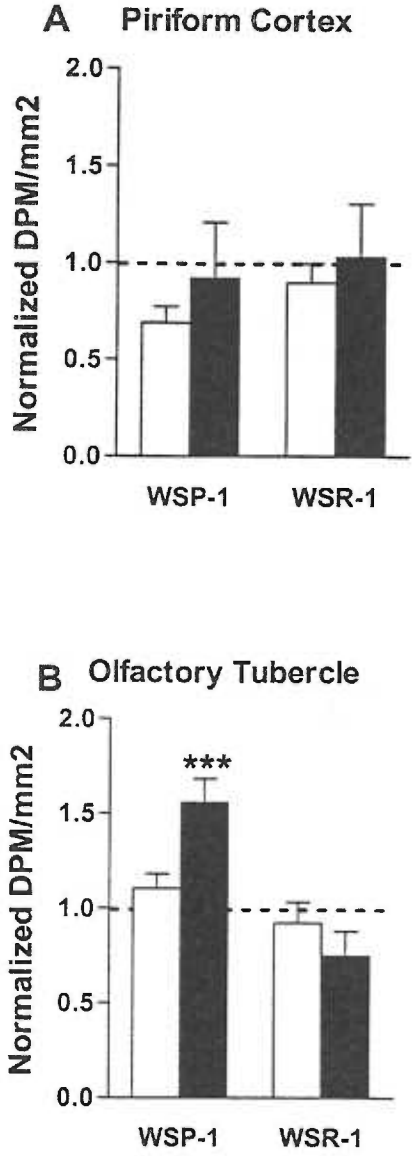
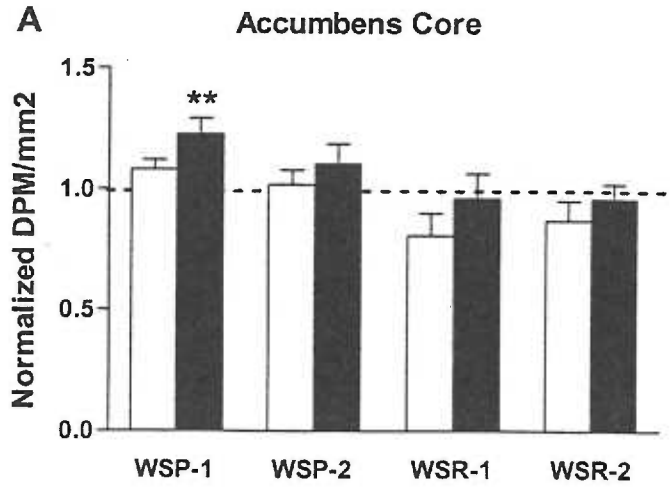
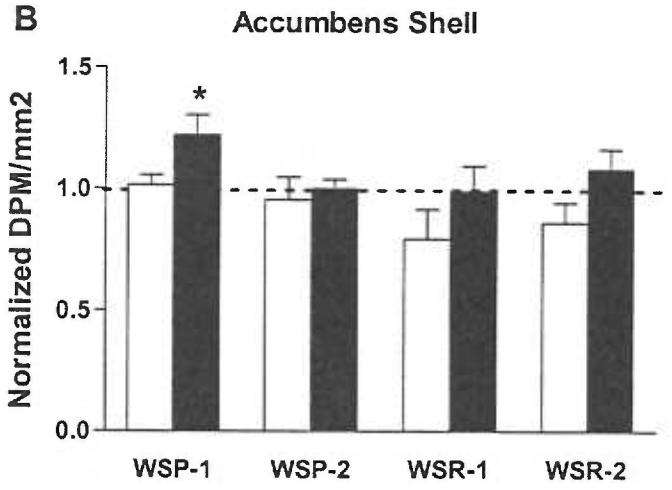


Figure 7. In situ hybridization analysis of prodynorphin mRNA abundance in piriform cortex and olfactory tubercle of replicate one WSP and WSR mice. Values are expressed as fold increase from control; control is arbitrarily set to one and is depicted by the dotted line. Open (□) bars represent data from the ethanol-intoxicated groups for the line and replicate indicated, while solid (■) bars represent ethanol-withdrawn groups. (A) ANOVA revealed no significant effects of treatment on prodynorphin expression in Pir of either WSP-1 or WSR-1 lines. (B) ANOVA revealed a significant increase in prodynorphin abundance in Tu of WSP-1 subjects withdrawn from ethanol compared to their controls [$F(2,24)=9.88$, $p<0.001$], (***$p<0.001$). No effects of treatment were observed in WSR-1 subjects, although there did appear to be a trend toward a decrease in prodynorphin abundance in withdrawn animals.


A
Accumbens Core
Normalized DPM/mm2
1.5
1.0
0.5
0.0
**
WSP-1
WSP-2
WSR-1
WSR-2


B
Accumbens Shell
Normalized DPM/mm2
1.5
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0.0
*
WSP-1
WSP-2
WSR-1
WSR-2

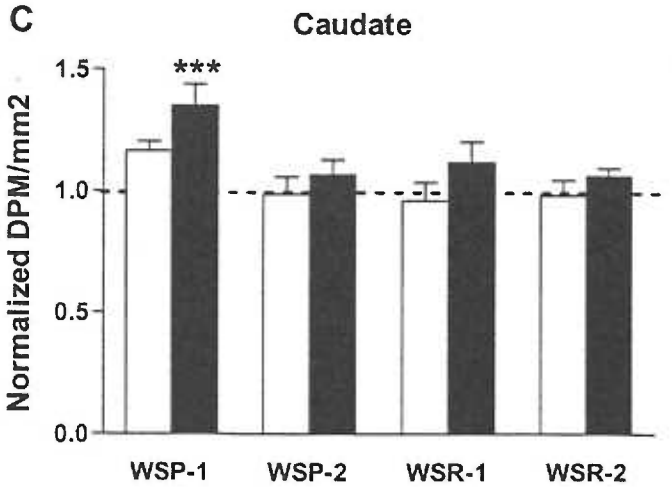
C
Caudate
Normalized DPM/mm2
1.5
1.0
0.5
0.0
***
WSP-1
WSP-2
WSR-1
WSR-2

Figure 8. *In situ* hybridization analysis examining effect of EtOH intoxication and EtOH withdrawal on *Pdyn* abundance in the nucleus accumbens core, nucleus accumbens shell, and caudate-putamen of WSP and WSR mice. Values are expressed as fold increase from control; control is arbitrarily set to one and is depicted by the dotted line. Open (□) bars represent data from the ethanol-intoxicated groups for the line and replicate indicated, while solid (■) bars represent ethanol-withdrawn groups. **(A)** Prodynorphin abundance was significantly increased in the nucleus accumbens core of WSP-1 mice during withdrawal from ethanol (**$p<0.01$) and there was a trend toward an increase in WSP-2 mice during withdrawal that did not reach significance. Note the small decrease in prodynorphin abundance within the nucleus accumbens core of ethanol intoxicated WSR lines. **(B)** Analysis of prodynorphin abundance within WSP-1 shell revealed a significant increase in prodynorphin abundance during ethanol withdrawal (*$p<0.05$). Examination of WSP-2 mice revealed no effect of treatment. No significant differences were observed in WSR mice. **(C)** ANOVA in caudate-putamen revealed a significant increase in prodynorphin abundance during withdrawal in WSP-1 mice (***$p<0.001$). There was also a slight increase of *Pdyn* in ethanol-intoxicated animals, but this did not reach significance. WSP-2 animals were not significantly affected by ethanol exposure or withdrawal. No significant effects of treatment were observed in WSR-1 or WSR-2 lines.

analysis using Dunnett's test revealed EtOH-6 WSP mice show increased *Pdyn* abundance compared to their controls. A significant main effect of treatment [$F(2,29)=4.87$, $p<0.05$] was present whenWSP-1 animals were examined by one-way ANOVA. Post-hoc testing showed *Pdyn* abundance was significantly increased within the AcbC of EtOH-6 WSP-1 mice compared to their Pyr controls ($p<0.01$); no significant effects were observed in EtOH-0 animals. ANOVA in WSP-2 animals alone revealed no significant effects of treatment. Thus, while there is a significant increase in *Pdyn* abundance in EtOH-6 WSP mice when collapsed across replicate, the effect in WSP-2 EtOH-6 groups does not reach significance on its own, suggesting WSP-1 mice are more dramatically affected by EtOH withdrawal than WSP-2 mice. Analysis of WSR-1 and WSR-2 mice for effects of replicate and treatment revealed no main effects of replicate or treatment, and no treatment by replicate interactions. Collapsing across replicate did not reveal a significant main effect of treatment in WSR mice ($p=0.10$). It is, however, interesting to note that the EtOH-0 groups in both WSR lines show a small non-significant decrease in *Pdyn* abundance.

Nucleus Accumbens Shell. Alterations in the AcbSh of WSP mice were similar to those observed in AcbC. EtOH-6 WSP-1 animals exhibited a 22% increase in *Pdyn* mRNA abundance, while no effects were present in either WSP-2 group. EtOH-0 WSR-1 mice displayed a 22% decrease in *Pdyn* levels, while WSR-2 animals exhibited a decrease of 14%. Two-way ANOVA in WSP mice revealed no main effects of replicate or treatment, and no replicate by treatment interactions. Collapsing the WSP data across replicate revealed only a trend toward an effect of treatment ($p=0.07$). However, *a priori* analysis of treatment effects in WSP-1 mice alone was significant [$F(2,26)=3.51$,

$p<0.05$). Identical to the findings within the WSP-1 AcbC, EtOH withdrawal increases *Pdyn* abundance in the AcbSh of WSP-1 animals ($p<0.05$), while intoxication had no effect on *Pdyn* levels in this brain region (Figure 8B, Accumbens Shell). One-way ANOVA examining treatment effects on *Pdyn* abundance within WSP-2 AcbSh did not reach significance. No significant main effects of replicate or treatment were present in the two-way ANOVA examining WSR mice, and there were no interactions. Analysis of WSR data collapsed on replicate resulted in no effect of treatment. Interestingly, WSR EtOH-0 groups again showed a small reduction in *Pdyn* abundance. Thus, EtOH withdrawal appears to increase *Pdyn* abundance within the prone WSP-1 line, but this effect is not significant in WSP-2 lines, and EtOH withdrawal has no significant effect on *Pdyn* abundance in the AcbSh of the WSR replicates.

Caudate-putamen. Examination of the effect of EtOH in CPu of WSP and WSR mice revealed that EtOH withdrawal again increased *Pdyn* mRNA abundance in WSP mice (Figure 8C, Caudate-putamen). In WSP-1 animals the increase was 35%, while in WSP-2 mice this effect was a much smaller 6%. EtOH exposure also produced a 16% increase in *Pdyn* in WSP-1 mice, but no effects were present in WSP-2 animals. EtOH intoxication had no effect in either WSR replicate, however, EtOH withdrawal increased *Pdyn* expression in WSR-1 animals by 12%, and in WSR-2 animals, 8%. Two-way ANOVA in WSP lines revealed a significant effect of replicate [$F(1,54)=9.21$, $p<0.005$] and treatment [$F(2,54)=6.07$, $p<0.005$], but only a trend toward a replicate by treatment interaction ($p=0.07$). *A priori* one-way ANOVA in WSP-1 animals revealed a significant main effect of treatment [$F(2,30)=8.63$, $p=0.001$]. Post-hoc analysis using Dunnett's test revealed *Pdyn* expression was significantly increased in the CPu of EtOH-

6 WSP-1 mice ($p<0.001$) (Figure 8C). *Pdyn* expression within this brain region was not significantly altered in EtOH-0 WSP-1 mice. While the ANOVA did not reach significance in the WSP-2 line, note that the alterations in *Pdyn* abundance in these animals are again in the same direction as that of the WSP-1 line. Two-way ANOVA on WSR mice revealed no effect of replicate or treatment, and no replicate by treatment interaction. When analyzed collapsed across replicate, there was still no effect of treatment in WSR mice.

Kappa opioid receptor autoradiography

The brain regions analyzed and representative images for the KOP-R AR are shown in Figure 9. Incubation with radioligand in the presence of 1 μM naloxone resulted in no detectable binding, thus the binding was specific for KOP-R. Within the brain regions analyzed, effects of EtOH exposure and EtOH withdrawal on KOP-R abundance were minimal. However, EtOH related changes were observable in the CPu, *l*AcbSh, Tu, and Cg. Interestingly, binding was also high in the Cl, DEn, and medial AcbSh, but the abundance of KOP-R in these brain regions was unaffected by EtOH treatment. Instead, we observed inherent differences in KOP-R abundance across the WSP and WSR lines. This suggests that differences in KOP-R abundance may have emerged during the selective breeding process.

Basal differences in KOP-R abundance across replicate selected lines

Caudate-putamen. Findings in the CPu suggest that differences in KOP-R abundance do not correlate with withdrawal-related phenotypic differences observed between the WSP and WSR lines. Additionally, differences in KOP-R abundance

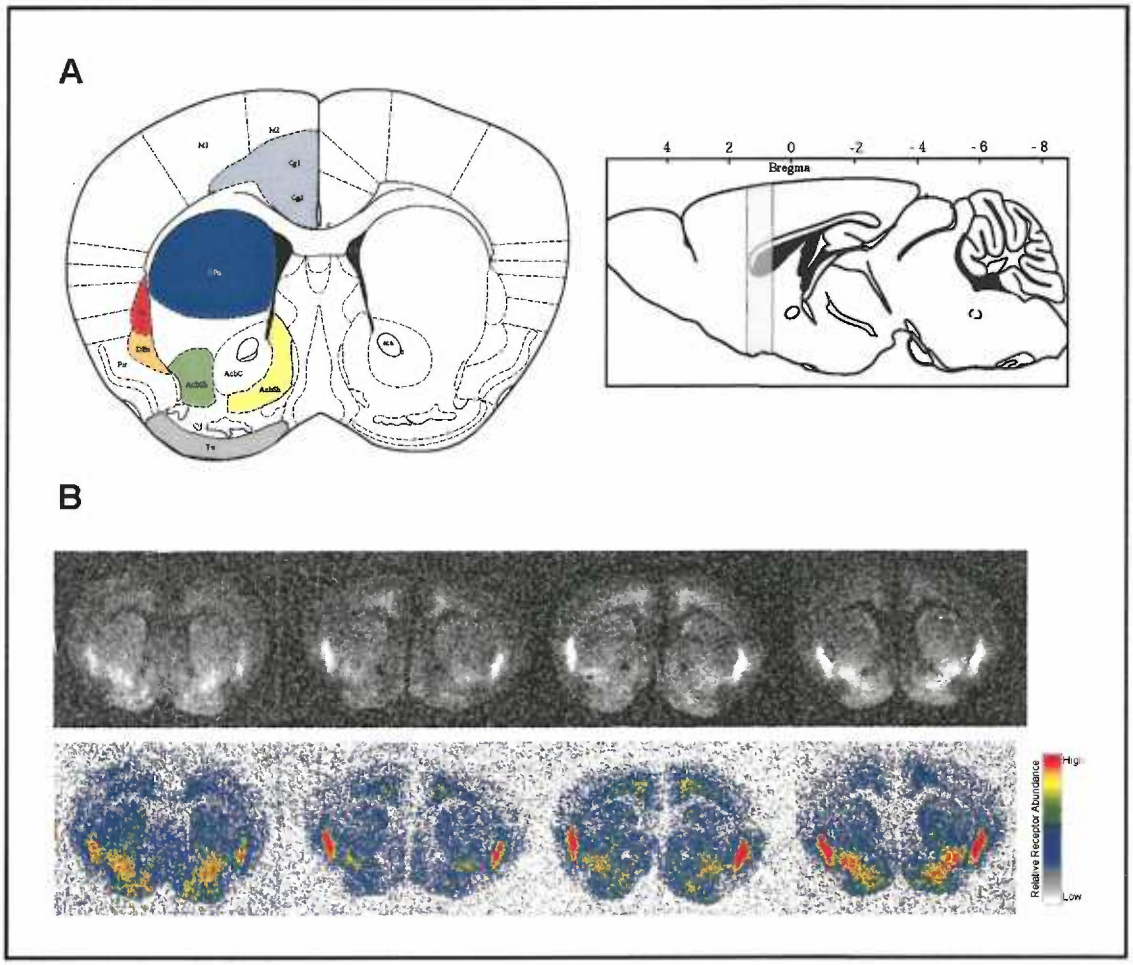

Figure 9. Kappa-opioid receptor autoradiography (KOP-R AR) using ^{3}H-CI-977. **Drawing and representative images from the four replicate lines highlighting kappa-opioid receptor expression in the dorsal endopiriform nucleus. The brain regions to be analyzed were identified as described for *in situ* hybridization.** (A) **Line drawings adapted with permission from *The Mouse Brain In Stereotaxic Coordinates, second edition*, (Paxinos and Franklin, 2001). Left: Coronal section showing the brain regions analyzed by kappa-opioid receptor autoradiography. Regions analyzed were the cingulate cortex (Cg, light blue), caudate-putamen (CPu, blue), claustrum (Cl, red), dorsal endopiriform nucleus (DEn, orange), olfactory tubercle (Tu, gray), medial nucleus accumbens shell (*m*AcbSh, yellow) and lateral nucleus accumbens Shell (*l*AcbSh, green). Right: Saggital section indicating regions from which coronal sections were gathered for KOR AR.** (B) **Representative images in (L to R): WSP-1, WSP-2, WSR-1 and WSR-2 control mice. Images were gathered by scanning film autoradiographs using a ScanMaker 9800XL Scanner with transparency adaptor (Microtek, Carson, CA) at 1200 dpi. Top panel: Images have been converted to darkfield format to more clearly delineate discrete brain regions displaying KOR binding. Bottom panel is the same data presented in pseudocolor format to highlight the increasing abundance of kappa-opioid receptor in the dorsal endopiriform nucleus of control animals (WSP-1<WSP-2<WSR-1 WSR-2).**

between WSP-1 and WSP-2 lines would not appear to contribute to the baseline HIC differences between these two lines. Analysis by two-way ANOVA for line and replicate effects revealed significant main effects of line [$F(1,51)=5.98$, $p<0.05$] and replicate [$F(1,51)=4.19$, $p<0.05$] but no line by replicate interactions. Post hoc analyses using Bonferroni's test revealed WSR animals showed significantly higher KOP-R expression than WSP mice overall, and replicate-2 animals had significantly higher KOP-R abundance than replicate-1 one animals overall. Analysis of the replicates individually revealed only small, non-significant differences between the WSP-1 and WSR-1 lines ($p=0.09$) and the WSP-2 and WSR-2 lines ($P=0.13$) (Figure 10, CPu). Examination of WSP lines revealed no differences between the replicates, but a trend toward a difference between the WSR replicate lines was present ($P=0.07$). Thus, while it appears that WSR-2 mice express greater levels of KOP-R than the remaining lines, this effect was not significant.

Lateral Nucleus Accumbens Shell. WSR-2 mice exhibited 50-58% higher basal KOP-R abundance ($p<0.001$) in the *l*AcbSh compared to WSR-1 mice and both WSP lines. Two-way ANOVA on controls examining baseline differences revealed significant effects of line [$F(1,51)=19.23$, $p<0.001$] and replicate [$F(1,51)=27.97$, $p<0.001$] as well as a significant line by replicate interaction [$F(1,51)=23.76$, $p<0.001$]. Post hoc analysis of the interaction revealed WSR-2 mice express significantly greater amounts of KOP-R than all the other replicate lines ($p<0.001$ vs. WSP-1,WSP-2,and WSR-1), while WSP-1 and WSR-1 KOP-R levels were not significantly different from each other. However, WSR-2 controls expressed significantly greater levels of KOP-R than WSP-2 animals ($p<0.001$) (Figure 10, *l*AcbSh). This is moderate support for a genetic correlation

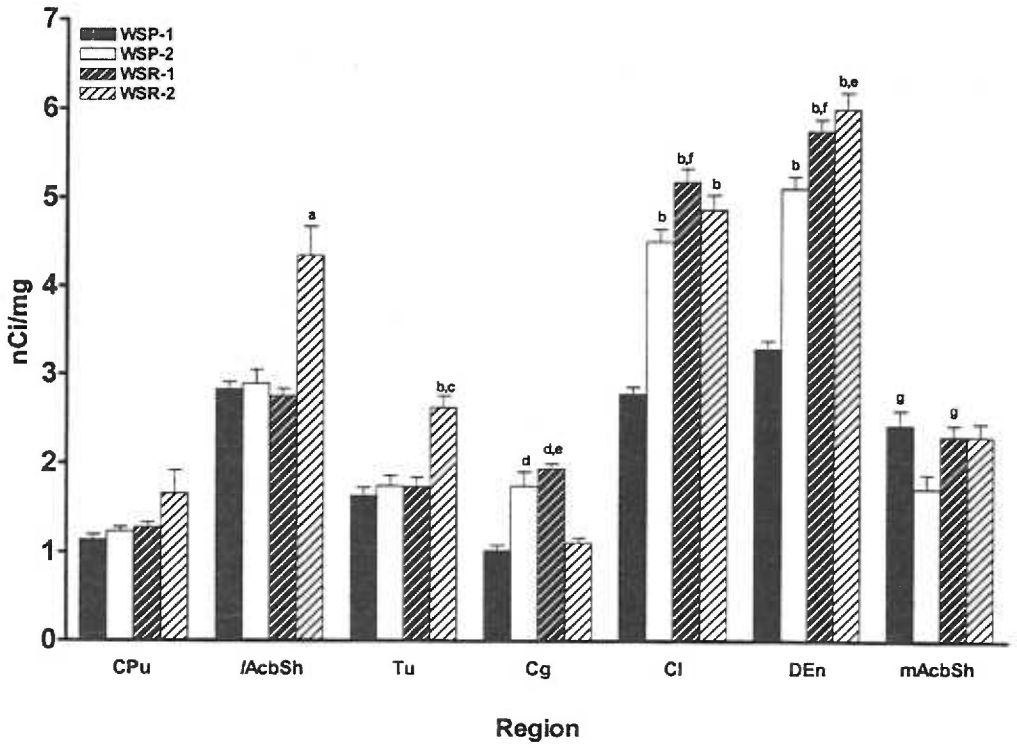

Figure 10. Basal differences in kappa-opioid receptor levels across multiple brain regions of WSP-1 (■), WSP-2 (□), WSR-1 (▨) and WSR-2 (▨) lines. *Caudate-putamen (CPu):* There was a trend for WSR-2 animals to express greater levels of receptor in caudate-putamen, however this effect did not reach significance. *Lateral accumbens shell (lAcbSh) and Olfactory tubercle (Tu):* WSR-2 animals exhibited significantly greater levels of kappa-opioid receptor in lateral accumbens shell and olfactory tubercle than all other groups ($^{a}p<0.001$ vs. WSP-1, WSP-2 and WSR-1; $^{b}p<0.001$ vs. WSP-1; $^{c}p<0.005$ vs. WSP-2 and WSR-1). *Cingulate cortex (Cg):* WSP-1 and WSR-2 mice did not differ in their expression of kappa-opioid receptor in cingulate cortex however, these two lines expressed significantly less kappa-opioid receptor than both WSP-2 and WSR-1 lines ($^{d}p<0.001$ vs. WSP-1 and WSR-2). WSR-1 mice expressed significantly greater kappa-opioid receptor levels than WSP-2 mice ($^{e}p<0.001$ vs. WSP-2). *Claustrum (Cl):* Within the claustrum, WSP-1 mice displayed significantly lower levels of kappa-opoid receptor than all the other groups ($^{b}p<0.001$ vs. WSP-1). WSP-2 mice expressed less kappa-opioid receptor than WSR-1 mice ($^{f}p<0.005$ vs. WSP-2). *Dorsal endopiriform nucleus (DEn):* WSP-1 mice again exhibited significantly lower levels of kappa-opioid receptor compared to the other three groups ($^{b}p<0.001$ vs. WSP-1). While WSP-2 mice expressed levels of receptor significantly greater than WSP-1 mice, KOR abundance in WSP-2 mice was significantly less than both WSR lines ($^{e}p<0.001$ vs. WSP-2; $^{f}p<0.005$ vs. WSP-2). *Medial Accumbens Shell (mAcbSh):* WSP-2 mice express significantly less kappa-opioid receptor than WSP-1 and WSR-1 mice ($^{g}p<0.05$ vs. P2). While there was a trend toward a difference between WSP-2 mice and WSR-2 mice, the difference did not reach significance (p=0.06).

between inherent KOP-R abundance in the *l*AcbSh and the phenotypic responses seen in WSP and WSR animals. Differences were not present between WSP-1 and WSP-2 lines, suggesting there was no influence of KOP-R abundance in the *l*AcbSh on the observed differences in basal HIC severity in the WSP replicate lines.

Medial Nucleus Accumbens Shell. Within the *m*AcbSh we observed a 25-30% decrease in WSP-2 KOP-R compared to WSP-1 and both WSR lines. Examination of inherent KOP-R abundance across WSP and WSR replicate lines by two-way ANOVA revealed no effect of line, but a significant main effect of replicate [$F(1,83)=2.09$, $p<0.05$], and a significant line by replicate interaction [$F(1,83)=4.88$, $p<0.05$]. Post hoc analysis of the interaction revealed WSP-2 mice expressed significantly lower levels of KOP-R abundance compared to WSP-1 ($p<0.05$) and WSR-1 ($p<0.05$) lines, but there was only a trend for the same effect when compared to the WSR-2 line ($P=0.06$) (Figure 10, *m*AcbSh). No other significant differences were observed. Thus, significant differences in KOP-R levels were not observed in either replicate within the *m*AcbSh. The absence of any significant differences between the replicates suggests that alterations in KOP-R levels in the *m*AcbSh do not participate in the selected and correlated responses of these lines.

Olfactory Tubercle. Inherent differences in KOP-R abundance in the Tu were observed between WSP and WSR lines, with WSR-2 exhibiting a 50-60% increase in basal KOP-R abundance compared to both WSP lines, and the WSR-1 line. Two-way ANOVA with line and replicate as factors revealed significant main effects of line [$F(1,41)=13.20$, $p<0.001$] and replicate [$F(1,41)=13.45$, $p<0.001$] and a significant interaction [$F(1,41)=8.24$, $p<0.01$]. Post hoc analysis of the interaction again indicated

WSR-2 animals expressed significantly greater levels of KOP-R than WSP-1 ($p<0.001$), WSP-2 ($p<0.01$) and WSR-1 lines ($p<0.01$) (Figure 10, Tu). No other significant differences were observed. Thus, WSP-2 and WSR-2 lines express significantly different levels of KOP-R, with WSR-2 again expressing greater levels than WSP-2. No differences were observed between replicate one, and WSP-1 and WSP-2 KOP-R abundance did not differ. This again provides moderate evidence for a contribution of inherent differences in KOP-R abundance in the Tu to the phenotypic differences observed between the selected lines. No differences between WSP-1 and WSP-2 KOP-R abundance exists in the Tu, thus the data does not suggest that differences in KOP-R abundance within this brain region contribute to the differing HIC response in naïve WSP animals.

Cingulate Cortex. Findings in the Cg suggest there is no contribution of KOP-R differences in this region to the withdrawal related phenotypic differences observed between these lines. Two-way ANOVA on Cg data revealed no effect of line or replicate, however a significant line by replicate interaction was present [$F(1,54)=84.20$, $p<0.001$]. Post hoc analysis of the interaction revealed WSP-1 mice express significantly less KOP-R than both WSP-2 and WSR-1 lines while WSP-2 KOP-R mice express significantly greater amounts of KOP-R than WSR-2 mice. Finally, WSR-1 KOP-R abundance is significantly greater than that of WSR-2 mice (Figure 10, Cg).

Claustrum. Significant differences in KOP-R abundance in the Cl were restricted to replicate one animals. Two-way ANOVA in Cl revealed a significant main effect of line [$F(1,155)=98.37$, $p<0.001$], and replicate [$F(1,155)=25.92$, $p<.001$], and a significant line by replicate interaction [$F(1,155)=53.84$, $p<0.001$]. Post-hoc analysis of the line by

replicate interaction revealed WSP-1 mice express significantly less KOP-R in Cl than WSP-2, WSR-1, and WSR-2 mice. In addition, WSP-2 mice express significantly less KOP-R than WSR-1 mice, but the difference between WSP-2 and WSR-2 mice was not significant (Figure 10, Cl). Thus, replicate one WSP and WSR mice exhibit significantly different KOP-R abundance in Cl with higher levels observed in the WSR-1 line. However, this effect is not observed in replicate two animals. Additionally, WSP-1 mice express significantly less KOP-R than WSP-2 mice in Cl. These results provide moderate support for a possible correlation between inherent differences in KOP-R abundance and the differing HIC severity, drinking and initial response to the aversive effects of EtOH in these selected lines. The observed difference between the WSP-1 and WSP-2 lines also may contribute to the observed differences in baseline HIC severity between the two WSP lines.

Dorsal Endopiriform Nucleus. Differences in KOP-R abundance were observed between both replicate lines in the DEn. Two-way ANOVA revealed a significant main effect of both line [$F(1,154)=153.65$, $p<0.001$] and replicate [$F(1,154)=58.18$, $p<0.001$] as well as a significant interaction [$F(1,154)=33.37$, $p<0.001$]. Post-hoc analysis of the interaction revealed WSP-1 KOP-R abundance is significantly lower than that of WSP-2, WSR-1 and WSR-2 animals ($p<0.001$ for WSP-1 vs. all lines). In addition, KOP-R abundance in DEn of WSP-2 animals is significantly lower than that of both WSR lines ($p<0.005$, WSP-2 vs. WSR-1; $p<0.001$ vs. WSR-2). Finally, analysis of WSP replicates revealed WSP-1 mice express significantly less KOP-R than WSP-2 mice [$F(1,76)=128.29$, $p<0.001$] (Figure 10, DEn). Thus, there is again a difference between WSP-1 and WSP-2 KOP-R abundance, with WSP-1 expressing significantly less than

WSP-2; this difference may contribute to the baseline differences in HIC severity observed between the two replicate lines. The levels of KOP-R expressed in WSP and WSR mice, from least to greatest is WSP-1<WSP-2<WSR-1=WSR-2. The presence of significant differences in KOP-R abundance between the lines for both replicates provide the strongest evidence for participation of a specific brain region and the KOP-R system as important in mediating EtOH withdrawal severity as an inherent selected difference.

EtOH effects on KOP-R Abundance in WSP and WSR lines

After characterizing basal expression of KOP-R in WSP and WSR animals, we examined the effects of EtOH exposures and withdrawal on KOP-R abundance in these lines. Effects of EtOH were observed in the CPu, *l*AcbSh, OT, and Cg, while no effects of EtOH were observed in the Cl, DEn or *m*AcbSh.

Caudate-putamen. EtOH exposure increased KOP-R abundance by 35% in WSP-1 animals; during peak withdrawal the abundance of KOP-R remained elevated by 27% compared to control. No effects of EtOH were observed in the remaining lines in CPu. Three-way ANOVA examining the effects of line, replicate and treatment on KOP-R abundance in CPu revealed no effect of line or replicate. A trend toward an effect of treatment was observed ($p=0.07$), and there was a significant interaction between line and replicate [$F(2,139)=6.80$, $p=0.01$]. No other interactions were present. Given the significant line by replicate interaction, each replicate line was analyzed individually. In addition, based on the *a priori* assumption that we would observe differential effects of EtOH between the selected lines, each seizure-prone and seizure-resistant replicate line was also analyzed for replicate and treatment effects. Two-way ANOVA in WSP mice revealed a significant main effect of replicate [$F(1,67)=22.02$, $p<0.001$], and treatment

[F(2,67)=3.62, $p<0.05$] as well as a significant replicate by treatment interaction [F(2,67)=5.60, $p<0.01$]. One-way ANOVA in WSP-1 mice revealed a significant main effect of treatment [F(2,37)=7.94, $p<0.005$]. Dunnett's test revealed EtOH intoxication significantly increased KOP-R abundance in the CPu of WSP-1 mice ($p<0.01$) compared to control. EtOH withdrawal also resulted in a significant increase in KOP-R abundance ($p<0.05$) compared to control (Figure 11A, Caudate-putamen). No effects of treatment were observed in the WSP-2 line when analyzed by one-way ANOVA. Two-way ANOVA in WSR-1 and WSR-2 lines revealed a significant main effect of replicate [F(1,72)=9.76, $p<0.01$] but no effects of treatment and no replicate by treatment interactions were observed.

Lateral Nucleus Accumbens Shell. EtOH withdrawal increased KOP-R abundance by 16% in WSP-1 mice ($p<0.05$). Three-way ANOVA revealed no effect of line or replicate, but a trend toward an effect of treatment was observed (p=0.07). No significant interactions were present. *A priori* analysis of each line independently for effects of replicate and treatment revealed a significant main effect of replicate [F(1,72)=5.13, $p<0.05$] and treatment [F(2,72)=3.24, $p<0.05$] in WSP lines, but no replicate by treatment interaction was observed. One-way ANOVA in WSP-1 groups revealed a significant effect of treatment [F(2,40)=4.29, $p<0.05$]. Post hoc analysis using Dunnett's test revealed the abundance of KOP-R in the EtOH-6 group was significantly increased from controls ($p<0.05$) (Figure 11B, lateral Accumbens Shell). One-way ANOVA in WSP-2 groups revealed no effects of treatment. Two-way ANOVA in WSR lines revealed no effect of replicate or treatment and no replicate by treatment interaction; collapsing on replicate did not result in an effect of treatment. Thus, in the *l*AcbSh, there

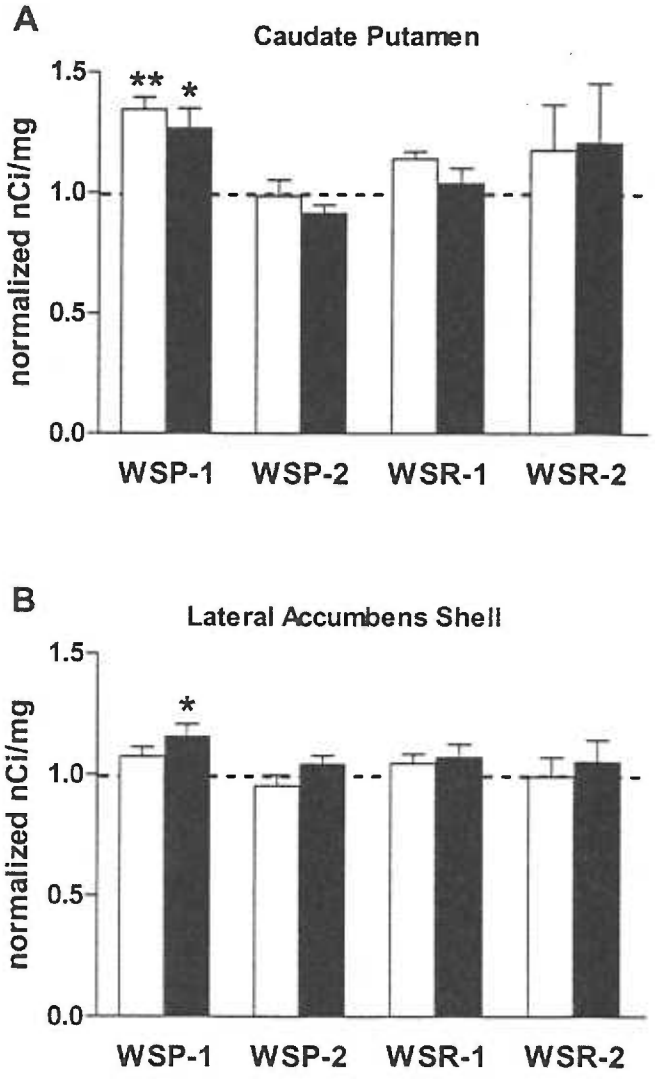

Figure 11. **Kappa opioid receptor abundance in CPu and *l*AcbSh of WSP and WSR mice.** Values are expressed as fold increase from control; control is arbitrarily set to one and is depicted by the dotted line. Open (□) bars represent data from the ethanol-intoxicated groups for the line and replicate indicated, while solid (■) bars represent ethanol-withdrawn groups. **(A)** Intoxicated WSP-1 mice exhibited significantly increased kappa-opioid receptor levels in the CPu (**$p<0.01$). This effect persisted during withdrawal in WSP-1 CPu (*$p<0.05$). Replicate two WSP mice were unaffected by treatment, and both WSR replicates showed no alterations in kappa-opioid receptor abundance during intoxication or withdrawal. **(B)** Kappa-opioid receptor abundance is significantly increased in the *l*AcbSh of WSP-1 mice during ethanol withdrawal, (*$p<0.05$). Ethanol intoxication had no effect on kappa-opioid receptor abundance in WSP mice; kappa-opioid receptor abundance in WSR mice was unaffected by intoxication or withdrawal.

was an increase in KOP-R abundance during withdrawal in WSP-1 mice, but no other effects of EtOH were observed in this brain region.

Olfactory Tubercle. In the Tu, EtOH withdrawal increased KOP-R abundance 24% from control levels in WSP-1 mice ($p<0.05$). Three-way ANOVA revealed no effect of line or treatment, however a significant effect of replicate was observed [$F(1,90)=5.74$, $p<0.05$]. No main effect or interactions were observed. When WSP mice were analyzed separately for replicate and treatment effects a trend for an effect of replicate ($p=0.06$) was observed, but there was no effect of treatment and no interaction. ANOVA on the WSP data collapsed across replicate resulted in a main effect of treatment [$F(2,51)=4.35$, $p<0.05$]. Dunnett's test revealed this effect was due to a significant increase in KOP-R abundance in EtOH-6 WSP mice compared to control (Figure 12A, Olfactory Tubercle). Analysis of each line and replicate individually revealed a significant main effect of treatment in WSP-1 animals [$F(2,29)=4.46$, $p<0.05$]. Post-hoc analysis using Dunnett's test showed EtOH-6 WSP-1 mice expressed significantly greater levels of KOP-R than controls ($p<0.05$). There was no significant difference between control and EtOH-0 WSP-1 animals. ANOVA on WSP-2 animals revealed no effect of treatment, suggesting again, that the effects of EtOH withdrawal on WSP-1 mice are more dramatic than the effects observed in WSP-2 mice. ANOVA in WSR mice revealed no effects of replicate or treatment, and no interaction. Collapsing WSR groups across replicate again revealed no effect of treatment. Thus, the absence of replicate differences in WSP mice and the resulting increase in KOP-R abundance in WSP mice when collapsed across replicate suggest that EtOH significantly increases KOP-R abundance in EtOH-6 WSP mice. However, this effect is again much stronger in

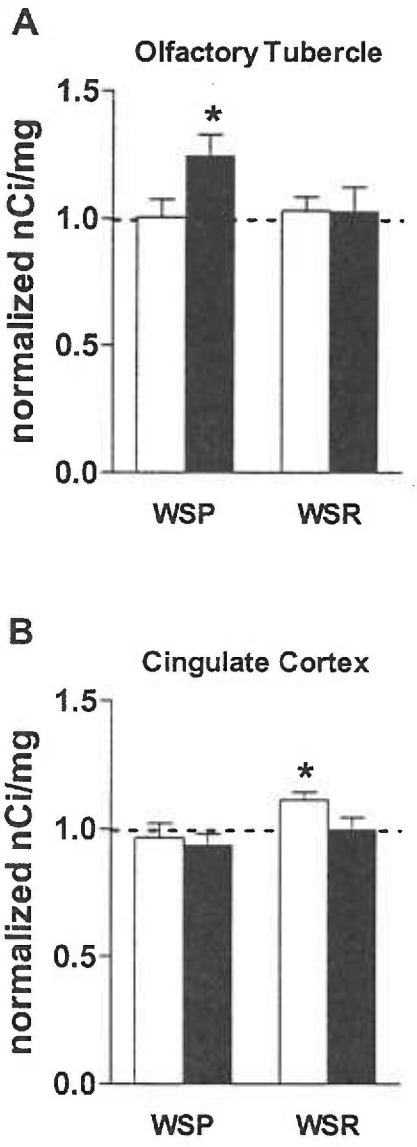

Figure 12. Kappa opioid receptor abundance in the Tu and Cg of ethanol intoxicated and ethanol withdrawn WSP and WSR mice. Data are collapsed across replicate, and the values are expressed as fold increase from control; control is arbitrarily set to one and is depicted by the dotted line. Open (□) bars represent data from the ethanol-intoxicated groups for the line indicated, while solid (■) bars represent ethanol-withdrawn groups. **(A)** Kappa-opioid receptor abundance was significantly increased in the Tu of WSP mice during withdrawal from ethanol (*$p<0.05$). There was no effect of ethanol intoxication on receptor abundance in WSP mice, and neither intoxication nor withdrawal affected receptor levels in WSR mice. **(B)** A significant increase of KOR abundance was observed in Cg of EtOH intoxicated WSR mice collapsed on control ($p<0.05$). No effects were present in withdrawing WSR mice, and WSP mice were unaffected by exposure or withdrawal.

the WSP-1 line, as it remained significant only in the WSP-1 lines when both replicates were examined individually.

Cingulate Cortex. A modest 11% increase ($p<0.05$) of KOP-R abundance overall in EtOH exposed WSR mice was present in the Cg. Analysis of Cg by three-way ANOVA revealed a significant main effect of line [$F(1,143)=3.88$, $p=0.05$] and a trend toward an effect of replicate ($p=0.06$). No effect of treatment was observed. However, a significant line by replicate interaction was observed [$F(1,143)=5.16$, $p<0.05$] as well as a three-way interaction of line, replicate and treatment [$F(2,143)=3.56$, $p<0.05$]. Analysis of each line separately for effects of replicate and treatment revealed a significant effect of replicate [$F(1,70)=6.30$, $p<0.05$], no effect of treatment, and a significant interaction [$F(2,70)=3.52$, $p<0.05$] in the WSP lines. When WSP-1 and WSP-2 lines were analyzed separately by one-way ANOVA, no treatment effects were observed. Two-way ANOVA on WSR lines revealed no effect of replicate and a significant main effect of treatment [$2,73)=4.38$, $p<0.05$]. Further analysis of WSR lines collapsed on replicate again revealed a significant main effect of treatment [$F(2,76)=4.36$, $p<0.05$] (Figure 12B, Cingulate Cortex). Post hoc using Dunnett's test revealed this was due to a significant increase in KOP-R abundance in EtOH exposed WSR mice.

Discussion

WSP and WSR mice are selectively bred lines that show severe and mild HICs respectively during withdrawal from EtOH (Crabbe et al., 1983a; Crabbe et al., 1985). In addition, these lines display differences in EtOH drinking, CPP and CTA (Kosobud et al., 1988; Crabbe et al., 1992; Chester et al., 1998). Because they have been selected over multiple generations, the genes contributing to their phenotype have been fixed, making the lines a rich resource for examining potential genes that contribute to EtOH withdrawal severity and correlated responses to selection. Based on data showing a role for the KOP-R system in seizure generation and aversion, our results supporting an anti-seizure response with pharmacological manipulation of the KOP-R system in WSP and WSR mice (see chapter Four), and our observations of increased *Pdyn* expression in whole brain of WSP mice, we identified this system as a possible contributor to the phenotypes in WSP and WSR mice. In this report we utilized *Pdyn in situ* hybridization analysis and receptor autoradiography to examine the effects of EtOH exposure and withdrawal on the KOP-R system.

Examination of *Pdyn* abundance revealed that EtOH withdrawal increased expression across multiple brain regions in WSP mice, with no change, or non-significant reductions in *Pdyn* within the WSR lines. The analysis of *Pdyn* mRNA abundance following EtOH exposure or EtOH withdrawal revealed that the WSP-1 line appeared to be most sensitive to EtOH regulation of *Pdyn* mRNA. Throughout the majority of the brain regions we analyzed, specifically the AcbC, AcbSh, CPu and Tu, a significant increase in *Pdyn* occurred during withdrawal from EtOH; the Pir was the only brain region analyzed that showed a non-significant reduction in *Pdyn* in EtOH treated WSP-1

mice. Increased *Pdyn* abundance was also observed in the WSP-2 mice, but these effects did not reach statistical significance. Small non-significant changes in *Pdyn* abundance within WSR mice were also observed in some of the brain regions analyzed. In contrast to the EtOH withdrawal related effects in the WSP lines, EtOH related effects in WSR mice were present primarily during EtOH exposure rather than withdrawal, and tended to reflect a reduction, rather than an increase in *Pdyn* expression. These findings are consistent with data previously gathered in our laboratory using Northern blot analysis, where significant increases in *Pdyn* were observed during EtOH withdrawal in whole brain of WSP-1 and WSP-2 mice, with non-significant increases observed in the WSR lines. The effects observed in the WSP lines using Northern blot analysis were also greater in the WSP-1 mice compared to WSP-2 mice. In addition, administration of a KOP-R antagonist to WSR mice decreased the EtOH withdrawal seizure-resistance observed in this line, while agonist administration non-specifically decreased HIC severity in WSP mice (Beadles-Bohling, manuscript submitted, see also Chapter four). Thus, there is accumulating evidence to suggest that alterations in *Pdyn* mRNA abundance are associated with, and may contribute to, the phenotype(s) observed in these lines which additional analyses using both systemic and site-specific pharmacological interventions should help to further elucidate.

The brain regions analyzed for differences in *Pdyn* and KOP-R abundance in WSP and WSR mice can be broadly divided into two categories, those involved with the affective components of withdrawal (i.e., dysphoria and aversion) and those involved with physical withdrawal (i.e., seizures). Those brain regions believed to contribute primarily to affective states include the Acb, Cg, Tu and the CPu, although this final

brain region could arguably be placed into either category. The Cg is a small region located within the prefrontal cortex that has been implicated in the control of mood states (Phillips et al., 2003). The Cg, as well as the Tu and CPu all receive inputs from the ventral tegmental area (VTA) (Koob et al., 1998a; Gianoulakis, 2004), which is the source of the dopaminergic afferents for the mesocorticolimbic system that also project to the Acb.

The Acb is considered a key participant in drug reward and aversion. Nearly all drugs of abuse, including EtOH, are rewarding, an effect likely brought about by enhanced extracellular levels of dopamine in the Acb (Di Chiara and Imperato, 1988b). This increase is the result of increased firing of dopaminergic neurons within ventral tegmental neurons that terminate in the Acb (Diana et al., 1993a). Conversely, removal of EtOH as well as other drugs of abuse results in reduced extracellular levels of dopamine in the Acb (Diana et al., 1993b; Simonato and Romualdi, 1996), and aversive withdrawal symptoms. Administration of KOP-R agonists into the Acb via microdialysis results in significant decreases in dopamine (Spanagel et al., 1990, 1992) and produces conditioned place aversion in rats (Bals-Kubik et al., 1993). Thus, the observed increases in *Pdyn* abundance within the core and shell of the Acb may contribute to the production of aversive states in WSP mice.

It has also been suggested that differences in dynorphin peptide levels in the accumbens may contribute to the motivation to drink alcohol. This hypothesis was supported by observations made in other EtOH preferring and avoiding selected lines. For example, examinations of basal *Pdyn* mRNA, dynorphin peptides, and KOP-R abundance within EtOH preferring B6 mice revealed significantly lower levels of every

product measured in the accumbens when compared to EtOH avoiding D2 mice. These authors suggested the differences in basal *Pdyn* and KOP-R might contribute to the differences in drinking between the alcohol preferring B6 and the alcohol avoiding D2 inbred strains. Finally, naïve alcohol-preferring (AA) rats have significantly lower levels of dynorphin A and dynorphin B peptide in this region when compared to their alcohol-avoiding (ANA) counterparts. An interesting suggestion made by the authors of this study was that this reduction may render the AA rats more sensitive to the dopamine increases in this brain region as a result of EtOH, thus making EtOH more rewarding to these animals (Nylander et al., 1994). Thus, the increased levels of *Pdyn* in WSP-1 mice might contribute to the production of a negative affective state that promotes EtOH avoidance.

Brain regions that were examined which may play a role in seizure activity included the DEn, Cl, Pir, CPu, and the Acb. The suggestion that alterations in the KOP-R system of the accumbens may participate in seizure activity as well as affective states stems from more recent data suggesting a role the for Acb in seizure circuitry. The exact role for this region in seizure activity has yet to be elucidated, although activity at metabotropic glutamate receptors has been suggested to influence EEG activity in the accumbens of rats (Popoli et al., 1999). The connections of this brain region with areas implicated in seizure activity also support a role for the accumbens in the generation or propagation of seizure activity (Leung et al., 2000; Zhang et al., 2001; Ma and Leung, 2002).

Using an immunocytochemical approach, it has also been observed that following induction of limbic seizures using kainic acid, there was an increase in Fos IR in the

AcbC, suggesting this brain region might be sensitive to seizure activity. However it is not yet clear how this region might participate in the production or maintenance of seizures. It is interesting to note that when the $GABA_A$ receptor agonist muscimol was administered to the same animals, the level of Fos in this brain region decreased (Zhang et al., 1997). $GABA_A$ agonists are anticonvulsant and it has recently been shown that administration of muscimol in combination with U50,488H, a KOP-R agonist, reduces maximal electroshock seizures severity in mice to a greater extent than the seizure protection produced by U50,488H alone. Thus, it is possible that the increased *Pdyn* observed reflects deficient dynorphin levels. This combined with decreased GABAergic activity contributes to the increased susceptibility to seizures in the WSP lines. While every effort was made to avoid inducing HICs in these animals, it is possible that subconvulsant seizure activity occurred in these mice. Finally, the observed increases in mRNA in WSP mice may be a response of the KOP-R system to depletion of dynorphin stores following this subconvulsive seizure activity. An increase in *Pdyn* synthesis would aid in the restoration of dynorphin to pre-seizure levels.

Kindled seizures generated by deep pre-Pir electrical stimulation have been shown to produce an increase in *Pdyn* mRNA in the CPu of rats (Xie et al., 1989), which is consistent with our observation of increased prodynorphin abundance in seizure-prone mice. It is also interesting to note that increased GABAergic activity in the striatum of rats results in decreased *Pdyn* mRNA abundance. This suggests that *Pdyn* expression may be under inhibitory GABAergic control in this brain region (Reimer and Hollt, 1991). Lower levels of α-1 $GABA_A$ receptor subunits have been observed in both EtOH naïve and EtOH intoxicated WSP mice when compared to WSR mice (Buck et al.,

1991b; Keir and Morrow, 1994). While subunit expression was not examined specifically in the CPu of these animals, it has been suggested that decreased abundance of this subunit may result in altered receptor abundance or function, which may be correlated with the increased HIC severity observed in withdrawing WSP mice. Thus, a reduction in the activity at $GABA_A$ receptors during EtOH withdrawal may also contribute to the observed increase in *Pdyn* abundance in the CPu of WSP mice.

Both ligand (*Pdyn*) and receptor (KOP-R) were examined in these studies. Our analyses of the KOP-R examined both basal and EtOH induced differences in the abundance of this receptor. A particularly interesting finding in the analyses of basal expression differences was the difference in KOP-R abundance observed in the DEn across the lines. Many studies suggest the DEn participates in circuitry important for seizure generation and propagation. For example, the DEn receives input from the amygdala, and it is believed that the DEn, along with the Cl, may serve as a gateway through which amygdala-kindled seizures are able to propagate activity to the motor system. It has been shown that increases in GABAergic activity within the DEn result in an increased afterdischarge threshold (i.e., decreased seizure susceptibility) to amygdala-kindled seizures in rats. In addition, this activity shortens the duration of these seizures (Stevens et al., 1988; Majak et al., 2002). The DEn is also reciprocally connected to the Pir, a region believed to be a "key structure in the 'tempestas response' of Piredda and Gale (1985)," (Piredda and Gale, 1985; Behan and Haberly, 1999) due to its many projections to brain regions involved in temporal lobe seizures. It has been suggested that these connections might act as a "substrate for regenerative positive feedback interactions" that would serve to propagate seizure activity (Behan and

Haberly, 1999). Our data revealed that the DEn of the WSP-1 line contained significantly less KOP-R than the WSP-2 line; this difference may contribute to the differences in HIC severity observed between the WSP lines. In addition, expression of KOP-R was significantly less in each of the WSP lines compared to their respective WSR lines. Thus, the levels of KOP-R expressed in WSP and WSR mice, from least to greatest is WSP-1<WSP-2<WSR-1=WSR-2, which is similar to the "graded" seizure severity of these lines during EtOH withdrawal when corrected for baseline HIC differences, where seizure responses from least to greatest are WSR-1=WSR-2<WSP-2<WSP-1. These observations are especially interesting because the lower levels of KOP-R in the WSP line compared to WSR was present within *both* replicates. This finding represents strong evidence for KOP-R participation in the EtOH withdrawal-related phenotypes of WSP and WSR mice, and suggests the DEn is an intriguing target for further studies in WSP and WSR mice.

Within the *l*AcbSh, WSR-2 mice exhibit significantly higher basal KOP-R abundance than WSR-1 mice as well as both WSP lines. Inherent differences in KOP-R abundance in the Tu were also observed between WSP and WSR lines, with the WSP-2 and WSR-2 lines expressing significantly different levels of KOP-R. WSR-2 mice again expressed greater levels of inherent KOP-R than WSP-2 mice. These findings provide moderate support for a genetic correlation between inherent KOP-R abundance in the *l*AcbSh and Tu and the phenotypic responses seen in WSP and WSR animals. Finally, differences were not present between the WSP-1 and WSP-2 lines in either of these regions, suggesting there was no influence of KOP-R abundance in the *l*AcbSh or the Tu on the observed differences in basal HIC severity in the WSP replicate lines.

Significant differences in KOP-R abundance in the Cl were restricted to replicate one animals. Thus, replicate one WSP and WSR mice exhibit significantly different KOP-R abundance in Cl with higher levels observed in the WSR-1 line. Additionally, WSP-1 mice express significantly less KOP-R than WSP-2 mice in Cl. These results provide moderate support for a possible correlation between inherent differences in KOP-R abundance and the differing HIC severity, CPP and CTA in these selected lines. The observed difference between the WSP-1 and WSP-2 lines in the Cl may contribute to the variation in baseline HIC severity between the two WSP lines.

Finally, the absence of any differences in KOP-R abundance within the Cg and the *m*AcbSh suggest that inherent differences in KOP-R system activity within these regions likely do not play a role in the phenotypic differences between the lines.

We observed less dramatic effects of EtOH on the density of KOP-R binding, although increased KOP-R levels were present in the CPu, *l*AcbSh, Tu and Cg. Similar to the effects of EtOH on *Pdyn* abundance, these changes were again observed primarily in WSP-1 mice. The effects of EtOH in the CPu were unique because KOP-R abundance was already increased during EtOH exposure, and remained high through withdrawal. In the *l*AcbSh increased KOP-R binding was present only in withdrawing WSP-1 animals. It is particularly interesting to note that in the Tu, no replicate effects were observed between the WSP lines, and when collapsed across replicate, this alteration in KOP-R abundance persisted, suggesting the increased KOP-R in the Tu is present in both WSP selected lines. Finally, alterations in KOP-R were observed in the Cg, however, this effect was unique from our previous observations in that this effect was present in WSR

mice rather than WSP mice, and changes in the KOP-R were detected during EtOH exposure rather than withdrawal.

Within the Cl and DEn we only measured KOP-R levels. In contrast to both the EtOH specific and drug independent differences in KOP-R abundance we had observed elsewhere, the only effects on KOP-R abundance that were present in these two regions were inherent differences in KOP-R abundance between the WSP and WSR lines. In the Cl both WSP lines showed significantly lower KOP-R levels compared to WSR-1 mice, while they were similar to those seen in WSR-2 mice, providing moderate evidence for participation of these inherent differences in the EtOH withdrawal response of WSP and WSR mice.

Of course, the observed alterations in *Pdyn* and KOP-R abundance in each brain region are a subset of many differences. Both known and unknown, highly complex neurocircuitry results in multiple interactions between the brain regions examined here as well as the remainder of the nervous system. Thus, while we can speculate regarding the role of alterations in *Pdyn* mRNA and KOP-R abundance within a brain region, and the outcome of these changes, it is likely that connections and mechanisms not yet characterized may also contribute to the severity of EtOH withdrawal. However, these observations do provide insight into the neuroanatomical targets of EtOH exposure and withdrawal, and suggest that many of the brain regions that have been shown to participate seizures generated by other disease states may also contribute to the susceptibility to EtOH withdrawal seizures.

Based on the brain regions exhibiting alterations in *Pdyn* mRNA and KOP-R abundance and our current knowledge of the functional role of these brain regions in

physiological and behavioral responses, we suggest that changes in the KOP-R system may influence EtOH withdrawal severity, drinking, and initial sensitivity to the aversive properties of EtOH. Further behavioral and biochemical analyses of each of these regions should help us to more specifically dissect the role of each of these brain regions in EtOH related responses, and should ultimately provide greater understanding of the brain mechanisms that contribute to alcohol dependence and withdrawal.

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Chapter IV
Anticonvulsive Effects of Modulation of Kappa Opioid Receptor Signaling in an Animal Model of Mild and Severe Ethanol Withdrawal

Preface

The following chapter represents a manuscript in preparation. The data presented here has been presented in part at Annual Meetings of the Research Society on Alcoholism.

Abstract

The neurochemical mechanisms contributing to alcohol withdrawal seizures are poorly understood. Withdrawal seizures likely reflect neuronal hyperexcitability resulting from adaptive responses to chronic alcohol consumption, unmasked upon removal of EtOH's depressant effects. Alterations in KOP-R signaling have been observed in multiple seizure types, however this system has not been systematically examined for its role in EtOH withdrawal seizures. Thus, we examined whether KOP-R manipulation alters withdrawal severity in mice selectively bred for differences in EtOH withdrawal severity, measured by handling induced convulsions (HICs). Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) mice were made physically dependent using chronic EtOH vapor inhalation, and then were examined for the effects of the KOP-R antagonist nor-binaltorphimine (nor-BNI) and agonist U-50,488H (U50) on HICs. Pretreatment with nor-BNI resulted in significantly increased HIC severity in withdrawing WSR mice, with no observable effects in withdrawing WSP mice. In contrast, U50 produced a significant decrease in HIC severity in WSP mice, with no observable effects in WSR mice. During late withdrawal (i.e., hours 12+), a rebound hyperexcitability was observed in WSP mice given KOP-R agonist. Thus, administration of a KOP-R antagonist increased HIC severity in a mouse line normally resistant to withdrawal seizures, while administration of a KOP-R agonist reduced HIC severity in animals susceptible to withdrawal seizures. These observations suggest the KOP-R system may be an interesting therapeutic target for management of EtOH withdrawal seizures, but also underscore the importance of determining the potential for rebound increases in withdrawal severity during later withdrawal episodes.

Introduction

Chronic exposure to EtOH can result in physical dependence, a response believed to be the result of neuroadaptive processes that occur at the cellular level in an attempt to maintain homeostasis within the organism (Himmelsbach, 1943; Solomon and Corbit, 1974). Upon removal of chronic EtOH, rebound hyperexcitability of the nervous system can often be observed in the form of a characteristic withdrawal syndrome (Himmelsbach, 1943). Unlike withdrawal from some drugs of abuse, alcohol withdrawal can be a life-threatening event. Some of the most severe symptoms include EtOH withdrawal related seizures and delirium tremens. While a great deal of progress has been made in our attempts to understand the neurotransmitter systems contributing to the development of EtOH dependence, we know far less about the neuroadaptive processes that occur to produce severe withdrawal following chronic exposure and subsequent removal of EtOH.

It is believed that withdrawal convulsions are the result of generalized tonic-clonic seizure activity (Simonato and Romualdi, 1996), however, the mechanisms and circuitry through which EtOH withdrawal produces these seizures is poorly understood. To begin to examine these mechanisms, we utilized established selected lines of mice that exhibit mild and severe EtOH withdrawal convulsions following 72 hours of chronic EtOH vapor inhalation. These selected lines are known as the Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) mice (Crabbe et al., 1983a; Crabbe et al., 1985). Derived from HS/Ibg stock, an 8-way cross of well characterized inbred strains (McClearn et al., 1970), these lines display a greater than 10-fold difference in withdrawal severity as measured by the handling induced convulsion (HIC) (Crabbe et

al., 1983a; Crabbe et al., 1985). The HIC is a measure of withdrawal severity that has been used extensively and is a reproducible and quantitative method for determining withdrawal severity (Goldstein and Pal, 1971; McSwigan et al., 1984; Crabbe et al., 1990a). To control for the random fixation of alleles unrelated to the trait of interest, the WSP and WSR lines have been bred in replicate, and are referred to as WSP-1, WSR-1, WSP-2 and WSR-2 lines. Because selected lines are bred for a particular phenotype, they represent an extremely useful, genetically enriched model for the study of these traits.

Many pharmacological studies have been performed in these lines in an attempt to identify neurotransmitter systems and other modulators of neuronal function that contribute to EtOH withdrawal severity. For example, NMDA (Crabbe et al., 1991a; Kosobud and Crabbe, 1993; Finn and Crabbe, 1999) and GABA (Feller et al., 1988; Buck et al., 1991a; Reilly et al., 2000) agonists and antagonists as well as alterations in calcium currents (Perez-Velazquez et al., 1994) have been examined for their role in the seizure susceptibility of these lines (for a recent review of systems and traits examined in WSP and WSR mice, see Metten and Crabbe, 1996). One interesting candidate that has not been well characterized pharmacologically in these lines, but may contribute to their withdrawal severity, is the KOP-R system.

The KOP-R is a member of the opioid receptor family, which also includes MOP and DOP receptors. Each of these receptors has endogenous ligands that bind to them with high affinity: dynorphins, endorphins, and enkephalins, respectively. Cloning and sequencing of these receptors (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Yasuda et al., 1993) confirmed that their predicted peptide products consisted of seven trans-membrane domains, characteristic of G protein-coupled receptors (GPCRs)

(Connor and Christie, 1999). Interestingly, activation of KOP-R modulates the activity of calcium (Ca^{2+}) (De Sarro et al., 1992; Hjelmstad and Fields, 2003) and potassium (K^{+}) channels (Shen and Crain, 1990) as well as GABA receptors (Hjelmstad and Fields, 2003). Recent evidence also suggests that this receptor interacts with NMDA receptors (reviewed in Mao, 1999). In addition, it has been proposed that EtOH produces some of its rewarding effects via either direct or indirect activation of DORs and MORs (for review, see Herz, 1997; Gianoulakis, 2001). In contrast, KOP-R activation appears to produce aversion (Mucha and Herz, 1985; Bals-Kubik et al., 1989; Sante et al., 2000) and may contribute to aversive states produced during EtOH withdrawal (Przewlocka et al., 1997). Thus, KOP-R signaling modulates receptor systems that likely participate in withdrawal and that are altered in WSP and WSR mice, making the KOP-R system an extremely interesting candidate for influencing withdrawal severity in these lines.

Studies examining the KOP-R system have been conducted in animal models of EtOH drinking. For example, B6 and D2 mice are inbred lines that show strikingly different EtOH drinking patterns in a free-choice drinking paradigm (B6>D2), and also exhibit disparate responses to EtOH withdrawal as measured by HICs (D2>B6) (Crabbe et al., 1983b; Belknap et al., 1993). Brains of naïve B6 and D2 mice contain significantly different levels of KOP-R and prodynorphin mRNA, as well as differences in the abundance of dynorphin peptide and the KOP-R, with D2 mice exhibiting significantly greater levels of these products in nearly every case (Jamensky and Gianoulakis, 1997; Winkler and Spanagel, 1998; Ploj et al., 2000). The authors suggested these basal differences were due to the divergent drinking behavior of the two strains, however, it is

also possible that these differences stem from the dissimilar withdrawal severity of the strains.

The effects of EtOH consumption on the KOP-R system have also been explored. Following 7 days of EtOH liquid diet consumption, B6 mice exhibited a significant increase in prodynorphin mRNA abundance in nearly all the brain regions examined when compared to isocalorically fed B6 controls, suggesting a role for this system in EtOH neuroadaptation (Gulya et al., 1993). The authors attributed these alterations in prodynorphin abundance to the EtOH consumption of these animals, but it is also likely that administration of the EtOH by liquid diet resulted in multiple withdrawals from EtOH throughout the exposure. Thus, these changes may be related to the withdrawal phenotype observed in B6 mice rather than, or in addition to, their drinking. Consistent with this hypothesis, rat lines selected for EtOH preference (Sardinian preferring, sP) and EtOH aversion (Sardinian non-preferring, sNP) exhibited no changes in prodynorphin abundance following 30 days of free access to EtOH (Fadda et al., 1999). This finding suggests that the KOP-R system may not simply be involved with drinking behavior per se.

Several studies during EtOH withdrawal have demonstrated alterations in the KOP-R system. Decreased dynorphin peptide immunoreactivity in multiple brain regions were present five days after the withdrawal of 10% EtOH that was administered to B6 mice via four weeks of voluntary two-bottle choice access (Ploj et al., 2000). A rebound increase in peptide was observed 21-days post EtOH withdrawal, while no significant effects of EtOH consumption on dynorphin levels were observed. Wistar rats given free access to EtOH for one month followed by 24, 48 or 96 hours of withdrawal showed an

initial increase in prodynorphin mRNA 24 hours after EtOH withdrawal in the nucleus accumbens. This effect persisted through the 48-hour time point, but returned to control levels by 96 hours (Przewlocka et al., 1997). Thus, the emergence of changes in peptide and mRNA levels during EtOH withdrawal suggests that alterations in the KOP-R system may be related to withdrawal rather than, or in addition to, differences in EtOH drinking behavior that were exhibited by these animal models.

Because little is known about the mechanisms specifically contributing to EtOH withdrawal HICs, one approach to examining their origin might be to identify those systems participating in other types of seizures. Thus, it is of interest that alterations in and/or manipulations of the KOP-R system appear to modulate seizure activity in other genetic animal models. When compared to their ddY parent strain, EL (epilepsy-like) mice exhibited significantly higher KOP-R binding in multiple brain regions. This effect was present in naïve animals as well as those that had experienced seizures (Kai et al., 1998). Another seizure model, the WAG/Rij rat, which exhibits spontaneous seizure activity and is considered a model of absence epilepsy, responded to both intracerebroventricular (icv) and subcutaneous (sc) injections of KOP-R agonists with a reduction in the number and severity of spike wave discharges (Przewlocka et al., 1995). Finally, the effects of opioid receptor agonists and antagonists, and the abundance of endogenous opioid receptor ligands have been examined in in selectively bred seizure sensitive (SS) Mongolian gerbils and their seizure resistant (SR) counterparts (Loskota et al., 1974). Following administration of the KOP-R agonist ketocyclazocine, a decrease in the severity and duration of seizures was observed in SS animals when compared to control; this effect could be blocked by prior administration of the opioid receptor

antagonist naloxone, suggesting a KOP-R specific effect (Lee et al., 1984). Brains of both pre-seizure and post-seizure SS gerbils contained an increased abundance of dynorphin like-immunoreactivity when compared to that observed in SR animals. These data suggest that selection for seizure susceptibility altered dynorphin levels. Interestingly, post-seizure SS gerbils showed a small but significant reduction in dynorphin-like immunoreactivity when compared to pre-seizure SS gerbils, which is suggestive of dynorphin release occurring in these animals during seizure activity (Lee et al., 1987).

Based on the findings described above suggesting a relationship between the KOP-R system and seizure susceptibility and withdrawal, we examined the KOP-R system at the molecular level in male WSP and WSR mouse lines following chronic EtOH vapor inhalation (Chapter two, Beadles-Bohling et al., 2000). This method is unique in that it allows for a clear temporal separation of the effects of EtOH exposure and EtOH withdrawal. Thus, we examined prodynorphin mRNA levels by Northern blot analysis in both intoxicated and withdrawn animals at two different time points: immediately upon removal from EtOH, and six hours later when these animals were experiencing peak withdrawal. Control animals exposed to air were also included in this design to assess basal differences. Interestingly, prodynorphin mRNA abundance was significantly increased in WSP mice during withdrawal when compared to intoxicated or air-exposed animals. In contrast, no effects of EtOH exposure or withdrawal were observed in WSR mice.

We also characterized expression of the KOP-R system in specific brain regions of male WSP and WSR mice using *in situ* hybridization and receptor autoradiography

(manuscript in preparation, see also Chapter three). Significant increases in prodynorphin mRNA in multiple brain regions of EtOH-withdrawing WSP-1 mice were observed, with similar trends in EtOH-withdrawing WSP-2 animals. In contrast, no significant changes in prodynorphin mRNA levels were observed in either of the WSR replicates during EtOH withdrawal. KOP-R abundance also was examined using receptor autoradiography, where basal and EtOH withdrawal related differences in KOP-R levels were observed between the lines. Combined, these findings suggest that alterations in the KOP-R system may contribute to the divergent withdrawal response observed in WSP and WSR selected lines, and further support a possible contribution of the KOP-R system to EtOH withdrawal severity.

Thus, alterations in the KOP-R system have been observed in both mouse and rat models of EtOH drinking, but many of these lines also show divergence in their withdrawal responses. Very little effort has been made to determine the effect of manipulations of the KOP-R system on EtOH withdrawal severity while evidence in other models of seizure activity suggests that this system might participate in EtOH withdrawal seizures. Our studies at the molecular level suggest that basal differences in KOP-R levels in distinct brain regions exist in WSP and WSR mice, and that EtOH withdrawal differentially alters KOP-R system expression in WSP and WSR mice. Given these results, we sought to determine whether such alterations in KOP-R and prodynorphin mRNA during EtOH withdrawal contribute to the line differences in withdrawal severity in WSP and WSR mice. Thus, the purpose of the present study was to examine the effects of pharmacological manipulations of KOP-R activity on EtOH withdrawal severity and duration in WSP and WSR mice. KOP-R activity was

modulated with the KOP-R agonist, (1S-*trans*)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzene-acetamide hydrochloride [(-)U-50,488H; U50], and the KOP-R antagonist, nor-binaltorphimine dihydrochloride (nor-BNI) and HICs were used as a behavioral measure of EtOH withdrawal severity. Alterations in the HIC responses following pharmacological manipulations of the KOP-R system in EtOH withdrawing animals would support a role for the KOP-R system in EtOH withdrawal severity. Pretreatment with nor-BNI resulted in significantly increased HIC severity in withdrawing WSR mice, with no observable effects in withdrawing WSP mice. In contrast, U50 produced a significant decrease in HIC severity in WSP mice, with no observable effects in WSR mice. Later in the withdrawal time course, a rebound hyperexcitability was observed in WSP mice given KOP-R agonist. Thus, administration of a KOP-R antagonist increased HIC severity in a mouse line normally resistant to withdrawal seizures, while administration of a KOP-R agonist reduced HIC severity in animals susceptible to withdrawal seizures. These observations suggest that the KOP-R system might be an interesting therapeutic target for management of EtOH withdrawal seizures, but also underscore the importance of determining the potential for rebound increases in withdrawal severity during later withdrawal episodes.

Materials and methods

All animal procedures and animal care were reviewed and approved by the Portland Oregon VA Medical Center Institutional Animal Care and Use Committee and met NIH guidelines for appropriate care and use of animals in research.

Animal subjects.

WSP and WSR mice were bred in the laboratory of J. C. Crabbe in Portland, OR. Drug-naïve adult male mice (age range 43-81 days) from selected generation 26 (filial generations G_{89}-G_{90}) were used. Mice of both replicates of the WSP (WSP-1 and WSP-2) and WSR (WSR-1 and WSR-2) lines were tested in these studies. Mice were maintained under a light/dark cycle of 0600-1800 light with water and Purina Lab Diet chow available *ad libitum*. Room temperatures were maintained at 22±1°C. EtOH exposure was initiated between 0800-0930 hr.

Drug sources and preparation.

Pyrazole HCl (Pyr) was purchased from Sigma Chemical Co. (St. Louis, MO). EtOH (ethyl alcohol, absolute, 200 proof) was purchased from Pharmco Products, Inc. (Brookfield, CT). Pyr was dissolved in saline (0.9%) and administered ip. EtOH was mixed with 0.9% saline (20% v/v) and injected ip or introduced without mixing as a vapor into the chambers. Nor-BNI was purchased from Sigma Chemical Co., (St. Louis, MO, USA), and was mixed in 0.9% saline to deliver a dose of 32 mg/kg in a volume of 0.10 ml/10 g mouse. This dose of nor-BNI was selected due to its inability to produce any obvious behavioral response on its own, and because this dose was capable of antagonizing the effects of the KOP-R agonist U50 for as long as four weeks (Endoh et

al., 1992; Broadbear et al., 1994). U50 was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in 0.9% saline to deliver one of three dose regimens: 80 mg/kg (single dose, experiment 2), 25 mg/kg (three doses resulting in cumulative exposure of 75 mg/kg, experiment 3a), and 75 mg/kg (single dose, experiment 3b) in a volume of 0.10 ml/10g mouse. Because no data was available pertaining to the effects of U50 on EtOH withdrawal convulsions, we chose to use a cumulative dose of 75-80 mg/kg that had been shown to be maximally effective against convulsions elicited with bicuculline (Yajima et al., 2000).

Chronic EtOH exposure and withdrawal.

Mice were made dependent upon EtOH as previously described (Beadles-Bohling et al., 2000). Briefly, on day one, male WSP-1, WSP-2, WSR-1 and WSR-2 mice were weighed, injected ip with EtOH and Pyr (an alcohol dehydrogenase inhibitor) and placed into EtOH vapor chambers. Controls were placed into air chambers and received Pyr only; a saline control was not included because previous data had shown that there was no difference between Pyr and saline treated animals with respect to prodynorphin gene expression (Beadles-Bohling et al., 2000). EtOH vapor concentrations within the chambers were periodically monitored using a gas chromatograph, and BECs were determined at hrs 24 and 48, in order to equate the EtOH exposure between the lines. Mean BECs were maintained between approximately 1.0-2.0 mg/ml, dependent upon whether the effects of the agonist or antagonist were being studied. At the 72-hr chronic exposure time-point, all mice were removed from the chambers, weighed, and had blood samples drawn from the tip of the tail for BEC determinations. Control animals were also restrained and their tails were snipped, however no blood was collected. Following

EtOH exposure, animals were moved to a quiet room, where they were scored hourly for HICs, as described in the following section.

Handling induced convulsion scoring.

Following removal from the EtOH vapor or air chambers, animals were scored for HICs as previously described (Crabbe and Kosobud, 1990; Crabbe et al., 1991b), beginning at hour 1 of removal. The animals were then scored hourly over the next 12-15 hours for HIC severity and again at 24 and 25 hours following removal from EtOH. If animals had not returned to baseline HIC levels by 25 hours, an additional score was obtained at 48 hours. The scale used (see Table 3) was identical to that published by Crabbe et al. (Crabbe et al., 1991b), which is a modified version of a scoring system originally described by Goldstein (Goldstein, 1972b).

Determination of BEC.

For analysis of BECs, the method described by Kosobud, et al. (Kosobud and Crabbe, 1986) was utilized. Briefly, 20 µl of blood was drawn from the tip of the tail and added to 50 µl of 3M $ZnSO_4$, mixed, and stored on ice until they were processed. After processing, the supernatant was analyzed for EtOH concentration by gas chromatography. A summary of the average BECs achieved in each study is presented in Table 6.

Area under the curve and peak HIC calculations.

Area Under the Curve (AUC) was a measure used to quantitatively evaluate withdrawal severity that was calculated using the trapezoidal method. Another index of withdrawal severity was the Peak HIC (PEAK HIC) score, which was calculated by

Table 6. Average BECs after exposure to 72 hours EtOH vapor in WSP and WSR mice

Experiment	Average BEC (mg/ml, mean±SEM)							
	WSP-1		WSR-1		WSP-2		WSR-2	
	EtOH (Groups combined)	EtOH + Drug (Groups combined)	EtOH (Groups combined)	EtOH + Drug (Groups combined)	EtOH (Groups combined)	EtOH + Drug (Groups combined)	EtOH (Groups combined)	EtOH + Drug (Groups combined)
1: Nor-BNI pretreatment	1.06±0.08	1.12±0.08	1.13±0.06	1.16±0.04	0.89±0.10	0.90±0.11	1.37±0.08	1.33±0.07
	(1.09±0.06)		(1.14±0.04[a])		(0.89±0.07)		(1.35±0.05[b,c])	
2: U50 just prior to peak withdrawal	0.90±0.05	1.02±0.06	1.20±0.05	1.20±0.08				
	(0.95±0.04)		(1.20±0.05[d])		(ND)		(ND)	
3a: U50 throughout EtOH exposure	2.32±0.06	2.54±0.15	2.11±0.33	1.42±0.18				
	(0.45±0.09[e])		(1.67±0.18)		(ND)		(ND)	
3b: U50 immediately following EtOH exposure	2.32±0.06	2.41±0.09	2.11±0.33	1.92±0.15				
	(2.37±0.06[f])		(1.99±0.15)		(ND)		(ND)	

[a]$p<0.01$ vs WSP-2, [b]$p<0.01$ vs WSP-1, [c]$p<0.001$ vs. WSP-2, [d]$p<0.001$ vs. WSP-1, [e]$p<0.05$ vs. WSR-1, [f]$p<0.001$ vs. WSR-1 in same experiment. ND, not determined

identifying the highest HIC for each individual animal; this score and the two flanking scores were then averaged to generate a PEAK HIC score. Because there were differences in baseline HIC severity between the WSP and WSR lines, as well as between the two replicate WSP lines, baseline corrected AUC scores were calculated. Following scoring, a group mean was determined for each hourly score in the control animals from each line and replicate. The mean value was then subtracted from the scores for each individual animal within a particular line and replicate, including the controls, to generate a corrected score. The result was a mean score of zero for the control animals. Negative values indicated a suppression of HIC severity from the measured baseline, while positive scores indicated an increase in the severity of HICs compared to baseline. These corrected values were used to calculate the various AUC scores; PEAK HIC scores were not corrected.

Statistical analyses.

Data are expressed as mean±SEM. Error bars have been omitted from graphs for clarity where indicated. The data were analyzed by one-, two- or three-way ANOVA (as indicated) followed by Bonferroni's post-hoc analysis when appropriate using the statistical package SYSTAT version 10 (Systat Software, Inc., Point Richmond, CA).

Experiment 1: The effects of pre-treatment with the KOP-R antagonist nor-BNI on EtOH withdrawal severity in WSP and WSR mice.

Following two baseline HIC measurements, both replicates of WSP and WSR mice were injected with either nor-BNI at a dose of 32 mg/kg or an equivalent volume of 0.9% saline at 5-6 days prior to the initiation of EtOH exposure. One day prior to the

initiation of EtOH exposure (4-5 days after nor-BNI injection) the animals were again scored for baseline HICs to determine whether nor-BNI altered baseline HIC responding. EtOH was administered via EtOH vapor inhalation with four treatment groups evaluated in this experiment: Pyr injection and placement in air only (Pyr-Control); pretreatment with nor-BNI followed by Pyr injection and placement into an air chamber (nor-BNI-Control); injection of an EtOH loading dose, Pyr injection and placement into an EtOH chamber (EtOH); and pretreatment with nor-BNI followed by an EtOH loading dose, Pyr injection and placement into an EtOH chamber (Nor-BNI+EtOH). Due to the lack of differences in the two control groups, Pyr-Control and nor-BNI-Control were collapsed into a single control group (Control), as discussed in the results. Following 72 hours of chronic EtOH vapor, animals were scored hourly for twelve hours, beginning at hour 1, and again at 24 and 25 hours of withdrawal. AUC and PEAK HIC scores were calculated as described. The injection and EtOH exposure time course for Experiment 1 is outlined in Figure 13A.

Experiment 2: Effects of the KOP-R agonist U50 on EtOH withdrawal severity when administered after the onset of EtOH withdrawal.

WSP-1 and WSR-1 mice were exposed to 72 hours of chronic EtOH vapor inhalation or air. Following the third hour of HIC scoring animals were injected with 80 mg/kg U50 or vehicle (0.9% saline). Four treatment groups were utilized: Pyr injected and placed into air chambers followed by a saline injection during withdrawal (Control); Pyr injected and placed into air chambers followed by U50 injection during withdrawal (Control-U50); EtOH loading dose, Pyr injected and placed into EtOH chambers followed by saline during withdrawal (EtOH); and EtOH loading dose, Pyr injected and

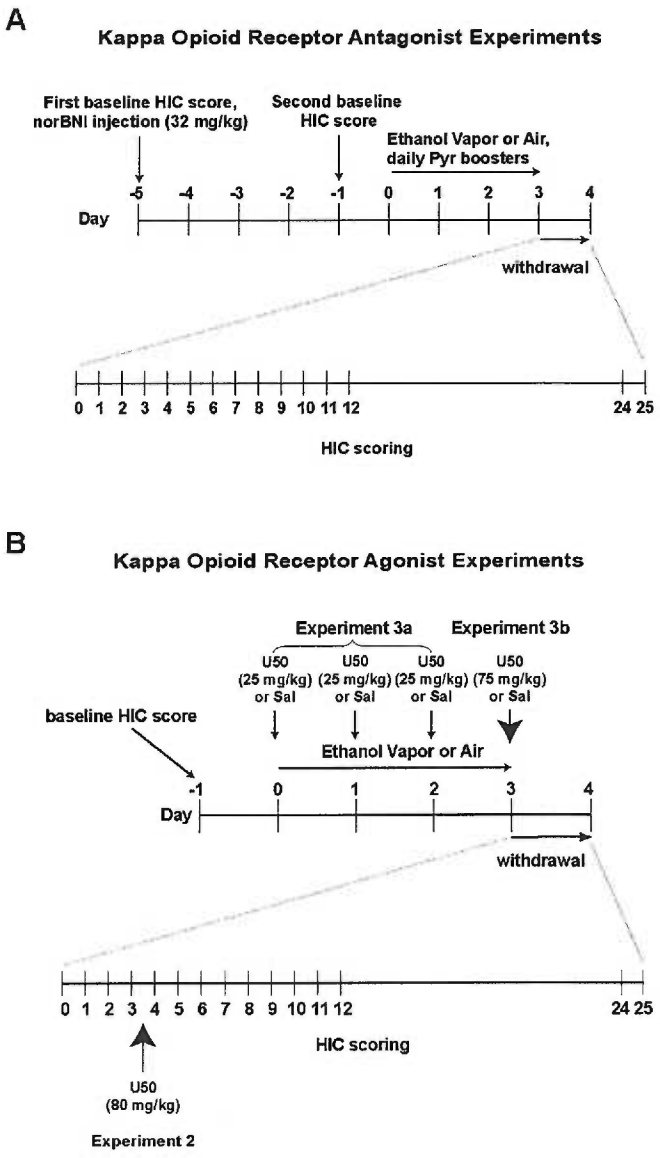


Figure 13. Injection and EtOH exposure paradigm for nor-BNI and U50 experiments. (A) Nor-BNI was injected 5-6 days prior to placement into EtOH or air chambers. Baseline HICs were measured immediately prior to nor-BNI injection, and again one day prior to chamber study initiation. EtOH loading doses containing Pyr were given to EtOH exposed animals prior to placement into EtOH chambers, air exposed animals were injected with Pyr only. Pyr boosters were administered daily as noted, HIC scoring occurred at the times indicted. **(B)** For the U50 experiments, three injection schemes were used, as indicated by experiment number (2, 3a and 3b). The time of injection and dose are noted. U50 was injected between hours three and four of withdrawal (experiment 2), throughout the chamber treatment (experiment 3a) or immediately upon removal from the chambers, prior to withdrawal onset (experiment 3b). Scoring occurred at the time points indicated on the diagram.

placed into EtOH chambers followed by U50 injection during withdrawal (EtOH-U50). Following removal from EtOH, the animals were scored hourly for HIC severity beginning at hour 1 of withdrawal. Scoring continued until 12 hours post EtOH withdrawal, and again at hours 24 and 25. The injection paradigm, along with the general EtOH exposure and HIC scoring paradigm for Experiment 2 is shown in Figure 13B.

Experiment 3: Effects of U50 administered prior to EtOH withdrawal.

Experiment three was conducted on replicate-1 WSP and WSR animals, and consisted of two studies: 3a examined the effects of U50 administration throughout the EtOH exposure, while 3b examined the result of administration of U50 just prior to the initiation of withdrawal. Control animals received daily injections of vehicle, as well as injections of Pyr, and were exposed to air in the chambers rather than EtOH vapor. At the conclusion of the chamber treatment, these animals received an additional vehicle injection so that the control group received an equivalent number of injections as the two experimental groups.

Experiment 3a: Effects of U50 administered concurrently with EtOH vapor exposure.

To examine whether agonist administration during EtOH exposure was capable of attenuating or blocking the expression of EtOH withdrawal HICs, U50 or 0.9% saline was administered daily to animals while they were exposed to either EtOH via vapor inhalation or air. Prior to the injection and placement into the EtOH vapor chambers on day 1, animals received either 25 mg/kg U50 or an equal volume of vehicle (0.9% saline). Thus, four groups were examined: saline and Pyr (injected at 0, 24 and 48 hours) and placed into air chambers (Control); 25 mg/kg U50 and Pyr (injected at hours 0, 24 and

48), and placed into air chambers (U50+Control); EtOH loading dose and Pyr and saline boosters injected at 0, 24, and 48 hours and placed in EtOH chambers (EtOH); and finally, 25 mg/kg U50, EtOH loading dose, Pyr boosters (at hours 0, 24 and 48), and placed into EtOH chambers (U50+EtOH). Thus, the U50+Control and U50+EtOH groups received a cumulative dose of 75 mg/kg U50, with the final dose given 24 hours before removal from the inhalation chambers. Upon removal from the EtOH vapor exposure all animals received an injection of saline to match the number and timing of injections with those given to the animals described in experiment 3b, which were run simultaneously. Animals were scored for HIC severity once per hour, beginning one hour after removal from the chambers over a span of 12 hours, and again at 24 and 25 hours post withdrawal. This injection scheme is labeled in Figure 13B as Experiment 3a.

Experiment 3b: Effects of U50 administered prior to the onset of withdrawal, immediately upon removal from EtOH exposure.

We were also interested in whether administration of a KOP-R agonist prior to the onset of EtOH withdrawal would result in a decrease in HIC severity in these animals. Thus, a second experimental group also was exposed to EtOH vapor with saline injections rather than U50 in order to match the number of injections given to all experimental groups. These experimental groups consisted of saline and Pyr (injected at 0, 24 and 48 hours) in air chambers, with a final saline injection at 72 hours (Control); Saline and Pyr (injected at 0, 24 and 48 hour) in air chambers followed by a 75 mg/kg U50 injection at 72 hours (Control+U50), EtOH loading dose and Pyr and saline boosters (injected at 0, 24, and 48 hours) placed in EtOH chambers with a final saline injection at

72 hours (EtOH); and EtOH loading dose, Pyr and saline boosters (at 0, 24 and 48 hours) placed in EtOH chambers, followed by a 75 mg/kg U50 dose at 72 hours (EtOH+U50) (see Figure 13B, experiment 3b). These animals were scored concurrently with those WSP-1 and WSR-1 mice in Experiment 3a that had received three injections of 25 mg/kg U50 over the 72-hour period. Thus, both drug treated groups received equivalent total doses of U50. Injection times and numbers were balanced across groups.

Results

Experiment 1: The effects of pre-treatment with the KOP-R antagonist nor-BNI on EtOH withdrawal severity in WSP and WSR mice.

HIC severity was initially compared by one-way ANOVA in the nor-BNI-Control animals versus Pyr-Control animals. There was no effect of nor-BNI on HIC severity in the WSP and WSR control mice (data not shown). Thus, the data from the Pyr-Control and the nor-BNI-Control groups were collapsed for subsequent statistical analyses and graphical presentation in the figures, and are referred to simply as Controls. The waxing and waning of HIC severity during EtOH withdrawal for WSP and WSR mice pretreated with nor-BNI or vehicle is presented in Figure 14 (A)WSP-1; and (B) (WSR-1); and Figure15 (A) WSP-2 and (B) WSR-2. Examination of the scores indicates that pre-treatment with nor-BNI increased EtOH withdrawal severity only in WSR-1 mice (Figure 14B).

Withdrawal severity was determined by calculating an AUC12 for each group (Figure 14C, Replicate-1; and Figure 15C, Replicate-2). Analysis by three-way ANOVA revealed significant main effects of line [$F(1,305)=64.89$, $p<0.001$], replicate [$F(1,305)=6.59$, $p=0.01$] and treatment [$F(2,305)=170.59$, $p<0.001$]. Significant two-way interactions were also observed between line and replicate [$F(1,305)=16.02$, $p<0.001$], and line and treatment [$F(2,305)=18.67$, $p<0.001$], with a trend toward an interaction between replicate and treatment ($p=0.11$). A significant three-way interaction was also present [$F(2,305)=6.15$, $p<0.01$]. Thus, subsequent analyses were conducted on each replicate line separately.

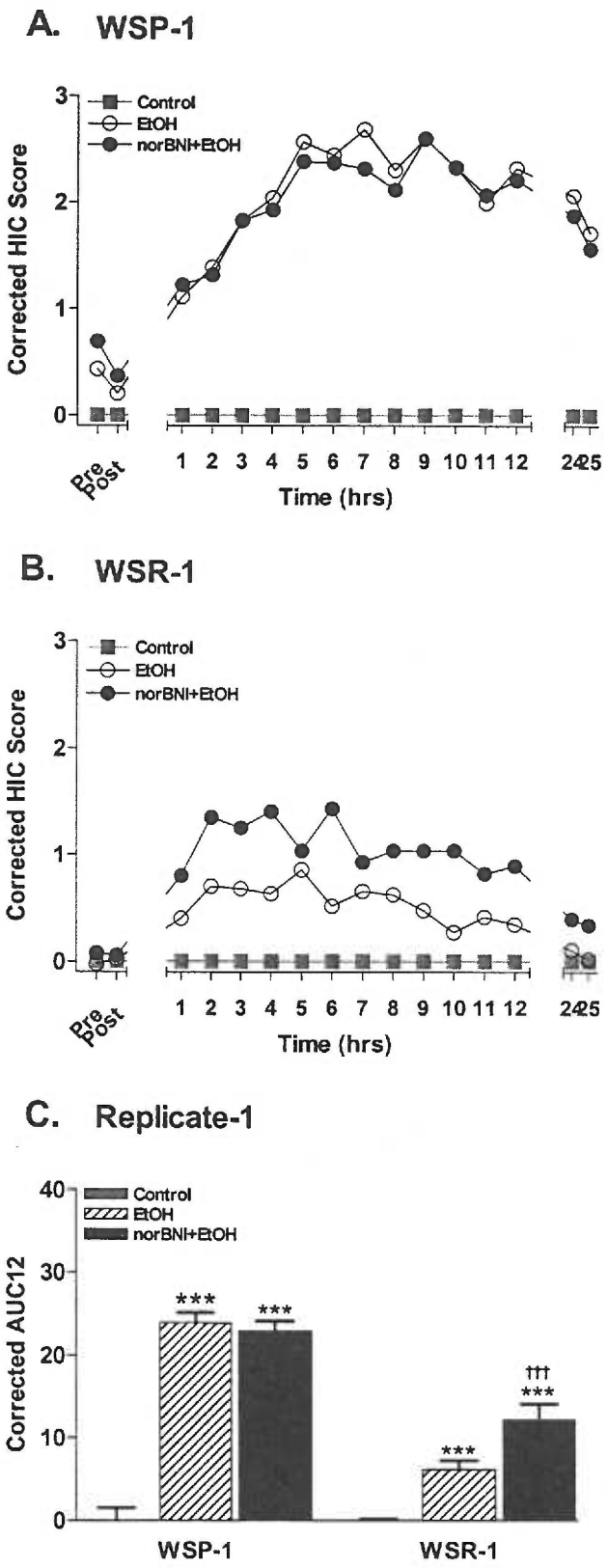

A. WSP-1
Control
EtOH
norBNI+EtOH
Corrected HIC Score
Pre
Post
1 2 3 4 5 6 7 8 9 10 11 12 24 25
Time (hrs)
B. WSR-1
Control
EtOH
norBNI+EtOH
Corrected HIC Score
Pre
Post
1 2 3 4 5 6 7 8 9 10 11 12 24 25
Time (hrs)
C. Replicate-1
Control
EtOH
norBNI+EtOH
Corrected AUC12
***
***
***
†††
***
WSP-1
WSR-1

Figure 14. Handling induced convulsion scores and AUC12 in replicate-1 WSP and WSR mice pretreated with nor-BNI. Scoring was performed as indicated in the methods. Two baseline values were recorded, one prior to nor-BNI administration (Pre), and a second following nor-BNI administration but before EtOH exposure (Post). The scores presented have been corrected for baseline differences between the lines and replicate. Data are expressed as group mean HIC scores for each time point and treatment group; standard errors are omitted for clarity. Filled gray squares (■), Control; Open black circles (○), EtOH; Closed black circles (●), nor-BNI+EtOH. No effect of nor-BNI was observed in Control animals, thus the Control data represents values in Pyr treated animals, collapsed across pre-treatment (saline or nor-BNI). AUC12 was calculated by summing the HIC scores over the first twelve hours of withdrawal. HIC scores in **(A)** WSP-1 mice (n=27-39) and **(B)** WSR-1 mice (n=18-22). **(C)** Withdrawal from EtOH produced a significant increase in the AUC12 for WSP-1 mice. This effect was unaffected by pretreatment with nor-BNI. Exposure to EtOH in WSR-1 groups produced a small but significant increase in AUC12. Pre-treatment with nor-BNI resulted in an additional increase in withdrawal severity in EtOH treated WSR-1 mice. $^{***}p<0.001$ vs. Controls, $^{\dagger\dagger\dagger}p<0.001$ vs. EtOH.

Replicate one. As shown in Figure 14C, EtOH treatment increased withdrawal severity as measured by AUC12 in WSP-1 mice. However, pre-treatment with nor-BNI did not significantly alter the EtOH withdrawal HIC severity as measured by the AUC12, nor did pre-treatment with nor-BNI potentiate the AUC12 in WSP-1 mice. Two-way ANOVA revealed a significant main effect of line [$F(1,187)=87.10$, $p<0.001$] and treatment [$F(2,187)=131.32$, $p<0.001$] as well as a line by treatment interaction [$F(2,187)=28.28$, $p<0.001$] on AUC12 scores. Because of the significant interaction, each line was further analyzed by one-way ANOVA. In WSP-1 animals, a significant main effect of treatment [$F(2,90)=104.99$, $p<0.001$] was seen. Post hoc analysis of treatment effects revealed that WSP-1 mice exhibited significant increases in AUC12 following EtOH treatment when compared to controls ($p<0.001$). This effect also was present in EtOH animals pre-treated with nor-BNI ($p<0.001$), and the values did not differ from EtOH alone values (Figure 14C). Similar to WSP-1 mice, one-way ANOVA on AUC12 of WSR-1 mice revealed a significant effect of treatment [$F(2,97)=33.51$, $p<0.001$]. Post hoc analysis revealed that WSR-1 mice treated with EtOH showed a significant increase in HIC severity as measured by the AUC12 when compared to their controls ($p<0.001$). In addition, EtOH withdrawing animals pretreated with nor-BNI displayed AUC12 scores that were significantly higher than Controls ($p<0.001$). However, in contrast to the effects observed in WSP-1 mice, nor-BNI pre-treatment resulted in a significant potentiation of the HIC response in the EtOH withdrawing WSR-1 group when measured using the AUC12 ($p=0.001$) (Figure 14C). Thus, the EtOH-induced increase in withdrawal severity in WSR-1 mice was potentiated by pretreatment with the KOP-R antagonist, nor-BNI.

Two-way ANOVA on PEAK HIC scores again revealed significant main effects of line [$F(1,187)=1072.70$, $p<0.001$] and treatment [$F(2,187)=94.78$, $p<0.001$] and a significant interaction [$F(2,187)=5.20$, $p<0.01$] (Table 7). Thus, one-way ANOVAs were performed for each line. A significant main effect of treatment was present in WSP-1 animals [$F(2,90)=64.79$, $p<0.001$]. Post hoc analysis revealed that PEAK HIC scores were significantly increased in EtOH and nor-BNI+EtOH treated groups of WSP-1 mice compared to their respective Controls ($p<0.001$). No additional exacerbations of PEAK HIC scores were observed in nor-BNI+EtOH WSP-1 versus EtOH WSP-1 mice. ANOVA in WSR-1 animals revealed a significant main effect of treatment [$F(2,97)=41.75$, $p<0.001$]. Post hoc analysis revealed that EtOH and nor-BNI+EtOH treated WSR-1 mice again exhibited significantly greater PEAK HIC scores than their Controls ($p<0.001$) and that the PEAK HIC score for nor-BNI+EtOH WSR-1 mice was significantly greater than that of EtOH WSR-1 mice ($p<0.01$). Thus, the nor-BNI+EtOH WSR-1 group again exhibited further potentiation of PEAK HIC scores versus EtOH WSR-1 mice.

The ANOVA examining AUC25 in replicate one (Table 7) also indicated significant main effects of line [$F(1,187)=106.16$, $p<0.001$] and treatment group [$F(2,187)=100.90$, $p<0.001$] as well as a significant line by treatment group interaction [$F(2,187)=33.06$, $p<0.001$]. One-way ANOVA in WSP-1 mice revealed a significant effect of treatment [$F(2,90)=79.12$, $p<0.001$]. Post hoc analysis indicated that both EtOH- and nor-BNI+EtOH-treated WSP-1 animals exhibited significantly increased AUC25 scores when compared to control ($p<0.001$). but these values did not differ from one another. In WSR-1 mice, one-way ANOVA on AUC25 revealed a significant effect

Table 7. Nor-BNI effects on EtOH withdrawal severity

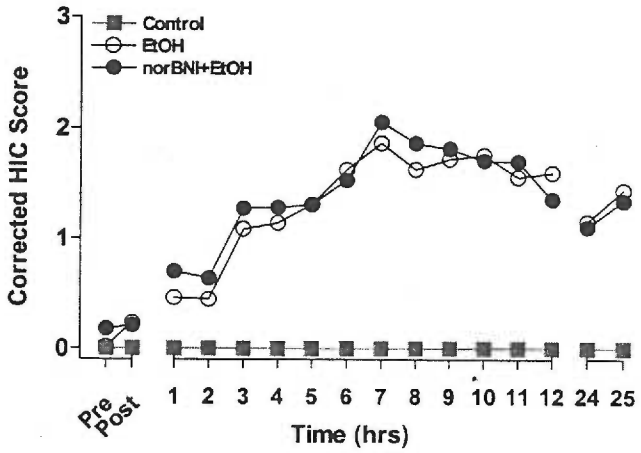
Withdrawal Measure	Line-Replicate	Control	EtOH	Nor-BNI +EtOH
AUC25	WSP-1	0.00±3.70	51.56±3.07***	48.79±2.83***
	WSR-1	0.00±0.14	8.94±1.68**	20.25±3.88$^{***,\#\#}$
	WSP-2	0.00±4.04	32.91±2.75***	32.17±2.99***
	WSR-2	0.00±0.66	21.00±4.03***	22.23±2.43***
PEAK HIC*	WSP-1	3.42±0.13	4.91±0.07***	4.74±0.08***
	WSR-1	0.18±0.05	1.17±0.15***	1.83±0.21$^{***,\#\#}$
	WSP-2	3.93±0.16	4.67±0.12***	4.78±0.13***
	WSR-2	0.24±0.07	1.46±0.21***	1.65±0.11***

*Note that all PEAK HIC scores are expressed as uncorrected values. Values represent the mean±SEM for the number of animals depicted in Figures 14 and 15. $^{***}p<0.001$ vs. Control, $^{**}p<0.01$ vs. Control, $^{\#\#}p<0.01$ vs. EtOH.

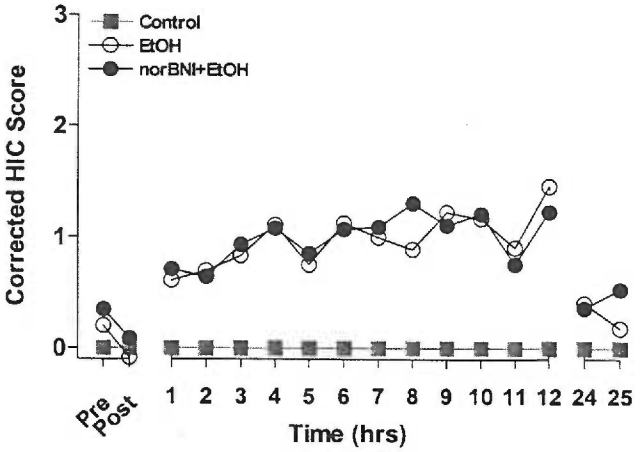
of treatment [$F(2,97)=24.70$, $p<0.001$]. Post hoc analysis again indicated that both the EtOH- and nor-BNI+EtOH-treated WSR-1 groups exhibited a significant increase in AUC25 scores compared to Controls ($p<0.01$ for Control vs. EtOH, and $p<0.001$ for Control vs. nor-BNI+EtOH). Nor-BNI+EtOH WSR-1 mice again had a significantly greater AUC25 than EtOH-treated WSR-1 mice ($p<0.01$). Thus, nor-BNI pretreatment potentiated withdrawal severity in WSR-1 mice throughout the 25-hour scoring period.

Replicate two. Analysis of the effects of nor-BNI on withdrawal in replicate-2 WSP and WSR lines yielded results that differed from those observed in replicate-1 (Figure 15). While two-way ANOVA on the AUC12 revealed a significant main effect of line [$F(1,118)=7.39$, $p<0.01$] and treatment [$F(2,118)=59.58$, $p<0.001$], no interaction was observed. One-way ANOVA revealed significant treatment effects in WSP-2 mice [$F(2,62)=37.84$, $p<0.001$]. Post hoc analysis revealed that EtOH and nor-BNI+ETOH groups both had significantly increased AUC12 scores compared to controls ($p<0.001$), but the two EtOH exposed groups were not different from each other. One-way ANOVA on the AUC12 for WSR-2 animals again revealed significant treatment effects [$F(2,56)=22.80$, $p<0.001$]. Post hoc testing again indicated that EtOH and nor-BNI+EtOH treated groups had significantly higher AUC12 scores than controls ($p<0.001$), but were again not different from one another. Thus, EtOH produced a significant increase in the AUC12 (Figure 15C) of WSP-2 and WSR-2 mice, and that effect persisted when animals were pretreated with nor-BNI. However, in contrast to replicate-1, nor-BNI did not potentiate the effects of EtOH in either WSP-2 or WSR-2 animals. Finally, as expected and based on the selected phenotype, the increase in the AUC12 was greater in WSP-2 than WSR-2 animals.

A. WSP-2



B. WSR-2



C. Replicate-2

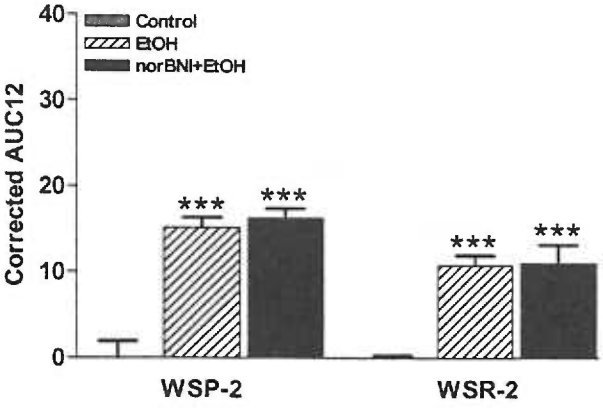


Figure 15. **Handling induced convulsion scores and AUC12 in replicate-2 WSP and WSR mice pretreated with nor-BNI.** Scoring was performed as indicated in the methods. Two baseline values were recorded, one prior to nor-BNI administration (Pre), and a second following nor-BNI administration but before EtOH exposure (Post). The scores presented have been corrected for baseline differences between the lines and replicate. Data are expressed as group mean HIC scores for each time point and treatment group; standard errors are omitted for clarity. Filled gray squares (■), Control; Open black circles (○), EtOH; Closed black circles (●), nor-BNI+EtOH. No effect of nor-BNI was observed in Control animals, thus the Control data represents values in Pyr treated animals, collapsed across pre-treatment (saline or nor-BNI). AUC12 was calculated by summing the HIC scores over the first twelve hours of withdrawal. HIC timecourse in **(A)** WSP-2 (n=21-23), and **(B)** WSR-2 animals (n=18-22). **(C)** EtOH withdrawal in replicate two WSP and WSR mice again revealed a significant increase in AUC12 scores, however, neither line was affected by pretreatment with nor-BNI. Note that the AUC12 score in WSR-2 mice treated with EtOH was greater than that of the EtOH treated WSR-1 line, but was very similar in magnitude to that of the nor-BNI+EtOH WSR-1 line. ***$p<0.001$ vs. Control.

Identical results were observed upon examination of the AUC25 and PEAK HIC severity in replicate-2 (Table 7). Two-way ANOVA on AUC25 revealed a significant effect of line [$F(1,118)=8.47$, $p<0.01$] with WSP-2 mice displaying significantly greater increases in AUC25 scores than WSR-2 mice. In addition, a significant main effect of treatment was present [$F(2,118)=54.40$, $p<0.001$], but there was no interaction. Post hoc testing of the treatment effects revealed that both the EtOH and nor-BNI+EtOH groups of replicate-2 exhibited significantly greater AUC25 scores than control. Two-way ANOVA on PEAK HIC scores in replicate-2 revealed a significant main effect of line [$F(1,118)=889.80$, $p<0.001$] and treatment [$F(2,118)=41.02$, $p<0.001$] as well as a significant interaction [$F(2,118)=3.02$, $p=0.05$]. Given the significant interaction, each line was examined by one-way ANOVA. A significant effect of treatment on PEAK HIC scores in WSP-2 mice was present [$F(2,62)=11.72$]. Post hoc analysis again showed that control WSP-2 mice had significantly lower PEAK HIC scores than EtOH ($p<0.001$) and nor-BNI+EtOH treated groups ($p<0.001$). One-way ANOVA on WSR-2 animals also revealed a significant effect of treatment [$F(2,56)=32.80$, $p<0.001$] in WSR-2 mice. Post hoc analysis again revealed that both EtOH and nor-BNI+EtOH treated groups had significantly greater PEAK HIC scores than control WSR-2 mice ($p<0.001$, control vs. EtOH and control vs. nor-BNI+EtOH). PEAK HIC scores were not different between EtOH and nor-BNI+EtOH WSR-2 groups. Thus, both EtOH treatment and pre-treatment with nor-BNI prior to EtOH resulted in significant increases in HIC severity in WSP-2 and WSR-2 groups, when compared to control animals. However, treatment with nor-BNI did not potentiate EtOH withdrawal severity in either WSP-2 or WSR-2 line (Table 7).

Three-way ANOVA on the average BECs (Table 6) for the EtOH treated animals revealed a significant main effect of line [$F(1,182)=22.43$, $p<0.001$], with WSR mice exhibiting a significantly higher average BEC than WSP mice. A significant line by replicate interaction [$F(1,182)=13.56$, $p<0.001$] was also present. There was no main effect of treatment with nor-BNI on average BEC, and no interactions of treatment with line or replicate. Post-hoc analysis of the line by replicate interaction revealed a trend for WSP-1 mice to have a higher average BEC than WSP-2 mice ($p=0.06$), while WSP-1 mice had a significantly lower average BEC than WSR-2 mice ($p<0.01$). Average BECs for WSP-2 mice were significantly lower than those of both WSR lines (WSP-2 vs. WSR-1, $p<0.01$; WSP-2 vs. WSR-2, $p<0.001$). WSR-1 mice also showed a trend for lower average BECs than WSR-2 mice ($p=0.06$). It is interesting to note that the WSR-2 group treated with EtOH displayed AUC12 scores similar to those observed in WSR-1 animals that had received both EtOH and nor-BNI. It is possible that these slightly higher BECs in the WSR-2 line produced a maximal withdrawal response in EtOH exposed WSR-2 animals that could not be further enhanced by nor-BNI.

Experiment 2: Effects of the KOP-R agonist U50 on EtOH withdrawal severity when administered after the onset of EtOH withdrawal.

We next examined the effect of modulation of the KOP-R system with a KOP-R agonist on withdrawal severity in WSP and WSR mice. Since the exacerbation of EtOH withdrawal severity by nor-BNI was only observed in replicate-1 mice, this study was performed on replicate-1 animals. Because the pharmacokinetics of U50 have not been well defined in the mouse, three time points for U50 administration were chosen in order

to examine the ability of U50 to reduce EtOH withdrawal severity. In experiment 2, U50 was administered in animals already undergoing withdrawal. By using this approach we hoped to achieve a maximal therapeutic level of U50 during peak withdrawal, with the goal of blocking or attenuating this peak response. Figure 16A and 16B depict the time course of hourly HIC scores for each group. Air-exposed WSP-1 mice showed a significant decrease in HIC scores that lasted for approximately 4 hours following an injection of 80 mg/kg U50, at which time HIC scores returned to baseline values (Figure 16A). WSP-1 animals treated with EtOH appeared to be insensitive to the anticonvulsant effects of U50. In contrast to the observation made in WSP-1 mice, administration of U50 to WSR-1 animals did not affect either control or EtOH-treated animals (Figure 16B).

To analyze the effects of U50 over the four hours following administration, the AUC4-8 was calculated (Figure 16C, WSP-1; 16D, WSR-1). An ANOVA was performed with the factors line, chamber treatment, and drug treatment. AUC4-8 was significantly affected by chamber treatment [$F(1,51)=69.43$, $p<0.001$] and drug [$F(1,51)=19.41$, $p<0.001$]. Significant two-way interactions of line and chamber treatment [$F(1,51)=45.60$, $p<0.001$] and line and drug [$F(1,51)=11.65$, $p<0.01$] also were present, with a significant three-way interaction between main effects [$F(1,51)=7.44$, $p<0.01$]. Post-hoc analysis of the three-way interaction revealed that control-U50 WSP-1 animals had a significantly reduced AUC4-8 when compared to their control counterparts ($p<0.001$). The AUC4-8 scores for both the EtOH treatment groups were significantly greater than that of the control group ($p<0.001$ control vs. EtOH; $p<0.05$ control vs. EtOH-U50). Finally, the EtOH-U50 group and the EtOH group both exhibited

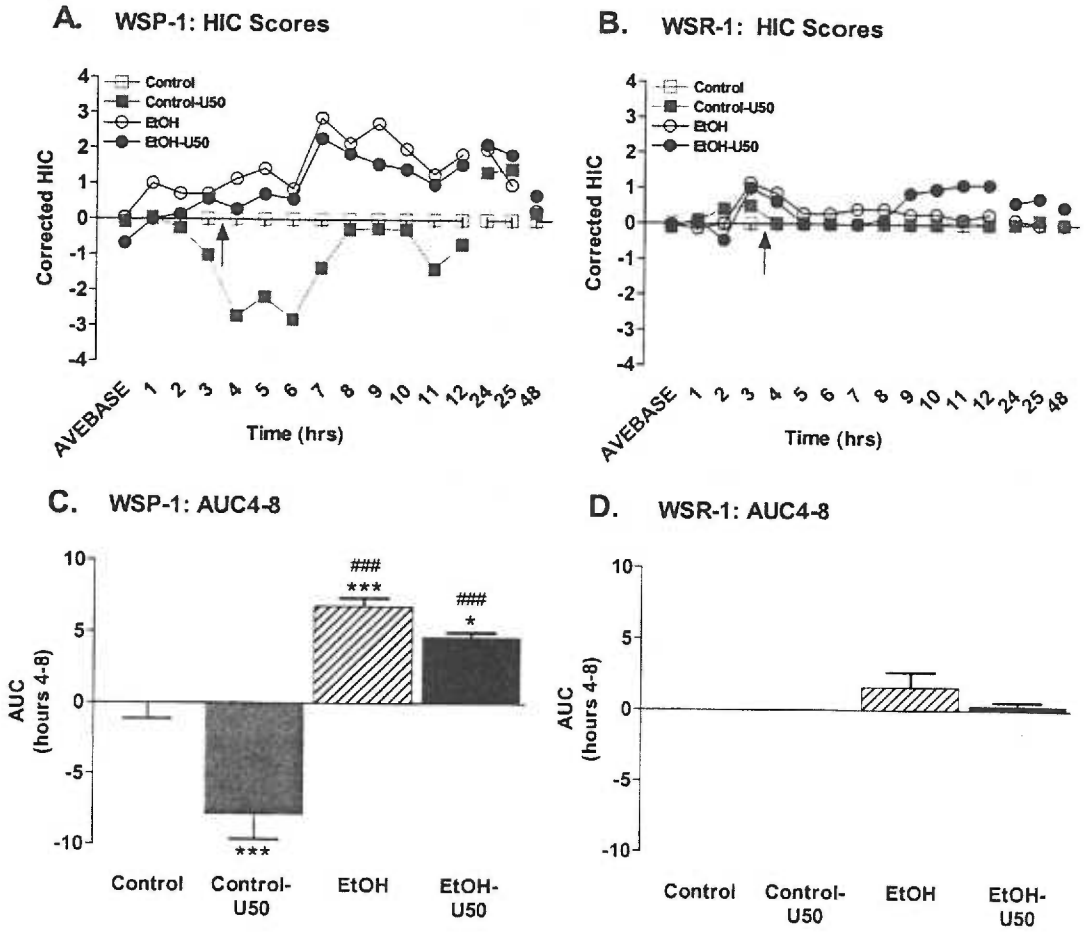


Figure 16. Plot of HIC time course and AUC4-8 in WSP-1 and WSR-1 lines given U50 during EtOH withdrawal. Data presented are baseline corrected. SEM is omitted for clarity in the time course plots. HIC timecourse is presented for **(A)** WSP-1 and **(B)** WSR-1 groups. Arrow indicates time of U50 injection. The AUC4-8 scores are presented in **(C)** for WSP-1 and **(D)** for WSR-1 groups. Open gray squares (□), Control; filled gray squares (■), Control-U50; Open black circles (○), EtOH; Closed black circles (●), EtOH-U50. **(A)** Control-U50 treated WSP-1 mice exhibited a decrease in HIC severity between hours 4 and 8 compared to control treated WSP-1 mice (n=7-8). A small rebound hyperexcitability was also observed between 12 and 48 hours. **(B)** WSR-1 EtOH-U50 groups did not appear to differ from EtOH treated WSR-1 animals between 4 and 8 hours of withdrawal (n=7-8). Note the small rebound increase in HIC severity in EtOH-U50 WSR-1 mice between 8 and 48 hours of withdrawal. **(C)** A significant decrease in the AUC4-8 was observed in Control-U50 treated WSP-1 mice compared to Controls. Both the EtOH and EtOH-U50 groups displayed AUC4-8 scores that were significantly elevated from control animals, but U50 treatment did not significantly alter HICs in the EtOH group. **(D)** No significant effects of U50 on the AUC4-8 were observed in the WSR-1 line. $*p<0.05$ vs. Control, $***p<0.001$ vs. Control, $^{###}p<0.001$ vs. Control-U50.

significantly elevated AUC4-8 scores when compared to the control-U50 WSP-1 animals as well ($p<0.001$ for both comparisons). No significant effects of EtOH or U50 were observed for AUC4-8 in the WSR-1 treatment groups.

Table 8 summarizes three additional measures of HIC severity that were analyzed. Examination of the AUC12-48 revealed a significant main effect of line [$F(1,51)=8.09$, $p<0.01$], chamber treatment [$F(1,51)=9.48$, $p<0.01$], and drug [$F(1,51)=4.19$, $p<0.05$], but no significant interactions. Thus, as expected, WSP-1 animals exhibited a significantly higher AUC12-48 than WSR-1 animals. However, EtOH withdrawal resulted in a significant overall increase in AUC12-48. Notably, the main effect of drug suggests that treatment with U50 produced a significant and non-selective increase in withdrawal severity late in the time course of withdrawal, when collapsed across line and chamber treatment groups, as measured by the AUC12-48.

Three-way ANOVA on PEAK HIC scores in these groups revealed a significant main effect of both line [$F(1,51)=252.36$, $p<0.001$] and chamber treatment [$F(1, 51)=12.44$, $p<0.001$], with no other main effects or interactions. As expected, the effect of line was due to the significantly greater PEAK HIC scores in WSP-1 animals compared to WSR-1 mice. In addition, EtOH withdrawal significantly increased the PEAK HIC score in these lines, but this effect was not affected by U50 administration. Given the significant main effect of line, each line was further analyzed independently for treatment and drug effects. Two-way ANOVA on WSP-1 animals revealed a significant main effect of both chamber treatment [$F(1,25)=34.25$, $p<0.001$] and drug [$F(1,25)=14.23$, $p<0.001$] but no interaction of these two factors. Post hoc testing showed that EtOH treatment significantly increased the PEAK HIC in WSP-1 animals

Table 8. Effects of the KOR agonist U50 given just prior to peak EtOH withdrawal severity in WSP-1 and WSR-1 mice

Withdrawal Measure	Line-Replicate	Control	Control-U50	EtOH	EtOH-U50
AUC12-48	WSP-1	0.00±11.63	24.57±9.48†	39.43±19.16**	53.86±6.51**,†
	WSR-1	0.00±0.00	1.50±1.50†	2.64±1.85	25.56±17.30†
PEAK HIC	WSP-1	4.00±0.07	3.54±0.19‡	4.86±0.10**	4.28±0.13**,‡
	WSR-1	0.33±0.19	0.67±0.39	1.19±0.36	1.17±0.50
AUC3	WSP-1	-0.39±0.24 (collapsed)		1.00±0.30 (collapsed)***	
	WSR-1	0.38±0.53 (collapsed)		0.28±0.37 (collapsed)	

Values represent the mean±SEM for the number of animals depicted in Figure 16. **EtOH withdrawal significantly increased the AUC12-48 overall compared to control animals ($p<0.01$). †U50 treatment produced a significant and non-selective increase in the AUC12-48 when collapsed on line and chamber treatment. ‡U50 treatment decreased the overall PEAK HIC score in WSP-1 animals compared to U50 naïve animals ($p<0.01$). ***$p<0.001$ vs. control WSP-1 group.

($p<0.001$), while treatment with U50 resulted in an overall decrease in PEAK HIC scores in WSP animals ($p<0.001$). Two-way ANOVA on WSR-1 animals revealed a trend toward an effect of chamber treatment ($p=0.09$), but no effect of drug, and no interaction. Thus, a small increase in PEAK HIC was observed in EtOH-treated WSR-1 mice when analyzed alone, but this measure did not reach significance.

Finally, we were interested in determining whether a measurable change in the withdrawal response was present in the first three hours of withdrawal for WSP-1 and WSR-1 mice, thus each line was examined by one-way ANOVA for effects of EtOH treatment on AUC3. The AUC3 data was collapsed across U50 treatment, since U50 had not yet been administered (Table 8). Because these animals had received a moderate EtOH exposure (Table 6) we expected that some signs of withdrawal might have begun to emerge. Indeed, in EtOH treated WSP animals the AUC3 was significantly increased from that of the air controls [$F(1,27)=13.67$, $p<0.001$] suggesting that at the time of U50 injection, these animals were already undergoing active withdrawal. In contrast, EtOH treated WSR-1 animals did not show any alteration in their AUC3 when compared to controls.

Experiment 3a: Effects of U50 administered concurrent with EtOH vapor exposure.

Given the observation that U50 was anticonvulsant in air controls, but did not modify EtOH withdrawal severity in WSP mice, we examined whether administration of U50 during EtOH exposure would attenuate or block the appearance of EtOH withdrawal seizures in WSP or WSR mice. Figure 17A shows the mean HIC scores for WSP-1 mice across time. There was a small increase in the EtOH withdrawal HIC scores of these groups when compared to the data presented in Figure 16, due to the higher BECs

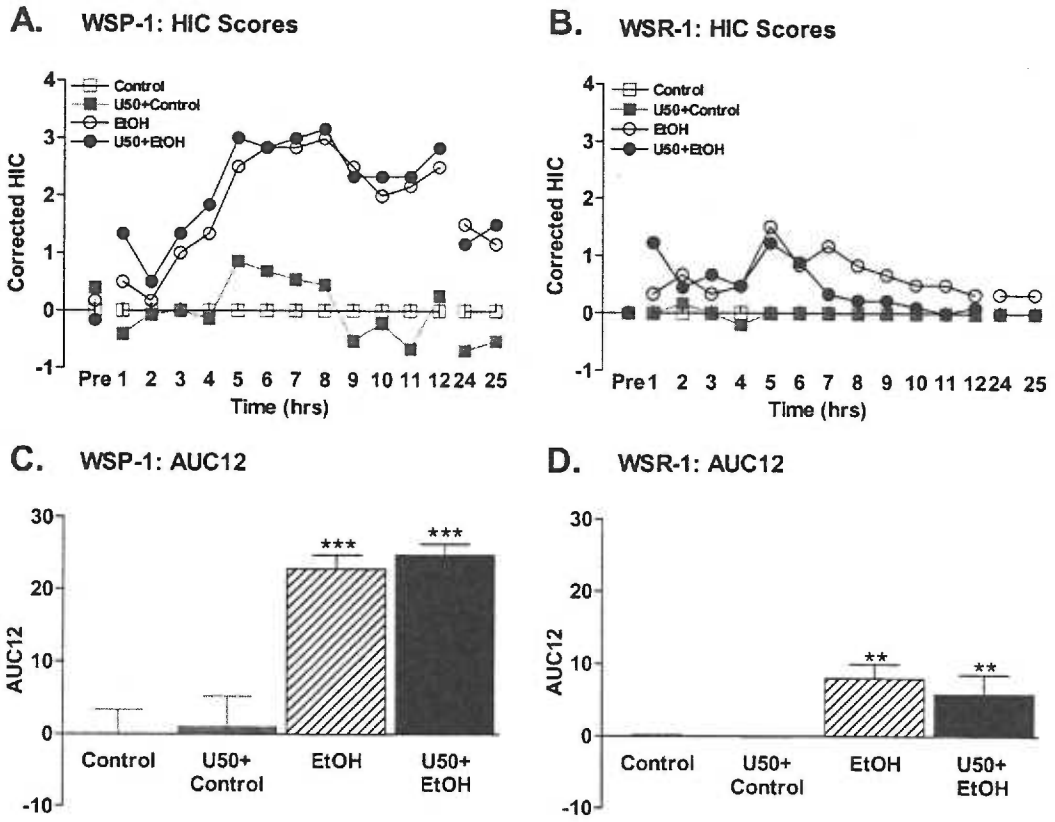


Figure 17. **The effects of U50 administration throughout ethanol exposure on ethanol withdrawal severity in WSP-1 and WSR-1 mice.** Data are presented as group means at each time point for each experimental group. SEMs are omitted from the time course plots for clarity. Open gray squares (□), Control; filled gray squares (■), U50+Control; Open black circles (○), EtOH; Closed black circles (●), U50+EtOH. **(A)** HIC scores in WSP-1 animals treated with EtOH were significantly increase compared to control animals (n=6-7). There were no effects of U50 treatment on HIC scores in any of the WSP treatment groups. **(B)** HIC scores in WSR-1 animals treated with EtOH showed a small but significant increase during withdrawal from EtOH; U50 administration during EtOH exposure had no effect on these scores (n=5-9). **(C)** AUC12 scores in WSP-1 animals were significantly increased in EtOH withdrawing animals compared to controls. No effects of U50 were observed in either WSP-1 group. ***$p<0.001$ vs. respective controls. **(D)** AUC12 scores in WSR-1 animals were significantly increased during EtOH withdrawal. U50 treatment had no effect on AUC12 scores. **$p<0.01$ vs. respective controls.

achieved in this study (Table 6). However, similar to the previous findings with U50 administration, there again appeared to be no effect of U50 in the EtOH treated WSP-1 group. In contrast to the findings in animals treated with U50 during withdrawal, no decrease from baseline HIC scores was apparent in control animals treated with U50. There was also no effect of U50 treatment on the HIC severity observed in WSR-1 mice (Figure 17B).

Three-way ANOVA on the AUC12 revealed significant main effects of line [$F(1,43)=23.56$, $p<0.001$] and chamber treatment [$F(1,43)=69.10$, $p<0.001$], but no effect of U50 treatment. An interaction between line and chamber treatment was also present [$F(1,43)=21.15$, $p<0.001$], thus each line was further analyzed by two-way ANOVA for effects of chamber treatment and U50 treatment. WSP-1 mice were significantly affected by chamber treatment [$F(1,21)=62.57$, $p<0.001$], but were unaffected by U50 treatment, and there was no interaction (Figure 17C). Results of the two-way ANOVA in the WSR-1 line again showed a significant effect of chamber treatment [$F(1,22)=10.11$, $p<0.01$]. There were again no effects of U50 treatment during EtOH exposure in WSR-1 mice, and there was no interaction between EtOH and U50 treatment (Figure 17D). Thus, EtOH withdrawal significantly increased the AUC12 in WSP-1 animals and WSR-1 animals compared to their respective controls, however, there were no effects of U50 treatment on this measure in either line.

We also examined EtOH withdrawal severity using the AUC25 and PEAK HIC scores (Table 9). Three-way ANOVA on AUC25 revealed a significant effect of line [$F(1,43)=25.49$, $p<0.001$] and chamber treatment [$F(1,43)=79.52$, $p<0.001$], as well as a line by chamber treatment interaction [$F(1,43)=38.46$, $p<0.001$]. There was no effect of

Table 9. Effects of the KOR agonist U50 on withdrawal severity when given throughout EtOH exposure

Withdrawal Measure	Line-Replicate	Control	U50+Control	EtOH	U50+EtOH
AUC25	WSP-1	0.00±5.07	-7.64±9.07	44.67±2.52***	47.42±2.24***
	WSR-1	0.00±0.20	-0.03±0.17	11.30±3.79**	6.58±3.36**
PEAK HIC	WSP-1	3.5±0.24	3.29±0.41	5.06±0.13***	5.06±0.06***
	WSR-1	0.07±0.07	0.06±0.06	1.28±0.29***	1.22±0.41***

Values represent mean±SEM for number of animals depicted in Figure 17. **p<0.01 vs. EtOH naïve controls. ***p<0.001 compared to EtOH naïve controls

U50 treatment on AUC25, and no other interactions were present. Given the significant interaction between line and chamber treatment, each line was further examined by two-way ANOVA. WSP-1 mice were significantly affected by chamber treatment [$F(1,21)=71.15$, $p<0.001$] but no effect of U50 treatment was present and there was no interaction. The same result was present in WSR-1 mice. EtOH exposure significantly altered the AUC25 score [$F(1,22)=8.78$, $p<0.01$], but U50 treatment had no effect on this measure and there was no interaction. Thus, EtOH withdrawing WSP-1 and WSR-1 mice exhibited significant increases in the AUC25 compared to their controls, but U50 treatment throughout the EtOH exposure did not alter this effect of EtOH. Finally, PEAK HIC scores were also analyzed. ANOVA revealed a significant main effect of line [$F(1,43)=280.37$, $p<.001$] and chamber treatment [$F(1,43)=44.75$, $p<0.001$], but no interaction. No main effect of U50 treatment was present, and there were no interactions with this factor. Thus, EtOH exposure significantly increased PEAK HIC severity in both lines, with a greater increase in WSP-1 vs. WSR-1 mice, but U50 did not significantly alter this measure in either line.

Experiment 3b: Effects of U50 administered prior to the onset of withdrawal, immediately upon removal from EtOH exposure.

Concurrent with the animals receiving U50 throughout their exposure to EtOH vapor or air, an additional group of animals received only vehicle injections throughout the EtOH or air exposure, but received 75 mg/kg U50 immediately upon removal from the inhalation chambers at 72 hours. The withdrawal response for WSP-1 mice is presented in Figure 18A, while HIC scores for WSR-1 mice are in Figure 18B. Note that following injection of U50, HIC severity decreased to below that of the control group in

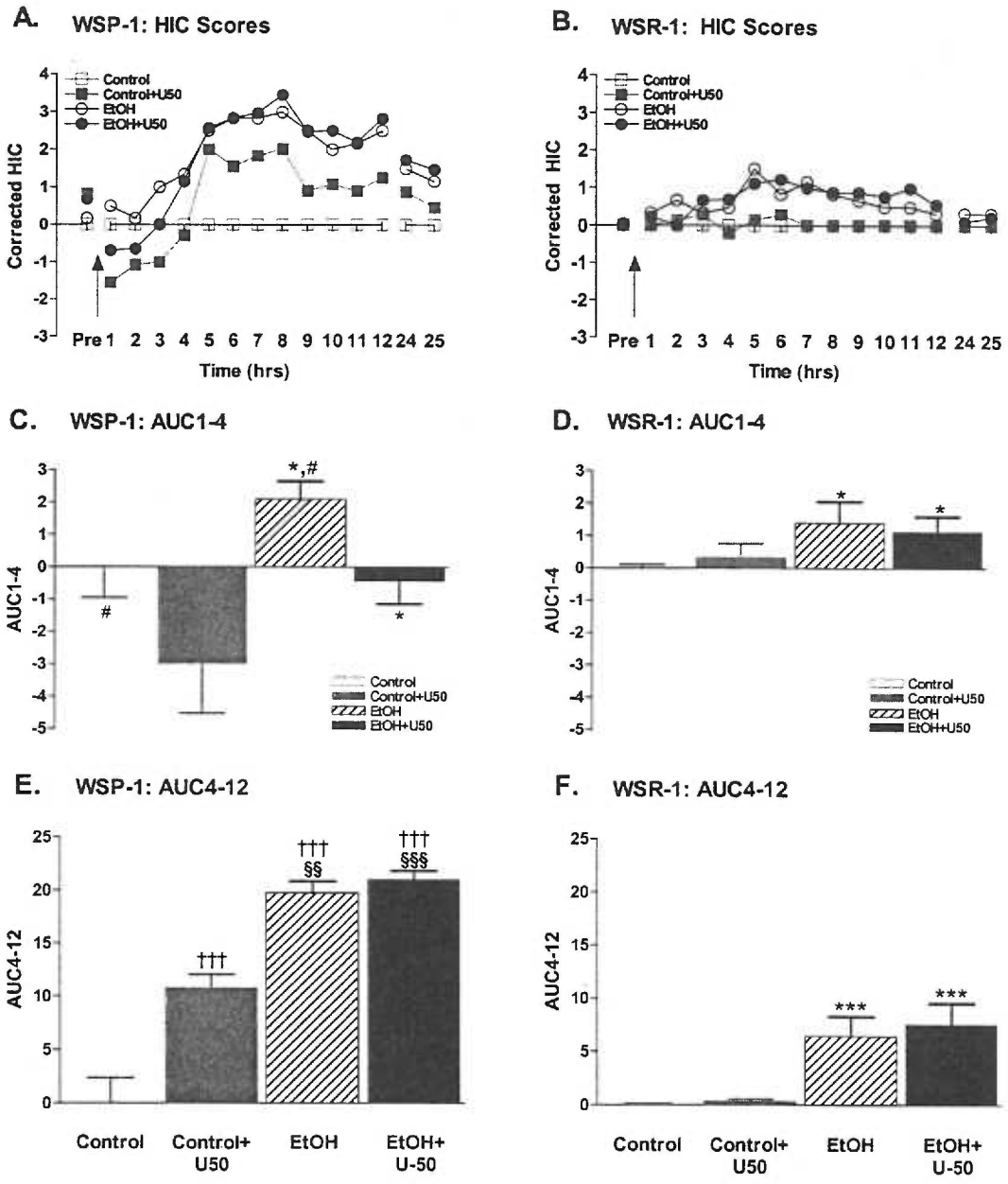

A.
WSP-1: HIC Scores
Control
Control+U50
EtOH
EtOH+U50
Corrected HIC
Pre 1 2 3 4 5 6 7 8 9 10 11 12 24 25
Time (hrs)
B.
WSR-1: HIC Scores
Control
Control+U50
EtOH
EtOH+U50
Corrected HIC
Pre 1 2 3 4 5 6 7 8 9 10 11 12 24 25
Time (hrs)
C.
WSP-1: AUC1-4
AUC1-4
*,#
#
*
Control
Control+U50
EtOH
EtOH+U50
D.
WSR-1: AUC1-4
AUC1-4
*
*
Control
Control+U50
EtOH
EtOH+U50
E.
WSP-1: AUC4-12
AUC4-12
†††
†††
§§
†††
§§§
Control
Control+ U50
EtOH
EtOH+ U-50
F.
WSR-1: AUC4-12
AUC4-12
***
***
Control
Control+ U50
EtOH
EtOH+ U-50

Figure 18. HIC time course, AUC1-4, and AUC4-12 in WSP-1 and WSR-1 lines treated with U50 immediately upon removal from EtOH exposure. The scores presented have been corrected for baseline differences between the lines and replicate. Data are expressed as group mean HIC scores for each timepoint and treatment group; standard errors are omitted for clarity. Open gray squares (□), Control; filled gray squares/ bar (■), Control+U50; Open black circles (○), EtOH; Closed black circles (●), EtOH+U50. Arrow in each time course plot indicates time of U50 injection. **(A)** HIC time course in WSP-1 mice (n=6-7). **(B)** HIC time course in WSR-1 mice (n=5-9). **(C)** Both control and EtOH treated WSP-1 mice showed a significant decrease in the AUC1-4 with U50 treatment. Control+U50 WSP-1 mice had AUC1-4 scores that were below baseline levels, while EtOH+U50 WSP-1 mice displayed HIC scores similar to those seen in EtOH naïve animals. $*p<0.05$ vs. respective controls, $\#p<0.05$ vs. U50 treated counterparts. **(D)** WSR-1 animals showed a small but significant increase in AUC1-4 scores with EtOH administration. These scores were unaffected by treatment with U50. $*p<0.05$ vs. respective controls. **(E)** Between 4 and 12 hours of withdrawal, EtOH treated WSP-1 mice showed similar AUC4-12 scores regardless of treatment with U50. In contrast, Control+U50 WSP-1 mice showed a significant increase in AUC4-12 when compared to Controls. This value was intermediate to the scores of the naïve and EtOH treated groups. $^{\dagger\dagger\dagger}p<0.001$ vs. Control, $^{\S\S}p<0.01$, and $^{\S\S\S}p<0.001$ vs. Control+U50. **(F)** WSR mice showed a significant increase in the AUC4-12 with EtOH treatment. Administration of U50 had no effect in either the Control or EtOH treated groups. $***p<0.001$ vs. respective control.

the WSP-1 animals, while there did not appear to be an observable effect in the WSR line. This effect again lasted for 4 hours. Three-way ANOVA on the AUC1-4 (Figure 18C-D), as a measure of that four-hour effect, revealed a trend for an effect of line (p=0.08), a significant main effect of chamber treatment [$F(1,45)=8.76$, $p<0.01$], as well as a significant main effect of U50 treatment [$F(1,45)=5.52$, $p<0.05$]. A significant line by U50 treatment interaction was also present [$F(1,45)=5.73$, $p<0.05$]. No other interactions were observed. Given the significant line by U50 treatment interaction, we further analyzed each line independently for EtOH and drug effects. Two-way ANOVA in WSP-1 animals revealed a significant main effect of chamber treatment [$F(1,22)=4.89$, $p<0.05$] and U50 treatment [$F(1,22)=6.80$, $p<0.05$]. No interaction of the two factors was present (Figure 18C). In contrast, ANOVA in WSR-1 mice revealed a significant main effect of chamber treatment only [$F(1,23)=4.82$, $p<0.05$], with EtOH treatment producing a small but significant increase in the AUC1-4 for WSR-1 animals ($p<0.05$). U50 treatment had no effect on the AUC1-4 in the WSR-1 line (Figure 18D).

Similar to the effects observed when U50 was administered during withdrawal, a rebound increase in HIC severity was observed when the AUC4-12 was examined (Figure 18E). Three-way ANOVA on this measure revealed a significant main effect of line [$F(1,45)=73.20$, $p<0.001$], chamber treatment [$F(1,45)=99.92$, $p<0.001$] and U50 treatment [$F(1,45)=9.53$, $p<0.01$]. Significant line by chamber treatment [$F(1,45)=14.14$, $p<0.001$], line by U50 treatment [$F(1,45)=5.89$, $p<0.05$], and chamber treatment by U50 treatment [$F(1,45)=4.04$, $p=0.05$] interactions were present, as well as a significant three-way interaction [$F(1,45)=5.61$, $p<0.05$]. Two-way ANOVAs on each line were performed as a result of the significant interactions. In WSP-1 mice, a significant effect

of both chamber treatment [$F(1,22)=107.40$, $p<0.001$] and U50 treatment [$F(1,22)=17.26$, $p<0.001$] were present, as well as a significant interaction [$F(1,22)=10.88$, $p<0.01$]. Post-hoc analysis revealed that the AUC4-12 in WSP-1 animals treated with EtOH was significantly greater than that observed in both control ($p<0.001$) and U50+Control ($p<0.01$) treated WSP-1 groups. WSP-1 groups treated with EtOH+U50 also exhibited AUC4-12 scores that were significantly greater than those of the control ($p<0.001$) and U50+control ($p<0.001$) groups. There were no differences between the scores of the two EtOH groups; thus, U50 did not affect AUC4-12 in EtOH treated WSP-1 mice. It is interesting to note that in the WSP-1 air controls, treatment with U50 significantly increased the AUC4-12 compared to controls ($p<0.001$), suggesting that U50 treatment produced a rebound increase in HIC severity in EtOH naïve WSP-1 mice. Two-way ANOVA on the AUC4-12 of WSR-1 animals only revealed an effect of EtOH treatment [$F(1,23)=17.45$, $p<0.001$]. U50 treatment had no effect, and there was no interaction between the two factors. Thus, EtOH treatment significantly increased the AUC4-12 in WSR-1 mice, but U50 treatment had no effect on this withdrawal measure (Figure 18F).

Two additional scores (AUC4-25 and PEAK HIC; Table 10) were examined in this group as well to assess withdrawal severity. Three-way ANOVA on AUC4-25 revealed significant main effects of line [$F(1,45)=71.38$, $p<0.001$], chamber treatment [$F(1,45)=91.67$, $p<0.001$], and U50 injection [$F(1,45)=5.93$, $p<0.05$]. In addition, a significant line by chamber treatment interaction was present [$F(1,45)=24.68$, $p<0.001$], and there were trends for both a line by U50 treatment interaction ($p=0.08$) as well as a three-way interaction ($p=0.09$). When the lines were analyzed separately, AUC4-25 was

Table 10. Effects of KOR agonist on withdrawal severity when given immediately upon removal from EtOH

Withdrawal Measure	Line-Replicate	Control	Control+U50	EtOH	EtOH+U50
AUC4-25	WSP-1	0.00±4.66	16.93±4.65$^{\#}$	41.58±1.77***	44.71±2.48$^{***,\#}$
	WSR-1	0.00±0.10	0.33±0.20	9.73±3.52***	12.57±3.76***
PEAK HIC	WSP-1	3.50±0.24	3.95±0.17	5.06±0.13***	5.29±0.15***
	WSR-1	0.07±0.07	0.29±0.13	1.28±0.29***	1.22±0.31***

Values represent the mean±SEM for the number of animals depicted in Figure 18. $^{***}p<0.001$, EtOH treatment produced a significant overall increase in the measure indicated when compared to EtOH naïve animals. $^{\#}p<0.05$, treatment with U50 resulted in a significant overall increase in the AUC4-25 when compared to those animals not receiving U50.

significantly influenced by chamber treatment [$F(1,22)=89.50$, $p<0.001$] and U50 injection [$F(1,22)=7.48$, $p<0.05$] in WSP-1 mice. While the interaction between chamber treatment and U50 injection did not reach significance, a tread toward an interaction was observed ($p=0.07$). Thus, EtOH treatment in WSP-1 mice significantly increased the AUC4-25 in these animals compared to EtOH naïve animals, and U50 injection results in a rebound increase in the AUC4-25, regardless of chamber treatment. AUC4-25 was significantly influenced by chamber treatment [$F(1,23)=12.85$, $p<0.01$] in WSR-1 mice, but there was no effect of treatment with U50, and no interaction of EtOH and drug treatment. Thus, in WSR-1 mice, EtOH exposure produced a small but significant increase in AUC25. However, U50 had no effect on the expression of withdrawal in these animals.

Analysis of PEAK HIC scores revealed a significant main effect of line [$F(1,45)=542.09$, $p<0.001$] and chamber treatment [$F(1,45)=61.60$, $p<0.001$], but no effect of U50 treatment (Table 10) and there were no significant interactions. Because a significant main effect of line was present, each line was further analyzed individually. In WSP-1 animals PEAK HIC was significantly influenced by chamber treatment [$F(1,22)=66.65$, $p<0.001$], with a trend for an effect of U50 treatment ($p=0.07$), and no interaction. Thus, treatment with EtOH significantly increased the PEAK HIC score for WSP-1 animals, while U50 administration following EtOH exposure resulted in a minor increase in PEAK HIC that did not reach statistical significance. Two-way ANOVA in WSR-1 animals revealed a significant main effect of chamber treatment [$F(1,23)=16.28$, $p<0.001$], but no effects of U50 injection, and no interaction between the two factors.

Thus, EtOH treatment increased the PEAK HIC score in WSR-1 animals. However, administration of a KOP-R agonist did not alter PEAK HIC scores in WSR-1 mice.

Discussion

We have examined the effect of pharmacological modulation of the KOP-R system using a KOP-R antagonist (nor-BNI) and agonist (U50) on EtOH withdrawal in a genetic animal model selected for mild or severe EtOH withdrawal seizures. Our data suggests that activation of the KOP-R system is anticonvulsant. Administration of nor-BNI produced a significant increase in HIC severity during EtOH withdrawal in WSR-1 but not in WSP-1 mice. On the other hand, administration of U50 to WSP-1 animals prior to the onset of withdrawal resulted in a brief but significant decrease in HIC severity in both control and EtOH treated animals. Thus, manipulations of the KOP-R system are capable of altering chronic EtOH withdrawal severity measured by HICs in WSP and WSR mice, suggesting a potential role for this system in the modulation of EtOH withdrawal seizure severity in these lines.

As expected, nor-BNI administered to control animals had no effect on basal HICs, a response that was predicted based on other reports showing nor-BNI did not alter chemically-induced convulsions on its own (Bausch et al., 1998; Yajima et al., 2000). However, following pretreatment with nor-BNI, EtOH withdrawal was significantly increased in WSR-1 mice but not in WSP-1 mice. This effect was present for all the withdrawal measures examined. HIC severity was increased in WSR-1 animals during early withdrawal as measured by the AUC12 and the magnitude of severity was increased, as measured by the PEAK HIC response. After 25 hours of withdrawal WSP and WSR mice generally show a return to their baseline HICs. The continued presence of significant differences between the nor-BNI+EtOH and the control and EtOH treated WSR-1 groups, also suggested an increase in the duration of withdrawal. However,

testing beyond the period reported here would be necessary to definitively make this conclusion. Together, these findings suggest that nor-BNI administration both intensifies the severity of withdrawal and increases its duration. This finding is particularly interesting because it occurs in WSR mice, animals normally resistant to EtOH withdrawal seizures. Thus, while the seizure response of WSR-1 mice to EtOH withdrawal is still far smaller than that observed in the seizure-prone lines, some protection against withdrawal is lost, and the response of WSR-1 mice more closely resembles that of WSP mice.

It is important to note that this effect of nor-BNI is not present in WSR-2 mice. While the effects of nor-BNI did not appear to be replicated in both WSR lines, there was an overall increase in EtOH withdrawal HIC severity in the WSR-2 animals. Rather than observing an enhanced EtOH response with pre-treatment with nor-BNI, the maximal HIC response may have already been present in the EtOH treated animals. That is, the magnitude of the response in WSR-2 mice treated with EtOH was similar to that of the nor-BNI+EtOH treated WSR-1 line, and might be the maximum HIC response that could be elicited from this line. Thus, a potential "ceiling effect" in WSR-2 mice may have masked the ability of nor-BNI to further potentiate HICs in these animals. The BECs of these animals provides one possible explanation for the greater response in EtOH exposed WSR-2 mice than that observed in WSR-1 mice. WSR-2 animals achieved a marginally higher BEC than their WSR-1 counterparts when exposed to the same EtOH concentrations (p=0.06, Table 6). This higher EtOH exposure may have contributed to the increased seizure response in the EtOH treated WSR-2 group since EtOH dose is positively correlated with EtOH withdrawal severity (Goldstein, 1972b). Regardless of

the cause for this differing response in WSR-2 mice, the presence of an effect in one of two replicate lines is moderate evidence for a correlated response to selection (Crabbe et al., 1990c), indicating that KOP-R signaling may be protective against enhanced severity of EtOH withdrawal seizures.

In contrast to the observed effects of nor-BNI in WSR mice, the KOP-R antagonist did not affect HICs in the WSP lines. EtOH withdrawal is already quite high in this line due to selection; thus a further increase in HIC severity might not be achievable due to the presence of a "ceiling" effect. An analysis of the effects of nor-BNI on EtOH withdrawal convulsions in this line following withdrawal from an acute administration of EtOH which produces less severe HICs, or in additional lines showing a more moderate HIC response will be very helping in verifying or discounting a role for the KOP-R in HIC severity.

While blocking KOP-R signaling with nor-BNI appeared to be pro-convulsant in WSR-1 mice, treatment with the KOP-R agonist U50 while withdrawal was in progress appeared to be anticonvulsant in EtOH-naive WSP-1 mice. This is consistent with the effects of this agonist that have been observed in other seizure models (reviewed in Tortella and DeCoster, 1994; Przewlocka et al., 1995; see also Bausch et al., 1998). However, the anticonvulsant effect of U50 was not present in animals exposed to EtOH. Multiple factors may explain this finding. First, the increased AUC1-3 in EtOH treated WSP-1 mice, compared to controls revealed that WSP-1 animals had already begun to actively experience withdrawal. As a result, it might have been difficult for the agonist to overcome the cascade of events that result in escalation of HICs once it has begun. Alternatively, it is possible that subconvulsive seizure activity within these lines before or

during EtOH exposure resulted in changes in KOP-R signaling and/or downstream targets of this receptor, such that U50 was no longer an effective anticonvulsant. Finally, it is also possible that the electrical and neurochemical activity contributing to the HIC response was great enough to overcome the anticonvulsant effects of U50 in this model.

To further test the hypothesis that prolonged EtOH exposure resulted in neuroadaptive changes in KOP-R system signaling, we performed an additional study in these lines where U50 was administered throughout the period of EtOH exposure. In this way we attempted to block the neuroadaptive response mediated by KOP-R signaling. In this paradigm, U50 administration had no effect on EtOH withdrawal HIC severity and also failed to alter HICs in naïve animals. Thus, it appeared that agonist administration did not block events that occured during EtOH exposure to render an animal more or less susceptible to EtOH withdrawal convulsions. It is possible this result was due to rapid clearance of the drug, as reflected by the brief period during which measurable changes in HICs were observed in experiment 1. The administration of a daily dose of 25 mg/kg for three days versus 75 mg/kg on one day may also have been less effective at blocking the development of hyperexcitability in WSP-1 mice. Finally, it is also possible that tolerance to the effect of U50 occurred over the three-day administration paradigm, since tolerance has been observed following repeated administration of U50 (Baker and Meert, 2002). Administration of U50 using a sustained release format in future studies will be required to test this hypothesis.

Finally, administration of a KOP-R agonist prior to the onset of withdrawal resulted in a non-selective decrease in HIC severity in WSP-1 mice. This finding was similar to that observed in animals given U50 while withdrawal was already in progress,

where we observed a decrease in the HIC severity of EtOH-naïve WSP-1 mice. However, this paradigm resulted in an anti-convulsant effect in EtOH treated animals as well. Note the similarity of the curves in Figures 16A and 18A; the major difference was a shift of the response curve temporarily to the left, consistent with the earlier administration of the drug. Therefore, when administered prior to the onset of withdrawal, U50 was capable of reducing HIC severity to values that were the same as or lower than those of control animals over the four-hour window in which U50 was effective. These differences in the anticonvulsant effect between the two studies suggest that upon emergence of withdrawal HICs, a cascade of events occured that could not be reversed by U50 treatment. This might be the result of constitutive activation of targets of KOP-R signaling, such as an increase in Ca2+ channel open time, depolarization of cells via blockade of K+ channels, activation of NMDA receptors, or blockade of GABA receptors.

The shift to the left of the withdrawal time course when U50 was administered immediately upon removal from exposure to EtOH or air also revealed a significant rebound hyperexcitability in U50-treated WSP-1 mice at later time points. While this effect was present when U50 was administered during withdrawal, the rebound increase in HICs was not as clearly observable because fewer measurements occurred over the time period that the animals exhibited this response. This rebound response is not unique to U50; administration of lorazepam to mice during multiple withdrawal episodes followed by an untreated withdrawal episode resulted in an initial decrease in HIC severity during the acute phase of withdrawal (hours 1-10) followed by an exacerbation of HICs during the later phase (hours 10-72, Becker and Veatch, 2002). Thus, it may be

that drugs that are initially seizure protective may later act to exacerbate these symptoms when administered during EtOH withdrawal. This possible side effect must be carefully examined if a drug is to be considered for the treatment of EtOH withdrawal hyperexcitability in clinical settings.

In conclusion, we have observed alterations of EtOH withdrawal severity by manipulation of the KOP-R system using agonists and antagonists in a mouse model displaying divergent EtOH withdrawal seizure severity. Pretreatment of seizure-insensitive WSR-1 mice with the KOP-R antagonist nor-BNI resulted in a significant increase in HIC severity in this line, thus, shifting the response of this line to more closely resemble the response of seizure-sensitive WSP mice. Complementary to this finding, when the KOP-R agonist U50 was administered immediately upon the initiation of withdrawal, a significant decrease in HIC severity was observed in both control and EtOH treated WSP-1 animals. This finding is consistent with the anticonvulsant activity demonstrated by this drug in other seizure models, and suggests that alterations in KOP-R signaling may contribute to seizure sensitivity during EtOH withdrawal. Thus, while the increased HIC severity following KOP-R antagonist administration was observed in only replicate one of the WSR line, the presence of a seizure protective effect following KOP-R agonist administration in WSP-1 mice also supports a role for this system in EtOH withdrawal seizure severity. Additional analysis of KOP-R agonist effects in replicate two animals will further support this hypothesis. In addition, it will be very interesting to examine the effects of KOP-R manipulations in additional genetic animal models that display mild and severe convulsions during EtOH withdrawal, such as the B6 and D2 strains. Further characterization of the role of the KOP-R system in EtOH withdrawal

should provide important insight into our understanding of the mechanisms participating in EtOH withdrawal severity, and should aid in the develop of better therapeutic treatments for the management of this potentially life threatening event.

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Chapter V
Conclusions

Whole Brain Analyses

The studies presented in this dissertation investigated potential KOP-R system involvement in modulating responses to chronic EtOH exposure in a mouse model of mild and severe withdrawal severity, the WSP and WSR selected lines, and indicate that enhanced KOP-R signaling might be anti-convulsant. Beginning with an analysis in whole brain as described in chapter two, *Pdyn* mRNA expression was examined in animals exposed to chronic EtOH followed by withdrawal. These studies revealed significant increases in *Pdyn* mRNA abundance in WSP mice withdrawn from EtOH, while EtOH intoxication non-significantly increased *Pdyn* abundance in WSP animals (Chapter Two, Beadles-Bohling et al., 2000). In contrast, both intoxicated and withdrawing WSR mice displayed minimal, non-significant increases in whole brain *Pdyn* mRNA abundance. The observation that significant effects were only present in the WSP lines suggests that increased *Pdyn* abundance might be associated with the increased EtOH withdrawal severity exhibited by WSP mice.

These findings were in contrast to observations made in B6 mice exposed to EtOH using a liquid diet paradigm (Gulya et al., 1993). In those studies, chronic EtOH exposure produced significant increases in *Pdyn* mRNA abundance across nearly every brain region examined. Thus, while the changes in *Pdyn* abundance observed in B6 mice were in the same direction (i.e., increased), our observed increase in whole brain *Pdyn* during EtOH withdrawal did not coincide with the timecourse for the increase in *Pdyn* in intoxicated animals that was previously documented in B6 mice. A possible explanation for these discrepancies may be found in the exposure paradigm used; vapor inhalation temporally separates exposure to EtOH from EtOH withdrawal in contrast to the

intermittent EtOH exposure provided by a liquid diet paradigm. Thus, we propose that our findings extend and clarify those of Gulya et al., (Gulya et al., 1993) and suggest that the effects observed on *Pdyn* abundance might be related more to withdrawal rather than EtOH exposure.

Based on previous studies examining *Pdyn* mRNA levels prior to, during and after seizures, I propose the increased *Pdyn* mRNA abundance in WSP mice is a response to either depleted or deficient dynorphin peptide levels in this line. During EtOH withdrawal there is increased neuronal hyperexcitability that produces seizure activity in the WSP mice. Dynorphin peptide might be released in an attempt to dampen seizure activity within the brains of the withdrawing animals. Alternatively, these animals might have minimal levels of dynorphin peptide due to selection, thus, the anticonvulsant effect of this peptide might be absent in this line. The increased *Pdyn* we observed might occur in order to replace these depleted stores of dynorphin in WSP mice. Unclear, however, is where in the brain these effects took place and, if these lines are not deficient in dynorphin, why the presumed release did not attenuate seizures exhibited by WSP mice. If the increased *Pdyn* mRNA levels are reflective of brain regions where dynorphin has been depleted, one would predict that those regions that have been implicated in the generation and propagation of seizure activity would show altered *Pdyn* levels during EtOH withdrawal in WSP mice compared to WSR mice. One possible explanation for the continued expression of HICs in the WSP lines is that they do not express adequate KOP-R numbers, thus, regardless of the amount of dynorphin released, there is insufficient activity at existing KOP-Rs to be seizure-protective and HIC severity remains high.

An alternative explanation for these findings is that the increased *Pdyn* abundance contributes to a correlated behavioral response to selection in WSP mice, rather than their selected HIC phenotype. For example, site specific injections of KOP-R agonists can produce aversion in rats and mice, and EtOH withdrawal often results in dysphoria, a negative affective state that may contribute to relapse to EtOH drinking. Thus, it might be that increased *Pdyn* mRNA levels in WSP mice contribute to the production of negative affective states in WSP mice, rather than physical withdrawal symptoms. Based on the relatively small changes in Pdyn abundance compared to the dramatic ten-fold difference in HIC severity exhibited by these lines, this is an attractive hypothesis that merits further testing. However, I believe that a role for the KOP-R system in the differences in HIC severity of WSP and WSR mice is likely, given the behavioral effects we observed with administration of agonist and antagonist for the KOP-R that will be discussed in greater detail later.

Finally, greater levels of *Pdyn* and other components of the KOP-R system have been measured in multiple brain regions of EtOH naïve D2 mice compared to naïve B6 mice (Jamensky and Gianoulakis, 1997). The greater *Pdyn* expression in D2 mice was suggested to participate in the lower levels of EtOH consumption exhibited by this genotype when compared to their EtOH preferring B6 counterparts. While neither of the WSP and WSR mice show EtOH preference, there are differences in the levels of EtOH consumed by the lines. Similarly, D2 and B6 mice display EtOH withdrawal HICs as well, but, analogous to the less dramatic differences in drinking behavior between WSP and WSR mice, the HICs of B6 and D2 mice are divergent, but are more mild than those of the WSP and WSR lines. However, if one compares WSP and WSR mice to D2 and

B6 mice, the WSP line is more similar to D2 animals, while WSR mice more closely resemble B6 animals both in terms of EtOH consumption and withdrawal severity. Thus, while we did not detect differences in the basal expression of *Pdyn* between the WSP and WSR lines, it is possible that the differences in EtOH consumption between WSP and WSR mice (WSP<WSR) may be due to basal differences in *Pdyn* mRNA abundance between the lines that we were unable to detect at the whole brain level.

Brain Region Specific Analyses

Because the Northern blot analyses did not answer important questions such as where the changes in *Pdyn* occurred, and why the release of dynorphin did not dampen HICs in WSP mice, the experiments described in chapter three expanded our analysis of *Pdyn* to specific brain regions and also included analyses of KOP-R binding via receptor autoradiography. Thus, these studies enabled us to test the hypothesis that the KOP-R system is functionally hypo-responsive in WSP mice and contributes to the severe HICs exhibited by these mice. Thus, the primary goal of this approach was to further clarify the role of KOP-R system changes in EtOH withdrawal severity, but again, it was possible that another phenotype was affected by the changes we documented. Therefore, the brain regions examined for *Pdyn* mRNA expression differences included the CPu, AcbC, AcbSh, Tu, and Pir. By including brain regions that participate in motor control (CPu, Pir and AcbC), as well as regions that appeared to participate in reward and aversion (AcbSh and Tu), we sought to identify whether *Pdyn* mRNA abundance was changing in brain regions likely to modulate seizure activity, affective states, or both. These studies confirmed our previous observations made at the whole brain level with respect to increased *Pdyn* mRNA, however, significant differences were detected

primarily in the WSP-1 line rather than in both WSP lines. In nearly every case, the observed effect was an increase in *Pdyn* abundance in withdrawing WSP-1 animals with respect to controls. It is important to note that in general, WSP-2 mice exhibited increased *Pdyn* compared to controls within these brain regions during EtOH withdrawal. Thus, while the data did not reach significance in WSP-2 mice, the observed change in the same direction (ie. an increase) was consistent with a role for signaling through the KOP-R system in the enhanced EtOH withdrawal severity of WSP mice. Some exceptions were present; non-significant decreases in *Pdyn* mRNA abundance were observed in both replicates of intoxicated WSR mice within the AcbSh and AcbC. Intoxicated WSP-1 mice showed a non-significant decrease in *Pdyn* in the Pir, while withdrawing WSR-1 mice displayed a non-significant decrease in *Pdyn* abundance within the Tu.

Basal KOP-R binding and alcohol exposure- and withdrawal-related changes in receptor density were examined in the CPu, Tu, *m*AcbSh, *l*AcbSh, Cl, DEn, and Cg. Of the regions studied, EtOH effects were present only in the CPu, Tu, *l*AcbSh and Cg. The EtOH-related effects on KOP-R abundance were more complex than EtOH's effects on *Pdyn* expression. However, the majority of the EtOH related responses again reflected increased KOP-R binding during withdrawal, with significant increases again present primarily in WSP-1 mice. Findings in the Tu were unique because the WSP-1 and WSP-2 lines did not differ, thus they were collapsed on replicate for analysis. EtOH withdrawal significantly increased KOP-R binding in the Tu of WSP mice. Within the CPu, KOP-R binding was again increased, however significant increases were present in both intoxicated and withdrawing WSP-1 animals. In the *l*AcbSh, a significant increase

in KOP-R was also observed; this increase was present only during withdrawal, and only in WSP-1 mice. These findings were in contrast to the effects observed in the Cg. Here, a significant increase in KOP-R abundance occurred only in intoxicated animals, and this effect was present in the WSR lines rather than WSP mice.

When basal KOP-R levels were analyzed, significant differences in basal expression were present in every brain region studied except the CPu. These analyses revealed that KOP-R density was generally higher in the WSR lines compared to WSP animals. These baseline differences in KOP-R density were actually more prevalent than the EtOH-related changes in KOP-R density and support the hypothesis that activity of the KOP-R system in WSP mice is not sufficient to protect the line from convulsive activity.

Claustrum , Dorsal Endopiriform Nucleus, and Piriform Cortex

Within the Cl and DEn only KOP-R levels were measured, and the only differences in KOP-R abundance that were present in these two regions were inherent differences in KOP-R abundance between the WSP and WSR lines. In the Cl both WSP lines had significantly lower KOP-R levels compared to WSR-1 mice, while they were similar to those seen in WSR-2 mice, providing moderate genetic evidence for participating of the Cl in the EtOH withdrawal response of WSP and WSR mice. The observations made with respect to KOP-R expression in the DEn are particularly interesting, in that the lower levels of KOP-R were present within both replicates of the WSP line compared to WSR. Thus, this finding represents the strongest statistical evidence for KOP-R participation in the divergent phenotypes of WSP and WSR mice. In addition, the DEn of WSP-1 mice contained significantly less KOP-R than the WSP-2

line, suggesting these differences might also contribute to the differences in HIC severity observed between the WSP lines. Thus, the line that exhibits the greatest exacerbation of HICs during EtOH withdrawal when corrected for baseline differences (WSP-1) also had the lowest level of KOP-R binding in the Den (Figure 19). KOP-R activation can decrease Ca^{2+} entry into cells, or increase K^{+} conductance, both of which result in inhibition of the target neuron (Satoh and Minami, 1995; Hjelmstad and Fields, 2001, 2003). In this way KOP-R activation is able to modulate the firing of both pre- and post-synaptic targets such as GABA and glutamate containing neurons. Reduced receptor density might therefore contribute to the greater seizure susceptibility of the WSP lines.

A great deal of literature suggests that the DEn and Cl participate in circuitry important for seizure generation and propagation. These brain regions are located in very close proximity to the 'area tempestas', a region demonstrated to be highly epileptogenic (Piredda and Gale, 1985). In addition, these two brain regions, along with the Pir, are anatomically close to one another and are likely to interact. Indeed, tracing studies revealed connections among these regions, with efferent connections observed from the Cl to Pir as well as from Cl to DEn (Behan and Haberly, 1999; Zhang et al., 2001). In turn, the DEn also has projections to the Pir (Behan and Haberly, 1999). Epileptiform excitatory post-synaptic potentials (e-EPSPs) have been measured in the Pir and the source of this activity was identified as cells within the En (Hoffman and Haberly, 1991, 1996). Further characterization using voltage sensitive dyes pinpointed the initial site of epileptiform discharge to the DEn (Demir et al., 1998), thus, the DEn appears to be the site of initiation of e-EPSPs which further propagate via the Cl and Pir. As noted in chapter three, the DEn also receives input from the amygdala. Thus, the DEn, along with

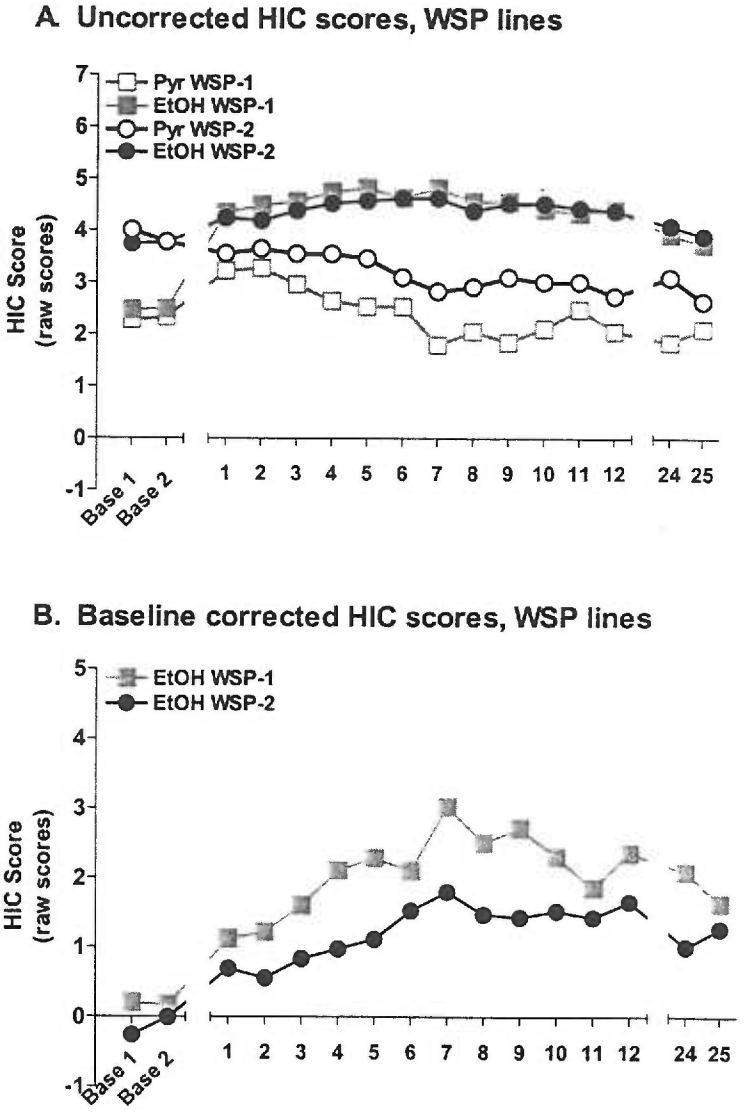

Figure 19. Representative HIC scores in WSP-1 and WSP-2 replicate lines. Open gray squares (□), Pyr WSP-1; filled gray squares (■), EtOH WSP-1; Open black circles (○), Pyr WSP-2; Closed black circles (●), EtOH WSP-2. (A) Raw HIC scores in Pyr and EtOH treated WSP-1 and WSP-2 lines. (B) Baseline corrected HIC scores in EtOH treated WSP-1 and WSP-2 mouse lines. See text for description of baseline correction methods.

the Cl and Pir, may serve as a gateway through which amygdala-kindled seizures are able to propagate seizure activity to the motor system. In fact, the interconnections of the DEn, the Cl, and the Pir, have led to the suggestion that a regenerative feedback loop may exist between these structures that contributes to the propagation of e-EPSPs within the Pir, DEn and Cl, and on to the hippocampus and cortex (Hoffman and Haberly, 1993; Behan and Haberly, 1999). Increased GABAergic activity within the DEn is anticonvulsant, while increased glutamate release in the Cl and DEn triggers epileptiform discharges from the DEn (Stevens et al., 1988; Hoffman and Haberly, 1991; Demir et al., 1998; Majak et al., 2002).

Kindling experiments further support a role for the Cl in seizure activity. Electrical stimulation within the Cl resulted in the rapid production of generalized seizure activity that later evolved to resemble limbic seizures generated via amygdala kindling (Zhang et al., 2001). The rapid development of kindling and the speed with which the authors observed generalization of the seizure activity led the authors to suggest that this brain region may be one of multiple forebrain structures that act as the gateway for propagation and generalization of seizure activity to motor regions of the brain. In keeping with this suggestion, tracing studies carried out by the same authors revealed in addition to connections with the Pir and DEn, efferents of the Cl project to the amygdala, the midline thalamus, and motor, prefrontal and limbic cortical regions (Zhang et al., 2001). The Cl also sends efferent projections to the AcbSh and the substantia nigra (SN), and there are reciprocal afferents to the Cl that originate in the amygdala, the medial prefrontal cortex, and the SN. Additional efferents to the Cl originate from the anterior Cg and VTA. These data reveal that the Cl connects with

many brain regions implicated in the generation and propagation of seizure activity. Most recently, kindling was performed in the Cl of rats followed by a 14-day break with no kindling. Upon retest, a single stimulation again produced a seizure that was as severe as those obtained prior to the suspension of the kindling (Sheerin et al., 2004). Thus, the Cl clearly participates in a forebrain network important for seizure generalization.

It is interesting to note that the Pir, Cl, and DEn connect to the hippocampus, and that dynorphin release within the hippocampus results in activation of KOP-Rs, which in turn decreases glutamate release in the hippocampus (Terman et al., 2000). While examinations of *Pdyn* abundance within the Pir did not reveal significant alterations in mRNA abundance within this brain region, a small decrease in *Pdyn* was present during EtOH exposure in the WSP-1 mice. This small decrease within the Pir may reduce the levels of dynorphin peptide available for release within the hippocampus. This could result in higher levels of hippocampal glutamate release that may contribute to the increased HIC severity observed in WSP mice while increased KOR abundance within the Cl and DEn of WSR mice might serve as a mechanism through which glutamate release in this region is reduced, thus conferring seizure protection in the WSR lines

Based on these observations, I propose that the higher levels of KOP-R in the Cl and DEn of WSR mice contributes to the decreased seizure severity observed in this line, with KOP-R activation resulting in seizure protection. The higher levels of KOP-R are activated by endogenously released peptide, and this activation in turn, acts to limit glutamate release within the Cl, DEn and Pir. The decreased glutamate release in these brain regions inhibits the initiation and propagation of seizure activity within the WSR

mice, thus conferring seizure-protection. In addition to the protective role of the increased KOR in WSR mice, the lower levels of *Pdyn* mRNA in the Pir along with the reduced density of KOP-Rs in WSP mice may result in decreased dynorphin levels in the terminals of Pir projection neurons. Given that these projections terminate in the hippocampal formation, the lower levels of dynorphin peptide would contribute to increased seizure susceptibility due to the inhibitory role dynorphin release plays in modulating glutamate release within the hippocampus. This effect, along with a generalized reduction in KOP-R density in WSP mice would contribute to the increased HIC severity of WSP mice. Together, the Cl, DEn, and Pir participate in the generation and expression of limbic seizures, which I propose is one brain circuit involved in the generation of HIC responses (Figure 20).

Important future experiments to address this hypothesis might involve a more extensive characterization of the brain regions that receive afferents from the Cl, Pir and DEn, as well as those that send afferent to these regions. Rather than an examination of Pdyn mRNA, a direct analysis of dynorphin levels would be important, since this hypothesis posits that WSP mice might have been selected for lower dynorphin peptide levels. An important target would the hippocampus, where dynorphin peptide is abundant, and which is intimately connected to these three regions. Furthermore, the hippocampus is a region that is highly susceptible to epileptogenesis, making it an attractive candidate for participation in HIC seizure circuitry. Finally, site specific injection of agonist and/or antagonist would allow us to directly test whether particular brain regions participate in HICs, and lesion studies might further help to elucidate the HIC seizure circuitry.

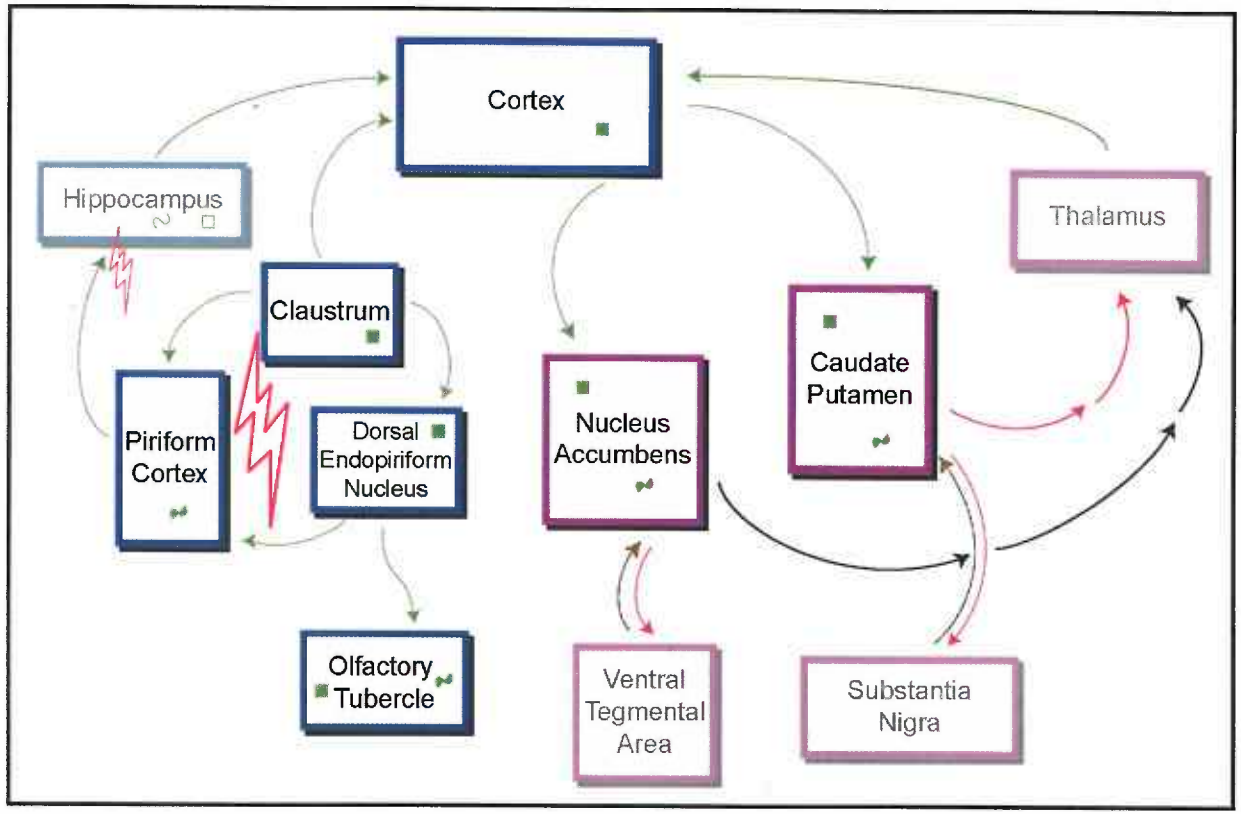

Figure 20. Circuitry Diagram. The brain regions analyzed using in situ hybridization or receptor autoradiography are depicted in dark blue and purple. The light blue and purple regions represent additional brain regions that may contribute to the generation and propagation of HICs but were not examined in this dissertation. Red lightning bolts identify brain regions and their projection targets that I propose are most likely to contrbute to the generation and propagation of HICs based on the data presented in this dissertation. The blue rectangles represent brain regions that are likely to contribute to forebrain seizure circuitry, and the purple rectangles represent brain regions identified as likely to participate in hindbrain seizure circuitry as defined by Gale (1988). Filled green squares and dark ribbons depict brain regions expressing KOP-R and *Pdyn* mRNA respectively that were examined in this dissertation. Open green squares and light ribbons identify brain regions known to express KOP-R or *Pdyn* that were not analyzed here.

Caudate-Putamen, Accumbens, Olfactory Tubercle, and Cingulate Cortex

Kindled seizures generated by deep pre-Pir electrical stimulation have been shown to produce an increase in *Pdyn* mRNA in the CPu of rats (Xie et al., 1989), which is consistent with our observation of increased *Pdyn* mRNA abundance in the CPu of seizure-prone mice. EtOH withdrawal has also been associated with increased extracellular glutamate in the striatum (Rossetti and Carboni, 1995). Activation of group I metabotropic glutamate receptors resulted in increased *Pdyn* abundance in striatal cell cultures, which may be one mechanism through which *Pdyn* mRNA abundance is increased in the CPu of WSP mice during EtOH withdrawal (Mao and Wang, 2001). *Pdyn* colocalizes in GABAergic neurons of the CPu, an observation that is very interesting in light of the connections of the CPu to the substantia nigra (SN), a brain region believed to be "capable of modifying the development and propagation of a seizure regardless of the neural mechanisms responsible for the seizure initiation" (Gale, 1988). It is possible that the increased *Pdyn* abundance in the CPu reflects a deficiency in dynorphin peptide in projection neurons of the CPu that terminate in the SN. This peptide deficiency could result in decreased KOP-R activation on SN target neurons, the net result of which would be disinhibition of GABAergic SN neurons. These SN GABAergic neurons terminate in the superior colliculus. Thus, the disinhibition of these SN neurons results in inhibition of GABAergic target neurons in the superior colliculus, and ultimately, decreased control over convulsive activity (Gale, 1988). Thus, a deficiency in dynorphin peptide in CPu projection neurons may be one mechanism that contributes to the generation of EtOH withdrawal seizures in WSP mice.

Application of KOP-R agonists decreases the levels of striatal glutamate in awake rats (Gray et al., 1999), and examination of KOP-R immunoreactivity in the striatum using electron microscopy supports the hypothesis that KOP-Rs act within the striatum to inhibit glutamate release (Meshul and McGinty, 2000). Thus, it is possible that the increases in KOP-R abundance in EtOH exposed and withdrawn WSP-1 mice may be a response to the alterations in GABA and glutamate neurotransmission during EtOH exposure and withdrawal, where the net effect of an increase in KOP-R levels during withdrawal might again be an attempt to increase anticonvulsant activity in WSP mice. To our knowledge, there is no evidence regarding glutamate levels in the CPu of WSP and WSR mice, however examinations in the hippocampus of seizure naïve animals that were not exposed to EtOH suggested there are greater levels of glutamate present in WSP mice that in WSR mice (Buckman and Meshul, 1997). Further investigation will be required to determine if these differences are also present in WSP and WSR CPu. Thus, the increase in *Pdyn* mRNA and KOP-R during EtOH withdrawal in WSP-1 mice might reflect a compensatory adaptive response to the increased seizure susceptibility of the WSP line.

It has been observed that following the induction of limbic seizures using kainic acid, there was an increase in Fos immunoreactivity in the AcbC. Since Fos immunoreactivity reflects neural activity, this finding suggests that the AcbC is sensitive to seizure activity. Like the CPu, the AcbC has projections to the SN, which might be a circuit through which AcbC activation could propagate seizure activity. In addition, the AcbC also projects to the pallidum (Zahm and Brog, 1992). The pallidum in turn has projections to the thalamus, where activation of a thalamo-cortical loop can result in

epileptic activity (Loscher and Ebert, 1996; Steinlein, 2004). Thus, the observed increases in *Pdyn* mRNA in the AcbC of WSP mice might again be a response to depleted peptide levels due to release or the result of an inherent deficiency in dynorphin that has been selected in this line. The increased *Pdyn* synthesis would aid in the restoration of dynorphin to pre-seizure levels.

In addition to *Pdyn* mRNA abundance, KOP-R abundance was also examined in Acb, but only in the shell region, as there is little expression in core. In *m*AcbSh it was observed that EtOH had no effect on KOP-R abundance in any of the lines. However, when examined for inherent differences in KOP-R levels, WSP-2 mice displayed significantly less KOP-R than the WSP-1 and WSR-1 groups. There was also a trend for this basal difference when compared to WSR-2 animals. Given the observations that KOP-R agonists tend to reduce seizures, it is possible that decreased receptor density resulted in reduced function of KOP-R in WSP-2 mice, and this rendered dynorphin peptide less effective as an anticonvulsant in the WSP-2 line.

Additionally, the *l*AcbSh was also observed to contain high levels of KOP-R binding. During EtOH withdrawal KOP-R levels in the *l*AcbSh were increased in WSP-1 mice, consistent with the effects observed in the dorsal region of the CPu. Thus, increased KOP-R levels in this brain region may also be a neuroadaptive response to peptide depletion in seizure susceptible WSP-2 mice. However, in contrast to the similar basal levels of KOP-R in the dorsal CPu of both the WSP and WSR mice, the *l*AcbSh brain region exhibited a significant increase in KOP-R abundance in WSR-2 mice. This higher level of receptor in WSR mice may contribute to overall reduced seizure susceptibility in this line.

The Tu also has been implicated as a potential participant in seizure circuitry. For example, the Tu receives efferent projections from the DEn where alterations in *Pdyn* abundance were also observed. As previously discussed, the DEn is a region that is likely to be involved with the initiation and expression of seizures (Behan and Haberly, 1999), thus the Tu might also be involved in seizure circuitry via its connections with the DEn. In addition, recent data documented epileptic seizures that appear to originate in limbic forebrain regions in mice deficient for the transcription factor SOX1 (Malas et al., 2003). When epileptiform discharges were measured in these animals it was determined that the Pir in this model was particularly prone to exhibit spontaneous epileptiform activity. Further characterization revealed that normal GABAergic neuronal development in the Tu of SOX1 deficient mice was absent. Thus, normally existing connections between the Pir and Tu of these animals were not present, suggesting that compensatory connections were formed during development leading to "abnormal intracortical connections within the olfactory region creating an overabundance of excitation in this area" (Malas et al., 2003).

The Tu is a component of the inhibitory striatonigral pathway described in the discussion of the CPu above (Newman and Winans, 1980), and it is believed that GABAergic neurons from this region also participate in the control of SN activity. Thus, a decrease in GABA release from this region would result in loss of the inhibitory control exerted by SN neurons that protect against convulsive activity. Increased activity of the Pir may be further potentiated in the absence of this anticonvulsant response (Malas et al., 2003). Our observations of increased *Pdyn* abundance within the Tu of WSP-1 mice during EtOH withdrawal, and the corresponding increase in KOP-R abundance within

both WSP lines during withdrawal are again consistent with the hypothesis that WSP mouse brain is depleted of endogeneous dynorphin stores during seizure activity. This attempt to dampen hyperexcitability would be unsuccessful due to inadequate receptor availability for the production of an anticonvulsant response. Thus increased expression of *Pdyn* and KOP-R might be occurring in these lines to compensate for the subtherapeutic levels that are normally present.

Findings characterizing increased KOP-R in the Cg are interesting for two reasons. First, this was the single region where the response to EtOH was specific to WSR mice, rather than WSP mice. In addition, while a significant increase in KOP-R was observed in EtOH exposed WSR mice compared to control WSR mice, the alcohol independent KOP-R levels were not consistent with basal expression in this brain region playing an important role in the phenotypes of WSP and WSR mice. WSP-1 mice displayed significantly less KOP-R compared to WSR-1 mice, while WSP-2 mice showed significantly greater levels of KOP-R than their WSR-2 counterparts. When independently selected replicate lines display differences between both the replicates that are in opposite directions, the data "strongly suggests the lack of a genetic correlation" (Crabbe et al., 1990c).

Behavioral Analyses

Thus, results in nearly all the brain regions examined for differences in *Pdyn* and the KOP-R of WSP and WSR mice are consistent with the proposition that decreases KOP-R system activity may participate in the disparate seizure activity of the two selected lines. In order to characterize the consequence of manipulation of KOP-R

activity on seizure severity, the final series of experiments we conducted examined the effect of direct manipulation of the KOP-R system on HICs, using both an antagonist and agonist in WSP and WSR mice. Administration of the KOP-R antagonist nor-BNI 5-6 days prior to the onset of withdrawal resulted in a significant increase in HIC severity in WSR-1 mice during EtOH withdrawal. In contrast, no effects of nor-BNI were observed in WSR-2 mice, and both WSP lines were unaffected by pretreatment with this KOP-R antagonist. However, it should be noted that in WSR-2 mice, HIC scores for EtOH withdrawing animals that had not received nor-BNI were already similar in magnitude to those observed in WSR-1 mice. Thus, it is possible that nor-BNI was unable to potentiate this response due to a "ceiling" effect on the severity of HICs attainable in this line. Further studies with lower EtOH exposures, or alternative methods of exposure should help to clarify the effects of nor-BNI in WSR-2 mice. Importantly, the effects of nor-BNI were only observed in animals that had been exposed to EtOH, thus the nor-BNI induced increases in HIC severity in WSR-1 mice appear to have been an EtOH-specific response, rather than a global change in HIC susceptibility in WSR mice following nor-BNI administration.

KOP-R agonist administration (U50) in WSP and WSR mice altered HICs in WSP mice, rather than in WSR mice. Thus, the observed effects of the agonist were complementary to those of the KOP-R antagonist. That is, while antagonist administration potentiated HICs in WSR mice, agonist administration attenuated HICs in WSP mice. Our inability to detect effects of U50 on HIC severity in either naïve or EtOH withdrawing WSR-1 mice were not surprising, given the absence of basal HICs in WSR-1 mice and the minimal HIC response exhibited by these animals during EtOH

withdrawal. In contrast to an EtOH-specific effect of nor-BNI, U50 administration resulted in a non-specific reduction in seizure severity. That is, both EtOH naïve and EtOH exposed WSP mice showed decreased HIC severity during withdrawal following administration of U50 consistent with an anti-convulsant effect of this drug. The results of these studies further support a modulatory role of the KOP-R system in EtOH withdrawal seizures. Based on the suppressive effect of the KOP-R agonist on basal HICs in the WSP mice, these results also suggest that this system may participate the general seizure activity of this line.

The data presented in this thesis provides converging molecular, pharmacological and behavioral evidence to suggest a modulatory role of the KOP-R system in the expression of basal and EtOH withdrawal-induced seizure activity in mice selectively bred to exhibit divergent HICs during withdrawal from EtOH. While it might be argued that the presence of significant differences in one replicate in some cases is only moderate evidence to support a role of the KOP-R system in EtOH withdrawal, the consistency of our findings across molecular, pharmacological and behavioral approaches makes a strong argument for a role of this system in the susceptibility to EtOH withdrawal HICs exhibited by these animals. Indeed, given the polygenic nature of alcohol-related traits, this finding is not surprising. Rather altered function of the KOP-R system is likely to be only one component in a large multitude of overlapping mechanisms through which EtOH exerts its effects on the nervous system and produces changes in nervous system function that result in hyperexcitability upon withdrawal. Further characterization of the KOP-R system in additional animal models of EtOH withdrawal will help determine the generalizability of these results to additional

populations, and will aid in further elucidating the role of the system in EtOH withdrawal severity. A better understanding of the role of the KOP-R system in EtOH withdrawal severity may eventually aid in the develop of novel therapeutic agents for the treatment of alcohol dependence and withdrawal.

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