THE ROLE OF PROTEIN KINASE A AND STRESS IN ETHANOL-INDUCED LOCOMOTOR SENSITIZATION

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ABSTRACT

JON R. FEE: The Role of Protein Kinase A and Stress in Ethanol-Induced Locomotor Sensitization
(Under the direction of Todd E. Thiele)

There is a growing body of literature dedicated to elucidating the mechanisms involved in behavioral sensitization. The following studies examined the ability of alterations in PKA signaling (via genetic knockout) and CRF signaling (via pharmacological blockade) to modulate ethanol-induced locomotor sensitization and restraint stress-induced cross-sensitization.

Experiment 1 utilized the RIIβ knockout model to determine if alterations in PKA signaling affect locomotor activation in response to ethanol as well as the development of ethanol-induced locomotor sensitization. $RII\beta^{-/-}$ mice consistently showed significantly greater ethanol-induced locomotor activity relative to $RII\beta^{+/+}$ mice. $RII\beta^{-/-}$ mice also showed increased sensitivity to ethanol-induced locomotor sensitization, an effect that may be dependent on genetic background and/or testing paradigm. Importantly, increased locomotor activity by $RII\beta^{-/-}$ mice was specific to ethanol injections, absent following saline injections, and not associated with altered blood ethanol levels.

Experiment 2 attempted to discern a few of the mechanisms underlying the restraint stress-induced cross-sensitized response to ethanol in mice. Chronic exposure to restraint stress (2hrs for 10 days) induced a cross-sensitized response to ethanol in two commercially

available inbred strains of mice, C57BL/6J and DBA/2J. The RIIβ knockout mouse model was also utilized to test if enhanced sensitivity to stress might be a factor contributing to the previously observed heightened sensitivity to ethanol-induced locomotor sensitization in these mice. $RII\beta^{-/-}$ and $RII\beta^{+/+}$ showed no differences in the effect of stress on ethanol-induced locomotor activity. Finally, pretreatment with CRF Receptor 1 antagonist, CP-154,526, prior to restraint stress sessions in male DBA/2J mice failed to block the acquisition of a cross-sensitized response to ethanol.

Experiment 3 used a CRF Receptor 1 antagonist, CP-154,526, in an attempt to block the acquisition and expression of ethanol-induced locomotor sensitization in male DBA/2J mice. Pretreatment with CP-154,526 was ineffective in blocking the acquisition of a sensitized response to ethanol. In contrast, pretreatment with CP-154,526 effectively blocked expression of a sensitized response in DBA/2J mice that had been previously sensitized to the locomotor stimulating effects of ethanol. These effects were not attributable to alterations in blood ethanol levels, and ethanol naïve DBA/2J mice when pretreated with CP-154,526 show no such reductions in locomotor activity in response to ethanol.

These experiments suggest a role for both PKA and CRF signaling in the mechanisms underlying ethanol-induced locomotor sensitization. Additionally, the ability of chronic restraint stress to elicit a cross-sensitized response to ethanol in mice is demonstrated. Ultimately, it remains unclear if alterations in PKA or CRF signaling are involved in the mechanisms associated with restraint stress-induced cross sensitization.

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LIST OF ABBREVIATIONS

$^{-/-}$	knockout
$^{+/+}$	wild-type
AA	Alko alcohol
AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
ANA	Alko non-alcohol
ANOVA	analysis of variance
Cα	catalytic subunit Alpha
cAMP	cyclic adenosine monophosphate
Cβ	catalytic subunit Beta
CeA	central nucleus of the amygdala
CMC	carboxymethylcellulose
CNS	central nervous system
CP-154,526	butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine
CREB	cAMP response element binding protein
CRF	corticotropin releasing factor
CRF1R	CRF receptor 1
CRF2R	CRF receptor 2
DNA	deoxyribonucleic acid
Drd2	dopamine D2 receptor
G-protein	guanine nucleotide binding protein

GABA	gamma-aminobutyric acid
g	gram
GRK	G protein receptor kinase
HAP	high alcohol preferring
HPA	hypothalamic-pituitary-adrenal
hr	hour
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.v.	intravenous
IS	inescapable stress
kg	kilogram
LAP	low alcohol prefering
µl	micro liter
min	minute
mg	milligram
ml	milliliter
mRNA	messenger RNA
PCR	polymerase chain reaction
pCREB	phosphorylated CREB
PKA	protein kinase A
PKC	protein kinase C
PVN	paraventricular nucleus of the hypothalamus
RIIβ	regulatory subunit II Beta

RIβ	regulatory subunit I Beta
RIIα	regulatory subunit II Alpha
RIα	regulatory subunit I Alpha
R	regulatory
S.E.M	standard error of the mean
VTA	ventral tegmental area
w/v	weight per volume

CHAPTER 1

GENERAL INTRODUCTION

Many neurotransmitters, neuromodulators, and hormones transduce their signal into cells by activating G-protein-coupled receptors. Following G-protein-coupled receptor binding, cAMP levels are either enhanced or inhibited via changes in adenylyl cyclase (AC) activation. This action leads to concomitant increases or decreases in cAMP-dependent protein kinase A (PKA) activity, respectively. PKA is a holoenzyme which consists of a regulatory (R) subunit homodimer and two catalytic (C) subunits (Brandon et al. 1997). In the mouse, PKA includes four regulatory subunits (RIα, RIβ, RIIα and RIIβ) and two catalytic subunits (Cα and Cβ) which are expressed in tissue-specific patterns (McKnight 1991). It has been shown that the RIIβ subunit is selectively expressed in brain and adrenal tissue in addition to adipose tissue (Sarkar et al. 1984). Sarkar and colleagues suggest that RIIβ is the predominant regulatory subunit of PKA in the central nervous system.

The RIIβ$^{-/-}$ mouse model exhibits reduced cAMP-stimulated protein kinase A (PKA) activity in various brain regions implicated in the pharmacological response to drugs of abuse, including the striatum, nucleus accumbens, amygdala, hippocampus and hypothalamus and generally in the cortex (Brandon et al. 1998; Thiele et al. 2000b). It has been proposed that the loss of the RIIβ subunit results in enhanced constituent PKA activation and in turn, enzymatic degradation of unbound catalytic subunit leading to a

functional loss of PKA activity. As a result, the relative distribution of other regulatory subunits up-regulates in an attempt to compensate for the loss of RIIβ (Amieux et al. 1997; Brandon et al. 1998). However, it stands to reason that this compensation is not full as evidenced by the reduced cAMP-stimulated PKA activity. These findings would suggest that the RIIβ$^{-/-}$ model is characterized by reductions in PKA signaling. The manipulation of PKA activity has been shown to have several interesting behavioral consequences. There is consistent evidence that deletion of the RIIβ subunit results in a mouse that will more readily consume ethanol solutions compared to wildtype littermate controls (Fee et al. 2004; Thiele et al. 2000b). In our most recent study, it was found that these genotype differences in consumption were not reliably predicted by basal levels of anxiety despite the observation that blunted PKA activity and phosphorylated CREB (pCREB) in the amygdala are associated with increased anxiety-like behavior (Pandey et al. 2003). RIIβ$^{-/-}$ mice consistently show reductions in their sensitivity to the sedative properties of ethanol (Fee et al. 2004; Thiele et al. 2000b). Recently, our lab discovered that RIIβ$^{-/-}$ mice are also more sensitive to the acquisition of ethanol-induced locomotor sensitization (Fee et al. 2006). These data are complemented by an earlier finding by McKnight and colleagues that RIIβ$^{-/-}$ mice are more sensitive to amphetamine-induced locomotor sensitization (Brandon et al. 1998). Taken together, these data would suggest that manipulation of PKA signaling via the deletion of the RIIβ subunit increases sensitivity to the sensitization that accompanies repeated exposure to drugs of abuse.

Manipulation of PKA signaling via deletion of the RIIβ subunit results in a mouse model that is more susceptible to the acquisition of locomotor sensitization following repeated amphetamine (Brandon et al. 1998) and ethanol (Fee et al. 2006) exposure.

Amphetamine exposure during the rat preweanling phase has been shown to produce persistent reductions in PKA activity in the accumbens and dorsal striatum (Crawford et al. 2000). Interestingly, these reductions in PKA function were not linked to a sensitized response to amphetamine, however, these rats only received a single dose of amphetamine and it is possible that the reduction in PKA activity could have promoted behavioral sensitization had repeated injections been administered. The ventral tegmental area (VTA) has also been implicated as a brain region in which PKA activity mediates the expression of amphetamine sensitization. Co-injection of the PKA inhibitor, Rp-cAMPS, blocked the sensitization induced by intra-VTA amphetamine injections and intra-VTA administration of the PKA activator, Sp-cAMPS, augmented the sensitization caused by peripheral amphetamine administration (Tolliver et al. 1999). These data suggest the possibility of regional specificity in PKA's involvement in the regulation of sensitivity to sensitization. A similar conclusion has been drawn concerning the role of PKA in modulating sensitivity to the sedative properties of ethanol (Rodan et al. 2002). Taken together, these observations suggest a role for PKA signaling in the mediation of behavioral sensitization caused by repeated application of drugs of abuse.

Repeated stress has been shown to blunt PKA activity in specific brain regions. It was recently discovered that rats exposed to inescapable stress (IS), a model of learned helplessness, show decreased PKA activity in the hippocampus and cortex. The continued presence of learned helplessness behavior following IS was linked to persistent reductions in PKA activity. Reductions in PKA signaling appear to be linked to reductions in the expression of RIIβ, Cα, and Cβ subunits (Dwivedi et al. 2004). Based on these data, it could be suggested that, at birth, RIIβ$^{-/-}$ mice resemble animals that have been exposed to repeated

stressors because both models show reduced cAMP-dependent PKA signaling in the cortex and hippocampus. In a related study, implantation of a corticosterone pellet in adrenalectomized rats decreased cAMP binding to PKA. Adrenalectomy led to increases in cAMP binding to PKA. Additionally, corticosterone treatment led to reduced expression of the PKA subunits RIIβ, RIα, and Cβ in the cortex and the hippocampus of rats (Dwivedi and Pandey 2000). These data also suggest that repeated or prolonged exposure to stress leads to persistent reductions in PKA activity. In contrast, it has been shown that repeated unpredictable stress increased levels of cAMP-dependent protein kinase activity in the nucleus accumbens (Ortiz et al. 1996).

PKA is involved in the regulation of receptors that mediate the stress response. PKA can phosphorylate and induce the transcription of the progesterone receptor (Denner et al. 1990). In addition, PKA has been shown to affect to the regulation of mineralocorticoid receptors (Massaad et al. 1999). Additionally, glucocorticoid receptor function in specific cell lines is believed to be mediated by cAMP-dependent protein kinase (Gruol and Dalton 1984). PKA can also enhance the DNA binding activity of glucocorticoid receptors (Rangarajan et al. 1992). Taken together these data suggest that PKA activity is important in the regulation of receptors involved in the stress response.

PKA has also been shown to play an important role in the regulation of the adrenal cortex. It has been suggested that PKA is critical in the growth and growth maintenance of the adrenal cortex, in that a single gene mutation for PKA results in hyperplasia (Keegan and Hammer 2002). Interestingly, this was one of the regions identified for its predominance of RIIβ subunit expression (Sarkar et al. 1984). These studies suggest a critical role for PKA and specifically the RIIβ subunit in the proper regulation of the hypothalamic-pituitary-

adrenal (HPA) axis as well as the cellular consequences of stress exposure. The HPA axis is the well characterized neuroendocrine system which responds to stress in a cascade of events resulting ultimately in corticosteroid release. A detailed assessment of the markers of HPA axis function in RIIβ$^{-/-}$ mice before and after exposure to stress would greatly benefit the field by determining how complete loss of the RIIβ subunit affects stress related signaling.

There is considerable evidence in the literature suggesting a role for PKA activity in the regulation of HPA axis activity. It has been suggested that the effects of diazepam on HPA axis activity are mediated via cAMP-dependant PKA activity in the hypothalamus (Vargas et al. 2001). PKA activity has also been put forward as a potential mechanism underlying the diminished hippocampal activity thought to be associated with the HPA axis hyperactivity in depression (Perera et al. 2001). Specifically, ethanol has been shown to increase PKA and cAMP activity and, in turn, increase corticotropin releasing factor (CRF) peptide secretion (Li et al. 2005). Additionally, PKA activity has been shown to play an essential role in CRF mediated decreased expression of CRF receptor 1 mRNA in part via the phosphorylation of cAMP response element binding (CREB) (Kasagi et al. 2002). However, other investigators have suggested a role for protein kinase C (PKC) and PKA in ACTH release (Iwabuchi et al. 1999), and a role for G protein receptor kinase (GRK) in the activity of CRF type 1 receptors (Dautzenberg et al. 2001).

A significant literature exists suggesting that direct manipulation of corticotropin releasing factor (CRF) will influence the acquisition and expression of drug-induced locomotor sensitization. Sensitization has been defined as the long-lasting and progressive enhancement of the locomotor and motivational responses to a drug following repeated administration (Kalivas and Stewart 1991). Intracerebroventricular (i.c.v.) administration of

the CRF antagonist, α-helical CRF, reduced stereotypic response to amphetamine following repeated restraint stress in rats (Cole et al. 1990b). In contrast, an augmentation of HPA axis signaling via pharmacological manipulation can result in the expression of sensitized behaviors. I.c.v. administration of CRF rather than subcutaneous administration of CRF leads to long-term locomotor sensitization to D-amphetamine (Cador et al. 1993). Similarly, microinfusion of CRF into the shell of the nucleus accumbens of rats led to a prolonged increase in general locomotor activity (Holahan et al. 1997). It also appears that a blockade of endogenous CRF signaling via administration of CRF receptor 1 antagonist, CP-154,526, will reduce cocaine-induced locomotor sensitization and relapse behavior (Przegalinski et al. 2005). These approaches have yielded fruitful results, and further investigation is necessary to more fully understand the role of CRF signaling and its effects within and outside of HPA axis activity in the acquisition and expression of cross-sensitized drug responses.

It is been shown that exposure to ethanol leads to an activation of the HPA axis (Ogilvie et al. 1997; Pruett et al. 1998). For this reason, it is important to consider stress-related signaling mechanisms when investigating the acquisition of ethanol-induced locomotor sensitization. It has been shown that restraint stress can substitute for ethanol injections in an ethanol sensitization paradigm suggesting that many of the same endogenous mechanisms are activated by ethanol and stressors. This study also found that pretreatment with the glucocorticoid receptor antagonist, RU38486, could block the expression of ethanol-induced locomotor sensitization (Roberts et al. 1995). This would suggest overlapping pathways between the molecular mechanisms involved in stress and ethanol exposure with respect to sensitization. This critical role for stress in the expression of sensitization is also found in the cocaine literature. Administration of a corticosterone synthesis inhibitor,

metyrapone, blocked the increased corticosterone levels and the sensitized locomotor response to cocaine in rats maintained on 90% food restriction (Marinelli et al. 1996). Repeated administration of various stressors (restraint, handling, and social defeat) has been shown to sensitize the locomotor response to morphine (Stohr et al. 1999). However, a sensitized corticosterone response is not necessary for the expression of long-term (3 weeks) amphetamine sensitization as evidenced by the lack of a correlational relationship between corticosterone levels and locomotor activity (Schmidt et al. 1999). Although corticosterone levels were not predictive, this does not necessarily mean that extended exposure to stress did not manifest itself in changes in receptor affinity or population. A larger picture of stress signaling, as provided by numerous markers, is necessary to elucidate the role of stress in the acquisition and expression of locomotor sensitization.

In 2001, Lessov and colleagues demonstrated that locomotor sensitization was possible in C57BL/6 mice. This was a model that had otherwise not shown sensitization and was used as an argument against the association between ethanol locomotor sensitization and ethanol reward based on the fact that C57BL/6 mice are notoriously high drinkers (Phillips et al. 1994). Pure C57BL/6 mice were shown to increase their ethanol preference after the acquisition of locomotor sensitization. There was an opposite effect on the preference of DBA/2J mice, a strain that is relatively easy to sensitize (Lessov et al. 2001). Additionally, a study of high alcohol preferring (HAP) and low alcohol preferring (LAP) mice found that high ethanol preference was predictive of the acquisition of ethanol-induced locomotor sensitization (Grahame et al. 2000). The RIIβ$^{-/-}$ mouse model is a third example of a high ethanol preferring mouse that exhibits heightened susceptibility to locomotor sensitization. In fact, ethanol-induced locomotor sensitization in RIIβ$^{-/-}$ mice maintained on a C57BL/6

background is possible using a less rigorous dosing schedule than that of the Lessov et al. (2001) study (lower dose, bi-weekly verses daily injections). Deletion of the dopamine D2 receptor (Drd2) also enhances ethanol-induced locomotor sensitization on a C57BL/6 background which resulted in the elimination of an initially reduced ethanol preference compared to wildtype mice. However, the effect of the Drd2 deletion had no effect on a 129 background suggesting an epistatic interaction (Palmer et al. 2003). With only a few exceptions, these studies suggest an interesting interaction between voluntary drug self-administration and the acquisition of sensitization or vice versa. Not only does the RIIβ$^{-/-}$ mouse readily self-administer ethanol, this model also lacks the critical subunit that has been implicated in the proper regulation of endogenous stress signaling. The RIIβ$^{-/-}$ mouse model allows for the very unique study of stress in ethanol-induced locomotor sensitization that is currently not available otherwise.

The following experiments will attempt to elucidate mechanisms (PKA and CRF) which we propose to be involved in ethanol-induced locomotor activation and sensitization. Given the availability of the RIIβ knockout model, a role for PKA activity in ethanol-induced locomotor sensitization will be investigated. Additional focus will be given to the ability of a CRF receptor 1 antagonist, CP-154,526, to block the acquisition and expression of ethanol-induced locomotor sensitization. Together these experiments will demonstrate a novel role for PKA signaling and CRF receptor activation in the sensitized locomotor responses that accompany repeated exposure to ethanol. A final set of experiments will look at the ability of stress to serve as a sensitizing agent in a series of restraint stress-induced cross-sensitized responses to ethanol.

CHAPTER 2

EXPERIMENT 1: INVOLVEMENT OF PROTEIN KINASE A IN ETHANOL-INDUCED LOCOMOTOR ACTIVITY AND SENSITIZATION

Introduction

Behavioral sensitization has been defined as the long-lasting and progressive enhancement of the locomotor and motivational responses to a drug following repeated administration (Kalivas and Stewart 1991). Over the past twenty years, many studies have demonstrated the ability of repeated ethanol exposure to elicit locomotor sensitization in mice (Crabbe et al. 1992; Cunningham and Noble 1992; Lister 1987; Phillips et al. 1995). Interestingly, a genetic predisposition to alcoholism has been associated with increased sensitivity to this phenomenon. Sons of alcoholics, when compared to sons of non-alcoholics, have been shown to be more sensitive to increases in locomotor activity that emerge over the course of repeated ethanol administrations (Newlin and Thomson 1991). It has been suggested that increased sensitivity to behavioral sensitization may be an underlying mechanism that increases the risk for developing drug dependence. According to this view, repeated exposure to a drug promotes neural reorganization leading to a hypersensitive state in brain reward circuitry (Robinson and Berridge 1993; 2000; 2001).

Studies of neural plasticity have shown that intracellular cAMP-dependent protein kinase A (PKA) modulates neurophysiological alterations that are responsible for the

sensitization associated with repeated exposure to noxious stimuli (Castellucci et al. 1980). As such, it is possible that PKA signaling also modulates the neural plasticity that is believed to be responsible for the expression of drug-induced behavioral sensitization. In fact, some of the neurochemical systems that have been implicated in drug-induced locomotor stimulation and behavioral sensitization involve G-protein-coupled receptors that recruit PKA signaling, including dopamine (Broadbent et al. 1995; Broadbent et al. 2005; Hamamura et al. 1991; Itzhak and Martin 1999; Lessov and Phillips 2003; Mattingly et al. 1994; Palmer et al. 2003), adenosine (Chen et al. 2003), serotonin (Auclair et al. 2004), opioid (Camarini et al. 2000) and GABA (Broadbent and Harless 1999). Theoretically, since PKA signaling is a basic neuronal mechanism influenced by different neurochemical pathways, neurobiological responses to drugs of abuse with different mechanisms of action on PKA may be a basis for drug cross-sensitization (Itzhak and Martin 1999; Lessov and Phillips 2003; McDaid et al. 2005; Muschamp and Siviy 2002).

It is currently unknown if PKA signaling modulates the stimulant effects and/or behavioral sensitization caused by ethanol administration. To address this question, we examined the effects of repeated ethanol administration on locomotor activity in a PKA-mutant mouse model. In the mouse, PKA includes four regulatory subunits (RIα, RIβ, RIIα and RIIβ) and two catalytic subunits (Cα and Cβ) which are expressed in tissue-specific patterns (McKnight 1991). Use of mice lacking the RIIβ subunit of PKA ($RII\beta^{-/-}$) allows for the assessment of locomotor sensitization in a model shown previously to have reduced cAMP-stimulated PKA activity in brain regions implicated in behavioral sensitization including the striatum and the nucleus accumbens (Brandon et al. 1998; Thiele et al. 2000b). We have previously shown that $RII\beta^{-/-}$ mice exhibit enhanced ethanol preference and

consumption as well as reduced sensitivity to ethanol-induced sedation when compared with wildtype littermate control mice (Fee et al. 2004; Thiele et al. 2000b). Several recent studies have suggested a positive correlation between ethanol preference and ethanol-induced locomotor sensitization in mice (Grahame et al. 2000; Lessov et al. 2001; Palmer et al. 2003). Thus, because RIIβ$^{-/-}$ mice show increased ethanol preference, we hypothesized that RIIβ$^{-/-}$ mice would show increased sensitivity to the stimulant effects of ethanol and enhanced ethanol-induced locomotor sensitization.

Because the expression of phenotypes can depend on the genetic background of the knockout mouse model (Fee et al. 2004; Palmer et al. 2003; Thiele et al. 2004), we evaluated ethanol-induced locomotor activity and sensitization in RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice maintained on differing genetic backgrounds. The sensitization paradigm developed in our laboratory involved bi-weekly ethanol injections. However, due to the fact that subtle differences in testing paradigm can lead to dramatic differences in observed behavior (Rustay et al. 2003), we also employed an alternate sensitization paradigm that is commonly used in other laboratories (Lessov et al. 2001; Meyer et al. 2005).

Methods

Animals

RIIβ$^{-/-}$ mice were created through the disruption of the RIIβ gene by homologous recombination in embryonic stem cells from 129/SvJ mice (Brandon et al. 1998). Chimeras were bred with C57BL/6J mice to obtain heterozygotes (50% 129/SvJ x 50% C57BL/6J). These heterozygotes were backcrossed with C57BL/6J mice over 8 generations to yield RIIβ$^{+/-}$ mice on a ~100% C57BL/6J genetic background. For some experiments described

here, non-littermate RIIβ$^{+/-}$ mice on the 100% C57BL/6J background were bred, to provide RIIβ$^{-/-}$ and RIIβ$^{+/+}$ F2 littermate mice. Additional experiments involved RIIβ$^{-/-}$ and RIIβ$^{+/+}$ F2 littermate mice on a 50% 129/SvEv x 50% C57BL/6J background that were created by crossing the RIIβ$^{-/-}$ mice with wild-type 129/SvEv mice. The genetic status of all mice was determined using polymerase chain reaction (PCR) procedures described elsewhere (Thiele et al. 2000b). Animals weighed approximately 20 g, were 3 to 6 months of age at the beginning of experiments, and were individually housed in polypropylene cages with corncob bedding. Mice had *ad libitum* access to water and standard rodent chow (Tekland, Madison, WI) except where noted. The colony room was maintained at approximately 22° C with a 12h:12h light:dark cycle with lights off at 3:00 pm. All procedures used in the present study were in compliance with the National Institute of Health guidelines, and all protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Ethanol-Induced Locomotor Activation: Test Chamber Injections

Drug-naïve RIIβ$^{-/-}$ (male, n = 14; female, n = 14) and RIIβ$^{+/+}$ (male, n = 13; female, n = 13) mice on a pure C57BL/6J background as well as RIIβ$^{-/-}$ (male, n = 12; female, n = 15) and RIIβ$^{+/+}$ (male, n = 13; female, n = 15) mice on a mixed 129/SvEv x C57BL/6J background were tested during the light phase of their light:dark cycle. All animals were transported to the testing room in their home cages and allowed to habituate for at least 35-min prior to testing. A fan provided masking noise in the testing room. Mice were removed from their home cages, given an intraperitoneal (i.p.) ethanol or equivolume saline injection according to the dosing schedule outlined in Table 2.1, and were placed into the center of an open-field

arena that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA). The open field arena measured 40.64 cm by 40.64 cm by 30.48 cm and was made of clear Plexiglas. Several cm of corncob bedding were placed into the open field chamber to aid in cleaning and to prevent the buildup of odor. Testing sessions were 20-min in duration and soiled bedding was removed from the chamber after each session. In summary, mice received three i.p. injections of isotonic saline (one injection per day) every 2 to 3 days to establish a locomotor baseline for activity and to allow the mice to habituate to the testing procedure. Following completion of baseline testing, mice received i.p. ethanol injection (one per day) two times a week for up to ten injections. Mice on the 129/SvEv x C57BL/6J genetic background were given a 1.4 g/kg dose while mice on the C57BL/6J genetic background were given a 2.0 g/kg dose (20% w/v solutions were mixed in isotonic saline). Doses of ethanol for each genetic background were chosen based on pilot observations in our laboratory. Higher doses where shown to have a significant sedative component. After ethanol injections, mice were tested following a single (129/SvEv x C57BL/6J mice) or two (C57BL/6J mice) i.p. saline injections (one injection per day) to examine specificity of the effects of ethanol on locomotor activity. Mice on the C57BL/6J genetic background received two additional ethanol tests immediately after saline injections. Finally, as an additional test for specificity of the effects of ethanol injection on locomotor activity, naïve RII$\beta^{-/-}$ (male, n = 10; female, n = 10) and RII$\beta^{+/+}$ (male, n = 10; female, n = 10) mice on the mixed 129/SvEv x C57BL/6J background were run in a study using procedures similar to those described above with the exception that only i.p. saline injections were given over 8 trials.

To determine blood ethanol levels, 6 μl tail nick blood samples were taken from 129/SvEv x C57BL/6J mice immediately after the 1st, 5th, and 10th 20-min test sessions that were run on ethanol injection days. For C57BL/6J mice, blood samples were collected following the 20-min session on the last (twelfth) ethanol injection day. Procedures for assessing blood ethanol levels are described below.

Ethanol-Induced Locomotor Activation: Home-cage Injections

Drug-naïve RIIβ$^{-/-}$ (male, n = 10; female, n = 10) and RIIβ$^{+/+}$ (male, n = 10; female, n = 10) mice on a pure C57BL/6J background were tested during the light phase of their light:dark cycle. An overview of the sensitization paradigm, adapted from (Lessov et al. 2001), is described in Table 2.2. This paradigm was used for its ability to induce locomotor sensitization in wildtype C57BL/6 mice. On test day 1-3, all mice received i.p. injections of isotonic saline (one injection per day) based on the equivalent volume for a 2.0 g/kg ethanol injection prior to being placed into the center of the open-field arena for 20-min sessions. On day 4, all mice received an i.p. injection of 2.0 g/kg ethanol prior to placement in the locomotor chamber in order to determine basal ethanol responsiveness during the 20-min session. Mice were then assigned to treatment groups equated for locomotor activity during the initial ethanol response (Day 4). Over the next ten days, mice received daily i.p. injections of 2.5 g/kg ethanol and were immediately returned to their home cage. On day 15, half of the mice received an i.p. injection of 2.0 g/kg ethanol and were immediately placed into the center of the activity apparatus to assess ethanol-induced locomotor sensitization.

The remaining mice received an i.p. injection of saline to test for general activity not attributable to ethanol.

Blood Ethanol Concentrations

6 µl tail nick blood samples were collected into capillary tubes and dispensed into 12x75 mm borosilicate glass tubes containing 375 µl of water and 0.5 g of NaCl. These liquid samples were capped and refrigerated until processing by gas chromatography. Liquid ethanol standards (also 6 µl, 0-200 mg%) and samples were similarly prepared and heated in a water bath at 55 °C for 10 min. Subsequently, a 1.5 ml sample of headspace gas was removed from the glass tubes with a plastic 3.0 ml syringe and injected directly into a SRI 8610C gas chromatograph (Torrance, CA) equipped with an external syringe adapter and 1.0 ml external loading loop. Samples were run at 140°C through a Hayesep D column and detected with FID at approximately 2 min post-inject. Hydrogen gas, carrier gas (also hydrogen), and internal air generator flow rates were 13.3, 25, and 250 ml/min, respectively. Areas under the curve for blood samples were analyzed with SRI PeakSimple software for Windows running on a Dell Inspiron 3500 ® laptop computer and converted to mg% in blood based on the curve generated for the standards.

Data Analyses

As a measure of the stimulant effects of ethanol on locomotor activity, the data for each ethanol test day data was expressed as change from average saline baseline (activity following ethanol injection – daily average of activity following initial saline injections). As a measure of sensitization to the effects of ethanol on locomotor activity, locomotor activity

on the first ethanol test day was subtracted from each of the subsequent ethanol test day data points (activity following the second and subsequent ethanol injection – activity following the first ethanol injection). Locomotor activity data (raw and converted) were analyzed using analyses of variance (ANOVA). All data are presented as mean $\pm$ S.E.M. and t-tests were used for planned comparisons (Winer et al. 1991). Significance was accepted at $P < 0.05$ (two-tailed).

Results

Ethanol-Induced Locomotor Sensitization: Test Chamber Injections

Locomotor activity data from mice maintained on the 129/SvEv x C57BL/6J are presented in Fig. 2.1. There were no differences between genotypes in locomotor activity following baseline saline injections or the saline injection given after the ethanol test days as determined by non-significant ANOVAs (Fig. 2.1A). Interestingly, the RII$\beta^{-/-}$ mice showed significantly greater locomotor activity over the 10 days of ethanol injections. A repeated measures ANOVA performed on ethanol-induced locomotor activity data revealed a significant main effect of genotype [$F(1, 51) = 7.18$, $p = .01$]. Post hoc tests indicated that the RII$\beta^{-/-}$ mice were significantly more active on ethanol injection days 1, 2, 4, 5, 6, 9, and 10 relative to RII$\beta^{+/+}$ mice. Similarly, when locomotor activity data were expressed as change from baseline activity (Fig. 2.1B), a repeated measures ANOVA run on ethanol test days showed a significant genotype main effect [$F(1, 51) = 6.88$, $p < .05$] and post hoc tests indicated greater locomotor activity by RII$\beta^{-/-}$ mice on days 2, 4, 6, 9, and 10. There were no genotype differences during the final saline injection. Surprisingly, when locomotor activity data were expressed as change relative to activity following the first ethanol injection - the assessment of locomotor sensitization (Fig. 2.1C), ANOVAs revealed no significant

genotype differences during ethanol test days or during the final saline injection. Taken together, these data suggest that while RIIβ$^{-/-}$ mice on the mixed 129/SvEv x C57BL/6J genetic background were more sensitive to the stimulant effects of ethanol relative to RIIβ$^{+/+}$ mice (Fig. 2.1A and B), there were no genotype differences in the development of behavioral sensitization (Fig. 2.1C). Fig. 2.2 shows blood ethanol levels following the 1st, 5th, and 10th days of ethanol testing. Repeated measures ANOVA revealed no significant genotype or day effects. Thus, differences in ethanol-induced locomotor activity between RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice are not associated with altered blood ethanol levels. Importantly, RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice on the mixed 129/SvEv x C57BL/6J genetic background did not differ in locomotor activity following 8 days (3 baseline days, averaged; 5 test days) of saline injection (Fig. 2.3).

Locomotor activity data from mice maintained on the C57BL/6J are presented in Fig. 2.4. ANOVA of average baseline locomotor activity following the initial saline injections revealed that relative to the RIIβ$^{-/-}$ mice, RIIβ$^{+/+}$ mice showed greater locomotor activity (Fig. 2.4A) [$F(1, 51) = 5.627$, $p < .05$]. However, there were no significant differences in locomotor activity following saline injections given between the 10th and 11th ethanol injection (as noted as S4 and S5 in Figure 2.4A). These injections were conducted to demonstrate the specificity of the locomotor response. Despite showing lower basal locomotor activity, RIIβ$^{-/-}$ mice on the C57BL/6J genetic background showed significantly greater ethanol-induced locomotor activity as revealed by a significant genotype main effect following a repeated measures ANOVA run on the first 10 days of ethanol testing [$F(1, 47) = 23.86$, $p < .001$]. Post hoc tests indicated that RIIβ$^{-/-}$ mice were significantly more active at each of the 10 days. Similarly, a repeated measures ANOVA run on ethanol tests days 11 and

12 showed a significant main effect of genotype [$F(1, 48) = 24.088$, $p < .001$] that reflected the increased activity of RII$\beta^{-/-}$ mice relative to wild-type animals. When expressed as change from baseline activity (Fig. 4B), repeated measures ANOVAs revealed genotype differences during the initial 10 ethanol injections [$F(1, 47) = 30.368$, $p < .001$] and during the two ethanol injections given at the end of the study [$F(1, 48) = 33.281$, $p < .001$]. Post hoc tests indicated that RII$\beta^{-/-}$ mice showed greater locomotor activity relative to RII$\beta^{+/+}$ mice at every ethanol test day. There were no genotype differences in change from baseline data following saline injections (S4 and S5). In general, the overall pattern of data in Fig. 2.4B indicates that RII$\beta^{-/-}$ mice are more sensitive to the stimulant effects of ethanol. However, it is important to note that following the first ethanol injection, both RII$\beta^{-/-}$ and RII$\beta^{+/+}$ mice showed reduced activity relative to baseline activity. Significant genotype differences at this point are consistent with previous observations indicating that RII$\beta^{-/-}$ mice are less sensitive to the sedative properties of ethanol (Fee et al. 2004; Thiele et al. 2000b).

C57BL/6J locomotor data expressed as change relative to the first ethanol injection are shown in Fig. 2.4C. In contrast to the mixed background mice, C57BL/6J RII$\beta^{-/-}$ mice exhibited significantly greater expression of ethanol-induced locomotor sensitization over the course of ethanol injections. This conclusion was supported by a repeated measure ANOVA that revealed a significant main effect of genotype [$F(1, 47) = 13.428$, $p = .001$]. Post hoc analyses showed significant genotype differences on ethanol test days 4-10, 11, and 12. Additionally, a repeated measures ANOVA run on the final two ethanol test days was significant ([$F(1, 49) = 11.674$, $p = .001$]), again indicating that RII$\beta^{-/-}$ mice showed greater expression of locomotor sensitization relative to RII$\beta^{+/+}$ mice. On the other hand, a genotype main effect following a repeated measures ANOVA revealed that RII$\beta^{+/+}$ mice showed

significantly greater locomotor activity relative to RIIβ$^{-/-}$ mice following saline injections (S4 and S5) [$F(1, 49) = 18.415$, $p < .001$]. Finally, there were no significant differences between RIIβ$^{-/-}$ (219.20 $\pm$ 6.59 mg%) or RIIβ$^{+/+}$ (217.27 $\pm$ 6.18 mg%) mice in blood ethanol levels immediately following the last ethanol test session on day 12. Thus, similar to mice on the mixed genetic background, RIIβ$^{-/-}$ mice in the pure C57BL/6J genetic background were more sensitive to the stimulant effect of ethanol relative to RIIβ$^{+/+}$ mice (Figs. 2.4A and B). Additionally, the greater increases in ethanol-induced locomotor activity by RIIβ$^{-/-}$ mice following repeated ethanol injections (Fig. 2.4C) indicated that RIIβ$^{-/-}$ mice on the C57BL/6J genetic background were also more sensitive to the development of locomotor sensitization.

Ethanol-Induced Locomotor Activation: Home Cage Injections

Locomotor activity data following home cage ethanol injections in mice maintained on a C57BL/6J genetic background are presented in Fig. 2.5. Similar to the previous experiment, RIIβ$^{-/-}$ mice on the C57BL/6J genetic background showed less average locomotor activity following baseline saline injections (Fig. 2.5A), as evidenced by a significant ANOVA [$F(1, 38) = 5.685$, $p < .05$]. Furthermore, an ANOVA run on locomotor data collected following the first ethanol injection (day 4) revealed that RIIβ$^{-/-}$ mice showed significantly greater activity relative to RIIβ$^{+/+}$ mice [$F(1, 38) = 9.311$, $p < .005$]. A two-way mixed factor ANOVA run on locomotor activity data from test day 15 showed significant main effects of drug [$F(1, 36) = 97.204$, $p < .001$] and genotype [$F(1, 36) = 7.681$, $p < .01$], and a significant interaction between these variables [$F(1, 36) = 5.245$, $p < .05$]. Post hoc tests indicated that while locomotor activity was enhanced by ethanol injection (relative to saline injection) in both genotypes, such increases were greater in RIIβ$^{-/-}$ mice.

Test day activity data expressed as change from average baseline are presented in Fig. 2.5B. A two-way mixed factor ANOVA revealed significant genotype [$F(1, 36) = 18.135$, $p < .001$] and drug treatment [$F(1, 36) = 106.113$, $p < .001$] main effects. Post hoc tests confirmed that RIIβ$^{-/-}$ mice show greater ethanol-induced increases, and less saline-induced decreases, in locomotor activity relative to RIIβ$^{+/+}$ mice. Test day activity data expressed as change from first ethanol injection are presented in Fig. 2.5C. A two-way mixed factor ANOVA revealed a significant treatment main effect [$F(1, 36) = 90.7$, $p < .001$], but the genotype main effect was not significant. Additionally, the interaction effect was significant [$F(1, 36) = 5.012$, $p < .05$]. Post hoc analyses revealed that this interaction could not be attributed to significant differences in locomotor activity in saline or ethanol treated RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice. Taken together, these data indicate that with the home cage injection paradigm, RIIβ$^{-/-}$ mice on the C57BL/6J genetic background are more sensitive to the locomotor stimulant effects of ethanol (Fig. 2.5B) but did not show enhanced sensitivity to behavioral sensitization (Fig. 2.5C) when compared with RIIβ$^{+/+}$ mice in this paradigm.

Discussion

In the present report, RIIβ$^{-/-}$ mice consistently showed significantly greater ethanol-induced locomotor activity relative to RIIβ$^{+/+}$ mice. RIIβ$^{-/-}$ mice also showed increased sensitivity to ethanol-induced locomotor sensitization, an effect that may be dependent on genetic background and/or testing paradigm. Importantly, increased locomotor activity by RIIβ$^{-/-}$ mice was specific to ethanol injections (and not seen following saline injections), and was not associated with altered blood ethanol levels. Normal blood ethanol levels and ethanol metabolism by RIIβ$^{-/-}$ mice has previously been documented (Fee et al. 2004; Thiele et al.

2000b). Since RIIβ$^{-/-}$ mice show blunted PKA activity in critical brain regions, such as the striatum and nucleus accumbens (Brandon et al. 1998; Thiele et al. 2000b), the present results suggest that normal PKA signaling is part of a mechanism that protects against ethanol-induced locomotor activity and behavioral sensitization.

A growing body of literature is emerging indicating that phenotypes, including neurobiological responses to ethanol, can depend on the genetic background of the knockout model (Bowers et al. 1999; Howe et al. 2002; Kelly et al. 1998; Palmer et al. 2003; Phillips et al. 1999; Simpson et al. 1997; Thiele et al. 2000a). It is possible that the protective role of the RIIβ subunit of PKA against ethanol-induced behavioral sensitization depends on epistatic interactions with other genes, interactions that may depend on the genetic background of the mouse. This argument is consistent with the observation in the present report that RIIβ$^{-/-}$ mice on the pure C57BL/6J, but not the mixed 129/SvEv x C57BL/6J, genetic background showed enhanced behavioral sensitization when repeated ethanol injections where given immediately before locomotor activity testing. Alternatively, the expression of enhanced behavioral sensitization by RIIβ$^{-/-}$ mice may have depended on procedural differences between experiments, rather than mouse genetic background. In fact, different sensitizing doses of ethanol were used in each of the three experiments reported here, ranging from a 1.4 to a 2.5 g/kg dose. In addition, C57BL/6J RIIβ$^{-/-}$ mice were given repeated ethanol injections either just before locomotor activity testing or in their homecage environment. Recent work has documented that the expression of phenotypes in mouse research can be sensitive to subtle differences in the testing procedures as well as the testing environment (Boehm et al. 2000; Crabbe et al. 1999; Rustay et al. 2003). Taken together, while the role of the RIIβ subunit of PKA in ethanol-induced behavioral sensitization may depend on mouse genetic background

(and thus epistatic interactions between genes), it is also possible that different outcomes between experiments resulted from procedural differences.

With the use of RIIβ$^{-/-}$ mice, the present results are the first direct demonstration that normal PKA signaling is protective against the stimulant effects of ethanol and ethanol-induced behavioral sensitization. This being said, caution is necessary when drawing conclusions because it is becoming increasing clear that interpretations of phenotypic data from studies with knockout mice are subject to several caveats (Gerlai 2001). One concern is that constitutive deletion of a gene could lead to compensatory processes (up or down regulation of other genes) during development. In fact, the relative distribution of other regulatory subunits up-regulate in an apparent attempt to compensate for the loss of RIIβ in the present model (Amieux et al. 1997; Brandon et al. 1998). However, it stands to reason that this compensation is not complete as evidenced by reduced cAMP-stimulated PKA activity in brain regions such as the striatum, nucleus accumbens, amygdala, hippocampus and hypothalamus (Brandon et al. 1998). A second concern pertaining to knockout models, as noted above, is the possibility that there are epistatic interactions between genes such that observed phenotypes may be dependent on the genetic background of the mouse. One way to address this concern is to test the knockout model on at least two genetic backgrounds as we have done in this report. While increased sensitivity to ethanol-induced behavioral sensitization may depend on the genetic background of RIIβ$^{-/-}$ mice, increased sensitivity to ethanol-induced locomotor activity was observed in RIIβ$^{-/-}$ mice on both genetic backgrounds tested. A third concern is that differences between knockout and wild-type mice may be related to genes other than the mutated gene, a problem that is exacerbated when mice are maintained on a hybrid genetic background (i.e., 129/SvEv x C57BL6J). One solution that

has been proposed to address this issue is to test mice that have been backcrossed to one genetic background. Here, we studied RIIβ$^{-/-}$ mice that were backcrossed to a C57BL/6J background over 8 generations.

We have previously shown that deletion of the RIIβ subunit results in a mouse that will more readily consume ethanol solutions compared to wildtype littermate controls in the absence of altered taste preference, caloric intake, or ethanol metabolism (Fee et al. 2004; Thiele et al. 2000b). Recently, we found that increased ethanol consumption by RIIβ$^{-/-}$ mice is not reliably predicted by basal levels of anxiety (Fee et al., 2004) despite the observation that blunted PKA activity and phosphorylated CREB (pCREB) in the amygdala are associated with increased anxiety-like behavior (Pandey et al. 2003). In addition to drinking more ethanol and showing increased sensitivity to the stimulant effects of ethanol, the RIIβ$^{-/-}$ mice consistently show reduced sensitivity to the sedative properties of ethanol (Fee et al. 2004; Thiele et al. 2000b) and in the present report RIIβ$^{-/-}$ mice were resistant to the sedative effects of an initial ethanol injection on locomotor activity that was evident in RIIβ$^{+/+}$ mice (Figs. 2.1A, 2.4A and B, and 2.5A). This latter observation raises the possibility that increased sensitivity to ethanol-induced locomotor stimulation and sensitization by RIIβ$^{-/-}$ mice are perhaps secondary to reduced sensitivity to the sedative effects of ethanol. While this issue cannot be completely ruled out, Phillips and colleagues have consistently demonstrated a dissociation between tolerance to the sedative/ataxic effects of ethanol and ethanol-induced behavioral sensitization (Meyer and Phillips 2003; Phillips et al. 1996). Furthermore, RIIβ$^{-/-}$ mice show enhanced behavioral sensitization to amphetamine (Brandon et al. 1998), a drug that does not induce sedation.

It has been suggested that increased sensitivity to the stimulant effects of drugs and behavioral sensitization that develops following repeated drug exposure may be an underlying common mechanism that increases the risk for developing drug dependence (Robinson and Berridge 1993; 2000; 2001). Consistent with this relationship, ethanol-preferring C57BL/6J mice showed increased ethanol preference after the acquisition of behavioral sensitization (Lessov et al. 2001) and a study with selectively bred high alcohol preferring (HAP) and low alcohol preferring (LAP) mice found that high ethanol preference was predictive of the acquisition of ethanol-induced locomotor sensitization (Grahame et al. 2000). Other manipulations of protein expression in mice have suggested a connection between ethanol preference and the development of locomotor sensitization following repeated ethanol injections (Szumlinski et al. 2005a). Here we extend these findings by showing a positive correlation between ethanol preference and ethanol-induced behavioral sensitization in the RII$\beta^{-/-}$ mouse model. At odds with this theoretical perspective is the observation that the DBA/2J strain of mice readily acquires ethanol-induced behavioral sensitization but strongly avoids consuming ethanol (Lessov et al. 2001; Phillips et al. 1994). However, a recent study by Camarini and Hodge found that repeated ethanol injections in DBA/2J mice significantly increased ethanol intake to levels similar to that observed in C57BL/6 mice (Camarini and Hodge 2004).

While the present results are the first direct demonstration that PKA signaling modulates the stimulant effects of ethanol and ethanol-induced behavioral sensitization, previous pharmacological and genetic studies have established that PKA signaling is involved with amphetamine- (Crawford et al. 2004; Crawford et al. 2000; Tolliver et al. 1999) and cocaine- (Miserendino and Nestler 1995; Park et al. 2000; Schroeder et al. 2004)

induced locomotor sensitization. Of direct relevance to the present report is the observation that RIIβ$^{-/-}$ mice are more susceptible to the acquisition of locomotor sensitization following repeated amphetamine exposure (Brandon et al. 1998). Interestingly, repeated amphetamine exposure reduces PKA activity in the nucleus accumbens and striatum (Crawford et al. 2004; Crawford et al. 2000). Thus, we speculate that reduced PKA signaling in these regions causes RIIβ$^{-/-}$ mice to be more sensitive to the stimulant effects of ethanol as well as ethanol- and amphetamine-induced behavioral sensitization. Because dynorphin, a neuropeptide that is reduced in the dorsal medial striatum of RIIβ$^{-/-}$ mice (Brandon et al. 1998), plays an inhibitory role in sensitization (Heidbreder et al. 1995), it can be speculated that increased ethanol- and amphetamine-induced behavioral sensitization in RIIβ$^{-/-}$ mice may be the result of low striatal dynorphin activity. However, we suggest that a degree of caution is necessary in that it is still unclear to what extent, if any, the neuronal mechanisms involved in ethanol- and amphetamine-induced locomotor sensitization overlap.

Collectively, these data provide the first direct evidence that normal PKA signaling, and specifically the RIIβ subunit of PKA, plays a protective role against the stimulant effects of ethanol and ethanol-induced behavioral sensitization. Future studies are required to define the specific brain regions in which PKA signaling influences behavioral sensitization and whether PKA signaling influences this phenomenon by affecting pre- and/or post-synaptic neuronal function.

Table 2.1 - Dosing schedule for ethanol-induced locomotor activity: test chamber injections.

129/SvEv x C57BL/6J Test Day 1-3	Test Day 4-13	Test Day 14	
Equivolume I.p. Saline	1.4 g/kg I.p. Ethanol	Equivolume I.p. Saline	
C57BL/6J Test Day 1-3	Test Day 4-13	Test Day 14-15	Test Day 16-17
Equivolume I.p. Saline	2.0 g/kg I.p. Ethanol	Equivolume I.p. Saline	2.0 g/kg I.p. Ethanol

Table 2.2 - Dosing schedule for ethanol-induced locomotor activity: home cage injections.

Treatment	Day 1-3	Day 4	Day 5-14	Day 15
Group	Habituation	Initial Ethanol	Conditioning	Test Day
EtOH	Saline - LC	2.0 EtOH - LC	2.5 EtOH - HC	2.0 EtOH - LC
Saline	Saline - LC	2.0 EtOH - LC	2.5 EtOH - HC	Saline - LC

LC = Locomotor Chamber, HC = Home Cage

Figure 2.1 Acquisition test for ethanol-induced locomotor activation and sensitization in129/SvEv x C57BL/6J RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice. (A) represents raw data for locomotor activity during a 20-minute activity session. Average baseline constitutes the numerical average for activity during the three habituation sessions. (B) represents locomotor activity corrected for basal activity (Ethanol trial activity 1-10 minus average baseline activity). (C) represents the acquisition of locomotor sensitization by presenting each ethanol exposure corrected for baseline ethanol exposure (Ethanol trial 2-10 minus Ethanol trial 1 activity). All values reported are mean ± SEM. There were significant genotype differences in ethanol-induced locomotor activity but not in ethanol-induced sensitization.

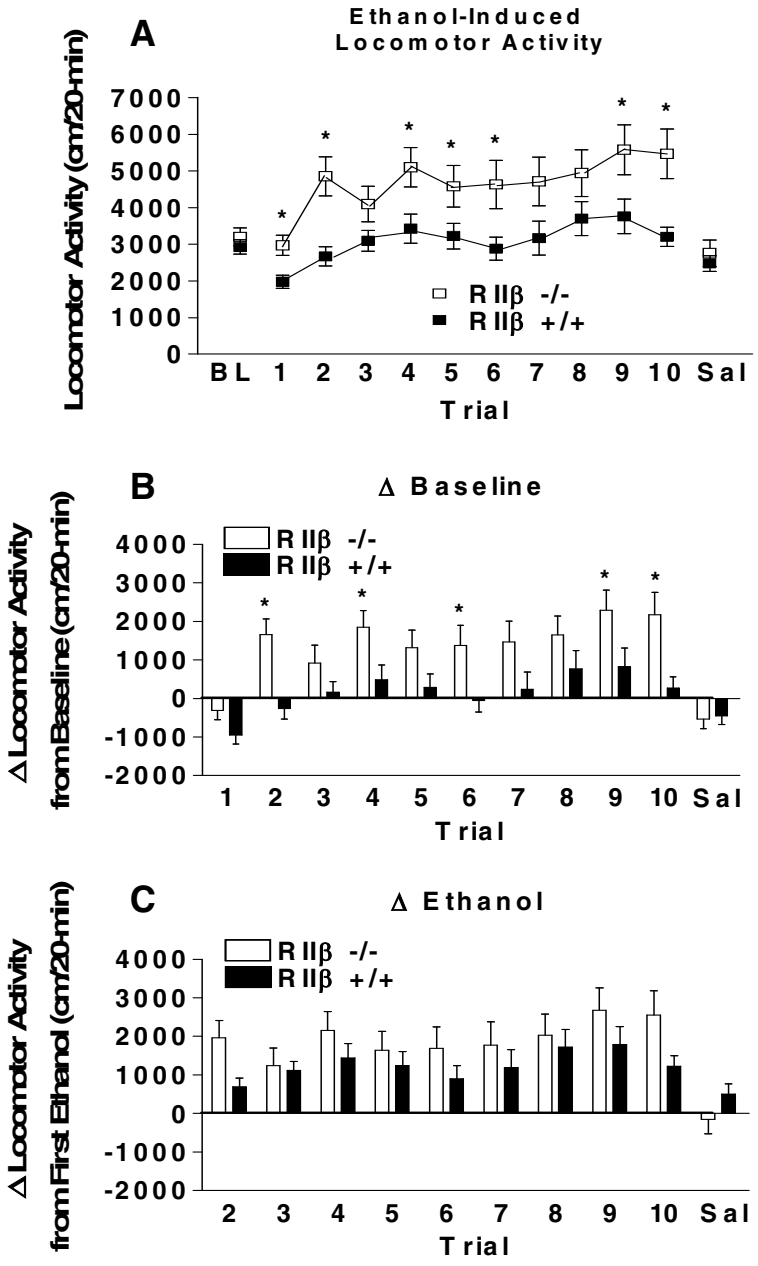


Figure 2.2 Blood ethanol concentrations (mg%) in 129/SvEv x C57BL/6J RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice. Immediately following ethanol test sessions 1, 5, and 10, 129/SvEv x C57BL/6J RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice had tail blood collected for analysis using gas chromatography to assess the possibility of alterations in ethanol pharmacokinetics over the course of the study. All values reported are mean ± SEM. There were no significant genotype differences.

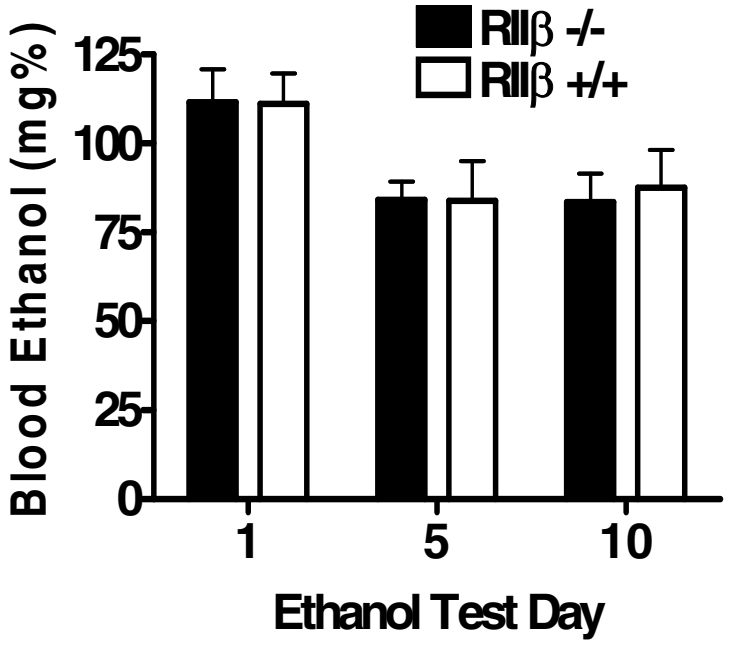
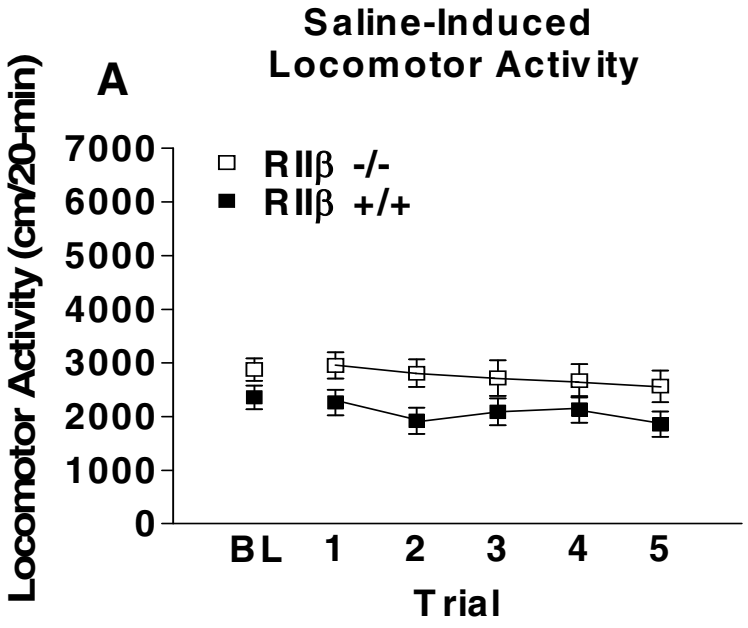


Figure 2.3 Locomotor activation and sensitization in129/SvEv x C57BL/6J RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice following repeated saline injections. (A) represents raw data for locomotor activity during a 20-minute activity session following saline injection equivalent in volume to a 1.4 g/kg ethanol injection. Average baseline constitutes the average activity for all three habituation sessions. (B) represents locomotor activity corrected for basal activity (Saline trial activity 1-5 minus average baseline activity). All values reported are mean ± SEM. There were no significant genotype differences in saline-induced locomotor activity.



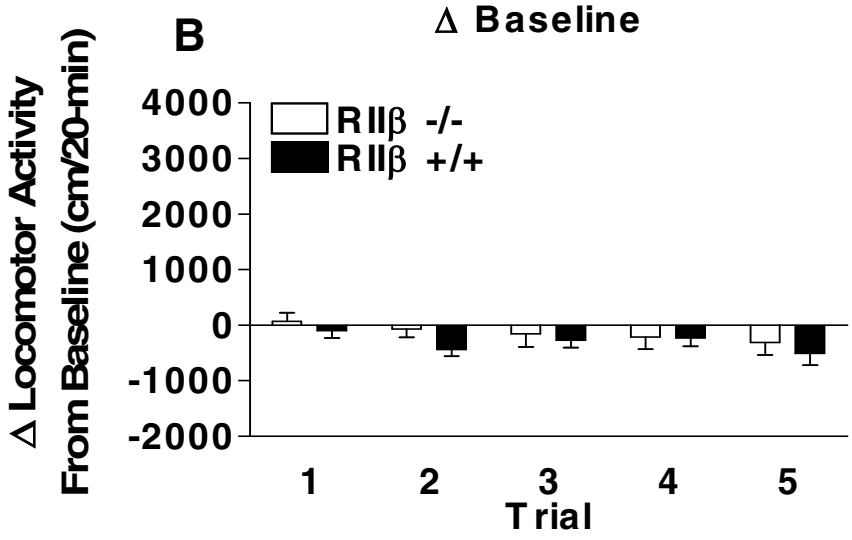


Figure 2.4 Acquisition test for ethanol-induced locomotor activation and sensitization in RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice maintained on a C57BL/6J background. (A) represents raw data for locomotor activity during a 20-minute activity session. Average baseline constitutes the numerical average for activity during the three habituation sessions. (B) represents locomotor activity corrected for basal activity (Ethanol trial activity 1-10,ethanol trial 11+12, and saline trial 4+5 minus average baseline activity). (C) represents the acquisition of locomotor sensitization by presenting each ethanol exposure corrected for baseline ethanol exposure (Ethanol trial activity 2-10,ethanol trial 11+12, and saline trial 4+5 minus Ethanol trial 1 activity). All values reported are mean ± SEM. There were significant genotype differences in ethanol-induced locomotor activity and ethanol-induced locomotor sensitization.

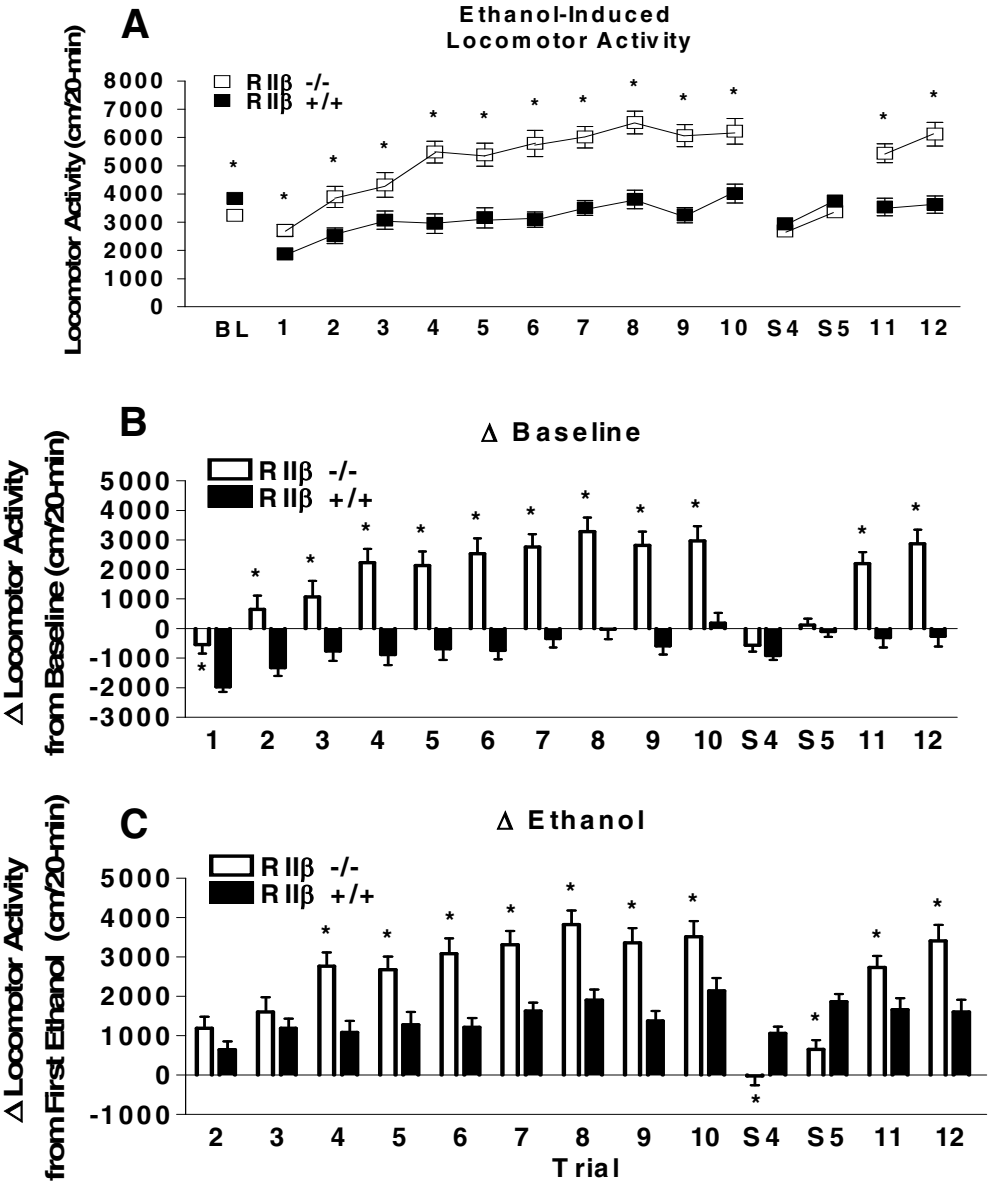
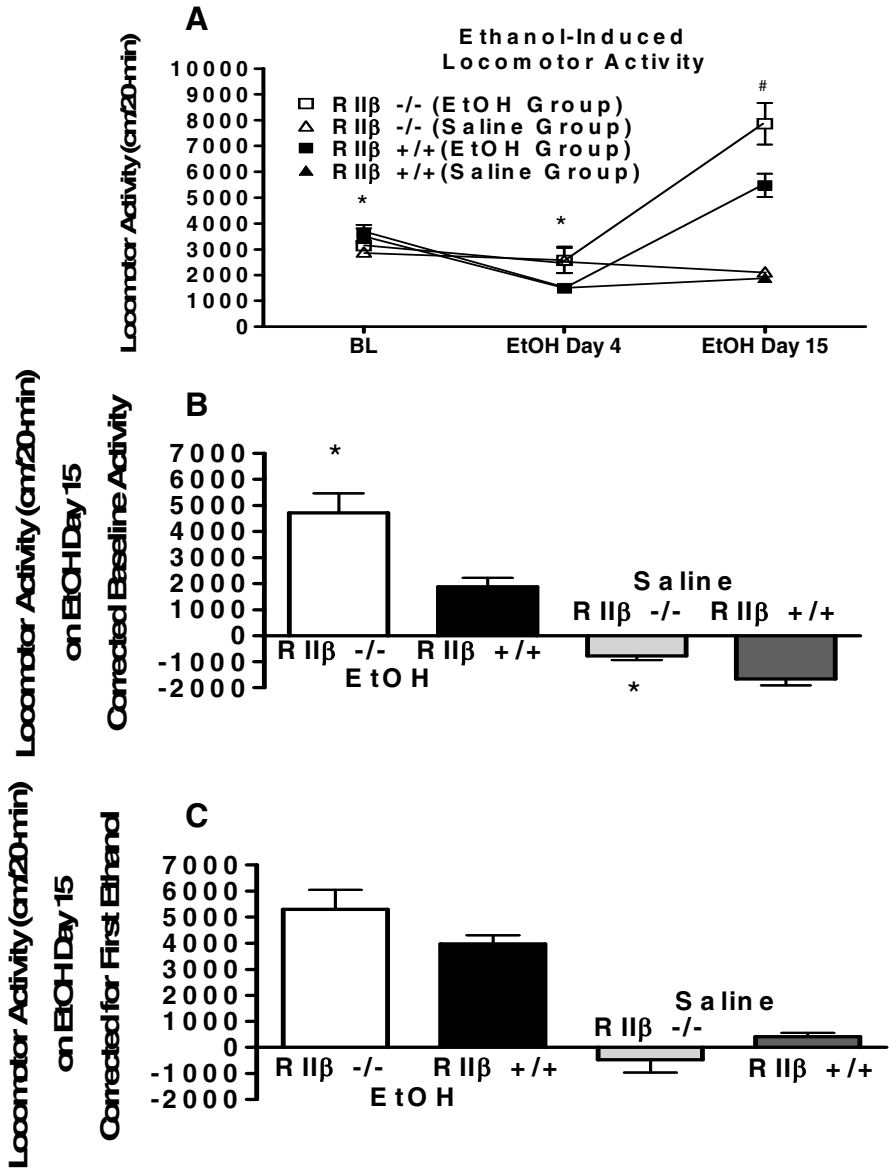


Figure 2.5 Expression test for ethanol-induced locomotor activation and sensitization in RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice maintained on a C57BL/6J background. (A) represents raw data for locomotor activity during a 20-minute activity session following first ethanol exposure (Ethanol Day 4) in locomotor chamber and final ethanol exposure (Ethanol Day 15). On days 5-14 ethanol was administered in the home cage and mice were not exposed to the locomotor chamber. Average baseline constitutes the numerical average for activity during the three habituation sessions. (B) represents locomotor activity on Day 15 corrected for basal activity (Ethanol Day 15 activity minus average baseline activity). (C) represents the acquisition of locomotor sensitization by presenting final ethanol exposure corrected for baseline ethanol activity (Ethanol Day 15 activity minus Ethanol Day 4 activity). All values reported are mean ± SEM. There were significant genotype differences in ethanol-induced locomotor activity but not ethanol-induced locomotor sensitization.



CHAPTER 3

EXPERIMENT 2: RESTRAINT STRESS-INDUCED CROSS-SENSITIZED RESPONSE TO ETHANOL: POSSIBLE CONTRIBUTION OF PROTEIN KINASE A SIGNALING AND CRF1R RECEPTOR ACTIVITY

Introduction

Behavioral sensitization is defined as the long-lasting and progressive enhancement of the locomotor and motivational responses to a drug following repeated administration (Kalivas and Stewart 1991). It is important to note that behavioral sensitization is a phenomenon that is not confined to any one particular class of drugs. The idea that sensitized responses to different drugs might involve similar mechanisms has been tested over the past decades creating more questions than answers. If overlapping mechanisms are indeed involved, then sensitization to one drug should confer enhanced sensitivity, or cross-sensitivity, to other drugs of abuse. Repeated treatment with morphine or cocaine induces a hypersensitive locomotor response to ethanol in mice (Lessov and Phillips 2003). It is of interest to note that mice repeatedly injected with ethanol did not show a similarly cross-sensitized response to morphine or cocaine (Lessov and Phillips 2003). A cross-sensitized response to cocaine is possible in ethanol-treated mice if exposure to testing chamber is kept to a minimum (Itzhak and Martin 1999). Repeated treatment with nicotine has also been shown to produce a cross-sensitized response to ethanol; an effect mediated by calcium

channel antagonists (Biala and Weglinska 2004). Genetic approaches have also proven useful in understanding mechanisms involved in cross-sensitization. Rats selectively bred for their ethanol preference, Alko Alcohol (AA) rats, exhibit behavioral sensitization following repeated morphine treatment; an effect that was absent in alcohol avoiding Alko Non-Alcohol (ANA) rats (Ojanen et al. 2007). Ojanen and colleagues (2007) also found that this heighten effect of behavioral sensitization in AA rats was accompanied by increased glutamate levels in the ventral tegmental area (VTA) but not in ANA rats. These studies suggest that a variety of drugs might share parallel or overlapping mechanisms with respect to the expression of locomotor sensitization. Clearly drug history as well as genetic factors both can play a role in this phenotype.

The administration of a varied number of stress models, including restraint stress (Roberts et al. 1995), social defeat (Yap et al. 2005), maternal separation (Kikusui et al. 2005), social isolation (Frances et al. 2000), and variable stress (Lepsch et al. 2005) have been shown to cause the acquisition of an enhanced sensitivity to the locomotor stimulatory properties of a variety of drugs of abuse. This stress-induced cross-sensitization is a phenomenon of particular interest given the hypothalamic-pituitary-adrenal (HPA) axis activation which accompanies the administration of abused drugs. Just as exposure to stress leads to a hypersensitive locomotor reaction to drug, cross-sensitization is also possible in the opposite direction; in other words, repeated exposure to drug will create a sensitized response to stress (Breese et al. 2005; Erb et al. 2003; Levy et al. 1994). These studies suggest the possibility that exposure to repeated stresses (or drug) leads to dysregulation of HPA axis signaling, which it turn, mediates the behavioral sensitization that accompanies subsequent drug exposure. In the human literature, individuals suffering from affective disorders can

exhibit a sensitized stress response mediated by activation of the HPA axis (Arborelius et al. 1999). It stands to reason that dysregulation of HPA axis signaling similar to that seen in chronic affective disorders might underlie the hypersensitivity to drug observed in behavioral sensitization. Recent rodent studies confirm that a depressant-like behavioral profile is associated with enhanced sensitivity to the development of sensitization (Alttoa et al. 2007; Wei et al. 2004).

Taking these findings one step further, investigators have attempted to use pharmacological and physiological interventions to gain a better understanding of the mechanisms that underlie stress and drug-induced cross-sensitization. There have been many promising findings in the literature but a clear and consistent pattern of results has yet to emerge. What follows is brief summary of the available literature. In examining a role for stress signaling in sensitization, the physiological endpoint, cortisol in humans and corticosterone in rodents, is often targeted. Adrenalectomy eliminates the synthesis and release of corticosterone, and has been shown to block the acquisition of cocaine sensitization if performed prior to the sensitization regime (Przegalinski et al. 2000). In contrast, if the sensitization paradigm is initiated prior to adrenalectomy, the expression of cocaine sensitization remains intact (Przegalinski et al. 2000). Prasad and colleagues also find a similar inability of adrenalectomy to alter the expression of cocaine-induced locomotor sensitization (Prasad et al. 1996). However, it is reported that adrenalectomy blocks the acquisition of sensitization when tested in acute withdrawal (24 hrs) but not in late withdrawal at 1 week (Prasad et al. 1996). Taken together these studies suggest the importance of glucocorticoid signaling in the acquisition of sensitized behaviors.

Similarly, administration of the glucocorticoid receptor antagonist, RU38486, prior to repeated restraint stress has been shown to block the acquisition of a cross-sensitized locomotor response to ethanol (Roberts et al. 1995). These findings point to the level of glucocorticoid signaling or receptor binding and activation as being critical to the development of behavioral sensitization. This is evidenced by the fact that elimination of corticosterone release with adrenalectomy or prevention of glucocorticoid receptor binding through pharmacological blockade prevent to the acquisition of a sensitized or cross-sensitized response. It is important to point out that additional studies have also suggested a role for glucocorticoid receptor signaling in the expression of a sensitized response. Pretreatment with the glucocorticoid receptor antagonist, mifepristone, blocks the expression of a sensitized locomotor response to amphetamine, while the drug had no effect on amphetamine responses in drug naïve rats (De Vries et al. 1996). Roberts et al. (1995) also found that RU38486 could block the acquisition of ethanol-induced locomotor sensitization in mice. Aside from the dissociation between adrenalectomy and receptor blockade, the aforementioned studies point to the importance of glucocorticoid signaling in both the acquisition and expression of behavioral sensitization.

Alterations in the activation of the HPA axis upstream of glucocorticoid have also been implicated in behavioral sensitization. Intracerebroventricular (i.c.v.) administration of the CRF antagonist, α-helical CRF, reduced stereotypic response to amphetamine following repeated restraint stress in rats (Cole et al. 1990b). In contrast, an augmentation of HPA axis signaling via pharmacological manipulation can result in the expression of sensitized behaviors. I.c.v. administration of CRF rather than subcutaneous administration of CRF leads to long-term locomotor sensitization to D-amphetamine (Cador et al. 1993). Similarly,

microinfusion of CRF into the shell of the nucleus accumbens of rats led to a prolonged increase in general locomotor activity (Holahan et al. 1997). It also appears that a blockade of endogenous CRF signaling via administration of CRF receptor 1 antagonist, CP-154,526, will reduce cocaine-induced locomotor sensitization and relapse behavior (Przegalinski et al. 2005). These approaches have yielded fruitful results, and further investigation is necessary to more fully understand the role of CRF signaling and its effects on HPA axis activity in the acquisition and expression of cross-sensitized drug responses.

The RIIβ knockout mouse model has been used to assess the role of protein kinase A (PKA) signaling on ethanol self-administration and a variety of other ethanol-related phenotypes (Fee et al. 2006; Fee et al. 2004; Thiele et al. 2000b). This is one of several genetic models to effectively demonstrate an enhanced sensitivity to drug-induced locomotor sensitization (Brandon et al. 1998; Fee et al. 2006; McDougall et al. 2005; Runkorg et al. 2006; Wei et al. 2004). The $RII\beta^{-/-}$ mice represent a unique opportunity to identify a role for PKA signaling in the acquisition of a stress-induced cross-sensitized response to ethanol. There is considerable evidence in the literature suggesting a role for PKA activity in the regulation of HPA axis activity. For example, Vargas and colleagues have suggested that the effects of diazepam on HPA axis activity are mediated via cAMP-dependant PKA activity in the hypothalamus (Vargas et al. 2001). PKA activity has also been put forward as a potential mechanism underlying the diminished hippocampal activity thought to be associated with the HPA axis hyperactivity in depression (Perera et al. 2001). Specifically, ethanol has been shown to increase PKA and cAMP activity and, in turn, increase CRF peptide secretion (Li et al. 2005). Additionally, PKA activity has been shown to play an essential role in CRF mediated decreased expression of CRF receptor 1 mRNA in part via the phosphorylation of

cAMP response element binding (CREB) (Kasagi et al. 2002). However, other investigators have suggested a role for protein kinase C (PKC) and PKA in ACTH release (Iwabuchi et al. 1999), and a role for G protein receptor kinase (GRK) in the activity of CRF type 1 receptors (Dautzenberg et al. 2001).

The following investigation sets out to use inbred strains of mice to reinforce the existing literature which suggests that repeated restraint stress can be used to induce a cross-sensitized locomotor response to ethanol. This study will also attempt to determine if enhanced sensitivity to stress is a factor underlying the previously reported heightened sensitivity to ethanol-induced locomotor sensitization observed in the RIIβ$^{-/-}$ mouse model (Fee et al. 2006). In a final experiment, the CRF receptor 1 antagonist, CP-154,526, will be used in an attempt to block the acquisition of restraint stress-induced cross-sensitization to ethanol. Taken together, the studies highlighted above will not only strengthen the literature suggesting the importance of stress mechanisms in behavioral sensitization, but the RIIβ study will also investigate a possible interaction between PKA signaling and stress in an animal model with a demonstrated sensitivity to behavior sensitization.

Methods

Preparation of CP-154,526

CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) was generously donated by Pfizer (Groton, CT). CP-154,526 was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF-1 receptor (K_i < 10 nM) and blocks CRF

stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al. 1996; Schulz et al. 1996).

Restraint Stress Application

Restraint is a common method for inducing stress in rodents. The procedure for the administration of restraint stress is very similar to the procedure used by Roberts et al. (1995). Mice were individually removed from their home cages and placed into plastic cylindrical restraint tubes. These tubes securely hold the mouse in place and prevent turning around within the tube. All mice were restrained for a period of 2 hours per each daily session. New tubes were used each session. Mice undergoing restraint stress were visually isolated from other mice. Additionally, mice were observed throughout the restraint session as a safety measure.

Restraint-Stress Cross-Sensitization in Inbred Mice

40 inbred DBA/2J mice and 40 inbred C57BL/6 mice approximately 3 to 6 months of age (Jackson Laboratories, Bar Harbor, ME) were put through the cross-sensitization paradigm described below. All animals are transported to the testing room in their home cages and allowed to habituate for at least 35-min prior to testing. A small fan provided masking noise in the testing room. On test day 1-3, all mice received isotonic saline (0.9% NaCl) injections based on the equivalent volume for a 1.5 g/kg ethanol injection (20% w/v solution) prior to being placed into the center of an open-field arena which automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA) for each of the habituation/basal locomotor activity sessions. The open field arena measures 40.64 cm

by 40.64 cm by 30.48 cm and was made of clear Plexiglas. Several cm of corncob bedding are placed into the open field chamber to aid in cleaning and to prevent the buildup of odor.

On day 4, all mice will receive a 1.5 g/kg ethanol injection prior to placement in the locomotor chamber in order to determine basal ethanol responsiveness. Mice were then assigned to treatment groups equated for locomotor activity during the initial ethanol response (Day 4). Mice underwent 10 consecutive days of 2.5 g/kg i.p. ethanol injection or 2 hr of restraint stress in their home cage. On day 15, half of the mice received a 1.5 g/kg i.p. ethanol injection to test for the effect of the respective stressor on ethanol-induced locomotor sensitization while the remaining mice received an equivolume i.p. saline injection to test for general activity not attributable to ethanol.

Restraint-Stress Cross-Sensitization in RIIβ$^{-/-}$ and RIIβ$^{+/+}$ Mice

40 RIIβ$^{-/-}$ and RIIβ$^{+/+}$ male and female mice approximately 6 months of age maintained on an approximately pure C57BL/6 background were put through the cross-sensitization paradigm. All animals are transported to the testing room in their home cages and allowed to habituate for at least 35-min prior to testing. A small fan provided masking noise in the testing room. On test day 1-3, all mice received isotonic saline (0.9% NaCl) injections based on the equivalent volume for a 1.5 g/kg ethanol injection (20% w/v solution) prior to being placed into the center of the open-field arena which automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA) for each of the habituation/basal locomotor activity sessions. On day 4, all mice received a 1.5 g/kg ethanol injection prior to placement in the locomotor chamber in order to determine basal ethanol responsiveness. Mice were then assigned to treatment groups equated for locomotor activity

during the initial ethanol response (Day 4). Next, half the mice underwent 10 consecutive days of 2 hr of restraint stress while the remaining mice were weighed daily (no stress) and returned to their home cages. On day 15, half of the mice received a 1.5 g/kg i.p. ethanol injection to test for the effect of restraint stress on ethanol-induced locomotor sensitization while the remaining mice received an equivolume i.p. saline injection to test for general activity not attributable to ethanol. On day 16, mice receiving ethanol on day 15 received an i.p. saline injection and vice versa.

Effect of CP-154,526 on the Acquisition of Restraint Stress-Induced Cross Sensitization

60 inbred DBA/2J mice approximately 3 months of age (Jackson Laboratories, Bar Harbor, ME) were put through the sensitization paradigm described below. Mice were each randomly assigned to one of the various treatment groups (n=10 per group: vehicle + Restraint; vehicle + No Restraint; 5 mg/kg CP-154,526 + Restraint; 5 mg/kg CP-154,526 + No Restraint; 10 mg/kg CP-154,526 + Restraint; or 10 mg/kg CP-154,526 + No Restraint). The doses were selected based on the slow elimination of CP-154,526, $t_{1/2}$ = 51h, as observed in a study of its basic pharmacokinetics (Keller et al. 2002) and attempted to minimize the possibility of excessive drug accumulation leading to aversive side effects (Arborelius et al. 2000a). Mice receiving restraint stress were tested for CP-154,526's ability to block the acquisition of restraint stress-induced cross sensitization while non-specific reductions in locomotor activity associated with chronic administration of CP-154,526 were assessed in non-restrained mice. On test day 1-10, mice received a pretreatment of CP-154,526 or vehicle based on treatment group assignment. Thirty minutes later, mice then received either 2 hr restraint stress session or no additional treatment. Restrained mice were then returned to

their homecages and left undisturbed aside from routine animal husbandry. On test day (day 11), all animals were transported to the testing room in their home cages and allowed to habituate for at least 35-min prior to testing. A small fan provided masking noise in the testing room. Mice were then injected with a 1.5 g/kg i.p. ethanol injection prior to being placed into the center of the open-field arena for the 20 minute locomotor activity sessions.

Data Analyses

Locomotor activity data were analyzed using analyses of variance (ANOVA). All data are presented as mean $\pm$ S.E.M. and t-tests were used for planned comparisons when applicable (Winer et al. 1991). Significance was accepted at $P < 0.05$ (two-tailed). For all experiments presented in this study, locomotor data was collect and analyzed for all 20 minute sessions in 5 minute bins. Further analysis reveled that the effect of CP-154,526 on locomotor sensitization, when present, occurred in the first 5 minute bin of locomotor activity. For this reason, only the data from this first bin will be presented in studies using CP-154,526.

Results

Restraint-Stress Cross-Sensitization in Inbred Mice

DBA/2J inbred mice show a robust sensitized response to ethanol following either 10 days of home cage 2.5 g/kg ethanol injections or 2 hrs of restraint stress (Figure 3.1). A two-way (treatment (restraint or no stress) X test day drug (ethanol or saline)) ANOVA revealed a significant main effect of test day drug [$F(1, 36) = 43.223$, $p < .001$] and non-significant main effect of treatment as well as a non-significant interaction between the two variables of interest. These data suggest that both DBA/2J mice receiving chronic restraint stress and

ethanol treatment both show a sensitized response to ethanol but not saline. Similar results were also observed in C57BL/6J inbred mice (Figure 3.2). A two-way (treatment (restraint or no stress) X test day drug (ethanol or saline)) ANOVA revealed a significant main effect of test day drug [$F(1, 36) = 27.568$, $p < .001$] and non-significant main effect of treatment. It is of interest to note there existed a significant treatment by test day drug interaction in C57/BL/6J mice, [$F(1, 36) = 6.369$, $p < .05$]. This effect appears to be attributable to the relative differences in the locomotor effects between saline and ethanol-treated mice in the restraint treatment groups.

Restraint-Stress Cross-Sensitization in RIIβ$^{-/-}$ and RIIβ$^{+/+}$ Mice

RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice exposed to chronic restraint stress show an enhanced locomotor response following ethanol injection when compared to RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice which received daily handling without restraint (Figure 3.3) for the first 5 min following ethanol injection. A two-way (genotype X treatment) ANOVA revealed a main effect of genotype [$F(1, 36) = 5.431$, $p < .05$] and treatment [$F(1, 36) = 5.418$, $p < .05$] but the genotype by treatment interaction failed to achieve significance. These data suggest that in general RIIβ$^{-/-}$ mice were more active following ethanol injection compared to their littermate wildtype controls. Additionally, mice of both genotypes receiving restraint stress displayed an enhanced response to ethanol compared to daily handled mice but this effect was not genotype specific.

CP-154,526 and Acquisition of Restraint Stress-Induced Cross Sensitization

Treatment with CP-154,526 prior to exposure to restraint stress failed to attenuate the increased locomotor activation that existed in restraint stress treated mice in comparison to non-restrained mice. A two way (treatment (restraint or no restraint) X dose (Vehicle, 5 and 10mg/kg)) ANOVA revealed a significant main effect of treatment [$F(1, 54) = 7.929$, $p < .01$] but not dose. The treatment by dose interaction also failed to achieve significance suggesting that neither dose of CP-154,526 altered the heightened locomotor response in restraint stress-treated mice (Figure 3.4).

Discussion

In the present report, the RIIβ knockout mouse model was utilized to determine if enhanced sensitivity to stress was a factor contributing to the expression of heightened sensitivity to ethanol-induced locomotor activation in these mice. A commonly employed restraint stress cross-sensitization paradigm was employed which has been previously demonstrated to produce a cross-sensitized locomotor response to ethanol in both our hands and other laboratories. $RII\beta^{-/-}$ mice failed to show a heightened sensitivity to the effects of restraint stress on the cross-sensitized locomotor response to ethanol. Chronic restraint stress induced a sensitized response to ethanol compared to non-stressed controls in both $RII\beta^{-/-}$ and $RII\beta^{+/+}$ mice with no genotype differences in the magnitude of this response. The ability of chronic exposure to restraint stress to induce a cross-sensitized response to ethanol was demonstrated in two commercially available inbred strains of mice, C57BL/6J and DBA/2J, further reinforcing the literature. In an attempt to discern a mechanism for the contribution of stress exposure to a cross-sensitized response to ethanol, pretreatment with CRF Receptor 1 antagonist, CP-154,526, prior to restraint stress sessions in male DBA/2J mice was employed

in an attempt to block the acquisition of cross-sensitization. Ultimately this approach was ineffective in blocking the acquisition of restraint stress-induced cross-sensitization; an effect that was unexpected given the efficacy of the glucocorticoid receptor antagonist, RU38486, in this aim (Roberts et al. 1995).

These data add to a rather small existing literature suggesting that repeated exposure to stress, specifically restraint stress, leads to a hypersensitive locomotor response to ethanol (Roberts et al. 1995). In a more general sense, these findings join a variety of empirical results suggesting that restraint stress exposure, both acute and chronic, lead to a cross-sensitization to a variety of drugs of abuse including cocaine (Lepsch et al. 2005), amphetamine (Badiani et al. 1992; Diaz-Otanez et al. 1997; Pacchioni et al. 2002), and morphine (del Rosario et al. 2002). These findings suggest the possibility that exposure to repeated restraint stress brings about alterations which ultimately confer a vulnerability to the abuse potential for a variety of drugs of abuse. However, a study by Haile and colleagues suggests that the predictability of the stressor is of ultimate importance in assessing its ability to produce a cross-sensitized response. Ten days of chronic unpredictable stress but not chronic predictable stress (restraint) led to a hypersensitive locomotor response to cocaine; an effect marked by enhanced corticosterone levels (Haile et al. 2001). The discrepancy between the aforementioned study and those previously cited suggest the possibility that any number of subtle environmental factors can ultimately contribute to a cross-sensitized response when restraint stress is the sensitizing agent.

Administration of a glucocorticoid antagonist, RU38486, prior to 10 daily administrations of 2-hr restraint stress has been shown to effectively block the acquisition of a cross-sensitized response to a 1.5 g/kg i.p. ethanol injection (Roberts et al. 1995). In the

present study, substitution of a CRF Receptor 1 antagonist, CP-154,526, for the glucocorticoid antagonist failed to have the same effect in DBA/2J mice. It is possible that the chosen dose range of CP-154,526 to block the acquisition of restraint stress cross-sensitized response to ethanol was too modest. The possibility remains that a higher dose of CP-154,526 could effectively block the acquisition of a cross-sensitized response to ethanol. However, higher doses were avoided based on the long half-life of CP-154,526 which has been previously published (Keller et al. 2002). Additionally, osmotic mini-pump treatment with a 32mg/kg/day dose led to visible malaise in rats and led to uninterpretable data in a previous study (Arborelius et al. 2000a). Doses were chosen that had been shown to produce anxiolytic effects but minimized the possibility that daily treatment would lead to drug accumulation and a similar malaise. As a possible explanation for lack of effect observed, it is interesting to note that chronic treatment (10 day) with an alternate CRF Receptor 1 antagonist, CRA1000, has been shown to have little effect on plasma concentrations of adrenocorticotropic hormone (ACTH) or corticosterone compared to vehicle treated rats at baseline and following acute immobilization stress when testing was done 26 hrs following the last CRA1000 treatment. In contrast, if CRA1000 is administered 2 hrs prior to restraint, it will significantly reduce ACTH and corticosterone levels in response to immobilization stress (Ohata et al. 2002). These data suggest the importance of the dosing schedule in assessing the ability of a CRF type 1 receptor antagonist to influence downstream neuroendocrine release. This should be considered in the design of future experiments aimed at altering CRF receptor function in the hope of blocking the stress-related contribution to sensitization. Using CRA1000 as a guide, it seems possible that CP-154,526 could

potentially have very different effects in its ability to block the acquisition verses the expression of a sensitized response.

Although the RIIβ study provides an additional example of restraint stress-induced hypersensitivity to ethanol, the present study failed to provide a potential mechanism for the genotype differences in ethanol and amphetamine-induced locomotor sensitization previously reported in this model. Given the hyperactivity and aggression observed during basic handling procedures (unpublished observations), enhanced sensitivity to stress was an attractive candidate for further investigation. Daily 2 hr restraint stress sessions caused a similar level of sensitivity to the locomotor activational effects of ethanol between $RII\beta^{-/-}$ and $RII\beta^{+/+}$ mice. If one views sensitized responses as an all or none phenomenon, it is possible that 2 hrs of restraint stress would not allow for discrimination between genotype, as it has been shown to produce sensitization in wildtype inbred C57BL/6 mice. An alternate approach calls for the use a sub-threshold dose of restraint stress (either in duration or number of days) in hopes of pulling apart genotype differences. Ultimately, it is possible that differences in HPA axis function are not responsible for the genotype differences in enhanced sensitivity to ethanol and amphetamine-induced locomotor sensitization.

Taken together, the present studies reinforce the literature by providing additional evidence that repeated exposure to restraint stress can cause a sensitized locomotor response to i.p. ethanol administration in mice. This replicated previous findings in DBA/2J mice (Roberts et al. 1995) and extended these findings to C57BL/6J mice. Additionally, this effect was also demonstrated in $RII\beta^{-/-}$ and $RII\beta^{+/+}$ mice maintained on an approximately pure C57BL/6J background. Pretreatment with CP-154,526 prior to restraint stress failed to block the acquisition of a sensitized locomotor response to ethanol; a finding that is particularly

interesting when considered in light of the fact that pretreatment with a glucocorticoid receptor antagonist is effective in achieving this aim. It is possible that compensatory changes in HPA axis signaling compensate for chronic CRF receptor 1 blockade, while this effect is not possible when the target of antagonism is the end result of neuroendocrine release as is the case with glucocorticoid antagonists or synthesis inhibitors. In summary, chronic restraint stress can produce a cross-sensitized response to ethanol, but the exact mechanisms involved in this phenomenon remain elusive. Further investigation of the effects of chronic verses acute antagonism of CRF and glucocorticoid receptor function should ultimately contribute to a greater understanding of this phenomenon.

Figure 3.1 Restraint stress-induced cross-sensitization to ethanol in male DBA/2J mice. This figure represents raw data for locomotor activity during 20-minute locomotor sessions. Prior to the first three locomotor sessions mice received saline injections. These data were averaged (Sal. Avg.) and serve as a measure of basal locomotor activity. Initial locomotor responses to 1.5 g/kg ethanol injection are also presented (Initial). Mice then received 10 days of either 2hr restraint stress of an i.p 2.5 g/kg ethanol injection. On test day, half of the mice from each condition received either a 1.5 g/kg ethanol injection or equivolume saline (Test Day). All values reported are mean ± SEM. Both chronic ethanol injection and restraint stress induced a sensitized response to ethanol. This hyperlocomotor effect was not present in saline treated mice.

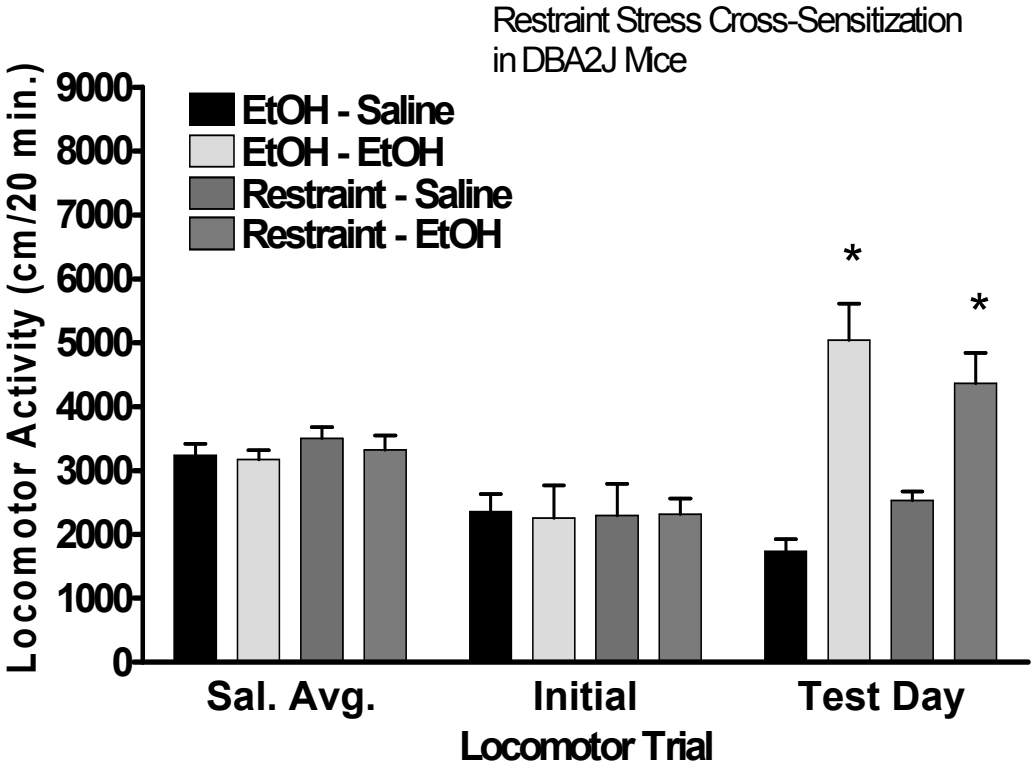


Figure 3.2 Restraint stress-induced cross-sensitization to ethanol in male C57BL/6J mice. This figure represents raw data for locomotor activity during 20-minute locomotor sessions. Prior to the first three locomotor sessions mice received saline injections. These data were averaged (Sal. Avg.) and serves as a measure of basal locomotor activity. Initial locomotor responses to 1.5 g/kg ethanol injections are also presented (Initial). Mice then received 10 days of either 2hr restraint stress of an i.p 2.5 g/kg ethanol injection. On test day, half of the mice from each condition received either a 1.5 g/kg ethanol injection or equivolume saline (Test Day). All values reported are mean $\pm$ SEM. Both chronic ethanol injection and restraint stress induced a sensitized response to ethanol. This hyperlocomotor effect was not present in saline treated mice.

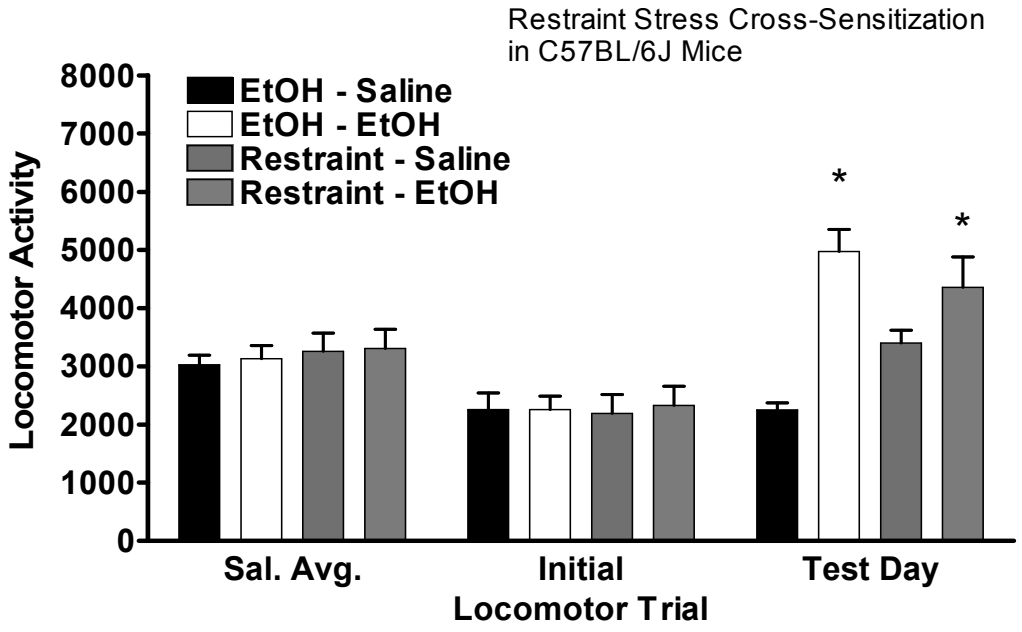


Figure 3.3 Effects of chronic restraint stress on locomotor responses to ethanol in RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice maintained on an approximately pure C57BL/6 background. Mice received either 10 days of either restraint stress (Restraint) or daily handling (Handle). Differences in restraint stress verses handled mice existed in the first 5 min bin of the 20 min locomotor session. In general, RIIβ$^{-/-}$ were more active in response to ethanol regardless of treatment condition compared to wildtypes. Restraint stress treated mice were significantly more active that handled mice regardless of genotype status. All values reported are mean ± SEM.

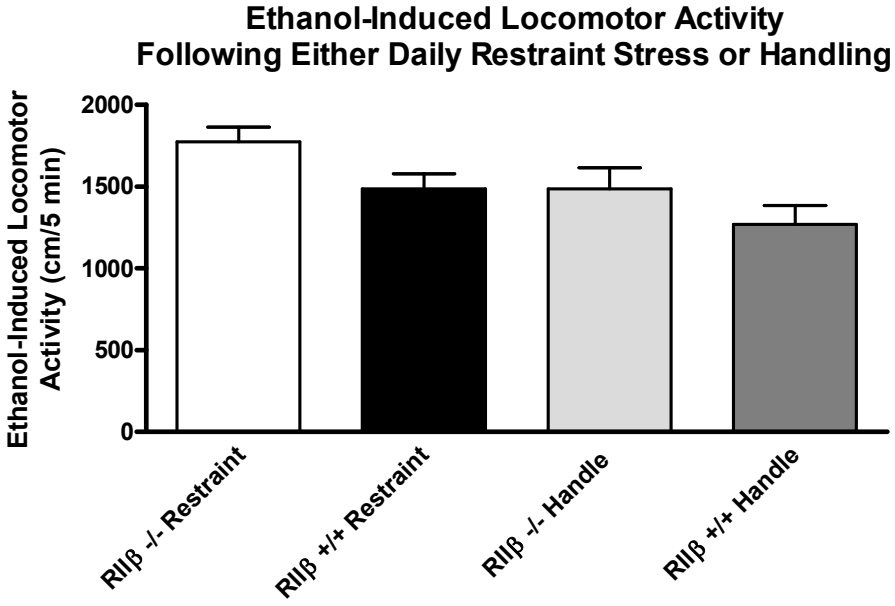
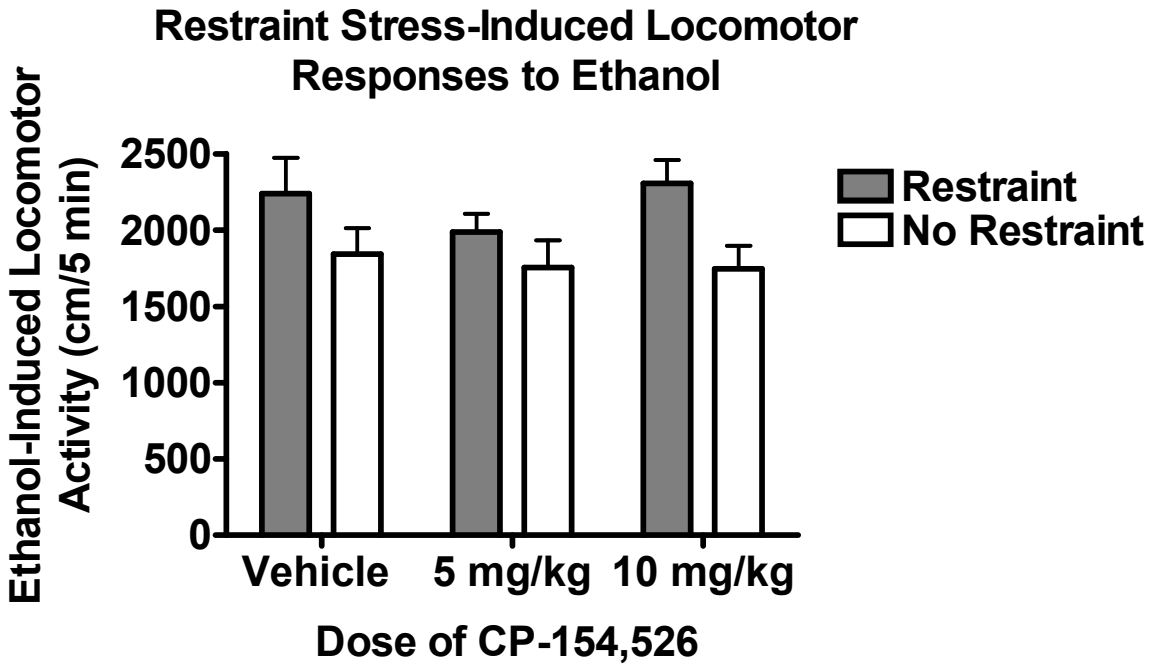


Figure 3.4 Effects of pretreatment with CP-154,526 on the acquisition of restraint stress-induced cross-sensitization to ethanol in male DBA/2J mice. Mice received pretreatment with CP-154,525 (Vehicle, 5 and 10mg/kg) prior to 2hr restraint stress or no restraint. One Test Day, mice received a 1.5 g/kg ethanol injection prior to placement in the locomotor chamber. Mice receiving chronic restraint stress were significantly more active than mice that did not receive stress. Pretreatment with CP-154,526 had no effect on the acquisition of cross-sensitization in animals receiving stress or general locomotor activity in unstressed mice. All values reported are mean ± SEM.



CHAPTER 4

EXPERIMENT 3: CORTICOTROPIN RELEASING FACTOR (CRF)-1 RECEPTOR ANTAGONIST, CP-154,526, BLOCKS THE EXPRESSION, BUT NOT ACQUISITION, OF ETHANOL-INDUCED BEHAVIORAL SENSITIZATION

Introduction

Behavioral sensitization is defined as the long-lasting and progressive enhancement of the locomotor and motivational responses to a drug following repeated administration (Kalivas and Stewart 1991). Over the past twenty years, many studies have demonstrated the ability of repeated ethanol exposure to elicit locomotor sensitization in mice (Crabbe et al. 1992; Cunningham and Noble 1992; Lister 1987; Phillips et al. 1995). It has been suggested that increased sensitivity to behavioral sensitization may be an underlying mechanism that increases the risk for developing drug dependence. According to this view, repeated exposure to a drug promotes neural reorganization leading to a hypersensitive state in brain reward circuitry (Robinson and Berridge 1993; 2000; 2001). To this end, a greater understanding of the underlying mechanism of behavioral sensitization may reveal the potential to slow or prevent the move to addiction as well as provide enhanced treatment options in drug-dependant populations.

Recent studies have done much to expand our understanding of this complex phenomenon but the neurochemical basis of ethanol-induced sensitization is still not fully

understood. A multitude of neurotransmitter systems have been implicated in ethanol-induced locomotor sensitization including dopamine (Broadbent et al. 2005; Palmer et al. 2003), GABA (Broadbent and Harless 1999), opioids (Camarini et al. 2000; Nestby et al. 1997a; Pastor and Aragon 2006), and glutamate (Broadbent et al. 2003; Kotlinska et al. 2006; Szumlinski et al. 2005b). The aforementioned neurotransmitter systems all involve guanine nucleotide binding protein-coupled receptors that recruit protein kinase A (PKA) signaling; an intracellular signaling mechanism which has been implicated in behavioral sensitization. Reduction of PKA activity via genetic knockout of the regulatory subunit IIβ has been shown to enhance sensitivity to both ethanol- (Fee et al. 2006) and amphetamine- (Brandon et al. 1998) induced locomotor sensitization in mice. Just as PKA activity represents a potential modulator underlying drug-induced behavioral sensitization, an organism's stress response mediated by the endogenous stress signaling system, or hypothalamic-pituitary-adrenal (HPA) axis, may represent a similar point of interest.

It has been suggested that individuals suffering from affective disorders can exhibit a sensitized stress response mediated by activation of the HPA axis (Arborelius et al. 1999). HPA axis activation also accompanies drug administration and the application of stress and/or central administration of corticotropin releasing factor (CRF) can induce a hyper-locomotive response (Lee and Tsai 1989; Lowry and Moore 2006). Given these data, it stands to reason that a dysregulation of the components of HPA axis signaling similar to that seen in chronic affective disorders might underlie the hypersensitivity to drug observed in behavioral sensitization. Recent rodent studies confirm that a depressant -like behavioral profile is associated with enhanced sensitivity to the development of sensitization (Alttoa et al. 2007; Wei et al. 2004) Furthermore, it seems unlikely that a sensitized drug response

could have evolved to the benefit of the organism; a more likely explanation would involve drugs of abuse usurping endogenous signaling mechanisms leading to dysregulations contributing to the development of sensitization. Similar dysregulations of CRF signaling have been shown to be critically important to ethanol self-administration in ethanol-dependant rats. CRF receptor antagonist, D-Phe-$CRF_{(12-41)}$, blocked excessive ethanol self-administration in rats made dependant on ethanol via prolonged exposure to ethanol vapor (Valdez et al. 2002). Taken together, these findings suggest dysregulations in the components of HPA axis signaling, specifically CRF, are important in the move from drug use to abuse.

Roles for the HPA axis and its associated neuropeptide, CRF, have also been implicated in sensitized behaviors but greater attention is required to determine the degree to which these versatile systems contribute to ethanol-induced locomotor sensitization. Enhanced sensitivity to the locomotor stimulatory properties of a variety of drugs of abuse has been observed following the administration of an equally varied number of stress models, including restraint stress (Roberts et al. 1995), social defeat (Yap et al. 2005), maternal separation (Kikusui et al. 2005), social isolation (Frances et al. 2000), and variable stress (Lepsch et al. 2005). Taking these findings one step further, investigators have attempted to block the effect of stress exposure on drug sensitivity and sensitization through the use of pharmacological interventions. Administration of glucocorticoid receptor antagonists has been shown to block the acquisition of ethanol-induced locomotor sensitization brought on by repeated restraint stress in mice (Roberts et al. 1995). Additionally, the intracerebroventricular (i.c.v.) administration of the CRF antagonist, α-helical CRF, reduced the stereotypic response to amphetamine following repeated restraint stress in rats (Cole et al. 1990b). In contrast, an augmentation of CRF signaling via pharmacological manipulation can

result in the expression of sensitized behaviors. I.c.v. administration of CRF rather than subcutaneous administration of CRF leads to long-term locomotor sensitization to D-amphetamine (Cador et al. 1993). Similarly, microinfusion of CRF into the shell of the nucleus accumbens of rats led to a prolonged increase in general locomotor activity (Holahan et al. 1997). These approaches have revealed the ability to modulate the acquisition and expression of sensitized drug responses via the manipulation of glucocorticoid and CRF receptor signaling.

CRF is a 41 amino acid neuropeptide that has been shown to play a critical role in the stress response and modulate a wide-variety of neurobiological responses to ethanol. On the most basic level, ethanol exposure has been shown to increase CRF release (Rivier et al. 1984; Rivier and Lee 1996). As previously mentioned, CRF has been shown to be particularly important in the self-administration of ethanol in ethanol-dependant rats (Valdez et al. 2002). Additional evidence is provided by the ability of CRF receptor 1 (CRF1R) antagonists (antalarmin, MJL-1-109-2, and R121919) to block excessive ethanol self-administration following acute withdrawal in this population (Funk et al. 2007). CRF also plays an important role in the anxiety-like behaviors associated with the cessation of ethanol use. Exogenous administration of CRF has been shown to potentiate, or sensitize, ethanol withdrawal in rats (Overstreet et al. 2004). In contrast, administration of the CRA1000, a CRF1R antagonist, blocks the reductions in social interaction characteristic the anxiety-like behavior accompanying ethanol-withdrawal (Knapp et al. 2004). Additionally, administration of CRF1R antagonists, CRA1000 and CP-154,526, during periods of ethanol withdrawal attenuates reductions in social interaction following subsequent ethanol withdrawals in rats (Overstreet et al. 2004). It is important to note that reductions in withdrawal induced

anxiety-like behavior do not appear to be confined to the CRF receptor 1 system as central administration of the potent CRF receptor 2 agonist, urocortin 3, attenuated withdrawal-induced increases in anxiety-like behavior as assessed by reduced time spent in the open arms of the plus maze as well as in heightened ethanol administration in rats (Valdez et al. 2004). The aforementioned examples demonstrate that CRF is intricately involved in all stages of ethanol exposure and an investigation of CRF's role in ethanol-induced locomotor sensitization is a logical extension of these findings.

Here we investigate if CRF1R signaling is involved in the CNS changes underlying behavioral sensitization brought on by repeated ethanol injection. To our knowledge, there has been no investigation of the ability of a CRF1R antagonist to diminish the expression or block the acquisition of ethanol-induced locomotor sensitization. However, there have been encouraging demonstrations that treatment with CRF1R antagonists (CP-154,526 and CRA1000) will block the sensitized anxiety-like response that comes with repeated ethanol withdrawals (Knapp et al. 2004; Overstreet et al. 2004) The following investigation sets out to determine if use of a CRF receptor 1 antagonist, CP-154,526, can be an effective pharmacological tool for blocking both the acquisition and expression of ethanol-induced locomotor sensitization. Taken together the experiments outlined above should not only add to the growing literature of ethanol-induced locomotor sensitization but also determine the efficacy of a pharmacological agent in attenuating a sensitized response to ethanol.

Methods

Animals

Subjects were 130 male DBA/2J mice (Jackson Laboratory, Bar Harbor, ME) obtained at 8 weeks of age and weighing between 20 and 26 grams at the beginning of the experiment. Mice were individually housed for two weeks with *ad libitum* access to standard rodent chow (Teklad, Madison, WI) and water and maintained at 22°C on a 12h:12h light:dark cycle. All experiments were conducted in compliance with the National Institutes of Health guidelines and protocols were approved by the University of North Carolina Animal Care and Use Committee.

Drug Preparation

CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) was donated by Pfizer (Groton, CT), and was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF-1 receptor (K_i < 10 nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al. 1996; Schulz et al. 1996). Importantly, systemic injection of CP-154,526 blocks anxiety-like behavior stemming from ethanol withdrawal in rats (Breese et al. 2005).

The Effect of CP-154,526 on the Acquisition of Ethanol-Induced Locomotor Sensitization

Mice were randomly assigned (n = 10 per group) to one of the 5 treatment conditions in which two intraperitoneal (i.p.) injections were administered with 30-minutes between each injection (CMC + Saline; CMC + EtOH; 5 mg/kg CP-154,526 + EtOH; 10 mg/kg CP-154,526 + CP-154,526 + EtOH; 10 mg/kg CP-154,526 + Saline). Groups that received i.p. injections of ethanol were compared to determine if pre-treatment with the CRF1R antagonist

would block acquisition of ethanol-induced locomotor sensitization while groups that received i.p. injection of saline rather than ethanol were compared to assess possible non-specific effects of CP-154,526 on locomotor activity. The general procedure is as follows: On days 1-10, mice were individually removed from their cage and pre-treated with an i.p. injection of CMC or CP-154,526 (5 or 10 mg/kg). Thirty minutes later, mice received an i.p. injection of either a 2.5 g/kg dose of ethanol (20% w/v solution, mixed in 0.9% NaCl saline) or an equivolume injection of isotonic saline (0.9% NaCl). Mice were then returned to their homecages and left undisturbed aside from routine animal husbandry. On day 11, all animals were transported to the testing room in their home cages and allowed to habituate for at least 35-minutes prior to testing. A small fan provided masking noise in the testing room. Mice were then given an i.p. injected of a 1.5 g/kg dose of ethanol prior to being placed into the center of an open-field arena that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA) for the 20 minute locomotor activity sessions. The open field arena measured 40.64 cm by 40.64 cm by 30.48 cm and was made of clear Plexiglas. Several cm of corncob bedding were placed into the open field chamber to aid in cleaning and to prevent the buildup of odor. Soiled bedding was removed after each locomotor activity session. Following the completion of locomotor activity testing, mice immediately received a tail nick for the collection of 6 μl of blood in Fisher Scientific heparinized micro-hematocrit capillary tubes (Pittsburgh, PA).

The Effect of CP-154,526 on the Expression of Ethanol-Induced Locomotor Sensitization

This paradigm used in this study has been described previously (Fee et al. 2006) and was adapted from a design commonly used to study ethanol-induced behavioral sensitization

(Lessov et al. 2001). All animals were transported to the testing room in their home cages and allowed to habituate for at least 35-minutes prior to testing. A small fan provided masking noise in the testing room. On test day 1-3, all mice received i.p. injection of isotonic saline, based on the equivalent volume for a 1.5 g/kg dose of ethanol, prior to being placed into the center of the open-field arena. On day 4, all mice received an i.p. injection of 1.5 g/kg dose of ethanol prior to placement in the locomotor chamber to establish a baseline of ethanol-induced locomotor activity. Mice were then assigned to 1 of 4 treatment groups (n = 10 per group) equated for locomotor activity following the initial ethanol injection (Day 4). All mice then underwent 10 consecutive days in which a 2.5 g/kg dose of ethanol (i.p.) was administered in the home cage (one injection per day). The site of i.p. injection (left or right) was alternated daily to minimize discomfort. On day 15, mice were transported to the testing room in their home cages 35-minutes prior to testing. Thirty-minutes before i.p. injection of a 1.5 g/kg dose of ethanol, mice were pretreated with CMC or a 5, 10, or 20 mg/kg dose of CP-154,526. The second (ethanol) injection was given contralateral to the pre-treatment injection to minimize discomfort. Following completion of the locomotor session, mice immediately received a tail nick for the collection of 6 µl of blood in Fisher Scientific heparinized micro-hematocrit capillary tubes (Pittsburgh, PA).

The Effect of CP-154,526 on Basal Ethanol-induced Locomotor Activity

All animals were transported to the testing room in their home cages and allowed to habituate for at least 35-minutes. A small fan provided masking noise in the testing room. On test day 1-3, all mice received i.p. injection of isotonic saline, based on the equivalent volume for a 1.5 g/kg dose of ethanol, prior to being placed into the center of the open-field

arena. Mice were then assigned to 1 of 4 treatment groups (n = 10/group) based on average locomotor activity over the 3 days with saline injections. On day 4, the mice were then pre-treated with an i.p. injection of CMC or CP-154,526 (5, 10, or 20 mg/kg). Thirty minutes later, all mice received i.p. injection of a 1.5 g/kg dose of ethanol (contralateral to the side of pre-treatment injection) immediately before locomotor activity testing. This study allowed for a determination of the effect of CP-154,526 on baseline ethanol-induced locomotor activity before the development of behavioral sensitization (i.e., after the first injection).

Assessment of Blood Ethanol Concentrations

Six µl of whole blood collected from tail nicks into capillary tubes and dispensed into 12x75 mm borosilicate glass tubes containing 375 µl of water and 0.5 g of NaCl. These liquid samples were capped and refrigerated until processing by gas chromatography. Liquid ethanol standards (also 6 µl, 0-400 mg%) and samples were similarly prepared and heated in a water bath at 55 °C for 10 min. Subsequently, a 1.5 ml sample of headspace gas was removed from the glass tubes with a plastic 3.0 ml syringe and injected directly into a SRI 8610C gas chromatograph (Torrance, CA) equipped with an external syringe adapter and 1.0 ml external loading loop. Samples were run at 140°C through a Hayesep D column and detected with FID at approximately 2 min post-inject. Hydrogen gas, carrier gas (also hydrogen), and internal air generator flow rates were 13.3, 25, and 250 ml/min, respectively. Areas under the curve for blood samples were analyzed with SRI PeakSimple software for Windows running on a Dell Inspiron 3500 ® laptop computer and converted to mg% in blood based on the curve generated for the standards.

Data Analyses

For all experiments presented in this study, locomotor data was collect and analyzed for all 20 minute sessions in 5 minute bins. Further analysis reveled that the effect of CP-154,526 on ethanol-induced locomotor sensitization, when present, occurred in the first 5 minute bin of locomotor activity. For this reason, only the data from this first bin will be presented. As a measure of sensitization to the effects of ethanol on locomotor activity, locomotor activity on the first ethanol test day was subtracted from each of the subsequent ethanol test day data points (activity following the second and subsequent ethanol injection – activity following the first ethanol injection). Locomotor activity data (raw and converted) were analyzed using analyses of variance (ANOVA). All data are presented as mean $\pm$ S.E.M. and LSD post-hot tests and t-tests (Winer et al. 1991) were used for planned comparisons. Significance was accepted at $P < 0.05$ (two-tailed).

Results

CP-154,526 and Acquisition of Ethanol-Induced Locomotor Sensitization

Daily treatment with CP-154,526 prior to a sensitizing dose of ethanol (2.5 g/kg) failed to block the development of locomotor sensitization in DBA/2J mice. A two-way (treatment (ethanol or saline) X dose (vehicle 5 or 10mg/kg)) ANOVA revealed a significant main effect of treatment [$F(1, 45) = 14.370$, $p < .001$] but not dose or their interaction (Figure 4.1A). This main effect of treatment suggests that repeated 2.5 g/kg ethanol injection, but not equivolume saline, successfully induced a sensitized response to ethanol. A post-hoc t-test comparing vehicle treated saline and ethanol treated groups confirms the existence of a sensitized response to ethanol [$t = -2.857$: $p < .05$]. These differences in locomotor activity

are not attributable to changes in ethanol metabolism as Figure 4.1B displays no significant differences in blood ethanol concentrations. Additionally, these data suggest that chronic treatment with CP-154,526 does not alter ethanol metabolism as no differences existed between any groups.

CP-154,526 and Expression of Ethanol-Induced Locomotor Sensitization

Figure 4.2A depicts locomotor response to a 1.5 g/kg ethanol injection following pretreatment with their respective dose of CP-154,526 in mice with a history of chronic ethanol injection (10 days of 2.5g/kg (i.p.)). Initial ethanol responses are provided as a frame of reference to visually demonstrate the presence of a sensitized response to ethanol. Pretreatment of 10 and 20mg/kg CP-154,526 prior to ethanol injection effectively reduces the ethanol-induced locomotor sensitized response observed in mice pretreated with vehicle or 5 mg/kg CP-154,526. A one-way ANOVA performed on test day data revealed a main effect of dose [$F(3, 36) = 4.502$, $p < .01$]. LSD post hoc test revealed significant differences in both 10 and 20 mg/kg responses compared to vehicle. There were no significant differences between vehicle and 5 mg/kg responses. To further confirm the ability of CP-154,526 to block the expression of ethanol-induced locomotor sensitization, pair samples t tests were performed comparing initial ethanol responses (1^{st} Ethanol) with test day responses (12^{th} Ethanol + CP-154,526) at the various doses. Vehicle and 5 mg/kg CP-154,526 treated-mice show a significantly enhanced response to ethanol, [$t = -6.319$: $p < .001$ and $t = -4.404$: $p < .005$] respectively. Mice treated with 10 and 20mg/kg CP-154,526 exhibited no such sensitized locomotor response to ethanol. Sensitized locomotor responses corrected for basal locomotor activity (locomotor activity on test day - basal ethanol locomotor responses) are presented in

Figure 4.2B. A one-way ANOVA revealed a main effect of dose [$F(3, 36) = 7.212$, $p = .001$]. LSD post hoc test revealed significant differences in both 10 and 20 mg/kg responses compared to vehicle. Figure 4.2C represents blood ethanol concentrations collected from ethanol-treated mice following locomotor activity. No significant differences in blood ethanol concentrations existed suggesting that reductions in locomotor activation following administration of CP 154,525 are not due to alterations in ethanol metabolism.

CP-154,526 and Basal Ethanol-induced Locomotor Activity

Pretreatment with CP-154,526 prior to exposure to an initial 1.5 g/kg ethanol injection failed to alter ethanol-induced locomotor activity (Figure 4.3). A one-way (dose) ANOVA revealed no significant main effect of dose and post-hoc LSD analysis similarly failed to find an effect of any of the doses tested (5, 10 and 20 mg/kg) in comparison to the 0.5% CMC vehicle.

Discussion

Here, we see an interesting pattern of results emerge with the use of a CRF Receptor 1 antagonist, CP-154,526, to block the acquisition and expression of ethanol-induced locomotor sensitization in male DBA/2J mice. Pretreatment with CP-154,526 was ineffective in blocking the acquisition of a sensitized response to ethanol. In contrast, pretreatment with CP-154,526 effectively blocked expression of a sensitized response in DBA/2J mice that had been previously sensitized to the locomotor stimulating effects of ethanol. These effects, or lack of effect as was the case with the acquisition study, were not attributable to alterations in blood ethanol levels. Additionally, ethanol naïve DBA/2J mice

when pretreated with CP-154,526 show no such reductions in locomotor activity in response to ethanol. These data guard against the possibility that CP-154,526 and ethanol in combination simply created a sedative effect and inhibited general locomotor activity. It is also important to note that the doses utilized in this study are in the range of published doses in rodents that have been demonstrated to produce an anxiolytic effect (Arborelius et al. 2000b; Griebel et al. 1998).

These data join a small but growing literature demonstrating the ability of pharmacological manipulations of neurochemicals associated with the HPA axis to block ethanol-induced locomotor sensitization. While the present results are the first direct demonstration that CP-154,526, a CRF Receptor 1 antagonist, can be used to block the expression of ethanol-induced locomotor sensitization, previous pharmacological studies have established a role for CRF receptor signaling in amphetamine- (Cole et al. 1990a) and cocaine- (Erb and Brown 2006; Przegalinski et al. 2005) induced locomotor sensitization. As it is often hypothesized that the various drugs of abuse share at least some common mechanisms in behavioral sensitization, CRF receptor 1 activity and/or activation of the HPA axis may represent a point of commonality. Direct examination of HPA axis function in future sensitization studies will help to determine if the observed results are attributable to CRF manipulations involved in HPA axis signaling, CRF receptor function located outside of the HPA axis, or both.

Although the current findings are promising, there are potential concerns that should be considered. When selecting doses to block the acquisition of ethanol-induced locomotor sensitization, a very modest range was selected. The possibility remains that a higher dose of CP-154,526 could effectively block the acquisition of a sensitized response to ethanol.

However, higher doses were avoided based on the long half-life of CP-154,526, $t_{1/2}$ = 51h, which has been previously published (Keller et al. 2002). Additionally, osmotic mini-pump treatment with a 32mg/kg/day dose led to visible malaise in rats and led to uninterpretable data in a previous study (Arborelius et al. 2000b). Doses were chosen that had been shown to produce anxiolytic effects but minimized the possibility that daily treatment would lead to drug accumulation and a similar malaise. The question also remains if the observed effects of CP-154,526 are due to the drug's peripheral or central actions. CP-154,526 is known to easily cross the blood brain barrier and achieve peak concentrations 20 minutes after intravenous (i.v.) administration (Keller et al. 2002) as well as produce anxiolytic effects 30 minutes following intraperitoneal administration (Griebel et al. 1998), but this fact does not eliminate the possibility that the observed effects of the compound are attributable to its actions in the periphery. However, CRF1R expression is more prominent in the CNS and it is the CRF type 2 Receptor (CFR2R) that is more widely distributed in the periphery (Chatzaki et al. 2006). Although this investigation cannot definitively address this concern, the existing literature on behavioral sensitization would suggest that this is not the case. Central administration of CRF via intracerebroventricular (i.c.v.) canulla promotes the development of locomotor sensitization to d-amphetamine (Cador et al. 1993) while i.c.v. administration of a CRF antagonist blocked the expression of stereotypy in response to amphetamine (Cole et al. 1990a). These studies suggest that CRF activity in the CNS is critical to the development and/or attenuation of a sensitized response to drug. For this reason, we conclude that the observed ability of CP-154,526 to block the expression of ethanol-induced locomotor sensitization is most likely due to its activity within the central nervous system.

Increased sensitivity to behavioral sensitization may be an underlying mechanism that increases the risk for developing drug dependence. According to one view, repeated exposure to a drug promotes neuronal reorganization leading to a hypersensitive state in brain reward circuitry, presumably increasing sensitivity to the positive reinforcing value of a drug (Robinson and Berridge 1993; 2000; 2001). Viewed this way, increased drug-seeking behavior is driven by the heightened reinforcement resulting from chronic drug intake. An alternative view of drug-induced neuronal reorganization is the allostasis model of drug dependence. The allostasis model suggests that neuroplastic changes following repeated, intermittent drug exposure increases negative emotional reactions in addicted individuals (Koob 2003; Koob and Le Moal 2001). Chronic exposure to stressors, including drugs, promote changes to the processes that maintain the system's emotional "set point", such that a "normal" emotional state can only be achieved in the presence of the drug. Thus, in addicted individuals, the negative emotional reaction resulting from the absence of drug drives drug-seeking behavior via negative reinforcement (i.e., drug alleviates the negative affective state) (Koob 2003).

Procedures that support behavioral sensitization involve repeated and intermittent exposure to ethanol, which may be considered analogous to the repeated cycles of excessive consumption and abstinence characteristic of alcohol dependent individuals. Viewed this way, behavioral sensitization may be a useful model for assessing neurochemical changes proposed by the allostasis model. While a CRF receptor antagonist has been shown to block the expression of cocaine-induced locomotor sensitization (Erb and Brown 2006), a role for CRF signaling in modulating ethanol-induced behavioral sensitization has yet to be examined. However, there are data in the literature suggesting a possible role for CRF. In

addition to increasing CRF activity (Koob et al. 1993; Rasmussen et al. 2000; Rivier et al. 1984), ethanol exposure has been shown to increase ACTH (Rivier et al. 1984; Rivier and Lee 1996) and corticosterone levels (Rivier et al. 1984), hormones, which together with CRF, constitute the stress-activated hypothalamic-pituitary-adrenal axis. Interestingly, restraint-induced stress can substitute for ethanol injections in the acquisition of behavioral sensitization in mice. Furthermore, administration of a glucocorticoid receptor antagonist, RU38486, blocks sensitized responses to ethanol (Roberts et al. 1995). In the present report, we determined if another component of the HPA axis, namely CRF, modulates ethanol-induced behavioral sensitization.

Given the ubiquitous expression of CRF throughout the rodent brain (Cummings et al. 1983), the current findings beg the question of the anatomical location for CP-154,526's effects. It stands to reason that an examination of the ethanol sensitization literature in combination with the CRF literature may provide some insight. Several candidate brain regions emerge when looking to the ethanol locomotor sensitization literature. A 1997 study by Nestby and colleagues found that repeated treatment with ethanol lead to increased reactivity of dopaminergic and cholinergic neurons of the nucleus accumbens and the striatum (Nestby et al. 1997b). Additional evidence for the involvement of the striatum in ethanol induced-locomotor sensitization is found in the dopamine literature. Mice expressing ethanol-induced locomotor sensitization show significant increases in dopamine D2 binding sites in the striatum compared to controls (Souza-Formigoni et al. 1999). The striatum and the nucleus accumbens both represent possible points of overlap between regions of known CRF expression and CRF neuronal innervation (Cummings et al. 1983) and brain regions previously implicated in ethanol-induced locomotor sensitization. However, the alternate

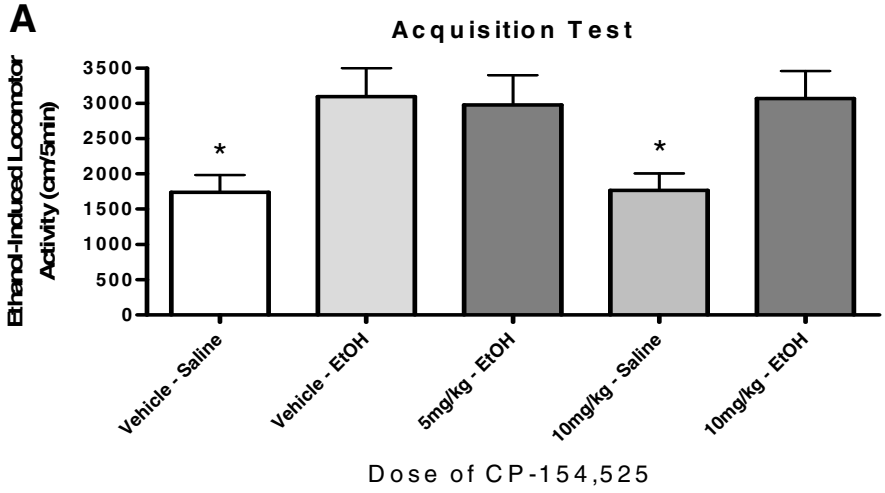
hypothesis still remains that CP-154,526 is modulating CRF Receptor 1 signaling in alternate region or regions that ultimately influences an ethanol sensitized response in the striatum or accumbens. The central nucleus of the amygdala (CeA) is one such brain region that has been demonstrated to play a critical role in CRF signaling (Cummings et al. 1983) but has also been implicated in a variety of behaviors associated with addiction. Increased cocaine-induced release of CRF occurs in the CeA of rats repeatedly treated with cocaine (Richter et al. 1995). It was also shown that corticosterone implantation in newborn rats (postnatal day 0-12) leads to higher locomotor activity in adulthood as assessed by plus maze activity (Roskoden et al. 2005). The aforementioned result was found to be accompanied by a decreased number of CRF containing neurons in the CeA of corticosterone treated rats (Roskoden et al. 2005). The ethanol literature also provides some interesting evidence for the involvement of the CeA in ethanol-dependant animals. Administration of the CRF antagonist, D-Phe-$CRF_{(12-41)}$, directly into the CeA blocked the increased ethanol self-administration of ethanol-dependant rats during acute withdrawal (Funk et al. 2006). Additionally, acute withdrawal is characterized by a reduced CRF immunoreactivity in the CeA suggesting a potentiated release of CRF in this region (Funk et al. 2006). Third party modulation of ethanol-induced locomotor sensitization by CRF in the CeA certainly represents a promising avenue for future investigations. Alternately, CRF's role in the paraventricular nucleus of the hypothalamus (HPA axis activation) could also serve as a fertile target when investigating if CRF Receptor 1 activity in the expression of ethanol-induced locomotor sensitization is site-specific.

These findings suggest that CRF Receptor 1 signaling may represent an interesting avenue in the treatment of alcoholism. CP-154,526 was effective in blocking a sensitized

locomotor response to ethanol; a behavior thought to be reflective of reward associated with drug administration. Unfortunately, pretreatment with CP-154,526 was unable to block the acquisition of a sensitized response in mice and therefore might not be the best target for a prophylactic treatment in high risk populations. However, a pharmacological intervention that is capable of blocking the expression of a sensitized response in drug dependant individuals might be more clinically useful than one aimed at blocking the acquisition of a sensitized drug response as the former population would be far easier to identify than the latter. More testing with CP-154,526 is necessary before theses aims can be realized but these data represent a promising step in this direction.

Taken together these data provide the first direct evidence a CRF Receptor 1 antagonist's, CP-154,526, ability to block the expression of ethanol-induced locomotor sensitization in DBA/2J mice. This was an effect that was not attributable to alterations in ethanol metabolism or a basic pharmacological interaction producing sedation. Future studies are required to determine the exact mechanisms by which this modulation of CRF signaling is capable of blocking the expression of a sensitized behavior. Additionally, these data evaluated in light of the finding by Roberts and colleagues (1995) which demonstrate a glucocorticoid receptor antagonist's ability to block the acquisition of ethanol-induced locomotor sensitization suggests that antagonism of receptors associated with HPA axis signaling is not one size fits all. Further investigation is necessary to determine why a blockade of corticosterone signaling and CRF1R signaling are not functionally equivalent with respect to their ability to block the acquisition of ethanol-induced locomotor sensitization.

Figure 4.1 Effects of CP-154,526 on the acquisition of ethanol-induced locomotor sensitization in male DBA/2J mice. (A) Mice received either pretreatment with CP-154,525 (Vehicle, 5, or 10mg/kg) prior to 2.5 g/kg ethanol or pretreatment with CP-154,525 (Vehicle or 10mg/kg) prior to equivolume saline for ten consecutive days. One Test Day, mice received a 1.5 g/kg ethanol injection prior to placement in the locomotor chamber. Mice receiving chronic 2.5 g/kg ethanol injections were significantly more active than mice receiving saline injection. Pretreatment with CP-154,526 had no effect on the acquisition of cross-sensitization in animals receiving stress and 10mg/kg CP-154,526 pretreatment had no effect on the general locomotor activity of saline-treated mice. (B) Blood ethanol concentrations (mg%) in DBA/2J mice. Immediately following locomotor activity assessment after final ethanol injection (acquisition test day), mice had tail blood collected for analysis using gas chromatography to assess the possibility of alterations in ethanol pharmacokinetics. There were no differences in any of the groups tested. All values reported are mean $\pm$ SEM.



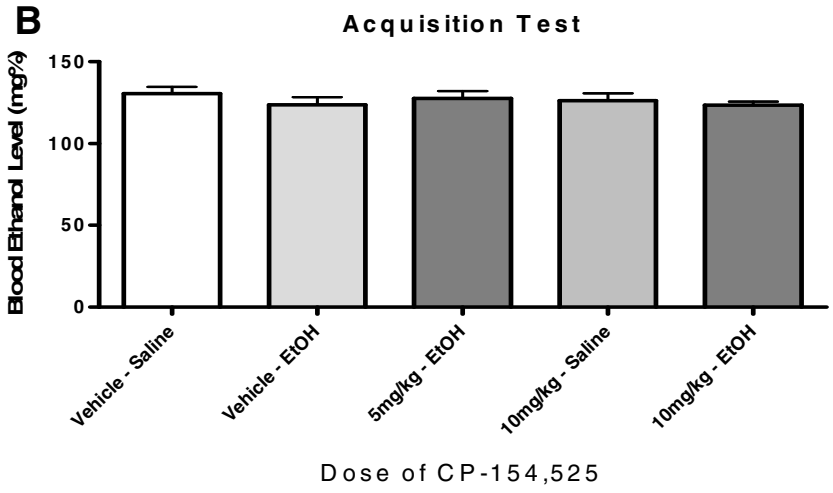


Figure 4.2 Effect of CP-154,526 on the expression of ethanol-induced locomotor sensitization in male DBA/2J mice. (A) represents locomotor responses were recorded following a 1.5 g/kg ethanol injection following pretreatment with their respective dose of CP-154,526 in mice with a history of chronic ethanol injection (10 days of 2.5g/kg (i.p.)). Initial ethanol responses are provided as a frame of reference to visually demonstrate the presence of a sensitized response to ethanol. Pretreatment of 10 and 20mg/kg CP-154,526 prior to ethanol injection effectively reduces the ethanol-induced locomotor sensitized response observed in mice pretreated with vehicle or 5 mg/kg CP-154,526. (B) represents the expression of locomotor sensitization by presenting test day ethanol exposure corrected for baseline ethanol activity (12^{th} ethanol activity minus 1^{st} ethanol activity). (C) Blood ethanol concentrations (mg%) in DBA/2J mice. Immediately following locomotor activity assessment of 12^{th} ethanol injection, mice had tail blood collected for analysis using gas chromatography to assess the possibility of alterations in ethanol pharmacokinetics. There were no differences in any of the groups tested. All values reported are mean $\pm$ SEM.

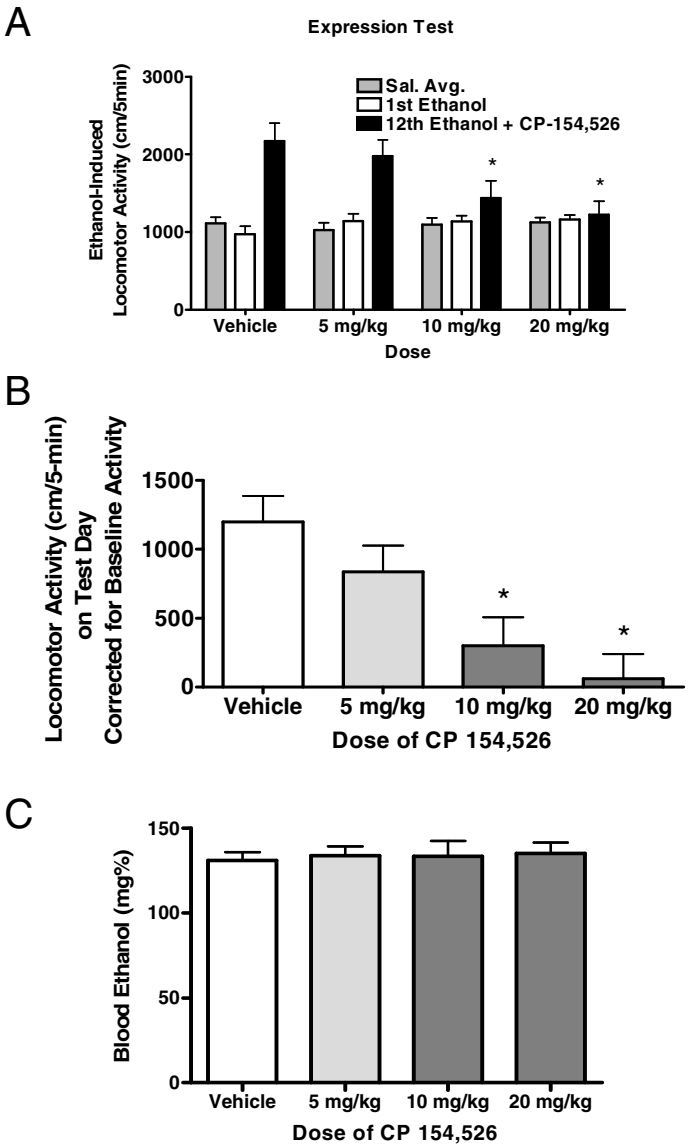
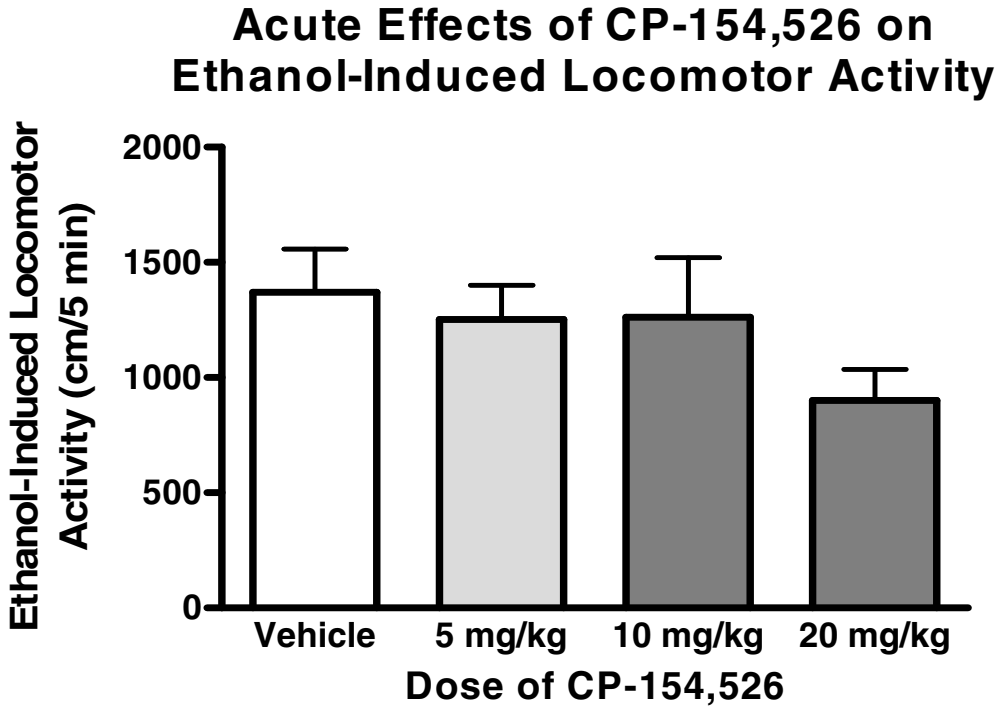


Figure 4.3 Acute effects of CP-154,526 on ethanol-induced locomotor activity in male DBA/2J mice. Pretreatment with CP-154,526 30 min prior to exposure to an initial 1.5 g/kg ethanol injection failed to alter ethanol-induced locomotor activity. All values reported are mean $\pm$ SEM.



CHAPTER 5

GENERAL DISCUSSION

Summary of Experimental Findings

In the present report, RIIβ$^{-/-}$ mice consistently showed significantly greater ethanol-induced locomotor activity relative to RIIβ$^{+/+}$ mice. RIIβ$^{-/-}$ mice also showed increased sensitivity to ethanol-induced locomotor sensitization, an effect that may be dependent on genetic background and/or testing paradigm. Importantly, increased locomotor activity by RIIβ$^{-/-}$ mice was specific to ethanol injections (and not seen following saline injections), and was not associated with altered blood ethanol levels. Normal blood ethanol levels and ethanol metabolism by RIIβ$^{-/-}$ mice have previously been documented (Fee et al. 2004; Thiele et al. 2000b)Since RIIβ$^{-/-}$ mice show blunted PKA activity in critical brain regions, such as the striatum and nucleus accumbens (Brandon et al. 1998; Thiele et al. 2000b), the present results suggest that normal PKA signaling is part of a mechanism that protects against ethanol-induced locomotor activity and behavioral sensitization.

To extend these findings, the RIIβ knockout mouse model was utilized to determine if enhanced sensitivity to stress was a factor contributing to the expression of heightened sensitivity to ethanol-induced locomotor activation in these mice. A commonly employed restraint stress cross-sensitization paradigm was employed which has been previously demonstrated to produce a cross-sensitized locomotor response to ethanol in our hands and in

other laboratories. RIIβ$^{-/-}$ mice failed to show a heightened sensitivity to the effects of restraint stress on ethanol-induced locomotor activation when compared to RIIβ$^{+/+}$ mice. Chronic restraint stress induced a hypersensitive response to ethanol compared to non-stressed controls in both RIIβ$^{-/-}$ and RIIβ$^{+/+}$ mice with no genotype differences in the magnitude of this response. Additional studies were performed demonstrating the ability of chronic exposure to restraint stress to induce a cross-sensitized response to ethanol in two commercially available inbred strains of mice, C57BL/6J and DBA/2J. In an attempt to discern a mechanism for the contribution of stress exposure to a cross-sensitized response to ethanol, pretreatment with CRF Receptor 1 antagonist, CP-154,526, prior to restraint stress sessions in male DBA/2J mice was employed in an attempt to block the acquisition of cross-sensitization. Ultimately this approach was ineffective in blocking the acquisition of restraint stress-induced cross-sensitization; an effect that was rather unexpected given the demonstrated efficacy of the glucocorticoid receptor antagonist, RU38486 (Roberts et al. 1995).

Despite the lack of effect observed when testing CP-154,526's ability to block the acquisition of a cross-sensitized response to ethanol, its ability to block both the acquisition and expression of ethanol-induced locomotor was investigated. This was a logical next step given the fact that RU38486 has also been shown to block the acquisition of ethanol-induced locomotor sensitization (Roberts et al. 1995). An interesting pattern of results emerges with the use of a CRF Receptor 1 antagonist, CP-154,526, to block the acquisition and expression of ethanol-induced locomotor sensitization in male DBA/2J mice. Pretreatment with CP-154,526 was ineffective in blocking the acquisition of a sensitized response to ethanol. In contrast, pretreatment with CP-154,526 effectively blocked a sensitized response in DBA/2J

mice that had been previously sensitized to the locomotor stimulating effects of ethanol. These effects, or lack of effect as was the case with the acquisition study, were not attributable to alterations in blood ethanol levels. Additionally, ethanol naïve DBA/2J mice when pretreated with CP-154,526 show no such reductions in locomotor activity in response to ethanol. These data guard against the possibility that CP-154,526 and ethanol in combination simply created a sedative effect and inhibited general locomotor activity. It is also important to note that the doses utilized in this study are in the range of published doses demonstrated to produce an anxiolytic effect in mice (Arborelius et al. 2000b; Griebel et al. 1998).

Proximate Mechanisms Investigated in Locomotor Sensitization

While the present results serve as the first direct demonstration that PKA signaling modulates the stimulant effects of ethanol and ethanol-induced behavioral sensitization, previous pharmacological and genetic studies have established that PKA signaling is involved with amphetamine- (Crawford et al. 2004; Crawford et al. 2000; Tolliver et al. 1999) and cocaine- (Miserendino and Nestler 1995; Park et al. 2000; Schroeder et al. 2004) induced locomotor sensitization. Of direct relevance to the present report is the observation that RIIβ$^{-/-}$ mice are more susceptible to the acquisition of locomotor sensitization following repeated amphetamine exposure (Brandon et al. 1998). Interestingly, repeated amphetamine exposure reduces PKA activity in the nucleus accumbens and striatum (Crawford et al. 2004; Crawford et al. 2000). Thus, we speculate that reduced PKA signaling in these regions causes RIIβ$^{-/-}$ mice to be more sensitive to the stimulant effects of ethanol as well as ethanol- and amphetamine-induced behavioral sensitization. Because dynorphin, a neuropeptide that is

reduced in the dorsal medial striatum of RIIβ$^{-/-}$ mice (Brandon et al. 1998), plays an inhibitory role in sensitization (Heidbreder et al. 1995), it can be speculated that increased ethanol- and amphetamine-induced behavioral sensitization in RIIβ$^{-/-}$ mice may be the result of low striatal dynorphin activity. However, we suggest that a degree of caution is necessary in that it is still unclear to what extent, if any, the neuronal mechanisms involved in ethanol- and amphetamine-induced locomotor sensitization overlap.

The RIIβ$^{-/-}$ model represents a unique opportunity to determine if alterations in PKA signaling will affect the expression of stress-induced cross-sensitization to ethanol in mice. There is considerable evidence in the literature suggesting a role for PKA activity in the regulation of HPA axis activity. The effects of diazepam on HPA axis activity are mediated via cAMP-dependant PKA activity in the hypothalamus (Vargas et al. 2001). PKA activity has also been posited as a mechanism underlying the diminished hippocampal activity believed to be associated with the HPA axis hyperactivity in depression (Perera et al. 2001). Specifically, ethanol has been shown to increase PKA and cAMP activity and, in turn, increase CRF peptide secretion (Li et al. 2005). Additionally, PKA activity has been shown to play an essential role in CRF mediated decreased expression of CRF receptor 1 mRNA in part via the phosphorylation of cAMP response element binding (CREB) (Kasagi et al. 2002). Ultimately, it appears the alterations in PKA signaling observed in the RIIβ knockout model which contribute to differences in ethanol-induced locomotor sensitization are not responsible for the alterations in ethanol responsivity that accompany chronic exposure to stress. However, it is possible the employed stress paradigm failed to reveal genotype differences because it effectively created a hypersensitive response in both knockout and

wildtype mice; a less intense stress paradigm could have allowed for genotype discrimination.

Although the RIIβ model failed to provide a specific mechanism for the contribution of stress to a cross-sensitized locomotor response to ethanol, further focus was given to a neuropeptide associated with the HPA axis, CRF, due to the demonstrated ability of pharmacological interventions in HPA axis signaling to alter the acquisition and expression of sensitized responses. In examining a role for stress signaling in sensitization, the physiological endpoint, cortisol in humans and corticosterone in rodents, is often targeted. Adrenalectomy eliminates the synthesis and release of corticosterone, and has been shown to block the acquisition of cocaine sensitization if performed prior to the sensitization regime. However, if the sensitization paradigm is initiated prior to adrenalectomy, the expression of cocaine sensitization remains intact (Przegalinski et al. 2000). Prasad and colleagues also find a similar inability of adrenalectomy to alter the expression of cocaine-induced locomotor sensitization, but it was also reported that adrenalectomy blocks the acquisition of sensitization when tested in acute withdrawal (24 hrs) but not in late withdrawal at 1 week (Prasad et al. 1996). Taken together these studies suggest the importance of glucocorticoid signaling in the acquisition of sensitized behaviors. Additionally, pretreatment with the glucocorticoid receptor antagonist, mifepristone, blocks the expression of a sensitized locomotor response to amphetamine, while the drug had no effect on amphetamine responses in drug naïve rats (De Vries et al. 1996). Roberts et al. (1995) also found that RU38486 could block the acquisition of ethanol-induced locomotor sensitization and restraint stress-induced cross-sensitization to ethanol in mice. These findings point to the level of glucocorticoid

signaling and subsequent receptor binding and activation as being critical to the development of behavioral sensitization.

Alterations in HPA axis signaling upstream of glucocorticoid release have also been implicated in behavioral sensitization. Intracerebroventricular (i.c.v.) administration of the CRF antagonist, α-helical CRF, reduced stereotypic response to amphetamine following repeated restraint stress in rats (Cole et al. 1990a). In contrast, an augmentation of HPA axis signaling via pharmacological manipulation can result in the expression of sensitized behaviors. I.c.v. administration of CRF rather than subcutaneous administration of CRF leads to long-term locomotor sensitization to D-amphetamine (Cador et al. 1993). Similarly, microinfusion of CRF into the shell of the nucleus accumbens of rats led to a prolonged increase in general locomotor activity (Holahan et al. 1997). Previous pharmacological studies with CP-154,526 have established a role for CRF receptor signaling in cocaine- (Erb and Brown 2006; Przegalinski et al. 2005) induced locomotor sensitization. After finding a prominent role for neurochemicals associated with HPA axis activity, specifically CRF signaling, in the sensitization literature, it was a logical step to determine if a role for CRF Receptor 1 signaling existed in the acquisition and expression of ethanol-induced locomotor sensitization.

CP-154,526 pretreatment in mice previously exposed to the sensitization paradigm effectively blocked the expression of ethanol-induced locomotor sensitization. This points to the idea that once established, neuroplastic changes associated with the development of sensitization can be modulated by CRF signaling. In contrast, it was interesting to find that chronic pretreatment with CP-154,525 failed to protect against the acquisition of locomotor sensitization that accompanies repeated ethanol exposure. This finding suggests the

possibility that the plastic changes associated with ethanol-induced locomotor sensitization can be achieved despite a pharmacological blockade of CRF 1 receptors. Taken together, these two pieces of evidence suggest that CRF receptor 1 signaling plays a complimentary role in the development and expression of sensitization. It appears that blockade of CRF1Rs will not prevent the development or reverse the changes associated with heightened behavioral sensitization, but it might help to modulate mechanisms associated with the expression of sensitized behaviors.

The question remains if the observed effects of CP-154,526 are due to the drug's peripheral or central actions. CP-154,526 is known to easily cross the blood brain barrier and achieve peak central concentrations 20 minutes after intravenous (i.v.) administration (Keller et al. 2002) as well as produce anxiolytic effects 30 minutes following intraperitoneal administration (Griebel et al. 1998), but this fact does not eliminate the possibility that the observed effects of the compound are attributable to its actions in the periphery. However, CRF receptor 1 expression is more prominent in the CNS and it is the CRF 2 Receptor (CFR2R) that is more widely distributed in the periphery (Chatzaki et al. 2006). Future studies examining the effects of peripheral verses central administration of CP-154,526 on ethanol-induced locomotor sensitization and the corresponding levels of ACTH and corticosterone may provide insight into the anatomical location of action.

It is curious that the more or less constant presence of CRF receptor 1 antagonism in the acquisition studies failed to block both ethanol-induced locomotor sensitization and the restraint stress-induced cross-sensitized response to ethanol. Considering these results in light of the findings by Ohata et al. (2002) which demonstrated the ability of a CRF1R antagonist, CRA1000, to attenuate restraint stress-induced ACTH and corticosterone release 2h but not

26h after final CRA1000 treatment, it seems possible that the observed results might be explained by the ability of CP-154,526 to exert short-term rather than long-term effects on CRF1R signaling within the HPA axis effectively reducing the release of ACTH and corticosterone. However, the paraventricular nucleus of the hypothalamus is just one site of CRF1R expression and the possibility remains that CRF receptor 1 signaling in any number of alternate brain regions could have mediated the observed reductions in the expression of ethanol-induced locomotor sensitization. Some of these regions will be discussed in the following section as they relate to proposed models of drug abuse and addiction.

Ultimate Mechanisms Related to Sensitization

In order for the aforementioned studies to be relevant in the broader sense of drug use and addiction, it is important to view sensitization in the context of the current theories of drug addiction. The incentive sensitization model put forth by Robinson and Berridge provides an explanation for the initiation of drug use, the continuation of this behavior, and the escalation of use that accompanies addiction (Robinson and Berridge 1993; 2000; 2001). On the other hand, the allostasis model offered by George Koob suggests that neuroplastic changes involved in our emotional regulation become dysregulated with chronic exposure and in turn, increase the likelihood of future use (Koob 2003; Koob and Le Moal 2001). A role for locomotor sensitization exists in both theories of drug use and abuse. However, the most important question still remains. Is the locomotor activation observed in locomotor sensitization purely a motor output free of psychological control or is it reflective of a psychological state (e.g. drug seeking/reinforcement)? The answer to this question will ultimately determine the relative importance of locomotor sensitization in the two models of

drug addiction and will help to predict which pharmacological interventions will be most affective in preventing the development and expression of these behaviors.

On the most basic level, the idea of drug-induced locomotor sensitization fits well with the incentive sensitization model of drug addiction. The locomotor activation in response to drug is believed to be predictive of its rewarding properties and repeated administration of drug, a schedule utilized in sensitization paradigms, leads to significantly enhanced locomotor activation. The sensitized locomotor response is therefore reflective of a sensitized reward system and individuals receiving a hypersensitized response to reward should be more vulnerable to future abuse and addiction. Physiological assessments of neurotransmitter release in brain regions associated with reward confirm this idea of a sensitized reward system. Amphetamine-locomotor sensitization is associated with enhanced stimulated dopamine release in the dorsal and ventral striatum (Paulson and Robinson 1995). Nicotine-induced behavioral sensitization is also associated with heightened extracellular dopamine release in the striatum and nucleus accumbens (Shim et al. 2001). Enhanced responsiveness of accumbal dopaminergic and cholinergic neurons has been observed following repeated administration of ethanol, morphine, cocaine and amphetamine (Nestby et al. 1997b). Chronic cocaine treatment has also been shown to produce a sensitized release of glutamate in the accumbens (Reid and Berger 1996). The functionality of striatal dopamine transporters has also been implicated in locomotor sensitization (Claye et al. 1995) suggesting that changes in extracellular neurotransmitter release may only be part of the emerging picture.

Although a significant subset of the behavioral sensitization literature points to sensitization of signaling mechanisms found within the prominent reward pathways; this is a

nuanced literature. Recent studies suggest that a basic locomotor response to drug is not always sufficient to establish this connection with reward. A study by Allen and colleagues demonstrates that rats selected for their low locomotor response to cocaine, but not rats exhibiting high locomotor responses, will later exhibit cocaine-induced locomotor sensitization and conditioned-place preference (Allen et al. 2007). It seems possible that mechanisms associated with reward are not of primary importance when sensitization is only possible in a population of rats selected for their initially low responding. Additionally, a mechanistic sensitization is not always a hallmark of locomotor sensitization. Behavioral sensitization following repeated ethanol has been observed in C57BL/6J and DBA/2J mice in the absence of an enhanced dopaminergic response in the nucleus accumbens of these mice (Zapata et al. 2006).This dissociation between reward and sensitization is also present in a study by Hodge and colleagues which found that mice exhibiting low levels of ethanol self-administration were most sensitive to ethanol-induced locomotor sensitization (Camarini and Hodge 2004). These studies, particularly those by Allen (2007) and Camarini (2004), suggest the possibility that enhanced initial sensitivity to the rewarding aspects of drug might not be the best predictor of a future sensitized response.

The incentive sensitization model places great focus on the rewarding aspects of drug use and abuse but the negative affect and emotional dysregulations that accompanies drug addiction is difficult to explain when focused purely on reward circuits. However, it is possible that negative affect and the resultant drive to eliminate said affect enhances the incentive salience of drug and encourages future drug use. Alternatively, the allostasis model put forth by Koob might provide more emphasis on the emotional component of drug use and the subsequent dysregulations of neuropeptide systems that is well documented in chronic

drug exposure (Koob 2003; Koob and Le Moal 2001). This model provides the opportunity to test a variety of compounds on behaviors associated with dependence. Antagonism of CRF, the major neuropeptide existing in a reciprocal balance with Neuropeptide Y (NPY), is a prime target for alleviating the negative affect associated with drug dependence and withdrawal. Administration of the CRF antagonist, D-Phe-$CRF_{(12-41)}$, directly into the CeA blocked the increased ethanol self-administration of ethanol-dependant rats during acute withdrawal (Funk et al. 2006). The ability of CRF antagonists to block dependence and withdrawal driven-behaviors certainly strengthens this idea of allostatic modulation of behavior. Similarly, the present study uncovers a role for CRF receptor signaling in the expression of ethanol-induced locomotor sensitization. Given this result, it seems possible that in addition to representing a model of a sensitized reward system, it may also be used as a fairly simple model of ethanol dependence. It is of interest to determine if pharmacological interventions aimed at alleviating the negative emotionality associated with dependence or compounds aimed at preventing a heightened reward state ultimately prove more efficacious in blocking sensitized behaviors.

The model of dependence upon which one focuses will suggest various brain regions as being of particular interest. The nucleus accumbens quickly presents itself in the reward literature. CRF activity in nucleus accumbens shell but not core has been linked to increases in general locomotor activity (Holahan et al. 1997). More directly related to drug taking behavior, pharmacological blockade of CRF1R signaling in the nucleus accumbens resulted in significantly reduced cocaine-induced dopaminergic overflow in the accumbens (Lodge and Grace 2005). I.c.v. infusion of CP-154,526 also reduced extracellular concentrations of dopamine in the nucleus accumbens and ventral tegmental area following cocaine injection

(Lu et al. 2003). Exogenous administration of CRF via microinjections into the medial shell of the nucleus accumbens enhanced cue-induced responding for sucrose (Pecina et al. 2006).

The amygdala has similarly demonstrated its importance in the modulation of emotionality as well as the emotional dysregulations that accompanies chronic exposure to drug and the resulting dependence. CRF signaling has been shown to play a critical role in amygdaloid function. CRF activity modulated via the exogenous application of a CRF receptor 1 and 2 agonist, urocortin, into the basolateral amygdala resulted in anxiety-like behavior in rats and was suggested to underlie the plastic changes in emotional dysregulations (Rainnie et al. 2004). In a related experiment, exposing sheep to predator stress (dog) results in increased CRF and GABA release in the amygdala as well as CRF release in the PVN (Cook 2004). CRF1R activity in the amygdala has also been implicated in the defensive posturing that follows acute social defeat in mice (Robison et al. 2004) and has been hypothesized to modulate the development of an exaggerated fear response following exposure to intense stress. As previously mentioned, CRF activity in the CeA has also been shown to be particularly important in the ethanol self-administration of dependant rats following ethanol-withdrawal (Funk et al. 2006). Alternatively, CRF function in the amygdala has also been associated with drug-related phenotypes typically associated with reward. Infusion of CP-154,526 into the amygdala as well as the nucleus accumbens of rats attenuates morphine-induced reinstatement of morphine conditioned place preference (Wang et al. 2006). Taken together the studies mentioned above all point to a critical role for CRF signaling in the amygdala, but it is also important to consider a role for amygdaloid function in behavioral sensitization. It is of interest to note that low-current electrical stimulation of the central nucleus of the amygdala significantly increased the magnitude of concurrent d-

amphetamine-induced locomotor sensitization (Gelowitz and Kokkinidis 1993). Related to stress-induced cross-sensitization, the heightened locomotor response to amphetamine in rats repeatedly receiving social defeat stress has been linked to enhanced Fos activity in the VTA and the amygdala (Nikulina et al. 2004). Given the wide-ranging role for the various sub-regions of the amygdala, it stands to reason that CRF activity in the amygdala could contribute to behaviors representing any number of underlying mechanisms associated with drug use and abuse.

This brief review of CRF1R's role in behavioral sensitization focuses on just a few of the brain regions which are potentially importance in the expression of ethanol-induced locomotor sensitization. Unfortunately, the studies described above are unable to determine the exact mechanism of action by which CP-154,526 achieves its attenuation of ethanol-induced locomotor sensitization. Site-specific infusion of CP-154,526 into various brain regions, including the paraventricular nucleus of the hypothalamus, amygdala, nucleus accumbens, VTA, and striatum, would assure the presence of drug at the proposed site of action and would also allow for a more systematic approach to the identification of brain regions essential to blocking ethanol-induced locomotor sensitization. The possibility remains that CRF1R function in any set of brain regions, alone or in concert, could be mediating the expression of ethanol-induced locomotor sensitization.

Ultimately, no one model of drug abuse and dependence will provide a complete explanation for a specific situation. Additionally, assessments of HPA axis function as well as stress history are modulating variables that can interact with each of the models discussed above. Just as there is no one explanation for the etiology of alcoholic abuse, sensitization research can fit anywhere in the continuum between the reward associated with initial drug

responses to a representation of the dysregulated emotionality of a drug-dependant state. Ultimately, the locomotor activation that accompanies sensitization would take on very different meanings given the respective model of dependence. This does not eliminate the possibility that the same behavior could be exhibited for very different reasons. However, regardless of its origin, locomotor sensitization provides a powerful screening tool to determine the efficacy of compounds associated with both models of dependence and will continue to due so far into the future.

Implications of Current Findings

Alterations in markers upstream of PKA signaling, adenylyl cyclase activity, have been observed in alcoholic populations (Pauly et al. 1999; Yamamoto et al. 2001). Additionally, alterations in PKA activity via the deletion of the regulatory subunit IIβ enhance sensitivity to ethanol-induced locomotor sensitization (Fee et al. 2006). These finding suggests the possibility that pharmaceuticals aimed at altering PKA signaling might be a useful tool in the treatment of alcohol and other drugs of abuse. However, the rather ubiquitous expression of PKA throughout the brain, the bi-direction nature of PKA signaling varying from brain region to region, and its involvement in virtually every g-protein coupled receptor all point to the unfortunate likelihood that the unintended and unpredictable side effects of these treatments preclude their viability.

Chronic exposure to stress imparts a powerful vulnerability to subsequent drug exposure. The ability of stress to reinstate both drug seeking (Erb et al. 2001; Wang et al. 2005) and the resumption of drug taking (Covington et al. 2005; Kabbaj et al. 2001) has been well documented. For these reasons, compounds exhibiting the ability to alter the stress

response and/or attenuate signaling associated with HPA activation could be invaluable tools in the treatment of drug addiction. Along these lines, Shaman and colleagues found that pretreatment with CP-154,526 attenuated footshock-induced drug-seeking in both heroin- and cocaine-trained rats (Shaham et al. 1998). The inability of CP-154,526 to block the acquisition of restraint stress-induced cross-sensitization to ethanol and ethanol locomotor sensitization contrasted with the efficacy of RU38486 suggest that glucocorticoid signaling may ultimately provide a more feasible target for the prevention of the acquisition of a sensitized response to drug. For this reason, in populations with a documented drug addiction diathesis, such as the children of alcoholics, prophylactic treatment with a glucocorticoid antagonist prior to drug exposure may help slow or prevent the progression from casual use to abuse. However, these proposed treatments may prove ineffective due to the rapid adaptation possible within the HPA axis, potential side effects (e.g. RU38486's efficacy as a contraceptive), and interactions due to the system's ubiquitous expression.

The presented findings suggest that CRF Receptor 1 signaling may represent an attractive avenue in the treatment of alcoholism. CP-154,526 was effective in blocking a sensitized locomotor response to ethanol; a behavior thought to be reflective of reward associated with drug administration. This idea is evidenced by the fact that mice with low ethanol self-administration put through a sensitization paradigm will consume high amounts of ethanol in future self-administration sessions (Camarini and Hodge 2004). Unfortunately, pretreatment with CP-154,526 was unable to block the acquisition of a sensitized response in mice and therefore might not be the best target for a prophylactic treatment in high risk populations. However, a pharmacological intervention that is capable of blocking a sensitized response in drug dependant individuals could be more clinically useful than one aimed at

blocking the acquisition of a sensitized drug response as the former population would be far easier to identify than the latter. It seems more likely that a patient will present after ethanol dependence or addiction has been crystallized rather than the critical but brief initial move from use to abuse. More testing with CP-154,526 is necessary before theses aims can be realized but these data represent a promising step in this direction.

Possible Future Directions

The RIIβ knockout remains an interesting model for the study of drug sensitization. Given the enhanced sensitivity to both ethanol and amphetamine, it stands to reason that RIIβ knockout mice will show enhanced sensitivity to sensitization when other drugs of abuse are administered. Ethanol is known for its wide-ranging effects on a variety of neurotransmitter systems. The use of drugs of abuse with more specific mechanisms of action in the examination of cross-sensitized responses to ethanol might help to elucidate the role of PKA signaling in ethanol-induced locomotor sensitization. Alternatively, the rescue of PKA signaling via the expression of RIIβ subunit via viral vector technology will provide a powerful tool in assessing the candidate brain regions involved in ethanol-induced locomotor sensitization as well as further validate the model.

The present findings demonstrating the ability of CP-154,526 to block the expression of ethanol-induced locomotor sensitization suggest a role for the activation of CRF1 receptors in sensitized locomotor behaviors. However, the neuroanatomical location of these rather diffusely expressed CRF1 receptors remains unclear. The hypothalamus, amygdala, nucleus accumbens, and striatum have all surfaced as potential candidate regions and site-specific treatment with CP-154,526 may uncover a regional specificity in CP-154,526's

effect on the expression of ethanol-induced locomotor sensitization. The difficulty of these experiments would be compounded by the possibility that CRF signaling in a concert of brain regions is critical to the expression of sensitized behaviors. Regardless, regional specificity of CRF1 signaling has been demonstrated in research investigating ethanol drinking in dependant rats and should be possible in ethanol-sensitization studies as well.

In summary, these data suggest a novel role for the involvement of PKA activity in the acquisition of ethanol-induced locomotor sensitization. Additionally, a role for CRF receptor 1 signaling was discovered in the expression of ethanol-induced locomotor sensitization. Together these findings suggest new avenues of research which will lead to a greater understanding of the mechanisms involved in behavioral sensitization.

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