Locomotor behavior, immediate-early gene profiles, and gene expression associated with acute and repeated injections of ethanol in mice.

A dissertation

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List of Abbreviations

IEG immediate early gene

Cg1 cingulate cortex area 1

Prl prelimbic cortex

NAc nucleus accumbens

Lat sept. lateral septum

BSTLP bed nucleus of the stria terminalis, lateral portion

MPA medial preoptic area of the hypothalamus

LH lateral hypothalamus

BLA basolateral amygdala

CeA; CeC, CeL, CeM central nucleus of the amygdala: capsular, lateral, and medial portions

Prh perirhinal cortex

Ect ectorhinal cortex

S2 secondary somatosensory cortex

VTA ventral tegmental area

EW Edinger-Westphal nucleus

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Abstract

This body of work examines the 1. locomotor behavior associated with repeated ethanol injections, 2. brain regions activated by this paradigm, and 3. genes associated with an ethanol-sensitized phenotype. To this end, two main experiments were performed. The first examined the time-course of behavior of DBA/2J (D2) mice in a fifteen-day repeated alcohol paradigm and the concurrent activation of various brain regions during this time-course. Previous work has shown that after a period of eleven-thirteen days of daily alcohol injections D2 mice show an increased motor response relative to mice given a single injection of ethanol (Phillips et al. 1994). Furthermore, work from this lab has shown that concurrent with a single injection of ethanol is an increase in c-Fos expression in the central nucleus of the amygdala (CeA; Hitzemann and Hitzemann, 1997). However, the brain regions activated by repeated ethanol administration in D2 mice are not well defined. Additionally, measurement of c-Fos expression to gauge regional activity following repeated drug application is thought not to be accurate, as levels of this protein tend to habituate with repeated perturbation. Recent literature has instead implicated another immediate-early gene (IEG), delta Fos B, whose levels correlate well with sensitized locomotor behavior following repeated cocaine administration to rats. This IEG has also been shown to accumulate in the nucleus accumbens (NAc), a brain region associated with the motivational and motor aspects of drug-taking. Thus, our hypothesis for the first experiment was that 1. mice given ethanol repeatedly would show a sensitized motor response, 2. c-Fos protein levels would habituate in these mice, and 3. increased Fos B expression would be observed in

the NAc. We were unable to observe ethanol-sensitized locomotor behavior in mice repeatedly-injected with ethanol. Furthermore, *both* c-Fos and Fos B expression were significantly *increased* in the NAc and basolateral amygdala, when compared to protein levels in these brain regions in mice acutely injected with ethanol.

By using the genetic diversity found in a heterogeneous stock (HS) of mice, we obtained two distinct behavioral phenotypes following an ethanol-sensitization paradigm: one that demonstrated locomotor sensitization to repeated ethanol administration, and one that did not. Staining for c-Fos and Fos B was performed in order to visualize regional activation following this paradigm. c-Fos expression in CeA was significant and evident in mice injected acutely with ethanol; however, mice injected acutely with ethanol did not demonstrate an increased motor response relative to saline control mice. In this mouse strain, acute ethanol-induced motor activity was not correlated with c-Fos expression in the CeA. Furthermore, Fos B expression was not significant between any of the groups, including sensitized and non-sensitized mice. In a third experiment, fos b (mRNA) regulation was examined via microarray analysis. Gene expression analysis also confirmed the lack of fos b regulation between sensitized and non-sensitized mice, thus mirroring the immunohistochemical results. However, differences in the expression of other transcription factors and potassium channels were noted between the two phenotypes.

It is possible that these differentially regulated transcripts would make better markers for sensitized and non-sensitized behaviors, however, confirmation analyses remains to be done. In closing, increases in Fos B expression following repeated ethanol administration were dependent on the mouse strain examined, and were localized to the basolateral amygdala and specifically, the core of the accumbens.

Chapter 1: Introduction

The set of experiments described in this document build upon prior studies examining ethanol-induced locomotor stimulation and sensitization in mice (e.g. Phillips et al. 1994; Hitzemann and Hitzemann 1997). The experiments also attempt to link sensitized behavior with regionally-specific patterns of brain activation as assessed by immediate-early gene expression profiles. The sections below describe the relationship(s) between sensitization and genetic factors as well as pharmacological profiles. Immediate-early gene expression is also discussed as it pertains to the brain regions involved in acute and chronic drug administration. Additionally, the putative neurotransmitter systems in the nucleus accumbens and the central nucleus of the amygdala thought to be involved in the sensitization response are briefly discussed. Finally, data from gene expression studies are presented as a method to complement other traditional genetic mapping approaches for multigenic traits such as alcoholism. By understanding the changes that occur in the brain with repeated alcohol use, we have the potential to develop more effective drug therapies that alone or when used in conjunction with counseling, may help to control the disease.

I. Sensitization

A. Definition of Sensitization

Numerous studies using rodents have observed an increase in motor activity following an acute injection of a psychostimulant (e.g. cocaine and amphetamine), an analgesic (e.g. morphine) and a sedative-hypnotic (e.g. ethanol and benzodiazepines) (Epstein and Altshuler, 1978, Broderick, 1991; Kalivas and Duffy, 1987; Papanicolaou and Fennessy, 1980; Hitzemann and Hitzemann, 1999). This increase in locomotor

behavior with an acute drug injection is significant when compared to the motor behavior of saline-injected, or non-drug, controls. Sensitized locomotor behavior can also be observed when these different classes of drugs are given repeatedly (Epstein and Altshuler, 1978; Shuster et al. 1975; Masur et al. 1986; Lister, 1987). With sensitized behavior, repeatedly-injected subjects demonstrate increased motor behavior in comparison to subjects that are acutely-injected with drug. Sensitization is a useful behavior to study as it can reveal the neuroadaptations that occur with chronic or repeated drug use. Recent literature has further strengthened the utility of understanding sensitization-related behaviors as they have been found to co-occur with increased drug craving in humans and rodents (Robinson and Berridge 2001; Lu et. al., 2002); in rodent models increased drug craving can be quantified as increased approach behaviors for drug-associated cues, or increased drug-seeking behaviors (See 2002).

B. Data from human studies: ethanol-induced and psychostimulant-induced sensitization

King and Schuster (2002) examined sensitization to ethanol using a group of high-drinkers [HD] (n=20, 16 men, 4 women; criteria: at least 10 drinks per week, with subjects having at least 5 drinks on any one occasion) and a group of light-drinkers [LD] (n=16, 10 men, 6 women; criteria: fewer than 5 drinks a week, with never more than 3 drinks at any one time). Sensitization was measured in this study via use of the BAES scale (Biphasic Alcohol Effects Scale) where subjects reported how they were feeling (for stimulation: elated, energized, talkative; for sedation: slow thoughts, sluggish, heavy-headed) at baseline, and at various time intervals following consumption of either a 0.4 g/kg, or a 0.8 g/kg dose of alcohol, or placebo. There were three subgroups within the HD and LD groups; each subgroup received a particular dose of ethanol or placebo in two

separate sessions. The doses were 0.4 g/kg of ethanol for the first subgroup, 0.8 g/kg of alcohol for the second subgroup, and placebo for the third subgroup. Subjects in the HD group showed greater stimulation as measured by BAES during the first 15 minutes following alcohol consumption. The effect was moderately higher with the larger dose of alcohol and was significant compared to the results from the LD group. Furthermore, the HD group showed less sedation on the descending limb of the blood alcohol curve than the LD group. Blood alcohol levels were similar between the HD and LD groups at each dose of ethanol. The authors proposed that the increase in stimulation and the decrease in sedation among individuals who consume greater quantities of alcohol might be a predisposing factor for developing alcoholism in their later years.

Newlin and Thomson (1990, 1991, and 1999) have also examined human alcohol-induced sensitization. Because alcohol has biphasic effects with stimulation appearing soon after beverage consumption (peaking after a half-hour) followed by a depression in motor activity, these investigators have stressed the importance of time in observing sensitization. These authors compared subjects who are at high-risk (family history positive for alcoholism, FH+) and at low-risk (FH-) for developing alcoholism. Subjects were not matched for their own previous drinking history. Regardless, the authors demonstrated that sons of alcoholic fathers (FH+) exhibited sensitized motor activity following three sessions with a 0.5g/kg dose of alcohol. This is in contrast to the FH- group that did not show sensitization. Additionally, FH+ subjects had sensitized finger pulse amplitude responses, while FH- subjects did not. FH+ subjects also showed an increase in sympathetic activity as measured by increases in heart rate, finger temperature, and cheek temperature. The authors interpreted these data as evidence of

increased arousal. There was a trend for tolerance to these measures in the FH- subjects. Overall, the authors concluded that the high-risk group responded with greater stimulation while the low-risk group responded with greater tolerance to specific behavioral and physiological measures of alcohol-related behaviors. Thus, these studies showed that an increased physiological response to ethanol was associated with a higher risk to develop alcoholism later in life.

Human data are also available for sensitization induced by psychostimulants. For instance, Strakowski and Sax (1998) demonstrated sensitization in eyeblink rate and activity/energy ratings in subjects that had no prior stimulant use and after three oral amphetamine administrations (0.25 mg/kg). This finding was significant in comparison to subjects given placebo. Imaging studies in humans have shown that when addicts were exposed to drug or to drug-associated cues, areas that receive projections from the VTA A10 dopamine neurons were activated (for a review see Robinson and Berridge, 2001). Two brain regions that showed consistent and large differences in activity were the nucleus accumbens and the amygdala, in both cocaine as well as in heroin addicts (as measured by changes in cerebral blood flow by magnetic resonance imaging [MRI]).

Studies examining sensitization in human samples show not only heightened motor responses but also sensitivity to similar drugs as evidenced by increases in neuronal activity. For example, Adinoff et al. (2001) used single photon emission computed tomography (SPECT) to show increased blood flow in the orbitofrontal cortex of cocaine addicts in response to an intravenous procaine challenge of 1.38 mg/kg, when compared to healthy, age-matched controls (Adamec et al. 1985). When given placebo, cocaine addicts showed decreased activity in this brain region as compared to controls.

Thus, in addicts that have used cocaine repeatedly, a heightened response to drug (i.e. procaine, an injectable anesthetic; trade name novocaine) was visible in the frontal cortex, while at baseline this area of the brain was hypoactive. Additionally, experimenters questioned subjects on their mood and feelings following drug administration via use of a drug assessment questionnaire. Addicts reported significantly more "good effects" following drug injection when compared to controls. Both control and addicted patients reported similar feelings with regard to the negative emotions induced by procaine injection (i.e. anxiety, depression, obsessive-compulsive symptoms). Thus, higher regional activity in orbitofrontal cortex was correlated with an overall positive mood in the experimental subjects following drug injection. Experimental subjects in this study had a seven-year average time of use for cocaine abuse and had abstained from drug use for 14-28 days prior to the start of the study.

When linking these clinical findings to work described in subsequent chapters it should be noted that heightened regional activity within the brain following drug injection does not always translate into heightened motor activity. This point will be discussed in further detail in the immediate-early gene section of the introduction. However it is important to note that evidence for sensitization is observed in clinical samples and thus the phenotype is not rodent-specific.

C. Genes underlying alcohol-related behaviors

1. Data from human studies

Support for a genetic basis for alcoholism has been demonstrated using linkage analyses. Briefly, lower frequencies of the isoforms of alcohol dehydrogenase enzymes (ADH) have been linked to a lower incidence of alcoholism in samples from East Asian

populations (Dick and Foroud 2003). Similar results have also been found in some European populations (Dick and Foroud 2003). The ADH isoforms are localized to chromosome 4q22 (see Dick and Foroud 2003--a good review for candidate genes involved in alcoholism in the human population). Low frequencies of acetaldehyde dehydrogenase class 2 enzymes (ALDH; responsible for the conversion of acetaldehyde to acetic acid) are in contrast, associated with lower rates of alcoholism; the gene for this enzyme is localized to chromosome 12. Additionally, Crabb et al. (2004) have shown that inheriting the high-activity isoform of ADH (ADH2*2) and the inactive isoform of ALDH (ALDH2*2) are correlated with a reduced risk of alcoholism. The beta-1 (chromosome 4) and beta-3 (chromosome 15) subunits of the GABA-A receptor have also been linked to alcoholism via studies in Southwestern American Indians and in studies examining the linkage between endophenotypes (i.e. event-related potentials and electroencephalograms in families of alcoholics) and incidence of alcoholism. Specifically, lower frequencies of the GABA-A beta 3 subunit have been associated with a greater severity of alcoholism (Dick and Foroud 2003).

Studies in humans concerning linkage effects of the dopamine D2 receptor, the dopamine transporter, and the serotonin transporter have produced mixed results (Dick and Foroud 2003). The variance among the studies has been postulated to arise from inconsistencies in classifying patients and subtyping them (Dick and Foroud 2003). Overall, alcoholism is a common multigenic disease afflicting up to 8% of the population (http://www.niaaa.nih.gov/faq/faq.htm). This profile suggests that the allele frequency of the alcoholism-associated genes will be high and difficult to detect.

2. Genetics underlying alcoholism, particularly sensitization, using rodent models

The C57BL/6J (B6) and DBA/2J (D2) mouse strains are two of the most commonly used strains in behavioral genetics research. D2 mice have a greater acute locomotor response to an intraperitoneal (i.p.) injection of ethanol (1.5-2 g/kg), when compared to saline-injected controls and acute-ethanol injected B6 mice (Hitzemann and Hitzemann 1997, Phillips et al. 1994, Crabbe et al. 1982). Use of the progeny from reciprocal D2xB6 crosses (i.e. F1, F2, and backcrosses), as well as a diallel cross from four inbred mouse strains have also provided evidence for an additive genetic influence on initial sensitivity to ethanol's locomotor stimulating effects (Dudek and Tritto, 1994; Dudek et al. 1991). Additionally, with repeated injections of ethanol (1.5-2 g/kg), D2 mice have been shown to sensitize to a greater extent than B6 mice (Phillips et al.1994, Lessov et al. 2001). Furthermore, this difference in reactivity to repeated ethanol between strains is not attributable to different blood ethanol concentrations. It is also worth noting that the greatest stimulant response following an acute as well as repeated ethanol injections is observed in the first five-to-fifteen minutes due to the biphasic nature of the ethanol response (i.e. both stimulant and depressant effects; Phillips et al. 1994). Furthermore, unlike the associative effects seen with psychostimulants, repeated ethanol administration in a specific context does not seem to produce conditioned locomotion following an acute injection of saline (Phillips et al. 1994, Broadbent et al. 1995).

D2 and B6 mice were used as the progenitor strains to create the BXD panel of recombinant inbred [RI] strains (Morse et al. 1979). The BXD RI strains have been both genotyped with >2000 markers and phenotyped for a wide variety of alcohol-related responses (Browman and Crabbe, 2000; Crabbe et al. 1999). Thus, the utility of the BXD

RI panel lies in being able to a.) detect genetic correlations among phenotypes and b.) detect putative quantitative trait loci (QTLs) without genotyping the animals. Phillips et al. (1995) used this panel to examine the QTLs associated with acute and chronic ethanol-induced locomotor behaviors. Two days of habituation to the testing environment and saline injections were included in the experiment, followed by a third day with an acute ethanol injection of 2 g/kg. As expected, the B6 strain showed no significant stimulation in activity while D2 mice had significantly increased motor activity with respect to both their saline counterparts and the B6 mice. Additionally, strains 8, 25, and 14 had levels of activity similar to D2 mice, while strains 24, 22, and 1 were similar to B6 mice in terms of locomotor activity. The analysis showed that loci on chromosomes 3 and 10 were positively associated with acute stimulation (i.e. the phenotype that was associated with these loci was similar to that of D2 mice), and loci on chromosomes 11, 12, 17, and 18 were negatively associated with this phenotype (i.e. the phenotype that was associated with these loci was similar to that of B6 mice). On odd numbered days from days 4 until 9, mice were injected with 2 g/kg of ethanol or saline. On day 11, testing for sensitization to the motor response was performed. For instance, BXD strains 23, 18, and 9 demonstrated marked sensitization. Loci on chromosomes 1, 8, and 10 were positively associated with sensitization. Another locus on chromosome 10 was negatively correlated with ethanol sensitization. The researchers went on to explore the correlation between the different activity measures (saline-, acute-, and repeated-ethanol induced activity) and found no significant correlations between any of the measures, concluding that neither saline-induced activity nor acute-ethanol induced activity were adequate

predictors of sensitized activity. This paper also supported an inverse genetic correlation between ethanol consumption and locomotor activity.

Cunningham in 1995 also analyzed QTLs for ethanol-induced sensitization using some of the same BXD RI strains. The dose of ethanol used in this paper was the same as above, 2 g/kg, and was administered four times. Cunningham found loci on chromosomes 2, 12, and 15 that were associated with the sensitized phenotype. The locus on chromosome 2 seemed the strongest, as it remained significant even when the data was corrected for activity following a saline injection. Palmer et al. (2002) also examined the commonalities between QTLs associated with allopregnanolone-induced activity and ethanol-induced activity. Allopregnanolone is a neurosteroid that is increased in brain following ethanol administration. Additionally, both compounds can have stimulant effects, depending on the dose used (Khisti et al. 2002, Finn et al., 2004). This analysis detected a QTL on chromosome 2 in a similar region as that found by Cunningham (1995) and a QTL on chromosome 17 in a similar region as that found by Phillips et al. (1995). While further fine-mapping studies will reveal the identity of the genes located in these intervals and functional studies will reveal causation between behavior and genes, it can be inferred that these QTLs can affect more than one type of stimulatory behavior.

D. Neural substrates implicated in ethanol- and psychostimulant-induced sensitization

Most of the sensitization studies from the Phillips lab have examined sensitized behaviors using ethanol doses in the range of 2-2.5 g/kg in either D2 or HS (heterogeneous stock) mice. Itzhak and Martin (1999) have shown that when Swiss Webster mice (an outbred strain) were given ethanol (2 g/kg) once a day for five days in their home cage, followed by a ten-day drug free period, and then challenged with 2 g/kg

ethanol, the mice demonstrated locomotor sensitization. The sensitization behavior was significant, relative to mice pretreated with saline and then challenged with ethanol. In this same paper, a separate set of mice were treated for five consecutive days with cocaine (20 mg/kg), and then following the ten-day drug-free period, challenged with ethanol. These mice also showed heightened activity relative to saline-pretreated mice challenged with cocaine. Cross-sensitization was also evident in the opposite situation such that mice pretreated with ethanol for five days, withdrawn for ten days, and then challenged with cocaine also showed heightened locomotor activation relative to saline pretreated mice challenged with cocaine. Thus, the two drugs were postulated to have some shared mechanisms of action, such as activation of the dopaminergic system. Lessov and Phillips (2003) showed somewhat similar results using HS female mice. The HS strain was formed by crossing eight different inbred strains of mice (McClearn et al. 1970). In this experiment, mice were repeatedly treated with cocaine (10 mg/kg) and demonstrated cocaine sensitization as well as cross-sensitization to a 2 g/kg ethanol challenge. However, Lessov and Phillips (2003) were unable to show cross-sensitization in the opposite situation; ethanol-sensitized mice did not cross-sensitize to the activating effects of a challenge dose of cocaine (either 5, 10, or 20 mg/kg). It should be noted that a slightly different paradigm was used by Lessov and Phillips (2003) than that used by Itzhak and Martin (1999); mice were repeatedly injected with drug for 12 days as compared to 5 days, and for 10 days out of the 12, a dose of 2.5 g/kg ethanol was used. Lessov and Phillips (2003) explained their results by implicating cocaine's associative effects. Cocaine-pretreated mice did not receive drug injections every day (in contrast to ethanol-treated mice) and on days that cocaine was administered, exposure to the activity

chambers took place for the measurement of motor scores. This was in contrast to ethanol-pretreated mice that were not exposed to the test apparatus following every single drug injection. Thus, there was no development of additional contextual conditioning with ethanol that might be important for cross-sensitization to cocaine. Additionally, the sensitization paradigms employed by Lessov and Phillips (2003) did not include a drug-free period, in addition to using a different mouse strain; these procedural variances might also contribute to the discordant findings.

Camarini et al. (2000) investigated the effects of an opioid antagonist on the development and expression of ethanol sensitization using three-month old Swiss-Webster male mice. In the development part of the study, naloxone (1 mg/kg; a non-selective opioid receptor antagonist) or saline pretreatment preceded an ethanol injection (2 g/kg) by 30 minutes for twenty-one days (habituation to the activity chambers was performed the week before). From days 21-24, only ethanol was given to this group followed by a challenge injection of the drug on day 25. The naloxone-pretreated group did not demonstrate ethanol sensitization, when compared to the saline-pretreated mice. The authors concluded that opioid receptor activation modulated the development of ethanol sensitization. To address the question of whether or not opiate receptor activation was required for the expression of the ethanol-sensitized response, mice were treated with either saline-saline or saline-ethanol for twenty-one days. On the twenty-second day, mice received either: saline pretreatment followed by ethanol, or naloxone pretreatment followed by ethanol. Activity was then measured. The authors found that naloxone administration on the challenge day did not affect the expression of ethanol sensitization.

Glutamate receptors are also thought to mediate psychostimulant-induced sensitization. For example, Gronig et al. (2004) administered amphetamine (1 mg/kg) to rats every other day for seven days. In one set of rats, amphetamine was paired with specific odor and contextual cues. On alternating days, the same set of rats were treated with saline and placed into their home cages. A second group of subjects had saline paired with the odor and context cues, but amphetamine was paired with placement into the home cage. On the test day (day eight), all rats were exposed to the odor and context cues. Half of the subjects in each group were pretreated with MK-801 (a non-competitive NMDA antagonist; 0.1 mg/kg) thirty minutes before the administration of amphetamine. The investigators observed that MK-801 attenuated locomotor behavior in the amphetamine/context-conditioned group but not in the group that received amphetamine paired with the home cage. Additionally, the researchers observed that dopamine levels in the nucleus accumbens were decreased in the MK-801 pretreated group of rats. MK-801 did not affect locomotor activity induced by an acute amphetamine injection, nor did it induce any significant locomotor effects on its own. Thus, MK-801 inhibited associative, amphetamine-induced sensitization behavior.

GABA agonists, particularly those that act at the $GABA_B$ receptor have been shown to inhibit the development and the expression of ethanol-induced sensitization in D2 mice. When baclofen, a $GABA_B$ agonist (5.0, 6.25, or 7.5 mg/kg), was administered in conjunction with ethanol (2 g/kg, once a day for four days), ethanol sensitization did not develop (Broadbent and Harless, 1999). This was in contrast to the findings with a $GABA_A$ receptor agonist, THIP. When this compound was administered with ethanol there were no effects on either the development or the expression of sensitization.

However, it should be noted that both agonists blocked the acute response to ethanol when administered on the first trial. The authors further demonstrated that neither of the compounds had effects on the metabolism of ethanol, thus the mechanism was purely pharmacodynamic.

The hypothalamic-pituitary-adrenal (HPA) axis has also been shown to mediate drug-induced sensitization. Male rats that were adenalectomized (ADX) and administered nicotine (0.4 mg/kg) for fifteen days exhibited significantly lower motor activity, when compared to rats that were sham-operated and treated with repeatedly with nicotine (Molander and Soderpalm, 2003). Local infusion via reverse microdialysis into the accumbens of apomorphine (100 uM), a dopamine D1 and D2 receptor agonist, also fostered cross-sensitization in the sham-operated/nicotine group. There was no significant motor effect of apomorphine in the ADX/nicotine group. Thus, several neural substrates, in addition to genetic factors, paradigm duration, and the type of procedure used have been found to influence drug-induced locomotor sensitization.

II. Immediate-early genes as a mapping tool for the brain regions activated by drugs of abuse

A. Using the immediate-early gene c-Fos to map the regions associated with acute drug administration.

Morgan and Curran (1987) appear to have been the first to use a c-Fos mapping strategy to determine the brain regions affected by acute pentylenetetrazole (a convulsant) treatment. The mapping strategy can utilize either gene expression (in situ hybridization) or protein expression (immunocytochemistry [ICC]). c-Fos is the prototypical immediate-early gene (IEG). c-Fos typically binds to a jun protein to form

an AP-1 complex (Marota et al. 1992). The AP-1 complex may then bind to the AP-1 site located in the promoter region of certain genes (Nestler 2001a). It is here that the complex interacts with RNA polymerase and other gene-regulatory proteins to affect gene transcription. c-Fos formation and its down-stream effects on gene transcription are some of the many neuroadaptions induced by both acute and chronic administration of drugs of abuse (Wilce et al. 1994). Additionally, c-Fos can be induced by a variety of stimuli such as stress, action potentials in neurons, and the application of numerous pharmacological agents. Thus it is important in any experiment to carefully control external influences so as to insure that the observed effects on c-Fos are actually due to the treatment in question (Ryabinin et al. 1995).

Morales et al. (1998) found that c-Fos was expressed in amygdalar neurons following acute ethanol administration (2 g/kg) to Sprague-Dawley rats. These investigators went on to further demonstrate that c- Fos was expressed in glutamic acid decarboxylase (GAD; the enzyme involved in GABA synthesis) positive neurons. Hitzemann and Hitzemann (1999) demonstrated similar findings in D2 mice with another sedative-hypnotic drug, chlordiazepoxide (CDP). CDP, like ethanol, causes behavioral stimulation in D2 mice at low to moderate doses. c-Fos was expressed in GAD-positive neurons in the central nucleus of the amygdala (particularly lateral and medial portions). Additionally, with specific doses of acute ethanol (0.25-4g/kg), increased c-Fos expression was observed in CeA of D2 mice. This increase in c-Fos expression is significant compared to levels seen in saline-treated D2 mice, and B6 mice treated with the same dose of acute ethanol (Hitzemann and Hitzemann, 1997). Thiele et al. (1997) examined c-Fos expression in ethanol-preferring (P) and non-preferring (NP)

selectively bred rat lines (Penn et al. 1978) following administration of an acute i.p. saline injection or 1 or 3 g/kg ethanol (25% w/v). Significant differences in c-Fos expression in specific brain regions between preferring P and NP rats were observed. At 1 g/kg ethanol, P rats expressed more c-Fos positive nuclei in the nucleus accumbens core than NP rats. With 3 g/kg ethanol, NP rats had significantly more c-Fos positive nuclei in the locus coereleus (LC) and nucleus tractus solitarious (NTS). The only saline-induced c-Fos difference noted was in the NTS with c-Fos staining more abundant in NP rats than in P rats. The authors also used another set of selectively-bred rat lines; AA (alcohol-preferring) and ANA (alcohol non-preferring) rats, to examine differences in c-Fos expression following drug administration (Eriksson, 1968). No differences in c-Fos staining were noted between the two lines following either a saline injection or an injection of 1 g/kg ethanol. With 3 g/kg of ethanol, ANA rats had significantly more c-Fos positive cells in the nucleus accumbens shell and in the LC than AA rats. Thiele et al. (1997) concluded that neurons in the nucleus accumbens, LC, and NTS might contribute to differences in alcohol consumption between the selected lines. As an additional precaution, the experimenters also examined blood ethanol concentrations (BECs) and found no significant differences in BECs between the P and NP, or between the AA and ANA lines at any of the doses of alcohol. Interestingly, there were no significant differences in c-Fos staining between the selected lines in the amygdala. There is some research to support that different routes of administration with the same drug induce different regional activation with c-Fos staining (Ogilvie et al. 1998; Knapp et al. 2001). However, in the three studies cited above, all used i.p. ethanol administration. It is most likely the dose of alcohol, the varying genetic backgrounds, varying selection paradigms,

the brain region examined, and the interaction between these factors contributed to the lack of amygdalar activation.

Increased c-Fos staining has also been observed in alcohol-consuming B6 mice (10% limited access for a half-hour for eighteen days) as compared to sucrose and water controls. Specifically, increases were found in the nucleus accumbens core, the medial posterioventral portion of the amygdala, and the Edinger-Westphal (EW) nucleus (Bachtell et al. 1999). Mean blood alcohol levels for the alcohol group at the end of the limited-access paradigm were 1.0 ± 0.16 mg/ml. Bachtell et al. (1999) examined the expression of two other immediate-early genes products, Fos B and Zif268. Significant differences in the numbers of Fos B-positive neurons for the alcohol-consuming group were found only in the EW nucleus. No significant differences in staining were found with Zif268 in any of the brain regions examined, or between any of the groups. It is important to note that although locomotor stimulation is not generally observed in B6 mice following an acute, low dose injection of ethanol (Phillips et al. 1994, Phillips et al. 1995), c-Fos activation is still observed in mice that are trained to self-administer ethanol or that have been given an acute injection of alcohol (Bachtell et al. 1999). Furthermore, the brain regions "activated" after i.p. versus self-administration appear to be quite different.

There is some overlap in the regional activation seen with ethanol and those observed with acute doses of other drugs of abuse such as cocaine; however, due to different mechanisms of action, activation of similar brain regions to different extents, or even activation of entirely different brain regions is also evident. For instance, when examining the dorsolateral striatum (lateral portion of the caudate putamen) and the

nucleus accumbens core and shell regions, Barrot et al. (1999) found differences between the Fos-like immunoreactivity associated with an acute 2 g/kg injection of morphine and those associated with an acute, 15 mg/kg injection of cocaine in male Sprague-Dawley rats. With morphine, the number of c-Fos positive cells could be ordered from highest to lowest with shell > core = caudate. In contrast, after cocaine administration, the order was slightly different: shell = caudate > core. Additionally, the group went on to show that the shell demonstrated the greatest change from its baseline in terms of extracellular dopamine (DA) levels following systemic injection of either morphine or cocaine, as measured by microdialysis. Hope et al. (1992) administered cocaine (15 mg/kg) or saline to Sprague-Dawley rats and euthanized subjects one hour later. Northern analyses showed increased expression of c-fos mRNA in the nucleus accumbens (core and shell regions were not differentiated in this study) of cocaine-injected versus saline-injected controls. Acute cocaine treatment also produced an increase in locomotor behavior above that seen with a saline injection. Both Hope et al. (1992) and Barrot et al. (1999) inferred from their data that the increase in c-Fos expression was occurring in neurons receiving input from the A10 VTA dopamine neurons. In a related study, Heilig et al. (1993) examined the effect of an intra- nucleus accumbens c-Fos antisense oligonucleotide injection on cocaine-induced locomotor activation. The oligo was injected in the morning; approximately 8 hours later, Wistar rats were placed into activity chambers for habituation to the environment. Motor activity was monitored during this time, and analyses did not show any significant differences between groups (vehicle, c-Fos sense oligo, c-Fos antisense oligo). Thirty minutes later, cocaine (10 mg/kg) was given to all subjects. The group that received c-Fos antisense had significantly lower activity scores,

when compared to rats that received a vehicle injection, or an injection of c-Fos sense oligos. Thus, c-Fos modulated the locomotor response to acute cocaine. The above studies show that an increase in c-Fos expression can be used to identify brain regions affected by acute drug treatments, helping to link neural substrates with a particular phenotype.

B. The use of other chronic Fos-related antigens to map brain areas associated with repeated drug administration.

The Hope et al. (1992) study cited above also examined the effects of chronic cocaine administration (twice daily for 15 days with 15 mg/kg cocaine/injection) on c-Fos levels in the nucleus accumbens. Their analysis revealed that chronically-treated subjects had c-fos mRNA levels comparable to those of control subjects while acutely-injected subjects showed a significant increase in c-fos mRNA. Thus, c-fos mRNA habituated in the nucleus accumbens following chronic cocaine treatment. Studies with acute and repeated methylphenidate (MP) have yielded similar results (Brandon and Steiner 2003). Acute MP administration induced increases in both c-fos and zif268 in the rostral striatum, both dorsal and lateral portions, and caudal striatum, both dorsal and lateral portions. Following chronic MP treatment (10 mg/kg once a day for seven days) c-fos mRNA levels were similar to those found in chronic vehicle-treated rats. A separate group of rats that received an acute injection of cocaine following chronic MP administration showed reduced stimulation of c-Fos expression, when compared to subjects that received chronic vehicle followed by an acute injection of cocaine. Thus, c-fos mRNA levels habituated in the striatum following chronic drug treatment (Brandon and Steiner 2003). The authors also examined levels of dynorphin, the endogenous kappa

opioid receptor agonist, following chronic MP treatment. Dynorphin-containing neurons constitute the direct striatal pathway---one of the major outputs of the nucleus accumbens. Significant increases in dynorphin mRNA were found in chronic-MP treated rats vs. chronic-vehicle treated rats. Interestingly, these increases in dynorphin were found in the lateral shell of the nucleus accumbens, dorsal regions of the middle striatum, and the dorsal region of the caudal striatum. Thus, slightly different regions of the striatum are activated depending on the type of drug chronically administered and the type of IEG or peptide examined.

Ryabinin et al. (1997) also found habituation of the c-Fos response following chronic ethanol administration. Rats that were given 1.5 g/kg ethanol once a day for fourteen days showed c-Fos habituation in most brain regions examined, when compared to acute-drug controls. However, the c-Fos response did not habituate in the following brain regions: NTS, medial preoptic area, lateral hypothalamus, and paraventricular nucleus of the hypothalamus (PVN), implying sensitization of c-Fos in these brain regions with repeated ethanol treatment. Following repeated administration of a lower dose of ethanol (0.5 g/kg) to a separate group of rats, significant increases in c-Fos expression were observed in the following brain regions: nucleus accumbens core, basolateral amygdala, lateral septum, EW, and ventral tegmental area (VTA). This study also showed that a novel environment could cause an increase in c-Fos expression and that the hippocampus was particularly sensitive to changes in environment. Similar results were obtained by acute ethanol administration (3 g/kg), where increases in the number of c-Fos positive neurons were evident in: bed nucleus of the stria terminalis (BNST), EW, central nucleus of the amygdale (CeA), LC, parabrachial nucleus and the

PVN (Chang et al. 1995). With repeated administration (3 g/kg two times/day for seventeen or for twenty-four days), c-Fos habituation was observed in these same brain regions.

Clearly, several factors influence c-Fos habituation following chronic drug administration. These factors include the schedule of drug administration, the route of administration, the dose of drug used, and the genetic background of the subjects.

Emerging evidence suggests that another chronic Fos-related antigen may serve as a more definitive marker for the brain areas activated by chronic drug treatment. Chronic Fos-related antigens (chronic FRAs) appear in significant amounts following several applications of a stimulus and are slow to degrade, persisting even after discontinuation of the stimulus (Nestler et al. 1999). In contrast, acute FRAs such as c-Fos appear after a single application of a stimulus, and degrade within hours. A prototypical chronic FRA whose presence has been recently linked to the long-term effects of cocaine is delta Fos B. Delta Fos B is actually a complex of proteins, made up of 37, 35, and 33 kD proteins, and is similar to the Fos B protein. Fos B is an unusual FRA in that it appears in significant amounts after a single application of a stimulus, but like chronic FRAs, will continue to accumulate with repeated stimulus applications (Nestler et al. 1999). Like c-Fos and other members of the Fos family, delta Fos B and Fos B need to dimerize with another protein (predominantly Jun D) to form an active AP-1 complex that can affect the transcription of certain genes (Nestler 2001b). The increase in delta Fos B as seen with repeated injections of cocaine to rats (22.5 mg/kg twice daily, for 14 days; Hope 1994) and to mice (10 mg/kg once a day for six days; Werme et al. 2002, Colby et al. 2003) occurs in the nucleus accumbens and is colocalized in neurons

that contain dynorphin. However, it is not clear as to whether the increase occurs in the shell or the core of the nucleus accumbens. Coincident with this increase in delta Fos B is behavioral sensitization to cocaine (Kelz et al. 1999). Delta Fos B accumulation has also been shown to occur following 10 days of treatment with a low amperage shock (Chen 1997). Lewis rats exposed to running wheels for thirty days showed significant accumulation of delta Fos B in the nucleus accumbens, when compared to control rats that had access to locked running wheels (Werme et al. 2002). In this same experiment, mice that were genetically engineered to overexpress delta Fos B in dynorphin-containing neurons ran significantly more than mice that carried the transgene for delta Fos B, but whose expression was suppressed.

Additionally, the neuronal cell-type in which delta Fos B is expressed affects reinforcement. For example, Werme et al. (2002) found that mice that conditionally overexpress delta Fos B in enkephalin-containing neurons in the striatum (these neurons constitute the output for the indirect pathway from the striatum [Brandon and Steiner 2003]) ran considerably less than their counterparts whose expression of delta Fos B was suppressed. Suppression was maintained using doxycycline (dox) and a tetracycline-sensitive promoter. Most recently, this group demonstrated that delta Fos B overexpressing mice (in dynorphin-containing neurons) will bar press more for lower doses of cocaine compared to doxycycline-maintained controls, suggesting that specific doses of drug were more rewarding in this line of mice. Additionally, there was no difference between groups in the number of bar presses on the inactive lever (Colby et al. 2003). Furthermore, the overexpressing mice had a greater break-point in a progressive ratio schedule for low and medium doses (250 and 500 ug/kg given intraveneously) of

cocaine than dox-maintained controls. Together these results suggest that delta Fos B-overexpressing mice demonstrated a greater level of drug-seeking behavior than their control counterparts. Thus, there is a correlation, and in two cases, a causal relationship between the chronic FRA, delta Fos B, sensitization, and cocaine self-administration. Therefore, it is believed that this protein mediates the long-term effects of chronic drug (i.e. cocaine) intake and/or administration.

III. The role of the nucleus accumbens in drug abuse

A. Connectivity of the nucleus accumbens and involvement of accumbens dopamine in reward of natural reinforcers and drugs of abuse

The ventral tegmental area (VTA) has both dopaminergic (DA) and GABA-ergic projection neurons that ascend to the nucleus accumbens (NAc) and the prefrontal cortex (PFC). The dopamine projection from VTA to NAc is termed mesolimbic, while the one from VTA to PFC is termed mesocortical. The medium spiny neurons of the NAc and neurons of the ventral pallidum also send GABA-ergic projection neurons back to the VTA. Mu-opioid receptors are located on GABA projection neurons and interneurons in the VTA, and when activated (by the administration of morphine or a similar agonist), inhibit the release of GABA. Because intra-VTA GABA neurons synapse on DA neurons, DA neuronal disinhibition (in the case of of mu receptor agonists), and subsequent release into the NAc and PFC can then occur (as summarized by Leite-Morris et al. 2004). The NAc also receives glutamatergic input from many other brain regions such as the PFC, amygdala, thalamus, and hippocampus (summarized by Li and Kauer 2004).

The NAc's role in drug abuse has been extensively studied. For instance, rats learn to lever press for microinjections of dopamine, amphetamine and dopamine re-uptake blockers such as nomifensine into the NAc (Wise 1998). Lesioning the NAc can block or attenuate the effects of intraveneous cocaine (Pettit et al. 1984). Cocaine injections into the PFC have been shown to increase dopamine turnover in the NAc (Goeders and Smith 1993). An increase in dopamine concentration in the NAc of P rats was found following five i.p. ethanol injections (1 g/kg); dopamine levels were not significantly altered in NP rats (Smith and Weiss 1999). Similarly, P rats showed a decrease in serotonin levels (measured by no-net flux microdialysis) in the nucleus accumbens following this ethanol regimen; this is in contrast to NP rats that showed an increase in serotonin. Thielen et al. (2004) have demonstrated that local perfusion of a serotonin receptor agonist in the NAc decreases dopamine levels in ethanol-exposed P rats. Thus, the affected neurotransmitter systems in the NAc were hypothesized to contribute to ethanol preference or avoidance. Leri et al. (2003) observed, via microdialysis, a dose-dependent increase in accumbens DA following fourteen days of treatment with heroin (administered via mini-pump in doses of 3.0, 7.0, or 14.0 mg/kg/rat/day). This increase in accumbens dopamine was also correlated with sensitized motor activity, as demonstrated by a separate group of chronic-drug administered subjects.

Cannon and Palmiter (2003) have approached the DA-reward integration from a different perspective. These investigators found that dopamine-deficient mice[1] (DD mice;

[1] DD mice lack the tyrosine hydroxylase gene. Because they lack this gene, they cannot synthesize catecholamines (dopamine, epinephrine, and norepinephrine). Catecholamines are vital for visceral function, motor coordination, and arousal; while in utero, mothers carrying DD mice must be treated with L-DOPA to rescue the DD pups.

Zhou and Palmiter, 1995) were able to consume sucrose (a natural reward), and did so in a higher quantity than DD mice given water. However, the pattern of consumption of the DD mice was significantly different from that of wildtype mice given access to sucrose. DD mice had significantly longer bouts, and more licks per bout, but the bout number of DD mice was significantly less than that of wildtype mice. These data suggest that reward is mediated in part by neurotransmitters other than dopamine. Additionally, the authors suggested that the lack of dopamine in DD mice lead to a lack of goal-directed behaviors.

More recently, evidence has shown that the primary targets of some drugs of abuse are the cholinergic neurons that project both rostrally and caudally within the medial forebrain bundle (MFB). The cell bodies of the cholinergic neurons within the MFB arise from the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus, areas of the basal hindbrain (Ericson et al. 2003). The cholinergic neurons projecting caudally may be able to activate VTA DA neurons in a secondary fashion, while those projecting rostrally may activate forebrain regions (Wise 1998). Ericson et al. (2003) demonstrated that ethanol applied specifically into the NAc via reverse microdialysis, at a concentration of 300 mM, could elevate extracellular levels of dopamine within this brain region. Mecamylamine (100 uM), a nicotinic receptor antagonist, was able to block the ethanol-mediated increase in accumbens dopamine, when perfused into the VTA. Thus, ethanol's effects on extracellular dopamine levels were modulated by cholinergic input into the VTA. Furthermore, acetylcholine and dopamine within the NAc help to mediate the reinforcing actions of drugs of abuse.

B. Shell and core involvement in drug-induced sensitization

Pierce and Kalivas (1997) proposed a model depicting the different brain regions involved in sensitization (figure 1.1). The authors hypothesized that glutamatergic projections from the PFC to the NAc were strengthened, as was the dopaminergic projection from the VTA to the NAc; additional inputs into the NAc included GABA-ergic projection neurons from the ventral pallidum as well as dynorphin-containing neurons from the VTA (figure 1.1). However, the authors did not discriminate between the shell and the core divisions of the NAc in their sensitization model. The section below discusses in more detail the roles of these two subdivisions of the NAc.

The shell and the core are the two major divisions of the NAc. The core is a region located more dorsolaterally while the shell is located more ventromedially (as summarized by Lecca et al. 2004). The outputs of each of these two structures have led to the idea that the subdivisions are involved in different behaviors. For instance, the NAc core projects to more motor nuclei than the shell; its projections include the globus pallidus and the ventral pallidum. Shell projections include the VTA, lateral hypothalamus (LH), ventral portions of the ventral pallidum, and brainstem autonomic centers (Lecca et al. 2004). Because the shell receives input from PFC and projects to motor as well as limbic regions, it can use information about stimuli to "select and execute goal-directed behaviors" (Lecca et al. 2004).

Parkinson et al. (2002) examined some of the functional differences in function between shell and core. Rats were trained to associate a light (CS) with the presentation of sucrose (US) and approach behavior was measured. Pre-surgery, all rats had similar levels of approach behavior and learned that the CS predicted a food-reinforcer.

Following excitotoxic amino acid lesions, core-lesioned subjects demonstrated impairment in approach behavior to the CS, while shell-lesioned subjects showed no change. The CS (light) was then used as a conditioned reinforcer in an instrumental conditioning task in which the number of lever presses on the reinforced lever (reinforced for the appearance of the light that was previously paired with sucrose) and non-reinforced lever (on which presses did not result in any consequences) was compared between lesioned and sham groups. Amphetamine was also administered intracerebroventricularly (i.c.v.) during the instrumental conditioning task at various doses to separate groups of lesioned and sham subjects since it has been demonstrated that systemic amphetamine could potentiate responding for a conditioned reinforcer. Thus, the authors used amphetamine to manipulate accumbens DA levels and determine the effects on Pavlovian and instrumental conditioning. The results showed that shell-lesioned animals did not acquire lever-pressing for a conditioned reinforcer. However, the core-lesioned animals did, but in a non-specific manner as their responses on the non-reinforced lever increased with increasing amphetamine dose. Similarly, locomotor testing with acute doses of amphetamine revealed that shell-lesioned animals did not display increased locomotion, while core-lesioned subjects did. The authors concluded that the shell needed to be intact for animals to respond to motivationally-relevant stimuli and to demonstrate drug-relevant motor behavior. Furthermore, the importance of the core's role in Pavlovian paradigms was also demonstrated.

Di Chiara (2002) stated a concurring opinion noting that NAc shell DA levels were released coincident with the presentation of a conditioned stimulus that had reinforcing properties. A related study emphasized the functional difference between the

core and the shell. Lecca et al. (2004) selected rats for rapid and slow acquisition of active avoidance. Rats that escaped readily were termed high-avoidance or HA rats. The HA rats, as compared to low-avoidance (LA) rats, had a significantly greater dopamine efflux in the shell of the NAc than in the core and in response to the administration of morphine, amphetamine and cocaine. There were no basal differences between HA and LA animals in the amount of dopamine present in either of the compartments of the NAc. These drug-induced differences in dopamine efflux also correlated with ambulation measures for each of the acute injections of the drugs. Thus, the HA line had higher motor activity measures with each of the drugs as compared to the LA line. The results of the above two studies contrast with other studies citing increases in core dopamine coincident with behavioral sensitization.

Cadoni et al. (2003) also detected differential roles of the core and the shell by examining stress, which exacerbated the behavioral response to repeated psychostimulant administration. Rats were food restricted to 80% of their body weight for one week and then administered repeated doses of psychostimulants. The food-restricted rats exhibited significantly greater behavioral sensitization to cocaine and to amphetamine, when compared to ad-libitum fed controls. This was not the case with groups of food-restricted rats that had been given morphine or nicotine repeatedly (all drug injections were given either i.p. or subcutaneously (s.c.)). Similarly, experimental rats that showed significant increases in behavioral sensitization to psychostimulants also had increased extracellular levels of dopamine in the core, and a reduction of dopamine in the shell, as measured by microdialysis. The authors concluded that concurrent with behavioral sensitization was an increase in extracellular dopamine levels in the core of the nucleus accumbens.

IV. The role of the central nucleus of the amygdala and the extended amygdala in drug abuse

A. Anatomical and neurochemical composition of the central nucleus of the amygdala (CeA)

The central nucleus of the amygdala has three major portions: capsular (CeC), lateral (CeL), and medial (CeM). The CeC receives projections from a number of brain regions including the occipital, temporal, perirhinal corticies, and the parabrachial nucleus. The CeL sends projections to the lateral and ventral portions of the bed nucleus of the stria terminalis (BNST), and the CeM (thus, it is believed that information is processed in a lateral to medial fashion in the CeA). Fewer fibers from the CeL project to the caudal portion of the NAc, parvicellular paraventricular nucleus of the hypothalamus, and portions of the lateral and medial regions of parabrachial (PB) nucleus. BNST connectivity is also reciprocal. Additionally, the CeL possesses a heterogeneous collection of neuropeptides: corticotropin-releasing hormone (CRH), neurotensin, enkephalin, and somatostatin (Petrovich et al. 1996). Further studies have determined that CRH and GABA are localized within the same neurons, and that methionine-enkephalin (met-Enk) and GABA are also co-localized; however, CRH and met-Enk are not in the same neurons (Veinante et al. 1997). The CeL receives a large dopaminergic innervation from the midbrain tegmental area (Alheid et al. 1998). The notion of the extended amygdala came from developmental studies which showed that the BNST and CeA arose as one structure in the fetus, and were later divided into a rostral (BNST) and caudal portion (CeA) by the fibers of the internal capsule (Fudge and Emiliano 2003). The concept of the "extended amygdala" remains controversial. Swanson and Petrovich

(1998) assert that the extended amygdala is actually an extension of the striatum and cerebral cortex, and functions largely to relay olfactory and autonomic information (Swanson and Petrovich 1998).

B. Drug effects on amygdalar-related processes

In both humans and mice the CeA is activated by emotionally-relevant information. Dopaminergic innervation to the CeA is thought to "filter" incoming stimuli, especially those with high emotional valence. Similarly, images captured via magnetic resonance imaging (MRI) and then superimposed on assembled positron emission tomography (PET) scans showed significantly more activation in CeA and anterior cingulate in abstaining cocaine addicts than in non-drug users when both groups were exposed to a video containing drug-related cues. There was no difference between experimental and control groups in the activity of the visual cortex and other brain regions chosen for control purposes (Childress et al. 1999). Thus, these data suggested that the CeA in part, encoded emotionally-valent stimuli.

Numerous studies point to the involvement of the basolateral amygdala (BLA) and CeA in alcohol and drug abuse. Lesion studies have shown differential roles of the CeA and BLA in response to conditioned stimuli. The study of conditioned stimuli (CS) is important in drug addiction because many addicts return to environments previously associated with drug-intake; these environments may play a role in relapse. In non-human animal models of drug addiction, the CS can reinstate responding for a drug as well as reinstate other drug-taking behaviors. For example, BLA lesions decrease responding for a CS associated with food or sex and eliminated second order Pavlovian conditoning in rats. This was in contrast to the effects of CeA lesions that decreased conditioned

approach behaviors as well as conditioned orienting and conditioned emotional responses (See 2002; Petrovich et al. 1996). Thus, while the two brain regions have differential roles in conditioning, both areas could modulate drug-associated behaviors (i.e. conditioned behaviors) in animals following an extinction period.

There is evidence to suggest that levels of corticotropin-releasing hormone (CRH) in the extended amygdala are disrupted during drug withdrawal. Most of the CRH within the CeA is thought to be concentrated in the CeL (Petrovich et al. 1996). Elevations in the mRNAs for both CRH and its binding protein (CRH BP) have been observed one day following cocaine withdrawal. In the BNST, CRH BP mRNA was increased three days post-cessation of cocaine (Erb et al. 2004). It is thought that CRH dysregulation contributes to the withdrawal dysphoria associated with abrupt drug (cocaine and ethanol) cessation (Weiss et al. 2001). However, it is unknown at the present time whether this increase in CRH during withdrawal contributes to dependence or initiates drug-seeking or maintenance behaviors. Weiss et al. (2001) also observed an increase in CRH staining via immunohistochemistry (IHC) six weeks into withdrawal in a group of mice that were treated chronically with ethanol and another group that was chronically treated with cocaine. The increased CRH staining was greater than that observed at baseline in drug-administering animals and control, or non-drug experienced, animals. However, it is not known whether Fos B was increased in the neurons that expressed CRH. Furthermore, CRH R1 receptors in the CeA mediate GABA release and ethanol-induced potentiation of GABA's effects in this brain region (Nie et al. 2004).

Post-natal stress (which presumably affects the CeA) also affected ethanol consumption when rats were tested at ten weeks of age (Ploj et al. 2003). Pups that had

been separated from their mothers for six hours a day, once a day, for twenty-one days (post-natal days 1-21) consumed more ethanol (6% or 8% ethanol) than those not separated, or those that were separated for only fifteen minutes per day. When testing was performed at 10 weeks of age, ethanol consumption in the rats separated for six hours as pups produced changes in kappa opioid receptor density (as shown by autoradiography) in the amygdala, substantia nigra, and periaqueductal gray (PAG) (Ploj et al. 2003).

Changes in the glutamate family of receptors have also been observed in CeA after chronic drug administration. For example, cocaine given three times a day for either one day or for up to five days at a moderate dose of 20 mg/kg increased NMDA R1 mRNA in the CeA and BLA of male Wistar rats. Similarly, morphine given at the same dose but once a day for ten days increased NMDA R1mRNA three hours and forty-eight hours into withdrawal (Turchan et al. 2003). Additionally, NMDA receptor current density has been shown to be increased in the lateral amygdala/BLA following chronic ethanol consumption in Sprague-Dawley rats (liquid diet; average daily consumption 11.2 ± 0.4 g/kg). Coincidentally, NR1 subunit mRNA expression was increased in these animals (Floyd et al. 2003). Thus, long-term drug administration affects many of the neurotransmitter systems located within the CeA.

Research has shown that not all drugs that induce sensitization have similar effects in the amygdala. For instance, chronic ethanol treatment did not affect cannabinoid receptor 1 (CB1) levels in CeA (both protein and mRNA levels were measured). In contrast, chronic morphine increased mRNA levels of CB1, but reduced the overall binding of the receptor in this brain region (Gonzalez et al. 2002).

There is growing support for the interaction between the amygdala and NAc in understanding the adaptations to drugs of abuse. For example, blockade of GABA-A receptors in the amygdala with an antagonist while administering a GABA-A agonist, muscimol, in the NAc, shifts the dose-response curve to the right in a two-lever discrimination procedure, such that a higher muscimol dose was required to substitute for ethanol (Besheer et al. 2003). It is thought that changes in these two brain regions during alcohol dependence are long-lasting and contribute to reinstatement of drug-seeking behaviors (Koob, 2003). Thus, these brain regions are excellent candidates for observing changes in gene expression coincident with repeated drug administration.

V. Microarrays

cDNA microarrays are one of the most common types of microarrays. Short stretches of DNA oligomers, (25 bases on the Affymetrix array) are spotted onto the array by means of a photolithographic process similar to that involved in the manufacturing of computer processors (Shalon 1998). Microarray technology is superior to older methods for identifying changes in gene regulation (such as slot-blot analysis) as only thirty-to-fifty transcripts could be examined in one slot blot apparatus. Today's microarrays allow for the investigation of the regulation of thousands of genes simultaneously. However, due to the high probability of false positives, verification of microarray results is the next stage in analysis. The most commonly used method of verification is RT-PCR due to its sensitivity (ability to measure the presence of low abundance transcripts), and reproducibility (www.ambion.com/basics/rtpcr). Biotechnology companies are also continuously manufacturing newer versions of their microarray arrays by incorporating more specific target sequences for a given gene as

well including oligos for newly discovered genes. This raises the issue of comparing gene expression across the different versions of the microarrays. It has been found that reproducibility of the results is dependent on the abundance of the transcript (the higher the abundance, the greater the correlation across arrays) and sequence similarity (Nimgaonkar et al. 2003). There are also a number of programs designed to assess the amount of gene expression for a given transcript on a microarrays. Most differ by how they determine and subtract noise (background) from the signal (Barash et al. 2004).

Occasionally, changes in mRNA abundance are reflected in protein concentrations. For instance, Ang et al. (2001) found that the mRNA expression for nuclear factor kappa b (NFkB) subunit 105 in the striatum of delta Fos B overexpressing mice was nine times greater than the mRNA levels observed in littermate controls maintained on doxycycline. Western analysis was also used in this experiment to examine protein concentrations. There was a two-fold increase in NFkB protein in the accumbens of delta Fos B overexpressing mice, and a 1.5-fold increase of NFkB in the caudate, when compared to dox-maintained controls. In the ICR outbred mouse strain, there was also a 200% increase in the concentration of this protein following a chronic cocaine paradigm (20 mg/kg for two weeks) above the levels observed following acute cocaine administration. Additionally, two other transcripts have been found to be increased in delta Fos B overexpressing mice; they are cdk5 and p35. These transcripts may be capable of mediating some of the long-term neuroadapataions to chronic cocaine.

Arlinde et al. (2004) used eight microarray chips (allowing for high amounts of reliability) per brain region of interest to identify differential gene regulation between selected lines of AA (alcohol-preferring) and ANA (alcohol-avoiding) rats under basal

(non-treated) conditions. The investigators found 48 genes that were differentially expressed between the selected lines. Many of the genes that were increased in expression in the NAc of AA rats (shell and core not differentiated) were involved in the MAP kinase pathway. Additionally the group observed a significant decrease in expression in beta-arrestin, a cellular trafficking protein. In contrast, there was a significant decrease in the expression of cytoskeletal-related genes in the CeA. Overall, these authors also stressed that significant changes in gene regulation were numerically modest (under a two-fold change) suggesting that small changes in transcription may be functionally relevant.

Kwon et al. (2004) found a similar regulatory element in the promotor region of all ethanol-responsive genes of C. elegans. The authors stated that this model was similar in terms of behavior to drosophilia, mice, and even humans, when exposed to alcohol. For instance, stimulatory behavior could be observed soon after exposure, with ataxia and sedation behaviors following. C. elegans were cultured and then exposed to 7% alcohol (w/v) in 200 ml of buffer for different lengths of time. Six hours following ethanol exposure, a group of affected genes was identified and further analyzed. It was determined that all of the six-hour genes had the following sequence in the promoter region: TCTGCGTCTCT. This sequence partially overlaps with those of heat-shock associated proteins. The authors inferred that this sequence might predict the responsiveness of certain genes to alcohol. While further investigation needs to be done, it is an exciting development that could potentially aid in the rapid identification of genes that underlie alcohol-related behaviors. Additionally, these findings stress the importance of examining non-coding regions as they might impact differential expression.

Studies examining the effects of chronic applications of ethanol are finding that significant changes in gene expression do not occur in neurotransmitter receptors, but occur in genes affecting cellular signaling or transcription (Daniels and Buck 2002; Hoffman et al. 2003). Thus, microarray analysis allows for the identification and elaboration of genes that have not been examined or considered as candidates in the drug abuse literature.

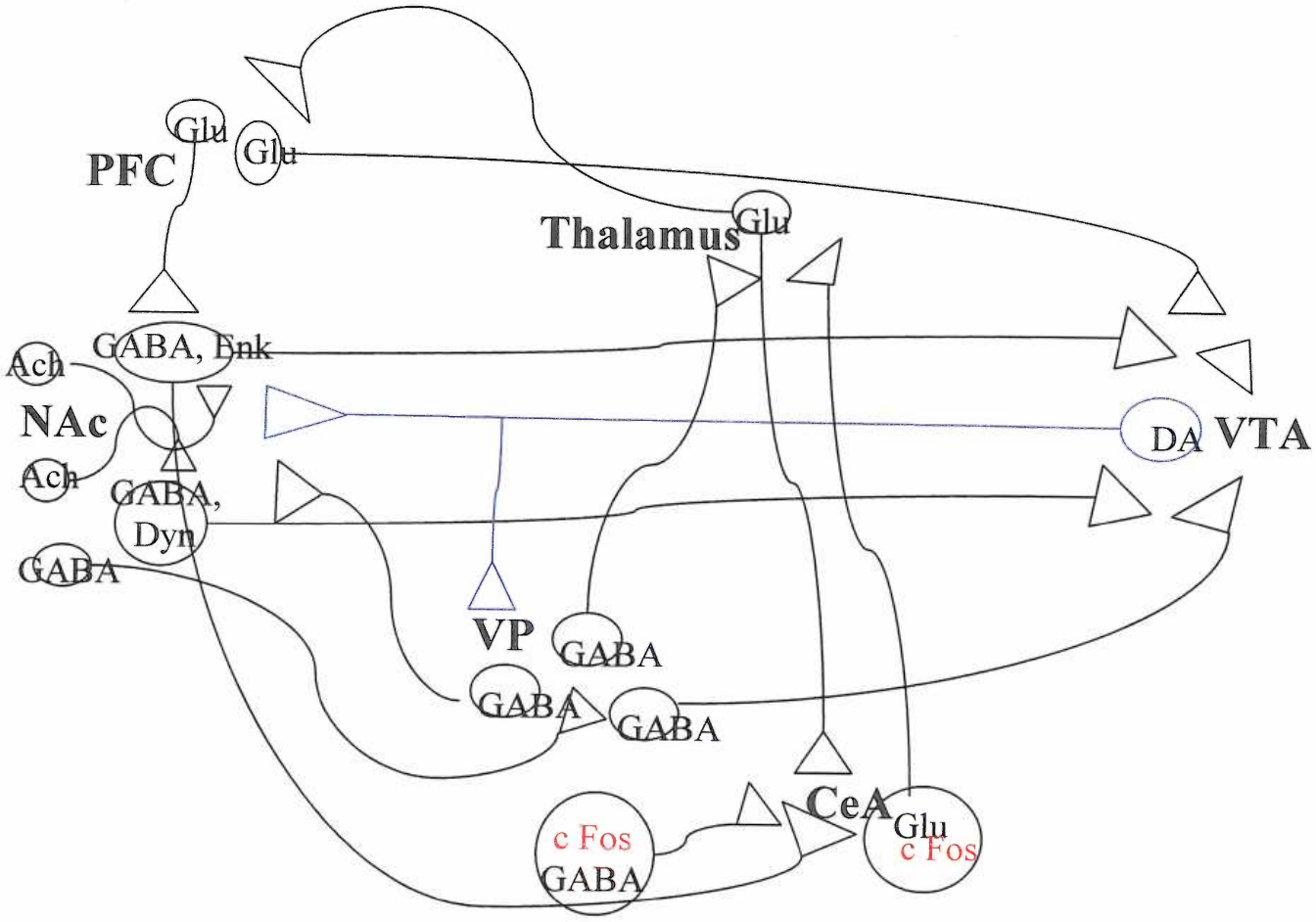
Many different types of microarrays are available today. For instance, pathway-focused arrays are custom-made arrays spotted with genes of interest implicated by previous studies in the research field. One major advantage of the pathway-focused array is that it helps to narrow down the amount of data generated by microarray analysis by focusing on a few key substrates. Pathway-focused arrays (PFAs) also provide a measure of the reliability of the hybridization procedure as researchers can compare results with those from broad-spectrum arrays. Konu et al. (2004) demonstrated the utility of the technology by using a PFA to identify specific components of the calcium-stimulated intracellular signaling pathway affected by a 48-hour nicotine exposure (1 mM) in a PC12 cell culture system. This system responds to the application of cholinergic agonists by secreting DA and other catecholamines. Affected messages included those that could be categorized into the following groups: protein modification/degradation/synthesis, cellular signaling, lipid metabolism/transport, and neuronal transmission. This research also verified the results found by a different set of investigators (Doornbos et al. 1999).

The following chapters detail IEG expression specifically in the NAc and BLA following both acute and chronic ethanol administration paradigms, and in different mouse strains. Additionally gene expression in the NAc from a subset of ethanol-

sensitized and non-sensitized mice was also examined in an effort to understand the neurochemical changes that were associated with these two ethanol phenotypes.

Figure 1.1 Activation of neurocircuitry by ethanol.

A. Acute ethanol administration.



B. Repeated ethanol administration.

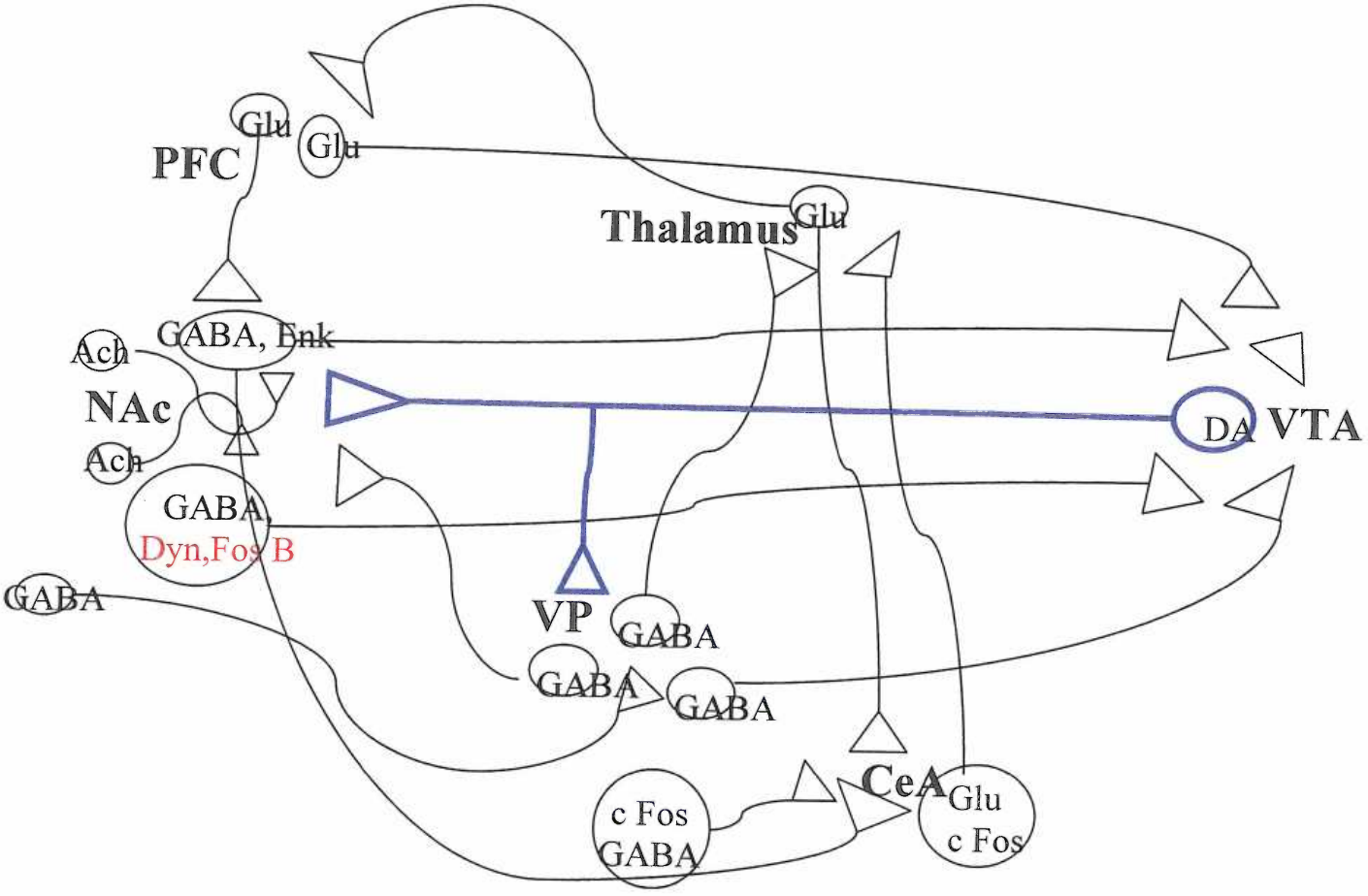


Figure 1.1 Activation of neurocircuitry by ethanol (a compilation of diagrams from: Zahm and Brog (1992) and Pierce and Kalivas (1997)). The ventral tegmental area (VTA) sends dopaminergic (DA) projection neurons to the nucleus accumbens (NAc) and ventral pallidum (VP). It is believed that with acute ethanol administration (panel a), locomotor stimulation is induced via the activation of VTA dopaminergic projections (Brodie and Appel, 2000), coincidental with an increase in c-Fos expression in many brain regions, particularly the central nucleus of the amygdala (CeA; Hitzemann and Hitzemann 1997, Hitzemann and Hitzemann, 1999). The VTA receives GABAergic projections from the NAc and VP, glutamatergic (Glu) projections from the prefrontal cortex (PFC) and cholinergic (Ach) projections from the pedunculopontine nucleus and lateral dorsal tegmental nucleus (not shown; Mesulam et al. 1983). The NAc is involved in behaviors directed towards obtaining natural reinforcers (Joseph and Hodges, 1990) and is believed to modulate the expression of psychostimulant-induced sensitized locomotion (Vezina 2004). In addition to receiving dopaminergic projections from VTA, the NAc also contains cholinergic interneurons and receives glutamatergic projections from PFC and GABAergic projections from VP. With repeated application of ethanol (panel b), increases in Fos B/delta Fos B are expected to occur in NAc while habituation of c-Fos expression is expected to occur within CeA. These neuroadaptions within the NAc may lead to increased firing of VTA dopaminergic neurons, and when summed together are thought to underlie ethanol-induced locomotor sensitization. Glutamatergic afferents from CeA project to the thalamus (Bauer et al. 2002). The VP and thalamus receive connections from limbic circuitry and thus provide a platform for limbic information to be translated into motor behavior (Zahm and Brog, 1992).

Chapter 2: Behavioral and immediate-early gene profiles associated with acute and repeated ethanol injections in DBA/2J male mice.

2.1 Abstract

Background: Sensitization is defined as an increased response to repeated administrations of a stimulus. It has been shown that repeated administration of drugs such as cocaine and amphetamine (both psychostimulants) as well as ethanol (a sedative-hypnotic) produce locomotor sensitization in rodents. In this study we investigated the neurobiological changes associated with repeated ethanol administration using DBA/2J (D2) mice.

Methods: Ethanol was administered intraperitoneally (i.p.) at a dose of 2 g/kg on days 3-15 in a fifteen-day sensitization paradigm (days 1 and 2 were saline habituation days). Behavioral testing for locomotor activity was performed using the Flex Field system (San Diego Instruments) on days 1, 2, 3, 6, 9, and 15. On days 3, 6, 9, and 15 groups of mice were euthanized one hour post-injection and brain tissue harvested. Immunohistochemistry (IHC) for the immediate early genes c-Fos and Fos B was performed on 30-um sections of brain tissue.

Results: Statistical analyses revealed an acute stimulatory effect of ethanol on behavior, but a lack of ethanol-induced sensitized locomotor behavior. Sensitization of the c-Fos and Fos B responses were evident following repeated ethanol injections in the NAc core and basolateral amygdala (BLA) on day 15 (twelfth ethanol injection) when compared to day 3 (first ethanol injection).

Conclusions: Significant increases in the expression of both c-Fos and Fos B were observed in repeated-drug subjects, despite a lack of sensitized locomotor behavior. The immunohistochemical results closely parallel those observed following repeated cocaine

administration to rats (Nestler et al. 2001). Thus, these two immediate-early genes may serve as markers for regional activation following repeated ethanol administration.

2.2 Introduction

Locomotor sensitization, or a progressive increase in motor behavior, is visible when certain drugs of abuse are administered repeatedly to rodents. In theory, locomotor sensitization is a useful model for studying the effects of drugs of abuse and their mechanisms because: 1. it is proposed that locomotor sensitization may be indicative of a risk for the development of drug abuse (Phillips, 1997) 2. motor behavior has a neural basis and therefore lends itself to the study of neural mechanisms underlying drug-induced changes in motor behavior 3. it is a behavioral phenomenon that is easy to measure in rodents.

Sensitization's relevance to the human addicted state is also a subject of active investigation. Currently, there is research suggesting that stimulant responses during the rising portion of the blood-alcohol curve are correlated with heavy or binge drinking in young adults 24-38 years of age (King et al. 2002). Certain subjective behavioral measures such as increased drug-craving, reward and euphoria as well as objective physiological measures such as heart rate, finger pulse amplitude, and skin conductance have been recorded as sensitized responses in certain high-risk populations (typically defined as subjects having a family history of alcoholism) during the rising portion of the blood alcohol curve (BAC). Similarly, less tolerance to certain behavioral measures has also been observed during the descending portion of the BAC in this same population (Robinson and Berridge 1993; Newlin and Thomson 1999). Thus, sensitization may be a predisposing factor for the development of addiction. Cocaine, morphine, and ethanol,

when administered acutely stimulate locomotion in rodents; when administered repeatedly these drugs can also induce sensitization. Similarly, cross-sensitization between these drugs can also occur, however, not always in a reciprocal fashion, suggesting that there are *some* shared mechanisms of action between these different classes of abused drugs (Lessov and Phillips 2003). In this paper we explored regional activation of the brain following repeated ethanol administration to DBA/2J (D2) mice using c-Fos and Fos B immunohistochemistry (IHC).

Morgan and Curran in 1987 showed that the c-Fos protein could be used as both a temporal and a spatial map for the brain areas associated with an acute perturbation (administration of pentylenetetrazol, a convulsant; Morgan et al. 1987). c-Fos is the protein product of the immediate-early gene (IEG) and transcription factor, c-fos. Since this time numerous studies have employed this regional mapping technique to find the brain regions activated with a particular treatment. Previous data from our lab have shown that with an acute ethanol injection of 1-2 g/kg in D2 mice, c-Fos protein levels were elevated significantly in the following regions: central nucleus of the amygdala (CeA; capsule, lateral, and medial portions), paraventricular nucleus of the thalamus (PVT), and ventral tegmental area (VTA) (Hitzemann and Hitzemann 1997), when compared to saline controls as well as ethanol-injected C57BL/6J mice. The CeA and PVT are part of the limbic and stress systems. However, they also send efferents to the motor system, specifically the VTA, nucleus accumbens, and thalamus (Pierce and Kalivas 1997). Important also is the fact that these doses of alcohol have stimulant effects in D2 mice. Mice injected acutely with 1-2 g/kg of ethanol have significantly higher

amounts of motor activity than their saline-injected counterparts (Phillips et al. 1994). Thus, c-Fos levels were correlated with acute ethanol-induced motor behavior.

There is much evidence to suggest that with a repeated stimulus of any kind (i.e. repeated restraint stress, repeated methylphenidate, dexfenfluramine, or ethanol administration) c-Fos levels in particular brain regions habituate; thus c-Fos may be used as a molecular marker for the brain areas activated by an acute stimulus but not a chronic stimulus (Medeiros et al. 2003; Chase et al. 2003; Chang et al. 1995; Ryabinin et al. 1997). Recent work by Nestler et al. (Hope et al. 1994; Kelz et al. 2000; Nestler et al. 2001) has shown that with repeated administration of cocaine to rats, an increase in delta Fos B was observed in the nucleus accumbens (NAc). Delta Fos B and Fos B are both members of the immediate-early gene (IEG) family. Fos B is a 45 kD protein, while delta Fos B is a protein complex made up of 33, 35, and 37 kD proteins and is missing 101 amino acids of Fos B at the c-terminus. While most immediate-early genes increase with an acute stimulation (acute fos-related antigens or acute FRAs) and then return to basal levels approximately 18 hours following the perturbation, both Fos B and Delta Fos B can be classified as chronic FRAs because they continue to accumulate over time with repeated perturbations. Immediate-early genes bind to each other to produce an AP-1 complex; this complex binds to the AP-1 site present in the promoter region of some genes to affect gene transcription. Specifically, increases in delta Fos B protein were found in the striatum of Sprague-Dawley rats given twice-daily i.p. cocaine injections (22.5 mg/kg); the protein returned to basal levels seven days following cocaine withdrawal. While the time course of this protein is shorter than some of the behavioral changes that can be observed in rodents who have been repeatedly exposed to drugs of

abuse (i.e. responding for drug in a two-lever choice paradigm), at the present time, this protein gives us the best insight for the long-term molecular changes associated with repeated drug exposure.

In this paper we investigated whether Fos B increases in the NAc and central nucleus of the amygdala would be correlated with ethanol-induced sensitization in D2 mice, and consequently serve as an accurate molecular marker for the long-term neurobiological changes associated with repeated alcohol exposure. To our knowledge, this is the first paper that contrasts c-Fos and Fos B staining across time and during an ethanol-sensitization paradigm.

2.3 Methods and Materials

Animals:

Five-week old DBA/2J (D2) male mice obtained from Jackson Laboratories, Bar Harbor, ME, were housed five to a cage with access to food and water *ad libitum* at the AAALAC-accredited animal facility at Oregon Health & Science University. Mice were allowed to acclimate to the colony room for one week before experiments commenced (lights were on a 12:12 light:dark cycle; on at 0600 hrs.).

Drugs:

The dose of ethanol (20% v/v, dissolved in saline) used in this study was 2 g/kg and was administered intraperitoneally (i.p.). Saline (0.9%) was given in an equivalent volume. A prior experiment from our lab showed that this dose of ethanol was effective in eliciting sensitized locomotor behavior in male D2 mice (Reed et al., unpublished observations).

Treatment and Testing Schedule:

The protocol used to elicit sensitization was modeled after Phillips et al. (1994). On test days, mice were moved from the colony room to the procedure room and allowed to acclimate to the environment for 25 minutes. Testing was always performed between 1100 and 1500 hrs. On non-test days, injections were given in the procedure room between 1100-1200 hours (table 1). It has been shown that the context of drug administration can affect the amount of observed sensitization for ethanol (Day et al. 2001).

Injection Controls:

The purpose of including these mice in the sensitization paradigm was to control for stress-induced sensitization (Marinelli and Piazza 2002; Phillips et al. 1997; Barr et al. 2002; Pacchioni et al. 2002). These research papers showed that exposure to psychostimulant drugs produced a long-lasting behavioral cross sensitization to stress. Additionally, it has also been shown that exogenous administration of corticosterone is able to facilitate the locomotor activity of psychostimulant drugs. Preliminary work in this laboratory suggested that repetitive handling and multiple saline injections increased the animal's acute response to ethanol; thus, the following groups of mice served as injection controls (table 2).

Activity Monitors:

Eighteen locomotor activity chambers were used in this experiment. The activity chambers were plastic cages lined with cobb bedding and measured 22(w) x 42(l) x 20(h) cm in dimension; these cages were surrounded by a metal frame housing a 4(w) x 8(l) array of photocells; the frame was situated 1 cm above the ground (Hitzemann et al. 2000). The software used to monitor the number of beam breaks was the Flex Field activity system designed by San Diego Instruments; beam breaks were recorded as soon as the animal intercepted a grid line. Vertical activity and repetitive horizontal activity were not measured.

Immunohistochemistry:

Twenty-four hours after taking brain tissue, the paraformaldehyde-fixed brains were transferred to 30% sucrose in phosphate buffer. Thirty-micrometer frozen coronal sections were cut on a microtome and collected in 10 mM phosphate buffered saline

(PBS). The sections were rinsed three times in PBS and treated with 0.3% H_2O_2 in PBS for fifteen minutes to block the endogenous peroxidase activity. The sections were rinsed in PBS six more times to remove the residual H_2O_2. Sections were then blocked for two hours in the immuno-reaction buffer (10 mM PBS containing 0.25% Triton X-100 and 3% goat serum) without antibody. Next, antibody (final dilution for c-Fos: 1:10,000, for Fos B: 1:1,000) was added and the incubation was continued for 48 hrs at 4°C. c-Fos antibody was obtained from Oncogene Research Products/Calbiochem (San Diego, CA); the antibody was raised in rabbit against residues 4 - 17 of human c-Fos protein. Fos B antibody was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and was raised in rabbit against human Fos B, residues 75-150.

Following incubation with the primary antibody, sections were rinsed three times in PBS and incubated with the biotinylated goat anti-rabbit IgG (1:200 of secondary antibody) in 10 mM PBS containing 0.3% Triton X-100 and 3% goat serum for two hours at room temperature. The sections were subsequently incubated with horseradish peroxidase avidin-biotin complex in 10 mM PBS for two hours at room temperature. The sections were rinsed three times in PBS and placed in 0.1 M Tris (pH 7.4) for 5 minutes. The chromatic reaction was completed with diaminobenzidine (50 mg/100 ml of 0.1 M Tris) in presence of 0.01% nickel ammonium sulfate solution and 0.035% hydrogen peroxide. The sections were mounted onto slides, dehydrated and cover-slipped with Permount. Only one section per region per mouse was analyzed for the Fos results (Hitzemann and Hitzemann, 1999). The regions of the brain that were examined for IEG expression were: cingulate gyrus, area 1 (Cg1), prelimbic cortex (Prl), nucleus accumbens (NAc) core, NAc shell, lateral hypothalamus (LH), paraventricular nucleus of

the hypothalamus (PVN), lateral septum (ventral portion), bed nucleus of the stria terminalis (BNST), basolateral amygdala (BLA), central nucleus of the amygdala (CeA): capsular(CeC), lateral(CeL), and medial divisions(CeM), perirhinal cortex (Prh), ectorhinal cortex (Ect), secondary somatosensory cortex (S2), and the Edinger-Westphal nucleus (EW). Some of these regions show increased c-Fos expression with an acute ethanol injection in D2 mice (Hitzemann and Hitzemann, 1997).

Statistical Analyses:

Data for behavior was first analyzed by a repeated measure ANOVA with day as the repeated measure and group as the categorical factor. If a significant interaction existed between day and group, one-way ANOVAs were performed to examine behavior across days for each group (a within-groups analysis), and to examine differences between groups on specific days of the time-course (between-groups analysis). Follow-up analyses were performed by using Neuman-Keuls post hoc tests. Data for IHC was separated on the basis of protein (c Fos and Fos B) and group (EE, SE) and then analyzed via a one-way ANOVA to examine differences in immediate-early gene expression across days for each group. Significant results were further analyzed by Neuman-Keuls post-hoc analyses. Significance was set at $p \leq 0.05$.

2.4 Results

Behavior:

Day 3 mice

Locomotor activity during the various timecourses is depicted in figure 2.1. A repeated measures ANOVA for day 3 mice revealed a significant group effect ($F_{(1,12)} = 5.6$, $p<0.05$) and a significant day by group interaction ($F_{(1,12)} = 18.6$, $p<0.01$; figure 2.1,

panel a). Day 2 activity was not included in the analysis due to a loss of data. A one-way ANOVA for behavioral differences between groups on day 3 revealed a trend for significance ($F_{(1,13)}$=4.5, p=0.054) such that the activity of group SE was higher than that of group SS. There were no significant between-groups differences on day 1.

A one-way ANOVA for group SE revealed a significant effect of day ($F_{(1,5)}$ = 7.5, $p<0.05$) such that follow-up post-hoc analysis showed that with an acute ethanol injection on day 3, mice exhibited greater locomotor activity in comparison to saline-induced activity on day 1 ($p<0.05$). Thus, there was a significant within-group effect of acute ethanol. There was also a significant effect of day for group SS ($F_{(1,7)}$=10.6, $p<0.05$) such that post-hoc analysis showed that activity of mice on day 3 was less than that observed on day 1 ($p<0.05$); this result suggests that habituation to the testing apparatus and injection procedure occurred in this group.

Day 6 mice

A repeated measure ANOVA for the group of mice euthanized on day 6 revealed a significant effect of day ($F_{(3,51)}$ = 6.4, $p<0.001$) and a significant day by group interaction ($F_{(6,51)}$ = 3.9, $p<0.01$; figure 2.1, panel b). Between-groups analysis via one-way ANOVAs revealed no significant group differences on days 1 or 2. On day 3, a one-way ANOVA revealed group differences ($F_{(2,17)}$ = 6.4, $p<0.01$), such that Neuman-Keuls post-hocs showed that the activity of group EE was greater than either SE ($p<0.05$) or SS ($p<0.05$) indicating acute ethanol's activating effects on horizontal locomotion. There were no significant between-group differences on day 6, indicating a lack of sensitized behavior.

There was a significant day effect for group EE ($F_{(3,21)} = 3.8$, $p<0.05$). Post-hoc analysis revealed activity on days 3 and 6 was significantly greater than the activity observed on day 2 following the second saline injection ($p<0.05$ for both days). Hence, there was acute stimulatory effect of ethanol on day 3, but this effect was unchanged on day 6. Thus, there were no further increases in activity coincident with the three additional ethanol injections given by day 6 to group EE. There was a significant effect of day for group SE as well ($F_{(3,21)} = 14.2$, $p<0.001$). Post-hoc analysis revealed that activity on days 1 and 2 did not differ; however, activity on day 3 was significantly lower than the activity observed on both these days ($p<0.01$ and $p<0.05$, respectively). This result indicates that it took three days to habituate to the testing/injection procedure instead of the two days included in the design. Furthermore, activity on day 3 was significantly lower than that seen on day 6 with an acute ethanol injection ($p<0.001$). Day 6 activity was also significantly higher than that seen on days 1 and 2. These results indicate an acute stimulatory effect of ethanol in group SE. There was no significant effect of day for group SS. Thus, the activity of this group remained unchanged throughout the paradigm.

Day 9 mice

A repeated measures ANOVA for the group of mice euthanized on day 9 revealed a significant group effect ($F_{(2,88)} = 14.1$, $p<0.001$), a significant day effect ($F_{(4,88)} = 3.9$, $p<0.01$), and a significant group by day interaction ($F_{(8,88)} = 4.8$, $p<0.001$; figure 2.1, panel c). Between-groups analysis showed no group differences on days 1 or 2. However, between-groups differences were evident on days 3 ($F_{(2,22)} = 15.2$, $p<0.001$), 6 ($F_{(2,22)} = 3.6$, $p<0.05$), and 9 ($F_{(2,22)} = 7.7$, $p<0.01$). On day 3, post-hoc tests revealed that the activity of group EE was higher than that of either group SE or SS ($p<0.001$ for both),

demonstrating again, the locomotor activating effect of acute ethanol. On day 6, the activity of group EE was higher than that of group SS ($p<0.05$). On day 9, the activity of group EE was greater than either SE ($p<0.05$) or SS ($p<0.01$). There was no effect of acute ethanol on group SE on day 9 because there was no significant increase in activity compared to the activity of group SS. Therefore, true between-groups sensitization did not occur on this day in this paradigm.

A one-way ANOVA for group EE revealed a significant effect of day ($F_{(4,36)} = 5.7$, $p<0.01$). Post-hoc analysis showed that activity on day 3, 6, and 9 was significantly greater than that observed on day 2 following the second saline injection ($p<0.01$ for all days). Additionally, there was no significant difference in activity on day 3, day 6, or day 9; thus, there was no development of locomotor sensitization. There was also a significant effect of day for group SE ($F_{(4,28)} = 7.1$, $p<0.001$). Post-hoc testing showed that activity following the first ethanol injection on day 9 was greater than the activity observed on day 2 and day 3 (saline injection days, $p<0.05$ and 0.01, respectively) but no different from the activity on day 6 or day 1. Because there was no difference between day 6, day 1, and day 9 activity, this suggests that habituation to the injection/testing procedure was decreasing. There was also a significant effect of day for group SS ($F_{(4,24)} = 3.1$, $p<0.05$) such that activity on day 3 was significantly lower than day 1 activity (initial procedure exposure; $p<0.05$). Again, because activity on day 6 and day 9 was not significantly different from day 1 this suggests a recovery from habituation.

Day 15 mice

A repeated measures ANOVA revealed a significant effect of day ($F_{(5,155)} = 7.2$, $p<0.001$) and a significant day by group interaction ($F_{(15,155)} = 4.7$, $p<0.001$) for the day

15 group of mice (figure 2.1, panel d). There was a significant effect of day for all groups. Between-groups analyses showed no group differences on days 1, 2, and 3. On day 6 there were significant between-groups differences ($F_{(3,31)} = 5.5$, $p<0.01$). Post-hoc tests showed that the activity of group EE was greater than that of group SS ($p<0.01$) and SE ($p<0.05$). On day 9, the results from the one-way ANOVA showed group differences ($F_{(3,31)} = 6.6$, $p<0.01$). Post-hoc tests showed that group ES demonstrated significantly higher activity compared to groups SE ($p<0.01$) and SS ($p<0.01$). Analysis for day 15 also showed group differences ($F_{(3,31)} = 5.6$, $p<0.01$), such that post-hoc tests revealed that both groups EE and SE had 1. similar activity levels and 2. significantly greater activity compared to groups ES ($p<0.05$, $p<0.001$, respectively). Additionally, the activity of group SE was significantly higher than that of group SS ($p<0.05$). As with the day 9 group of mice, there was no significant between-groups ethanol-induced sensitization with the day 15 mice.

For group EE ($F_{(5,35)} = 2.6$, $p<0.05$) analysis revealed that the activity on day 6 was significantly greater than the activity on day 2 ($p<0.05$). These results show that an acute stimulatory response was not observed on day 3 with the first ethanol injection, and that there was no evidence of within-groups sensitization as day 15 activity did not differ significantly from the activity of any other day. Post-hoc analysis following the significant day effect for group ES ($F_{(5,45)} = 9.1$, $p<0.001$) revealed an acute stimulatory effect of ethanol in this group on day 3 compared to activity on both days 1 and 2 ($p<0.05$ and $p<0.001$, respectively). Activity on day 6 and on day 9 was also significantly higher than that seen following a saline injection on day 2 ($p<0.01$ and $p<0.001$, respectively). However, activity on day 6 and on day 9 was not significantly different from that seen on

day 3 (i.e. no significant increases in activity following repeated ethanol administration). Activity on day 15 for group ES was significantly lower than activity on days 3, 6, and 9 suggesting that there was no overt contextual conditioning with this paradigm ($p<0.01$, $p<0.05$, $p<0.001$, respectively). Thus, group ES differed from group EE in that the acute stimulatory effect of ethanol was observed on day 3; however, both groups were similar with respect to the lack of development of an ethanol-sensitized locomotor response. There was a significant effect of day within group SE ($F_{(5,40)} = 6.0$, $p<0.001$). Activity on day 15 for this group was significantly greater than the activity observed on any other day ($p<0.01$ for all days) indicating an acute stimulatory effect of ethanol with the first ethanol injection. There was also a significant day effect for group SS ($F_{(5,35)} = 2.5$, $p<0.05$). Post-hoc tests revealed that activity on day 3 was nearly significant (i.e. greater) when compared to activity on day 1 ($p = 0.059$). Unfortunately, analyses for all day 15 groups revealed a lack of habituation on day 2 compared to day 1 ($p>0.05$).

The repeated measures and one-way ANOVAs for all of these groups of mice revealed that there was no evidence of ethanol-sensitization (neither within-groups nor between-groups) in any of the time courses for the group of mice administered ethanol repeatedly.

Injection-controls:

As is evident from figure 2.2, mice that were repeatedly treated with saline or simply handled on test days had similar amounts of locomotor behavior; thus, there was no effect of repeated vehicle injections on locomotion ($p>0.05$, ns).

Immunohistochemistry:

Although we were unable to observe sensitized behavior, we were still able to measure changes in c-Fos and Fos B expression. Immunohistochemistry results are pictured for two major brain areas: basolateral amygdala (BLA) and the nucleus accumbens core (NAc core). While the present experiments showed sensitization of the Fos response in BLA, Hitzemann and Hitzemann (1997 and 1999) did not report increased c-Fos expression in this brain region following acute ethanol treatment to either D2 or B6 mice. Results are reported for the NAc core, as this was the site where increased expression of delta Fos B was observed following a chronic cocaine paradigm (Hope et al. 1994).

c-Fos

A one-way ANOVA for group EE with day as the categorical factor revealed a sensitized c-Fos response in NAc core ($F_{(3,31)} = 5.2$, $p<0.05$; figure 2.3), and BLA ($F_{(3,26)} = 4.0$, $p<0.05$; figure 2.4) on day 15 (challenge day), when compared to all other days (days 3, 6, and 9). Additionally, there was a significant increase in c-Fos expression in Ect on day 15, when compared to day 3 ($F_{(3,25)} = 3.3$, $p<0.05$; data not shown). There was no change in the amount of c-Fos expression in any of the other brain regions examined for this group over time. These results contrast with those observed in NAc shell, when comparing groups EE and ES on day 15. Here, group ES had significantly more c-Fos in the NAc shell than group EE ($F_{(1,11)} = 24.3$, $p<0.01$).

c-Fos analyses for group SE showed that all brain regions except for one remained constant in their level of c-Fos expression over time. On day 15 there was a

significant increase in c-Fos expression in Cg1 compared to c-Fos levels in this region on day 3 ($F_{(3.26)}$=3.3, $p<0.05$).

Fos B

There was a significant increase in Fos B expression in several brain regions for group EE on day 15, when compared to Fos B levels on earlier days. For instance, in NAc core, the increase in Fos B expression on day 15 was significantly greater than the protein levels observed on days 3, 6, and 9 ($F_{(3,24)}$ = 4.3, $p<0.05$ for all days; figure 2.3). BLA ($F_{(3,27)}$= 3.7, $p<0.05$; figure 2.4), CeL ($F_{(3,29)}$ = 6.7, $p<0.01$; figure 2.5), CeM ($F_{(3,29)}$ = 6.0, $p<0.01$; data not shown) and S2 ($F_{(3,27)}$ = 10.9, $p<0.01$; data not shown) also exhibited a significant increase in Fos B expression on day 15, when compared to protein levels on day 3. However, for CeL and CeM, Fos B expression levels on days 15 and 9 were similar. Thus, Fos B levels in these brain regions peaked on day 9 and then plateaued. In Prh ($F_{(3,27)}$ = 6.8), Ect ($F_{(3,27)}$ = 7.0), and S2 ($F_{(3,27)}$ = 10.9), day 9 Fos B levels were significantly greater than those observed on all other days (day 15 included; $p<0.01$ for all regions; data not shown). Thus, the application of repeated ethanol increased Fos B expression in NAc core and BLA over that seen with a single ethanol injection. When comparing Fos B expression between groups ES and EE on day 15, there were significant increases for group ES in Fos B expression in Prl ($F_{(1,10)}$=5.1) and S2 ($F_{(1,11)}$=7.7) compared to group EE ($p<0.05$ for both regions; data not shown).

Interestingly, when Fos B expression was examined over time for group SE, all divisions of the CeA exhibited significant increases in Fos B on day 15 compared to all other days (CeC: $F_{(3,25)}$ = 6.0, CeL: $F_{(3,25)}$ = 5.7 (figure 2.5), CeM: $F_{(3,25)}$ = 6.5; $p<0.01$ for all regions; data not shown). The BSTLP also showed significant Fos B expression on

day 15 compared to day 3 ($F_{(3,24)}$=4.2, $p<0.05$; data not shown). Thus Fos B accumulation in these brain regions was associated with an acute locomotor response on day 15.

2.5 Discussion

Sensitized behavior following repeated ethanol administration was not observed in D2 mice. However, acute stimulatory behavior following a single injection of ethanol was almost always observed (with the exceptions of group SE on day 9 of the group 9 mice and group EE on day 3 in the group 15 mice). Thus, stimulatory behavior could be elicited, but increases above the acute response were not evident.

Ethanol-sensitization behavior did not develop in the present set of experiments. A brain region that is involved in the development of ethanol-induced sensitization is the hypothalamic arcuate nucleus (Miquel et al. 2003). Pups that were treated with monosodium glutamate (4 mg/g i.p.) to lesion the nucleus followed by ethanol at 10 weeks of age (2 g/kg every other day for twelve days) did not show an acute stimulatory response to ethanol and did not develop ethanol-sensitization when compared to control mice (treated with saline post-natally and then challenged with ethanol at 10 weeks). Cocaine-induced sensitization was also examined in a separate group of mice. These mice did express behavioral sensitization to cocaine; however, the amplitude of the effect was lower than that seen in control mice.

Co-administration of MK-801 at low doses (0.1 mg/kg) with ethanol (2 g/kg) has also been shown to have a facilitory effect on ethanol-induced sensitization (Meyer and Phillips, 2003). However, MK-801 doses of 0.2 g/kg or greater attenuated the expression of ethanol-induced sensitization (Meyer and Phillips, 2003) or even prevented its development (Broadbent and Weitemier, 1999). Authors of the former paper postulated

that the combination of the two drugs in larger doses may potentiate the drugs' sedative and ataxic properties.

There are several neuronal substrates that have been shown to mediate the expression of ethanol-induced locomotor sensitization. Swiss-Webster mice treated with 1.5 g/kg ethanol for seven days express locomotor sensitization (Itzhak and Martin, 2000). Once these mice were sensitized, a subset was treated acutely with a drug that inhibited the formation of nitric oxide (7-nitroindazole; 25 mg/kg) in combination with a challenge injection of ethanol. These mice demonstrated an attenuated sensitized response, implicating this molecule in ethanol-induced sensitization behavior. DBA/2J mice that had been sensitized to a 2 g/kg dose of ethanol (given once every day for four days) showed no locomotor sensitization when given ethanol in combination with MK-801 on a challenge day (NMDA receptor antagonist given at doses greater than 0.075 mg/kg; Broadbent et al. 2003). The same group also observed that when GYKI 52466 (an AMPA antagonist) was given acutely or in combination with ethanol to sensitized mice, locomotor sensitization was absent. The confound in this study was that both of these drugs were able to reduce the acute stimulatory effect of ethanol, in a separate group of subjects. Acamprosate (400 mg/kg) when given before ethanol challenge (2 g/kg) decreased the amplitude of ethanol-induced locomotor sensitization (Chester et al. 2001). Acamprosate is thought to block glutamate release during ethanol withdrawal (Dahchour et al. 1998) and to reduce calcium flux (Wilde and Wagstaff 1997). Finally, blockade of glucocorticoid receptors during the development phase of a sensitization paradigm also attenuates ethanol-induced sensitization (Roberts et al. 1995). While none of these drugs were administered in the present set of experiments, it is possible that these endogenous

systems may not have been activated to the proper extent in the current paradigm, therefore eliminating the expression of ethanol-sensitized behavior. Many of these neurotransmitter and receptor systems are widespread throughout the brain. However, it is has been demonstrated that activation of the NAc core is necessary for the expression of amphetamine-induced sensitization (see review by Vezina 2004).

The variable, and at times, increased motor behavior of repeated-saline treated mice (group SS) may also contribute to the lack of observed sensitization. The increased activity of SS mice decreased the difference in activity between all other groups. Difference scores for day 15 mice were also analyzed, however this method also revealed a lack of sensitized behavior. Stress may be a cause for the increased motor behavior of SS mice throughout the sensitization paradigms. Sprague-Dawley rats exposed to restraint stress (a type of handling stress) for 15 minutes increased basal levels of dopamine and norepinephrine (Marsteller et al. 2002). Dopamine's role in the motor response to an amphetamine sensitization paradigm has been investigated (Pierce and Kalivas, 1997). It would have been interesting to see if corticosterone levels at the end of the sensitization paradigm were higher than those on day 2, or day 3 for SS mice. There is little published literature on the effects of handling stress on basal locomotion; one study showed that a 3-hour restraint stress session once a day for four days *lowered* basal locomotion in male Wistar rats (Zebrowska-Lupina et al. 1990). This was in contrast to the effect of repeated handling on acute morphine-induced locomotion; here motor activity was increased above that seen in non-stressed rats given an acute morphine injection (Stohr et al. 1999). HR rats (exhibit greater motor activity in response to novelty than low-responder [LR] rats) exhibit greater activation to an acute morphine injection,

and show a longer-lasting increase in corticosterone in response to stress (Deroche et al. 1993).

Despite the lack of sensitized behavior, changes in c-Fos and Fos B expression were still measured. c-Fos sensitization was observed in the NAc core, BLA, and Ect of the EE group on day 15. Our hypothesis was that c-Fos expression would habituate within this group as it has been shown that c-Fos habituates with repeated stimulus applications (see section 1.4 in introduction). Additionally, both NAc core and BLA demonstrated increased Fos B expression on day 15 (relative to: all other days, day 3, respectively). These results suggest that the NAc core and BLA may contribute to the responses to repeated ethanol. This theory is further strengthened by the fact that no other group (ES, or SE) showed the same pattern of either c-Fos or Fos B expression within these two brain regions. A significant increase in locomotor behavior is observed on observed day 6 for group EE (see figure 2.1, panel d) while the significant Fos B accumulation within CeL appears later, on day 9. It is tempting to speculate that sensitized locomotor behavior may have been observed had Fos B expression in CeL on day 15 been significantly greater than protein expression on all other days for group EE. Alternatively, because the CeA is an area that is sensitive to both physical stressors (Hand et al. 2002) as well as fear (Hitchcock and Davis, 1991; Davis and Shi, 1999) large and significant increases in Fos B expression could represent a neuroadaptive response to stress.

However, it is perplexing that significant differences were found when examining c-Fos and Fos B expression between EE and ES groups on day 15. In terms of behavior the two groups were expected to differ (and they did); however, it was our hypothesis that

they would not differ with regards to immediate-early gene expression. The ES group, compared to EE mice, had significantly higher c-Fos expression in NAc shell. This is an interesting finding because it speaks to the different functions of the subdivisions of the accumbens, when combined with the NAc core results discussed above. The increase in c-Fos staining in NAc shell may represent a brain region that normally habituates to repeated ethanol application, but when the drug is removed, the activity within this brain region becomes hyperexcitable. With regards to Fos B, expression was increased in the prelimbic cortex as well as the somatosensory cortex. Both of these areas may have similar functions as the NAc shell, in that they habituate with repeated ethanol application and become hyperexcitable upon ethanol removal. Alternatively, the staining patterns of c-Fos and Fos B proteins in group ES may represent brain regions that act to inhibit the expression of associative, environmental conditioning.

As we did not measure c-Fos changes in group SE relative to group SS, we cannot definitively say that increased c-Fos expression in CeA was observed following acute ethanol injection. Most likely the increase did occur as past experiments from this lab have demonstrated this effect in D2 mice given acute ethanol (Hitzemann and Hitzemann, 1997 and 1999). SE mice also demonstrated a greater c-Fos response in cingulate gyrus on day 15 compared to day 3. The cingulate gyrus is considered part of limbic cortex and has been shown to possess neurons that fire specifically during alcohol-acquisition training (Alexandrov et al. 2001). Thus, this area of the brain may be involved in the acute adaptation to ethanol. Additionally, SE mice demonstrated an increase in Fos B expression in all areas of the CeA on day 15, when compared to all other days. In the BSTLP, there was also a significant increase in Fos B expression on day 15, when

compared to day 3. Thus, these brain regions are correlated with the acute stimulant response to ethanol, especially following 14 days of handling and saline injections. Indeed, it has been shown that following repeated social defeat and acute amphetamine injection in rats, c-Fos expression in the medial amygdala is increased significantly over levels of the protein seen with each treatment condition alone (Nikulina et al. 2004).

The IHC results of our experiment were slightly different than those reported for delta Fos B in cocaine-treated rats. In chronic cocaine-treated rats, increases in delta Fos B were localized to the accumbens. In our experiment, Fos B expression was localized to the core of the accumbens as well as the BLA. Additionally, Fos B was present in groups SE and ES, not only in the group repeatedly treated with ethanol. Furthermore, in NAc core and BLA, we observed both c-Fos sensitization as well as significant increases in Fos B expression relative to day 3. To our knowledge, this paper is the first of its kind to report changes in immediate-early gene reactivity, specifically comparing c Fos and Fos B protein levels, during a chronic ethanol administration paradigm.

In 1994, Hope et al. noted changes in AP-1 binding with chronic cocaine administration in rats. The investigators found that treating rats with cocaine (twice daily i.p. injections of 22.5 mg/kg cocaine) resulted in greater AP-1 binding on days 4 and 7; levels of AP-1 binding decreased after day 7 even if subjects were still administered drug. Furthermore, with chronic cocaine, increases in delta Fos B but not Fos B were found in the striatum of rats; instead, increased levels of Fos B were evident in parietal cortex with seven days of twice-daily cocaine treatment.

On a behavioral level, both Fos B knockout mice and delta Fos B inducible overexpressors have been developed and examined (Hiroi et al. 1997; Kelz et al. 1999).

While Fos B knockouts do retain some basal FRAs, upon acute administration of 20 mg/kg of cocaine they showed an exaggerated locomotor stimulation compared to wildtype controls. Additionally, sensitized behavior can be observed on the two days following an acute cocaine injection, but five days into a cocaine-sensitization paradigm, there is no within-groups sensitized behavior. Interestingly, these knockout mice were able to show an enhanced place preference at 10 mg/kg cocaine in comparison to wildtype controls, suggesting that they were still able to find a particular dose of cocaine reinforcing. However, a 20 mg/kg dose of cocaine induced a similar conditioned place preference in both wildtypes and knock-outs, when compared to baseline performance.

Delta Fos B overexpressors were modified to have greater protein abundance in the nucleus accumbens and caudate putamen than control mice. The overexpressors were able to demonstrate a higher acute locomotor response to 10 mg/kg cocaine and also had a higher sensitized locomotor response, when compared to single transgenic control littermates. The overexpressing mice also showed significant place preference at 5 mg/kg cocaine, while single transgenic controls did not; both groups of mice showed similar levels of place preference at 10 and 20 mg/kg of cocaine. It is unknown at the present time whether increases in delta Fos B (which we did not measure in the current set of experiments) in the accumbens and striatum are necessary for the expression of *ethanol*-sensitized locomotor behavior in D2 mice. These two animal models speak to the involvement of both of these immediate-early gene protein products in drug- (i.e. cocaine) sensitized behaviors.

This study shows that both the accumbens core and BLA were affected by repeated ethanol administration in D2 mice. Neither of these brain regions were

implicated in the amphetamine sensitization/motive circuit proposed by Pierce and Kalivas (1997). Furthermore, the Pierce and Kalivas (1997) article identified the extended amygdala (CeA, BNST, NAc shell) as modulating amphetamine-induced locomotor sensitization. More work needs to be done in order to determine the specificity of these molecular effects; for example, are the proteins elevated to the same extent and in the same regions following repeated stress? Furthermore, it would be beneficial to know if these molecular effects are visible with repeated application of other drugs within the sedative-hypnotic drug family. It would also be interesting to see if repeated psychostimulant administration resulted in similar levels of Fos B expression in these same brain regions. Finally, more investigation needs to be done to determine if the molecular changes within these brain regions modulate other types of drug-associated behaviors (i.e. self-administration, contextual cue association) as these behaviors can co-occur following repeated drug (pyshostimulant [amphetamine] and analgesic [morphine]) application (Lorrain et al. 2000; Lett, 1989).

Table 2.1: Treatment and testing schedule for DBA/2J mice.

Day	**1 Test**	**2 Test**	**3 Test**	4 Inject only	5 Inject only	**6 Test**	7-8 Inject only	**9 Test**	10-14 Inject only	**15 Test**
Group										
Day 3										
SS (n = 8)	S	S	**S**							
SE (n = 7)	S	S	**E**							
Day 6										
SS (n = 4)	S	S	S	S	S	**S**				
SE (n = 8)	S	S	S	S	S	**E**				
EE (n = 8)	S	S	E	E	E	**E**				
Day 9										
SS (n = 7)	S	S	S	S	S	S	S	**S**		
SE (n = 8)	S	S	S	S	S	S	S	**E**		
EE (n = 10)	S	S	E	E	E	E	E	**E**		
Day 15										
SS (n = 18)	S	S	S	S	S	S	S	S	S	**S**
SE (n = 19)	S	S	S	S	S	S	S	S	S	**E**
EE (n = 16)	S	S	E	E	E	E	E	E	E	**E**
ES (n = 19)	S	S	E	E	E	E	E	E	E	**S**

Table 2.1. Treatment and testing schedule for DBA/2J mice. The table depicts the treatment and testing schedule used for the sensitization paradigm; S=saline; E=ethanol (2 g/kg). Locomotor activity was monitored on days 1 and 2 (habituation days; i.p. saline injections), 3 (i.p. acute drug injection) and 6, 9, and 15 (challenge day). Each locomotor test session was 20 minutes in length. For day 15 mice, there was no locomotor testing on day 6. For day 3 mice, data from day 2 was lost. On days 3, 6, 9, and 15, mice were euthanized 1 hour after drug or vehicle injection, or 40 minutes after locomotor testing. Whole brains were extracted from these animals and placed in 4% paraformaldehyde for immunohistochemical analyses of c-Fos and Fos B.

Table 2.2: Treatment and testing schedule for injection controls.

	Day	1 (Test)	2 (Test)	3 (Test)	Days 4-8	Day 9 (Test)	Days 10-14	Day 15 (Test&Euthanize)
Group 1 (n=10)		Nothing	Nothing	Nothing	Nothing	Nothing	Nothing	S
Group 2 (n=11)		Nothing	Nothing	Nothing	Nothing	Nothing	Nothing	E

Table 2.2. Treatment and testing schedule for injection controls. S= saline, E= ethanol (both given at a concentration of 2g/kg). Motor behavior was monitored on days 1,2,3,9, and 15 for mice in the injection control groups (i.e., no day 6 monitoring). Furthermore, injection control mice were not euthanized until day 15 (no time-course study was performed on these mice) since this was when we expected to see maximum sensitization; brain tissue was then harvested from these mice for the detection of c-Fos and Fos B proteins.

Figure 2.1. Motor activity of D2 mice in an ethanol-sensitization paradigm.

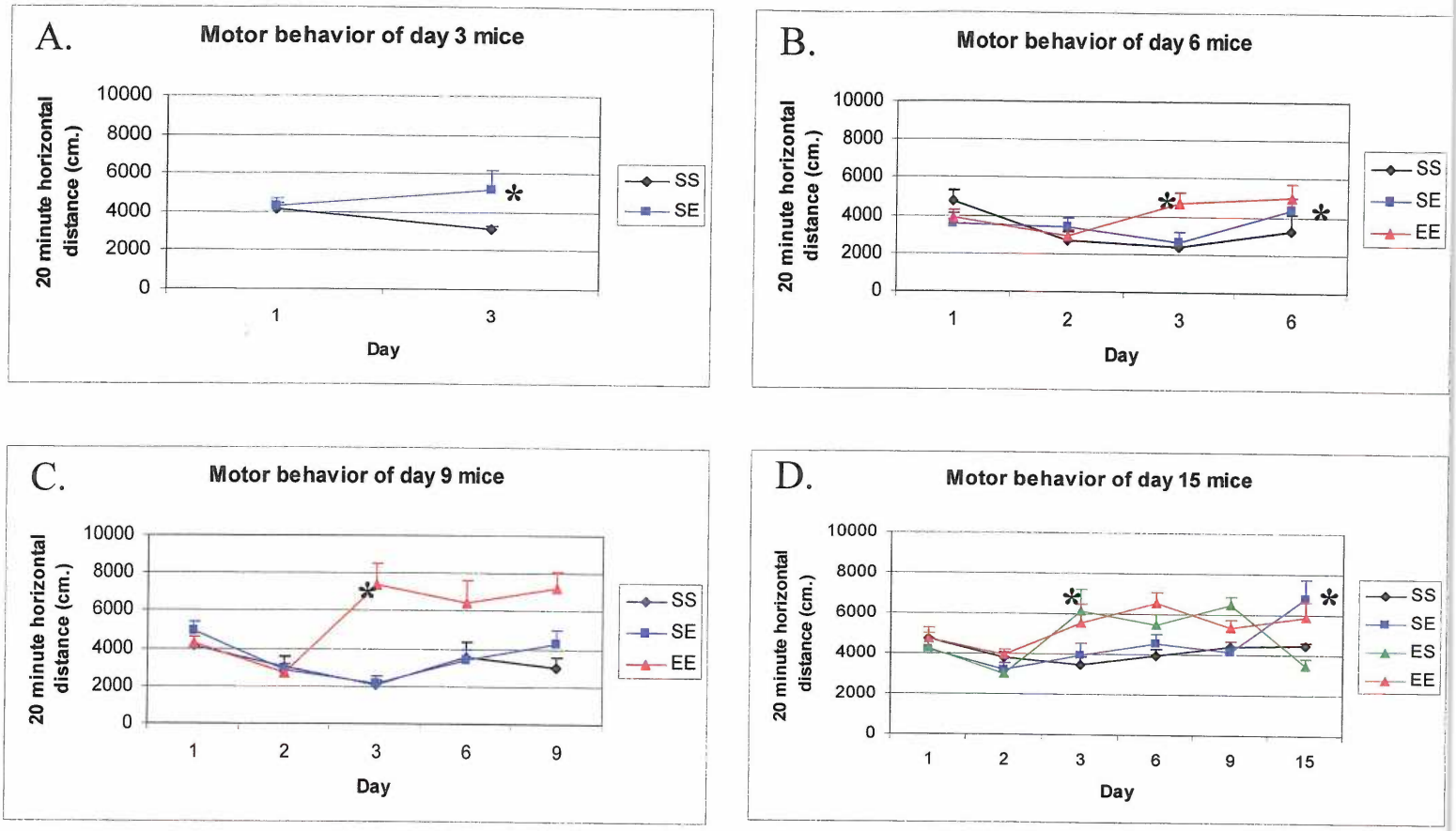
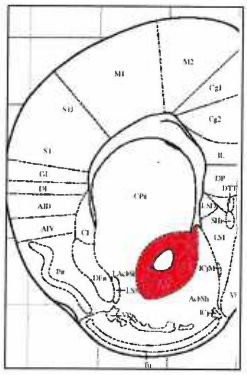
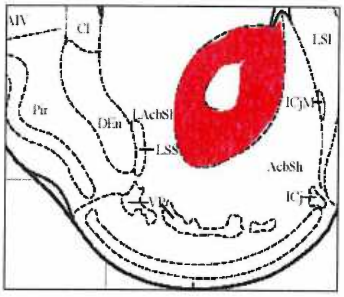


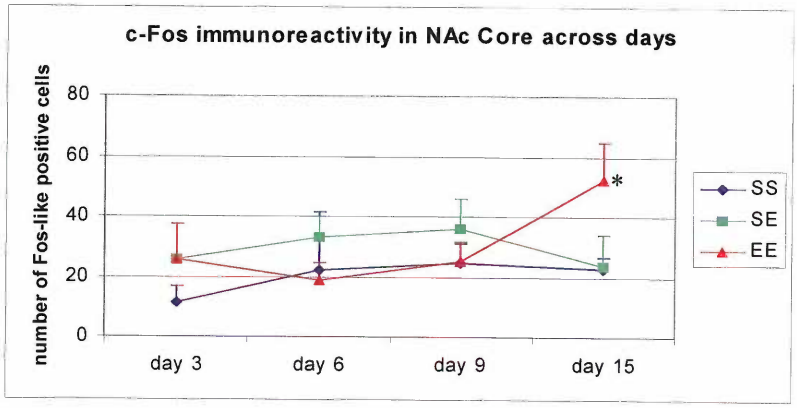
Figure 2.1. Motor activity of D2 mice in an ethanol-sensitization paradigm. Asterisks represent within group significant differences (ethanol-induced activity compared to saline-induced activity; for details and significance levels, please see text). On day 3, in panels a, b, and c, acute stimulatory activity was evident in group EE with the first injection of ethanol. In panel d, it was only the ES group that exhibited significant acute stimulatory behavior in response to the first ethanol injection on day 3. In panel b, acute stimulatory was also observed on day 6 in group SE with their first injection of ethanol. The same is true for group SE (panel d) on day 15; this group exhibited higher activity on day 15 with their first injection of ethanol compared to their saline-induced activity on all other days. No within- or between-groups locomotor sensitization was observed (with the repeated ethanol group; EE) in any of the time courses.

Figure 2.2. No effect of repeated injections on locomotor activity. Mice injected daily and mice that were handled on days 1-14 and injected only once on day 15 had similar levels of motor activity on day 15. Thus, the number of injections did not influence locomotor behavior. The mean $\pm$ SEM is depicted while numbers in parenthesis indicate group sizes.

Figure 2.3. Time-course of IEG immunoreactivity in NAc core.







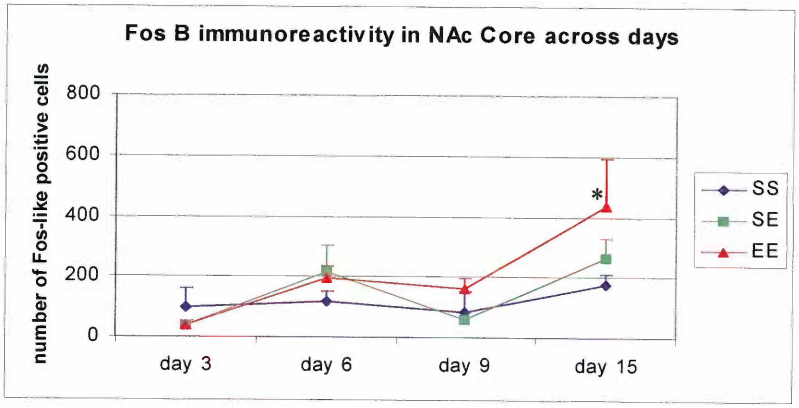
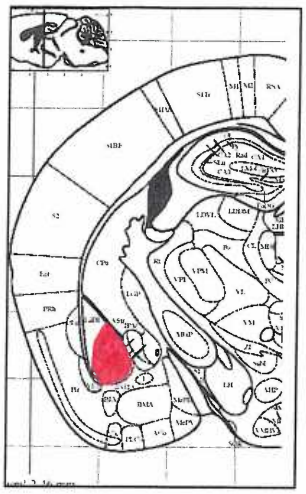
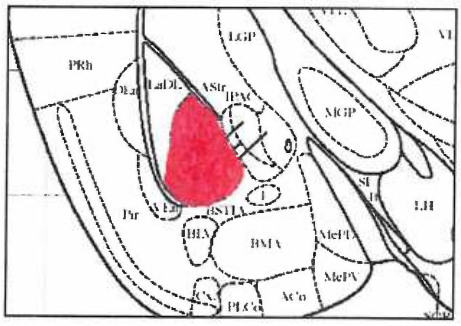


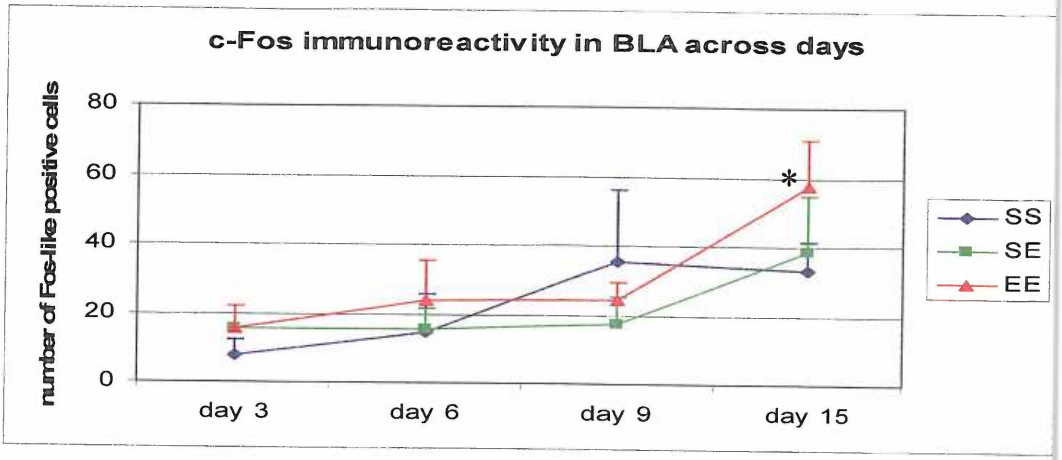
Figure 2.3. Time-course of IEG immunoreactivity in NAc core.

The inset to left of the graphs depicts the area from which Fos counts were obtained (plate 19 from Franklin and Paxinos (1997)). The panels to the right depict the mean $\pm$ SEM of c-Fos and Fos B immunoreactivity across days. Within the NAc core of group EE on day 15, there was significant c-Fos sensitization and a significant increase in Fos B expression relative to all other days (3, 6, and 9). c-Fos and Fos B immunoreactivity within the NAc core of ES mice was not significantly different from that of group EE (data not shown). c-Fos: n_{EE}=6-11; n_{SE}=5-11; n_{SS}=3-11; Fos B: n_{EE}=6-8; n_{SE}=5-7; n_{SS}=3-7 across days. Asterisks denote significance at $p<0.05$.

Figure 2.4. Time-course of IEG immunoreactivity in BLA.







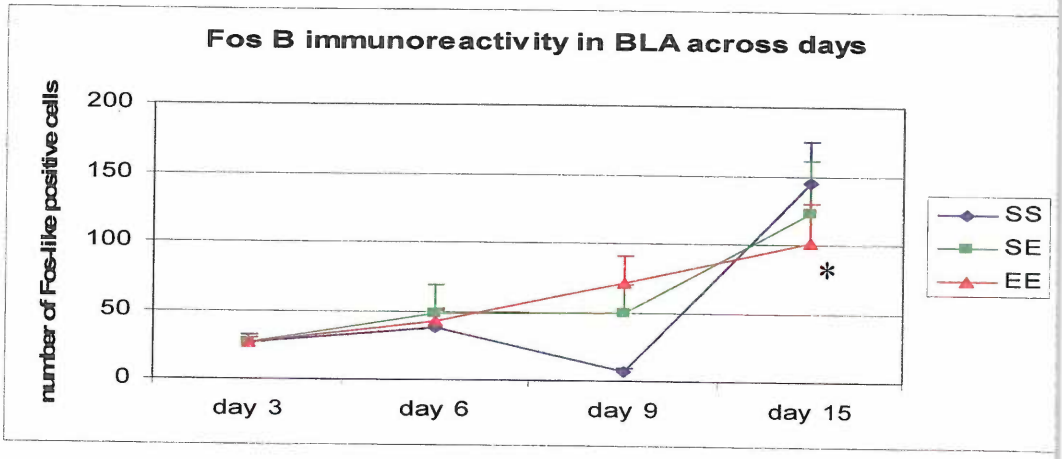
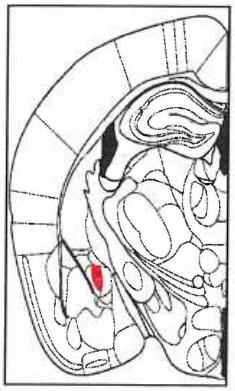
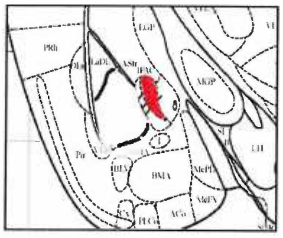
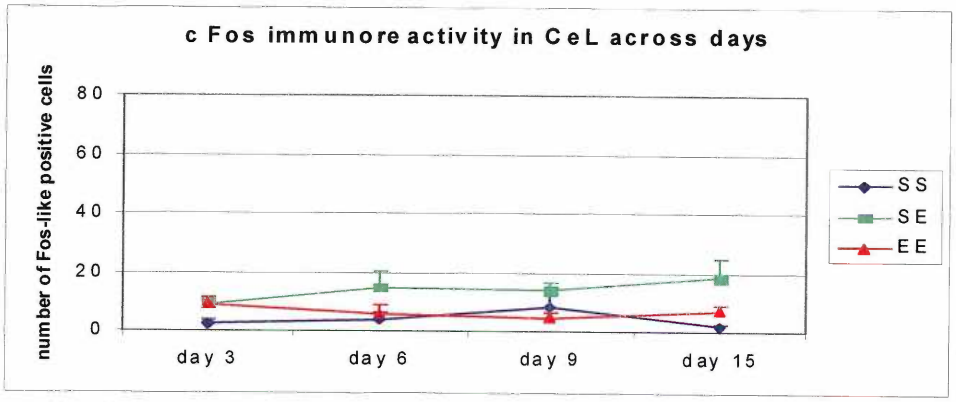


Figure 2.4. Time-course of IEG immunoreactivity in BLA. The inset on the left depicts the area from which counts were taken for the determination of immunoreactivity within this brain region (plate 42 from Franklin and Paxinos (1997)). The panels on the right depict the mean $\pm$ SEM of c-Fos and Fos B immunoreactivity across days. Data analysis showed significant increases in the expression of both c-Fos and Fos B in BLA of group EE on day 15 as compared to day 3 (asterisks denote significance at $p<0.05$). There were no statistically significant changes in c-Fos expression within the BLA of groups SS or SE across time. c-Fos: n_{EE}=6-9; n_{SE}=3-8; n_{SS}=3-9; Fos B: n_{EE}=7-8; n_{SE}=5-9; n_{SS}=2-8 across days.

Figure 2.5 Time-course of IEG immunoreactivity in CeL.







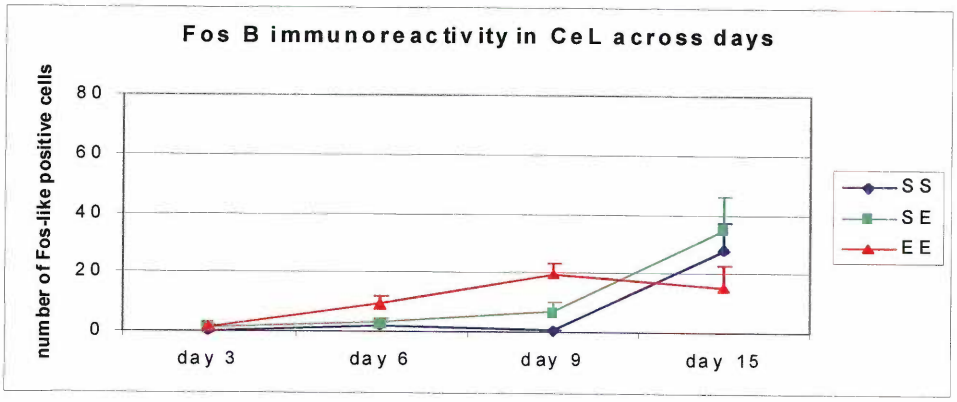


Figure 2.5. Time-course of IEG immunoreactivity in CeL.

The inset on the left depicts the area from which counts were taken for the determination of immunoreactivity within this brain region (plate 42 from Franklin and Paxinos (1997)). The panels on the right depict the mean $\pm$ SEM of c-Fos and Fos B immunoreactivity across days for groups SS, SE, and EE. There were no significant changes in c-Fos expression on day 15 compared to any of the previous days for either group SE or group EE. With respect to Fos B expression levels, group EE showed similar, increased levels of the protein on days 9 and 15, compared to levels on day 3. In contrast, Fos B expression levels on day 15 for group SE were increased significantly relative to Fos B levels for all other days (3, 6, and 9). c-Fos: n_{SS}=3-9; n_{SE}=3-8; n_{EE}=7-8. Fos B: n_{SS}=3-7; n_{SE}=5-10; n_{EE}=7-8.

Chapter 3: Ethanol-induced locomotor sensitization and Fos B regulation in HS mice

3.1 Abstract

Background: Locomotor sensitization results in an increased motor response relative to acute drug and saline controls and is observed in rodents following repeated application of ethanol, a sedative-hypnotic drug of abuse. In this study we used a genetically heterogeneous stock of mice to select for distinct phenotypes following a repeated ethanol paradigm. We examined the association between sensitized and non-sensitized phenotypes and immediate-early gene staining in the: nucleus accumbens (NAc) and central nucleus of the amygdala (CeA) and changes in gene expression via microarray analysis.

Methods: Ethanol was administered intraperitoneally (i.p.) at a dose of 2 g/kg on days 3-15 in a fifteen-day sensitization paradigm (days 1 and 2 were saline habituation days). Behavioral testing for locomotor activity was performed using the PASF system (San Diego Instruments) on days 1, 2, 3, 9, and 15. Following testing on day 15, mice were euthanized and brain tissue harvested. Half of the brain tissue was fixed and sectioned into 30-um slices and was used for immunohistochemical (IHC) analysis for the immediate early genes (IEGs) c-Fos and Fos B. The remaining half of the tissue was used for total RNA extraction via a phenol-chloroform extraction procedure. 4 ug of cRNA was generated by the Gene Microarray Shared Resource at OHSU, fragmented and hybridized to Affymetrix chip MOE 430 A. Analyses by MAS 5.0 identified genes that were detected in the samples and yielded globally scaled comparisons, while R and affy algorithms from the Bioconductor website were used for determining regulated

transcripts. The cut-off for regulation was a 1.4-fold increase or decrease in expression between samples.

Results: Statistical analyses revealed that a sensitized ethanol response could be detected on day 15 in a subset of mice repeatedly treated with ethanol; in contrast, a second subset of mice repeatedly treated with ethanol did not show sensitization. Additionally, IHC analyses revealed that c-Fos staining habituated in both repeated ethanol groups, regardless of phenotype, while acute drug control mice had significantly increased c-Fos staining in the central nucleus of the amygdala. Fos B staining did not differ between any of the groups. Furthermore, fos b mRNA was not differentially regulated between sensitized and non-sensitized HS mice. However, analysis did reveal a number of other transcription factors, channels, intracellular signaling factors, cytoskeletal proteins, and expressed sequence tags that were differentially regulated in the caudate and accumbens of sensitized and non-sensitized mice.

Conclusions: Fos B regulation, at the mRNA or at the protein level, did not correlate with an ethanol-sensitized phenotype in a genetically heterogenous mouse strain. However, it is entirely possible, given the genetic background of these mice, that other transcription factors could serve as molecular correlates of ethanol-sensitized behaviors.

3.2 Introduction

There is much evidence to suggest that certain aspects of alcoholism are heritable, and therefore have a genetic basis. For instance, much of the work by Schuckit and Smith (1996 and 2000) has demonstrated that non-alcoholic sons of alcoholics have a lower level of response to an alcohol challenge, when compared to sons of fathers that are negative for alcoholism; this low level of response (LR) can be used as a risk factor to

predict the likelihood of developing alcoholism later on in life (Soyka et al. 2004). Additionally, loci on chromosomes 10, 11, and 22 have been shown to be correlated with LR. Another gene linkage study performed on first-degree relatives of alcoholic and non-alcoholic families suggests that there are also loci on chromosomes 1, 2, and 16 are associated with the development of the disorder, as well as the development of concurrent depression (Nurnberger et al. 2001). Newlin and Thomson examined phenotypes in certain high-risk populations of men (those positive for a family history of alcoholism) and control subjects; their findings revealed that on the ascending portion of the blood alcohol curve, high-risk subjects were more stimulated with respect to both objective physiological measures and subjective mood ratings as compared to control subjects (Newlin and Thomson 1999). Thus, this study showed that a sensitized response to ethanol could be observed that in a prone human population.

Additionally, changes in gene expression in the brain are thought to underlie the long-term changes that lead to, or that are coincident with, addiction to several classes of drugs of abuse (i.e. cocaine and alcohol). Because much research suggests that drug addiction is a multigenic trait, microarrays are able to provide researchers with an efficient means of identifying a plethora of genes affected by the disease. For example, researchers have shown that there is differential gene expression in frontal cortex samples taken from alcoholics and non-alcoholic controls (Lewohl et al. 2000). In this study, a group of myelin-related genes, cell proliferation genes, and the beta subunit of the GABA-A receptor were all significantly decreased in expression in alcoholic subjects, when compared to controls. In contrast, genes involved in synaptic transmission were significantly increased in expression in alcoholic subjects versus controls. There was

some concern in this study about age-matched controls since the addicted individuals had a shorter life-span than the non-affected group. The authors addressed this issue by comparing their data with that from an aging study; only three of the proteins found to be differentially regulated in the above study were the same as in the aging study. Thus, this study provides us with useful information about associated gene expression changes in the brains of human alcoholics.

Microarray analyses techniques can be used to complement quantitative trait loci (QTL) analyses. Two papers of particular relevance to the present experiment have examined the loci associated with ethanol sensitization. Phillips et al. (1995) identified loci on chromosomes 1, 8, and 10 that were positively associated with sensitized behavior in a panel of BXD recombinant inbred (RI) strains; the locus for marker D10Mc1 was negatively associated with sensitized behavior. Cunningham (1995) also showed that loci on chromosomes 2, 12, and 15 were associated with ethanol-sensitized activity. Both of these studies used a 2 g/kg dose of ethanol as well as the BXD RI strains; however, slightly different durations of ethanol administration were used (Phillips et al: drug administered every other day for five days; Cunningham: drug administered every other day for four days).

Additionally, studies have identified putative QTLs on chromosomes 1 and 2 that influence acute ethanol-induced activity in different mouse strains (Hitzemann et al. 1998; Demarest et al. 2001; Hitzemann et al. 2002). Ethanol-induced activity is thought to reflect the drug's addictive liability as well as its euphoric properties (Phillips et al. 1997).

The aim of this paper was to extend our findings with a repeated ethanol paradigm with DBA/2J (D2) mice by using a sample of mice bred from a cross of eight different inbred strains (heterogeneous stock, HS). The eight different inbred strains that compose the HS stock are: C3H/J, BALB/cJ, AKR/J, A/J, C57BL/6J, DBA/2J, LP/J, and 129Sv/J. One of the major strengths in using HS mice is that subjects can have one of twenty-eight possible combinations of alleles at each genetic locus as opposed to the one type of allele found in an inbred strain. This allows for more genetic heterogeneity, and makes for a useful tool to examine the influences of genetics on certain behavioral traits.

The effects of acute ethanol in the individual strains of the HS stock are variable. BALB/Ibg (a different substrain of the BALB mice used in this study), and C57BL/6J mice have a polymorphism in their nicotinic acetylcholine receptor that does not respond with greater ionic efflux to an application of ethanol and nicotine, as compared to A/J, AKR/J, and C3H/Ibg mice (Butt et al. 2003). Additionally, A/J mice show significantly greater pressor responses in reaction to an acute, dose-dependent, intraperitoneal (i.p.) injection of ethanol, as compared to C57BL/6J, AKR/J, DBA/2J, and BALB/cJ mice (Hatton et al. 2000). Furthermore, C57BL/6J and BALB/cJ mice respond differently with respect to choline acetyltransferase (ChAT) activity in various brain regions following an acute 4.6 g/kg i.p. injection of ethanol (Hashemzadeh-Gargari and Mandel 1989). In our hands, only three of the eight strains in our HS stock show increased motor activation in comparison to their saline controls with an acute i.p. injection of 1.5 g/kg ethanol; these strains are C3H, BALB/cJ, and DBA/2J (unpublished observations; figure 3.1). With repeated ethanol injections of 2-2.5 g/kg for six to thirteen days, the DBA/2J mouse strain shows sensitized behavior, or increased motor activity relative to both acute-ethanol and

saline controls (Phillips et al. 1994). However, the activity of the other strains in the HS stock following repeated ethanol administration has yet to be determined. Most sensitization studies involving mice use the following lines or strains: Swiss-Webster (Camarini et al. 2000; Itzhak and Martin, 1999), DBA/2J (Broadbent and Weitemier 1999; Meyer and Phillips 2003), or HS/Ibg stock[1] (Lessov and Phillips 2003).

When using DBA/2J mice in an ethanol-sensitization paradigm, the majority of mice given repeated ethanol injections demonstrate sensitized locomotor behavior. In contrast, by using the genetically-heterogeneous HS stock, we expect to attain two divergent phenotypes; mice that sensitize to a fifteen-day repeated ethanol regimen (sensitizers, group S) and mice that do not sensitize (non-sensitizers, group NS). We also expect that immediate-early gene profiles would be altered in these two phenotypes such that sensitized mice would show enhanced Fos B staining in the NAc and central nucleus of the amygdala as compared to non-sensitized mice. Fos B is a peptide that is similar to delta Fos B; both peptides are chronic Fos-related antigens (FRAs), and by definition accumulate in neurons with repeated stimulation. For instance, delta Fos B is increased in staining and in abundance (demonstrated by Western analysis) in the NAc of rats treated chronically with cocaine (Werme et al. 2002). Furthermore, this protein continues to remain at significant levels (compared to chronic saline treated subjects) even after drug removal (Colby et al. 2003). It is thought that this protein may mediate some of the behavioral changes seen with and following chronic drug intake (Hope 1994; Chen 1997; Colby et al. 2003). Delta Fos B lacks the carboxy terminus found in Fos B, and it is thought that phosphorylation of its terminus adds to its stability (Nestler et al. 1999).

[1] This strain is slightly different than the one used in the present paper. Two of the strains, CBA/J and LP/J are not represented in the HS/Ibg stock; instead strains RIIIS and IBGS were used (McClearn et al. 1970).

Furthermore, we expected to see habituation of c-Fos staining in the NAc and CeA of both phenotypes (S and NS mice) as research from this lab has shown that repeated injection of a 2 g/kg ethanol dose to D2 mice results in c-Fos habituation (Guptaa et al. unpublished observations) while an acute injection of ethanol at a low-moderate dose results in an increase in c-Fos expression in specific brain regions in both the D2 and B6 mouse strains (Hitzemann and Hitzemann 1997). Additionally, D2 mice administered acute ethanol not only demonstrate increased c-Fos staining in the brain, but also demonstrate increased locomotion, relative to saline-injected controls (Crabbe et al. 1980). Thus, we expected HS mice acutely injected with ethanol to show both an increase in behavior and a corresponding increase in c-Fos staining, while HS mice that sensitized to ethanol would show an increase in Fos B staining. Additionally, differences in gene expression were also expected to correlate with phenotype, such that sensitized mice were expected to show an increased abundance of fos b mRNA relative to non-sensitized mice (c-Fos mRNA is not expected to differ between the two phenotypes due to habituation via repeated stimulation by ethanol).

3.3 Methods and Materials

Animals:

Seventeen breeding pairs (ten separate families) out of the fifty families maintained in our vivarium at OHSU were used to generate the subjects for this experiment. Mice were housed and treated in accordance with procedures set forth by AAALAC. Male mice were weaned at approximately three weeks of age, separated by family, and housed five to a cage, maximally. Food and water were available ad libitum in the colony room with lights on a 12:12 light:dark cycle (lights on at 0600 hrs.). Testing

was conducted on mice aged six to twelve weeks. The average age of the subjects undergoing testing was seven weeks.

To distinguish sensitized (S) from non-sensitized (NS) mice, the activity on day 15 was required to be greater than the habituated activity on day 2 as well as the acute activity on day 3. Thus, a positive value denoted sensitization while negative values represented mice that did not sensitize. Five families were represented in the group of mice that sensitized to ethanol; six were represented in group NS; two of these families were similar between the two groups. The two families therefore produced offspring that sensitized and those that didn't sensitize to ethanol. For RNA analysis, the NAc of five out of the twelve mice that sensitized were pooled; caudate putamens from the same five mice were also pooled for array analysis (mean difference in activity [day 15 – day 3] $\pm$ sem for the five mice used in array analysis was: 4856.4 $\pm$ 1374 cm). Out of the eleven mice that did not show sensitized motor behavior to repeated ethanol injections, the NAc from two mice were pooled for RNA analysis while five different subjects were utilized for gene expression analysis of caudate putamen (mean difference in activity [day 15 – day 3] $\pm$ sem for the five CPu mice: -1873.4 $\pm$ 1227.4 cm). Two mice were used for the NS NAc sample owing to difficulty in obtaining a pure RNA sample (260/280 OD reading < 1.8; likely protein contamination [Manchester, 1995]). All samples had an OD reading of 2.0 or greater as determined by the Gene Microarray Shared Resource at OHSU (GMSR).

Drugs:

The dose of ethanol (20% v/v, dissolved in saline) used in this study was 2 g/kg and was administered intraperitoneally (i.p.). Saline (0.9%) was given in an equivalent

volume. Studies from the Phillips lab have shown that 2 g/kg and 2.5 g/kg ethanol can be used to elicit sensitization in female HS mice (Lessov and Phillips 1998).

Treatment and Testing Schedule:

The protocol used to elicit sensitization was modeled after Phillips et al. (1994). On test days, mice were moved from the colony room to the procedure room and allowed to acclimate to the environment for 25 minutes. Testing was always performed between 1100 and 1500 hrs. On non-test days, injections were given in the procedure room between 1100-1200 hours. It has been shown that the context of drug administration can affect the amount of observed sensitization for ethanol (Day et al. 2001). The treatment and testing schedule used to elicit sensitization is depicted in table 3.1.

Eighteen locomotor activity chambers were used in this experiment. The activity chambers were plastic cages lined with cobb bedding and measured 22(w) x 42(l) x 20(h) cm in dimension; these cages were surrounded by a metal frame housing a 4(w) x 8 (l) array of photocells; the frame was situated 1 cm above the ground (Hitzemann et al. 2000). The software used to monitor the number of beam breaks was the Photocell Activity System designed by San Diego Instruments; beam breaks were recorded as soon as the animal intercepted a grid line. Vertical activity and repetitive horizontal activity were not measured.

Immunohistochemistry:

Twenty-four hours after taking brain tissue, the paraformaldehyde-fixed brains were transferred to 30% sucrose in phosphate buffer. Brain tissue was stored in this solution for approximately four days at 4°C. Thirty-micrometer frozen coronal sections were cut on a microtome and collected in 10 mM phosphate buffered saline (PBS). The sections

were rinsed three times in PBS and treated with 0.3% H2O2 in PBS for fifteen minutes to block the endogenous peroxidase activity. The sections were rinsed in PBS six more times to remove the residual H_2O_2. Sections were then blocked for two hours in the immuno-reaction buffer (10 mM PBS containing 0.25% Triton X-100 and 3% goat serum) without antibody; antibody (final dilution for c-Fos: 1:10,000, for Fos B: 1:1,000) was then added and the incubation was continued for 48 hrs at 4°C. c-Fos antibody was obtained from Oncogene Research Products/Calbiochem (San Diego, CA); the antibody was raised in rabbit against residues 4 - 17 of human c-Fos protein. Fos B antibody was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and was raised in rabbit against human Fos B, residues 75-150.

Following incubation with the primary antibody, sections were rinsed three times in PBS and incubated with the biotinylated goat anti-rabbit IgG (1:200 of secondary antibody) in 10 mM PBS containing 0.3% Triton X-100 and 3% goat serum for two hours at room temperature. The sections were subsequently incubated with horseradish peroxidase avidin-biotin complex in 10 mM PBS for two hours at room temperature. The sections were rinsed three times in PBS and placed in 0.1 M Tris (pH 7.4) for 5 minutes. The chromatic reaction was completed with diaminobenzidine (50 mg/100 ml of 0.1 M Tris) in presence of 0.01% nickel ammonium sulfate solution and 0.035% hydrogen peroxide. The sections were mounted onto slides, dehydrated and cover-slipped with Permount. Only one section was analyzed per region per mouse (Hitzemann and Hitzemann 1999).

Statistical Analyses for behavior and IHC:

Data for behavior was analyzed by a repeated measure ANOVA with day as the repeated measure. Follow-up analyses for significant interactions used one-way ANOVAs to examine each group's behavior across days (within-groups analysis) and between-groups behavior on each day (between-groups analysis). Neuman-Keuls post-hoc tests were used to elaborate on significant results. Data for IHC was separated on the basis of protein (c Fos and Fos B) and then analyzed via break-down ANOVA to check for significant brain regions with group as the categorical factor. Significant results were further analyzed by Neuman-Keuls post-hoc analyses. Significance was set at $p \leq 0.05$.

Total RNA isolation and precipitation:

Following locomotor activity testing on day 15, mice were returned to their home cage and left undisturbed for 40 minutes. Mice were then moved to a different procedure room and then euthanized. Half of the brain tissue was frozen in an RNase-free environment and on dry ice (RNase OUT, Geno Technology, St. Louis, MO). Both NAc core and shell, and caudate putamen were dissected using a Zivic Labs 1 mm acrylic brain block. Figures 15 through 24 from the Franklin and Paxinos mouse brain atlas were used (bregma 1.94mm to bregma 0.86mm) for the dissection of the accumbens and the caudate putamen (CPu) from the same 1mm slice. Cortex was removed; however, cortex contamination was noted in the caudate putamen sample from non-sensitized mice. For a diagram of the extracted brain regions, please refer to figure 3.4. Following extraction, slices were placed in 1.7 ml low-retention RNase-free tubes (Bioexpress, Kaysville, UT) and stored at -80°C until ready for RNA isolation. For RNA isolation, tissue samples from five mice for S NAc, S CPu, and NS CPu, and for two mice for sample NS NAc

were transferred from microfuge tubes to 15 ml plastic tubes (Elkay Laboratory Products, Inc., Shrewsbury, MA) for homogenization in 375 ul of Trizol (GibcoBRL, Carlsbad, CA; manufacturer recommends 750 ul of TRIzol per 50 –100 mg tissue). Samples were then incubated for five minutes at room temperature for the dissociation of nucleoproteins complexes, following which 100 ul of chloroform (Sigma-Aldrich, Milwaukee, WI) was added to each tube (200 ul per 50-100 mg tissue). Tubes were then capped and gently shaken by hand before incubating at room temperature for fifteen minutes. Samples were then centrifuged at 12,000 rpm (7300 x g) for fifteen minutes at 4°C. After spinning, the aqueous phase (supernatant) was pipetted into a clean centrifuge tube. 250 ul (500 ul for 50-100 mg tissue) of isopropyl alcohol was added to each sample to precipitate out total RNA. The samples were then incubated at room temperature for ten minutes, and centrifuged for an additional ten minutes at 12,000 rpm at 4°C. The isopropanol was then discarded, and 500 ul (1000 ul for 50-100 mg tissue) of 75% ethanol was added to each RNA pellet. Samples were then briefly vortexed and centrifuged at 7,500 rpm for five minutes at 4°C to wash the pellet. The pellet was then air dried for five-to-ten minutes, before dissolving in 50 ul of Rnase-free water (suspension volume recommended by Quiagen for cleaning up small amounts of RNA (Valencia, CA)). Spectrophotometric analysis was then performed; samples whose 260/280 ratio were lower than 1.5 were then precipitated in order to remove protein contaminants. If precipitation was not performed on the same day as the extraction, samples were frozen at -80°C until ready to use. For precipitation, samples were thawed before adding 1/10 the volume of 3 M sodium acetate (4.8 ul; pH 5.2) and 2 volumes of 95% ethanol (105.6 ul) to each sample. Samples were then incubated at -20°C for two hours-to-overnight. Tubes were then gently shaken and

spun at 12,000 rpm at 4°C for thirty minutes. The supernatant was then decanted and 0.5 mls of 70% ethanol was added to each sample. Samples were centrifuged at 12,000 rpm at 4°C for ten minutes. The supernatant was disposed of, and the pellet was air-dried before resuspension in the appropriate amount of RNase-free water to achieve a concentration of 1 ug/ul and a 260/280 OD reading of at least 1.8 (recommended concentration by the Gene Microarray Shared Resource Center for Affymetrix (GMSR) microarray analyses, West Campus OHSU, Beaverton, OR).

Microarray Chips:

The GeneChip Mouse Genome 430A Array (MOE 430A) by Affymetrix was used for the microarray experiment. There are 10,000 known mouse genes and 5,000 ESTs spotted onto this array. One of the major advantages in using this array is that in every probe set there are eleven multiple probe pairs; there are eleven perfect match probes or stretches of short oligos (25 base pairs) representing various lengths of the gene sequence or EST; for every perfect match probe there is also a complementary mismatch probe where one base has been changed to control for non-specific hybridization. Thus, the array allows for multiple replications within a single experiment by using these internal controls. In addition to the perfect match and mismatch controls, there are also other probe controls on the chip. For instance, there are hybridization controls, poly-A controls, and housekeeping genes spotted onto this array. The high sensitivity of detection (1, in 100,000 genes) for this array also allows for more confidence while interpreting the data. Further information on the microarray can be found at:

http://www.affymetrix.com/products/arrays/specific/mouse430a_2.affx,and

http://www.affymetrix.com/support/technical/datasheets/mogarrays_datasheet.pdf .

Microarray Hybridization:

After analyses on the Agilent Bioanalyzer by the GMSR at OHSU, 4 ug of RNA from each sample were reverse transcribed. An in vitro transcription reaction was then performed on the cDNA using an Enzo IVT kit that simultaneously labeled the product, cRNA, with biotin. Prior to hybridization, the cRNA was fragmented to reduce the number of base pairs per strand to 25-200. Following this step, cRNA was added to hybridization cocktail, injected into the Genechip hybridization chamber and allowed to incubate for 16 hours at 45°C. Chips were then stained with a fluorescent molecule (streptavidin-phycoerythrin) that binds to the biotin. The signal was amplified, the chip scanned, and the signal distribution pattern recorded.

Statistical Analyses for Microarray:

Because each pooled sample was only run on one chip, no standard errors for the detected transcripts were generated. In order to control for false positives a comparison was done using three different types of data analyses: MAS 5.0 by Affymetrix, Affy, and Robust Multichip Average (RMA) both by Bioconductor/R. Bioconductor and the associated R program allows users to compare data from different studies (and therefore, different labs) by using a MAS 5.0 algorithm similar to Affymetrix's, as well as other programs for analyzing data; the R software is freely available to all via a link at the bioconductor website (http://www.bioconductor.org/).

Affymetrix uses the following corrections in determining signal intensities for each gene spotted onto the array: a background calculation (based on two factors: the lowest 2% of cell intensities in each of the zones in the array and the distance of each cell to the center of its zone; a smoothing factor is then applied to transition from zone to

zone within the array), noise correction (adjusting each of the cell intensities for background; determining floor and threshold intensities), and finally, an adjustment of intensities at perfect match (PM) probes (by subtracting the log of the intensity at the mismatch probe (MM) from the log of the intensity at the PM when intensity MM < intensity PM, or by subtracting the mean difference of mismatch probes from perfect match probes for an entire probe set; this corrects for "stray signal" at the PM probe). For further specific information on the statistical parameters Affymetrix uses to generate raw intensities for the probe sets please refer to:

http://www.affymetrix.com/support/technical/whitepapers/sadd_whitepaper.pdf .

The mas/affy program available through the bioconductor website via R, is slightly different from the one Affymetrix uses in its analysis of its chips; the main difference between the two algorithms comes from rounding; additionally, the number of probe sets and the identity of the probe sets called increased, decreased, or unchanged should be similar between the two programs. Finally, the RMA algorithm used to generate signal intensities for each transcript is distinct from Affymetrix and R-affy in three important ways: the data are normalized in a quantile manner (fitted to an exponential curve) as opposed to the linear analysis used in MAS 5.0; there is no mismatch probe correction for each perfect match complement (thus, the stray signal component is not accounted for); and finally, the data output is given as log2 expression values.

The criteria used to determine if the gene was significantly different between the two phenotypes was agreement between the three different algorithms; additionally, the change in expression had to be >40% (i.e., >1.4-fold increase or decrease in expression).

This number is admittedly arbitrary; however, this criterion was in concordance with two papers examining gene expression in mice and rats following alcohol or heroin treatment and provided a starting point for analyses (Tabakoff et al. 2003, Jacobs et al. 2004).

3.4 Results

Behavior:

On day 15, analyses were conducted to determine groups of sensitized and non-sensitzed mice following the repeated ethanol paradigm. The EE or repeated drug group was composed of 23 mice; 12 mice were classified as sensitized (group S) and 11 as non-sensitized (group NS) on the basis of this criteria (see materials and methods). A repeated measures analysis for all groups' behavior across days revealed a significant day effect ($F_{(4,196)}$=10.0, $p<0.001$) and a significant day by group interaction ($F_{(16, 196)}$= 4.16, $p<0.001$). A depiction of the groups' behavior across days is shown in figure 3.2. Between-groups analyses showed no group differences on days 1 or 2. On day 3, there were significant group differences ($F_{(4,49)} = 3.1$, $p<0.05$), where post-hoc tests revealed that group NS demonstrated greater activity than group S ($p<0.05$). There were also significant between-groups differences on day 15 ($F_{(4,49)} = 8.3$, $p<0.001$), such that post-hoc tests revealed that sensitized mice had greater levels of activity compared to all other groups of mice ($p<0.01$ for all comparisons). Thus by the end of the paradigm, a subset of mice given ethanol repeatedly were able to demonstrate ethanol-induced locomotor sensitization.

A one-way ANOVA for the sensitized group of mice revealed a significant day effect, such that Neuman-Keuls analysis revealed that day 15 behavior (following receipt of the thirteenth ethanol injection) was greater than the activity observed on day 2

(following the second saline injection and apparatus exposure), day 3 (delivery of the first ethanol injection) and day 9 (the seventh ethanol injection; $p<0.01$ for all effects). Day 3 and day 9 activities did not differ from each other, suggesting that sensitization behavior did not develop during the first seven days of ethanol injection. Additionally, day 3 activity (first ethanol injection) was not significantly different from day 2 activity (habituation day, second saline injection), suggesting a lack of an acute stimulatory response by ethanol for the group, overall. Thus, group S demonstrated within-groups sensitization behavior, as well as between-groups sensitization behavior.

A one-way ANOVA also revealed a significant day effect for the group of mice repeatedly treated with ethanol, but that did not demonstrate locomotor sensitization (group NS; $F_{(4,40)} = 4.6$, $p<0.01$). Neuman-Keuls analysis revealed that activity on day 2, day 9, and day 15 was significantly lower than that seen on day 1 ($p<0.01$ for the above effects). Additionally, day 3 activity was not significantly different from any other day. These results suggest that: 1. there was no overall acute stimulatory effect of ethanol in this group, and 2. there was no sensitized behavior in this group. There was also a significant day effect for group ES ($F_{(4,36)} = 3.4$, $p<0.05$). Post-hoc analysis revealed that day 15 activity was significantly lower than that seen on day 1 (first exposure to apparatus, saline injection) and on day 9 (seventh ethanol injection; $p<0.05$ for both days). These results suggest that there was no contextual conditioning in this paradigm. There were no other differences between days for this group. A one-way ANOVA for group SE also revealed a significant effect of day ($F_{(4,36)} = 4.9$, $p<0.01$). Neuman-Keuls post-hoc testing showed that activity on day 2, day 3, and day 9 was significantly lower than that observed on day 1, suggesting that habituation to the procedure occurred

($p<0.05$ for day 2, and $p<0.01$ for days 3 and 9). Activity on day 15 following the delivery of an ethanol injection was not significantly different from any other day, suggesting a lack of an acute stimulatory effect of ethanol. There was also a significant effect of day for group SS ($F_{(4,40)} = 2.9$, $p<0.05$). Post-hoc analysis showed that activity on day 2, day 3, and day 9 was significantly lower than that observed on day 1 ($p<0.05$ for all days), suggesting that habituation occurred (lower activity on day 15 almost reached significance with $p=0.054$).

Immunohistochemistry:

The brain regions analyzed for c Fos and Fos B staining were: cingulate gyrus, area 1; prelimbic cortex, NAc core and shell, caudate putamen, lateral septum-ventral portion, bed nucleus of the stria terminalis-lateral portion, lateral hypothalamus, medial preoptic area of the hypothalamus, central nucleus of the amygdala, secondary motor cortex, entorhinal cortex, perirhinal cortex, and the Edinger-Westphal nucleus. Analyses revealed that there was significantly more c-Fos in all parts of the central nucleus of the amygdala in group SE as compared to all other groups on day 15 (capsule (CeC): $F_{(4, 36)}=3.6$, lateral (CeL): $F_{(4, 36)}=5.7$, medial (CeM): $F_{(4, 31)}=4.4$; $p<0.05$ for CeC, and $p<0.01$ for CeL and CeM). Figure 3.3 below depicts c-Fos staining in CeL on day 15 for all groups. The pattern seen in CeL of SE mice is similar to that seen in other parts of the CeA. Because SE mice did not show an increase in motor behavior on day 15 following an acute ethanol injection of ethanol, motor behavior was not correlated with an increase in c-Fos staining. Of interest also, is the observation that groups S and NS did not differ in c-Fos staining from each other (while there were differences in behavior), or from group SS. Therefore, with repeated ethanol injections, habituation in c-Fos staining occurred in the CeA of HS mice; additionally, c-Fos staining was not correlated with ethanol-sensitized motor behavior.

In contrast, no significant differences in Fos B staining were detected between any of the groups (i.e., SS, SE, ES, NS, and S) in any of the brain regions examined. In a separate analysis, S and NS mice were collapsed and the analysis re-done on the following groups: SS, SE, ES, and EE. The results of this analysis also did not reveal any significant differences in Fos B expression in any of the brain regions examined.

Microarray:

More transcripts were down-regulated as opposed to increased in expression in both the CPu (65 transcripts) and the NAc (300 transcripts) in NS mice relative to S mice. Thus, the non-sensitized phenotype was correlated with a general decrease in gene expression in these brain regions. However, for simplicity, only the transcripts similarly regulated in accumbens and caudate are shown in tables 3.2 and 3.3 for sensitized mice and tables 3.4 and 3.5 for non-sensitized mice. Most of the regulated genes affect transcription, intracellular signaling mechanisms, or cytoskeletal components; quite a few are also estimated sequence tags (ESTs), whose function is yet to be determined. Of particular note was the lack of regulation found by all of the three programs in NS or S mice of c Fos, Fos B, cdk5, or p35. cdk5 and p35 are cofactors that serve to dampen activation of the dopamine D1 receptor, via a phosphorylation protein, DARPP-32. Additionally, cdk5 and p35 have been shown to be increased in transgenic mice engineered to overexpress delta Fos B and treated with chronic cocaine (Bibb et al. 2001). It is thought that the increase in cdk5 and p35 serve as a brake on the increased motor behavior seen with cocaine sensitization and the induction of delta Fos B protein (Bibb et al. 2001). Additionally, mRNA for delta Fos B, the chronic FRA increased by repeated cocaine treatment was not spotted on this array, due to its instability as an mRNA species (i.e. quickly degraded; Nestler 2001b).

Interestingly, three subfamilies (shaker: maps to chromosome 6 at 61 cM in the murine genome; shab: maps to chromosome 2 at 97 cM; and a large conductance calcium activated channel which maps to chromosome 14 at 11.2 cM) of potassium channels were increased in both the caudate and accumbens of sensitized mice, as compared to non-

sensitized mice. It is possible that an increase in potassium channel expression may serve to maintain homeostasis by hyperpolarizing neurons, and lessening the likelihood of neuronal excitability; in effect serving a similar purpose as cdk5 and p35. Unfortunately, the potassium channel found in this study is located more distally on chromosome 2 than the QTL identified by Cunningham in 1995 (71-73 cM via marker D2Bir1). Thus, it is likely that the potassium channel in the present paper was not captured in the QTL identified by Cunningham (1995). However, this does not take into account potential epistatic effects of the Kcnb1 on other genes.

For the most part, transcript regulation was in the same direction in the accumbens and the caudate; however, there are three transcripts that serve as exceptions to this. Two of these transcripts are involved in transport (affy id: 1417714, 1423287; gene symbols: Hba-a1, Cbln1) and one is involved in intracellular signaling (affy id: 1423444; gene symbol: Rock1). All three of these transcripts were decreased in the accumbens of non-sensitized mice and increased in the caudate of sensitized mice.

3.5 Discussion

This set of experiments showed that by using a repeated ethanol paradigm and monitoring motor activity it was possible to observe two distinct phenotypes in group EE of HS mice on day 15: sensitized (S) and non-sensitized (NS) mice. NS mice on day 1 had a greater activity than all other groups, suggesting a greater reaction to novelty. By day 2, however, this group had habituated to the saline injection and the environment in a manner similar to all other groups. On day 3, no significant differences in activity were observed among groups. This result was different than what Lessov et al. (1998) found with female HS mice undergoing an ethanol sensitization paradigm. Here, the group of

female mice given an acute ethanol injection on day 3 showed a stimulatory response in comparison to saline-injected controls. Mice given an acute ethanol injection on day 3 in the present experiment (groups S, NS, and ES) did not show acute motor stimulation. Two main factors could account for the disparate results: 1. female mice were used in the 1998 study as opposed to males in the present study, and 2. a different heterogeneous mouse strain was used in the present study than in the 1998 study. For instance, Calihol and Mormede (2000) and Haney et al. (1994) found that female rats repeatedly injected with cocaine (10 mg/kg) had a greater sensitized motor response than male rats. This was true regardless of the type of rat strain used (e.g. spontaneous hypertensive (SHR), Wistar-Kyoto (WKY) and the RHA/RLA lines). In the Lessov and Phillips (1998) study, female HS mice acutely injected with 2.0 g/kg of ethanol had an estimated horizontal distance of 5000 cm in a ten-minute period; in the present study male HS mice moved a similar distance, but in a twenty-minute period. However, data analyzed from Demarest et al. (2001) which used mice from the same HS strain as in the current set of experiments showed a significant effect of sex (male vs. female) with *males* having a significantly higher motor score than females and a significant effect of drug (ethanol vs. saline) with ethanol-stimulated activity being greater than saline-induced activity; however, there was no interaction between the two variables. Thus, with the present data set, sex can likely be eliminated as a factor contributing to the lack of acute motor activity on day 3. Additionally, data analyses from Demarest et al. (2001) show that mice from the HS strain used in the present set of experiments were able to show stimulation with an acute 1.5 g/kg injection of ethanol. It is a possibility then, that a 1.5 g/kg dose of ethanol could stimulate motor activity to a greater extent than a 2.0 g/kg injection.

Despite the overall lack of stimulation on day 3 in any of the ethanol-treated groups in the present study, a subset of mice repeatedly treated with ethanol expressed ethanol-induced motor sensitization on day 15, relative to their day 3 activity scores and the activity of all other groups on day 15. It is of some interest to note that the Lessov and Phillips (1998) study used a different sensitization paradigm than the one used in the present set of experiments. Starting on day 4, mice were injected (but not tested until the end of the paradigm, day 13) with 2.5 g/kg of ethanol. On the test day mice were injected with 2 g/kg of ethanol. It would be interesting to see if the Lessov and Phillips (1998) paradigm would result in more robust sensitization in our sample of HS mice.

There were no significant correlations between day 3 and day 15 activity scores for either S or NS groups; group NS, however, did show a trend for a positive correlation between these two days ($r=0.57$, $p=0.06$). The lack of significant correlation between motor behavior on acute and sensitized test days mimics those results observed with RI strains undergoing an ethanol-sensitization paradigm; neither acute ethanol-stimulated nor basal locomotor activity was correlated with sensitized behavior (Phillips et al. 1995).

It is interesting to note that acute-ethanol control mice (group SE) had significant increases in c Fos staining on day 15 in all parts of the CeA, relative to all other groups. This same group, however, did not show locomotor activation following an acute ethanol injection. Thus, there is a dissociation between the increase in c-Fos staining and acute-ethanol induced motor activity in HS mice. This finding is further supported by examining the behavior of individual subjects within group SE. Mice that had increased locomotor activity with an acute ethanol injection on day 15 compared to their motor activity on days 2 and 3 with saline injections had significantly lower levels of c-Fos

expression in CeL compared to mice that didn't express acute stimulation with ethanol on day 15 ($F_{(1,4)}$=12.8, $p<0.05$). This is in contrast to what is observed in DBA/2J mice (Hitzemann and Hitzemann 1997), and what is observed in FAST mice that have been selectively bred (from a slightly different HS population as ours) to be acutely stimulated by a 2 g/kg dose of ethanol. Demarest et al. (1999 a) found that after an acute 1.5 g/kg dose of ethanol, *both* FAST and SLOW mice (selectively bred for sensitivity and resistance to a stimulatory response to an acute injection of ethanol, respectively) demonstrated an increase in c-Fos staining in the lateral portion of the central nucleus of the amygdala (CeL). However, it was only the FAST mice that showed an increase in the number of c-Fos-like neurons in the capsular portion of the central nucleus of the amygdala; thus the increased c-Fos staining in CeC was a correlated response to selection in FAST mice. While we examined many of the same brain regions in this study as in the Demarest paper, we were unable to uncover a correlation between c-Fos staining and activity. While we did not examine c-Fos staining on day 3, it is tempting to hypothesize in light of the present results, that group EE on day 3 would demonstrate increased c-Fos staining relative to SS mice, regardless of the behavioral response to an acute ethanol injection.

Importantly, with regards to c-Fos, it was evident that staining of this protein habituated in repeated ethanol mice (group EE or groups S and NS), as compared to acutely-injected controls. It is also interesting to contrast the brain regions activated via c-Fos staining in HS mice with those activated in D2 mice. Acute-ethanol (SE) D2 mice showed increased c-Fos in the following brain regions: prelimbic and cingulate gyrus (area 1) corticies, bed nucleus of the stria terminalis-lateral portion, capsular, lateral and

medial areas of the central nucleus of the amygdala (CeA) and somatosensory cortex, while acute ethanol HS mice only showed increased c-Fos staining in all portions of the CeA. Levels of activity following the repeated ethanol administration paradigm were also comparable between these two strains. These results speak to the dampening of c-Fos activation in brain regions other than the CeA by inclusion of seven other inbred strains in HS mice. However, the results from the HS and D2 studies again evidence amygdalar involvement in the actions of acute ethanol. Studies have shown that following an acute injection of chlordiazepoxide, an anxiolytic similar in its behavioral properties to ethanol, c-Fos is co-localized with GAD, an enzyme involved in the synthesis of the inhibitory neurotransmitter, GABA (Hitzemann and Hitzemann 1999). It is unknown at the present time whether amygdalar involvement following an acute ethanol injection is necessary for the development or the expression of a sensitized motor response to ethanol.

Because recent experiments have shown an increase in delta Fos B expression in NAc that was coincident with a cocaine-sensitized motor response, it was expected that S mice would have greater Fos B expression in the NAc, when compared to NS mice. However, the Fos B results from IHC and microarray experiments did not support this hypothesis; neither protein nor mRNA levels of Fos B were correlated with a sensitized phenotype in HS mice. Additionally, even lowering the cut-off for regulation (1.3 or 1.2-fold) did not allow for differential fos B expression between the two phenotypes. It is also feasible that another transcription factor or gene could serve as marker for accumbens-related changes associated with repeated ethanol administration and sensitized behavior. For instance, in the tables below, different subunits of the eukaryotic translation factors were differentially regulated between NS and S mice. One of the binding proteins

(Eif4ebp2) for this translation factor maps to chromosome 10 at 32 cM. The binding protein affects cAMP-mediated signaling, and the insulin receptor pathway via substrate binding. Phillips et al. (1995) found a locus on chromosome 10 at 64 cM that was positively associated with sensitization behavior in a panel of BXD RI mice. More work needs to be done to see if the findings of these two studies are in fact the same, i.e. replication of the current microarray experiment, and further fine-mapping of the region found in the QTL study. Furthermore, it would be interesting to see if selection pressure on these two phenotypes would cause more robust differences in gene expression; to our knowledge there is not a selected line of mice for ethanol-sensitized locomotor behavior, possibly because of laboriousness of these experiments. The closest phenotype to this is the existing FAST mice that have been selected for a high locomotor stimulant response to ethanol (1.5 or 2 g/kg depending on the selection generation; Phillips et al. 1991). However, at the present time, there is no published data on differential gene expression profiles between the FAST and SLOW selected lines of mice.

Finally, one might be able to observe more robust changes in staining if we had used an antibody directed against delta Fos B. Work from Nestler et al. (Chen et al. 1997) showed that this protein is specifically increased by the repeated administration of many different types of stimuli (different classes of drug, electrical stimulation) and that this effect is long-lasting. In fact, protein levels of delta Fos B are elevated for several months following cessation of drug. Although we did not perform withdrawal studies in this experiment, it is expected that staining for Fos B could still be observed for at least two days into drug withdrawal. Fos B does not have as long a half-life as its phosphorylated compound, delta Fos B. It is thought that post-translational modifications to delta Fos B,

such as phosphorylation, help to make this protein much more stable in vivo (Chen 1997).

In conclusion, these results show that sensitized behavior can be elicited in mice of this genotype. However, the sensitized locomotor behavior was not correlated with an increased expression of either c-Fos or Fos B in any of the brain regions examined. It is unlikely that a different circuitry is activated with repeated ethanol administration in this genotype as the one of the founder lines for these mice is the D2 strain. The D2 strain exhibits increased c-Fos and Fos B expression in the accumbens core and basolateral amygdala following repeated i.p. ethanol administration (see chapter 2 results) and also exhibits locomotor sensitization (Phillips et al. 1994). It may well be that another protein, as revealed by microarray analysis, may serve as a better correlate for ethanol-sensitized behavior.

Table 3.1: Treatment and testing schedule for HS mice.

Day	**1 Test**	**2 Test**	**3 Test**	4-6 Inject only	**9 Test**	10-14 Inject only	**15 Test**
Group							
SS (saline controls)	S	S	S	S	S	S	**S**
SE (acute ethanol controls)	S	S	S	S	S	S	**E**
EE (repeated drug group)	S	S	E	E	E	E	**E**
ES (conditioning controls)	S	S	E	E	E	E	**S**

Table 3.1. Treatment and testing schedule for HS mice. Days in bold indicate days on which locomotor activity was monitored; days 1 and 2 (habituation days; i.p. saline injections), 3 (i.p. acute drug injection), 9, and 15 (challenge day). Each locomotor test session was 20 minutes in length. On day15, groups of approximately n=10 mice were euthanized one hour after drug or vehicle injection, or 40 minutes after locomotor testing. Whole brains were extracted from these animals and placed in 4% paraformaldehyde for immunohistochemical analyses of c-Fos and Fos B.

Figure 3.1. Acute-ethanol stimulated activity in the 8 different inbred strains used to generate HS mice.

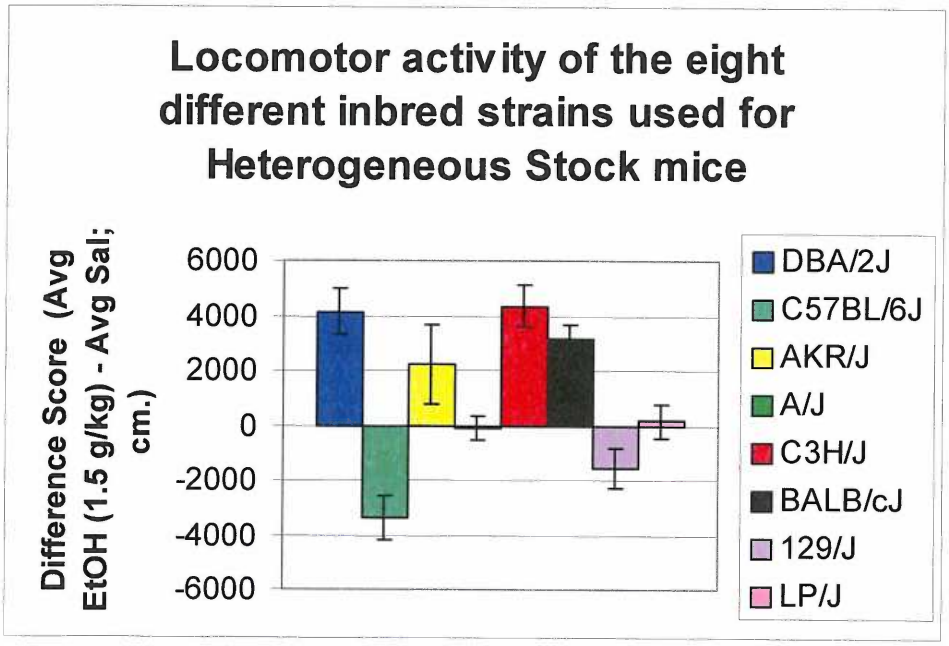


Figure 3.1 Locomotor activity (difference scores) of the 8 different inbred strains used to generate HS mice. Values represent the mean $\pm$ SEM for n=8/strain. The dose of ethanol used was slightly different than that typically used for sensitization paradigms (1.5 vs. 2 g/kg); however, 3 of the 8 strains showed significant stimulation over saline scores: DBA/2J, C3H/J, and BALBc/J. Data courtesy of B. Malmanger.

Figure 3.2. Motor activity of HS mice across days.

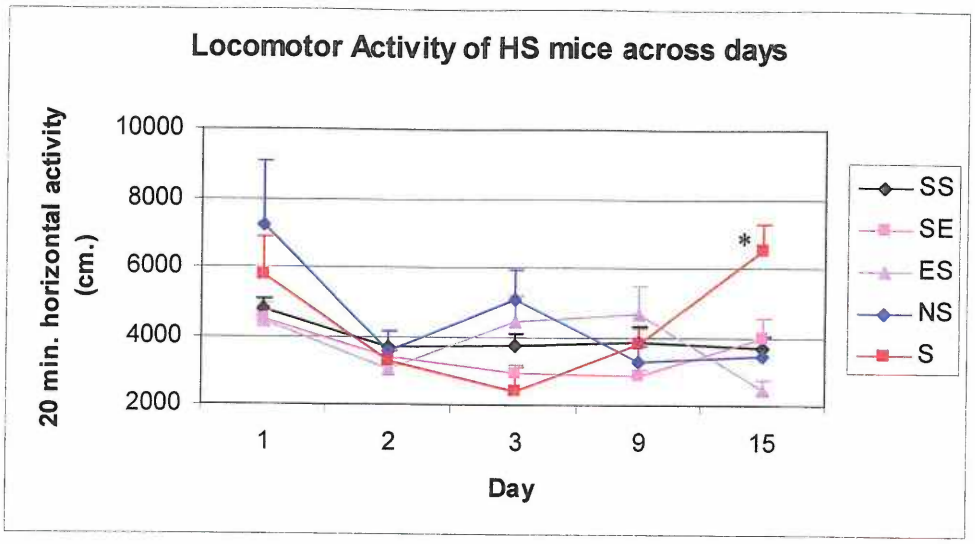


Figure 3.2. Motor activity of male HS mice across days. On day 3, none of the groups that received ethanol (S, NS, or ES) for the first time demonstrated a significant increase in locomotor activity compared to their saline-induced activity on day 2. However on day 15, S, or sensitized mice demonstrated significant within-group locomotor sensitization. Values represent the mean $\pm$ SEM; number of subjects/group: S=12, NS=11, ES=10, SE=10, SS=11.

Figure 3.3. HS c-Fos IHC in CeL on day 15.

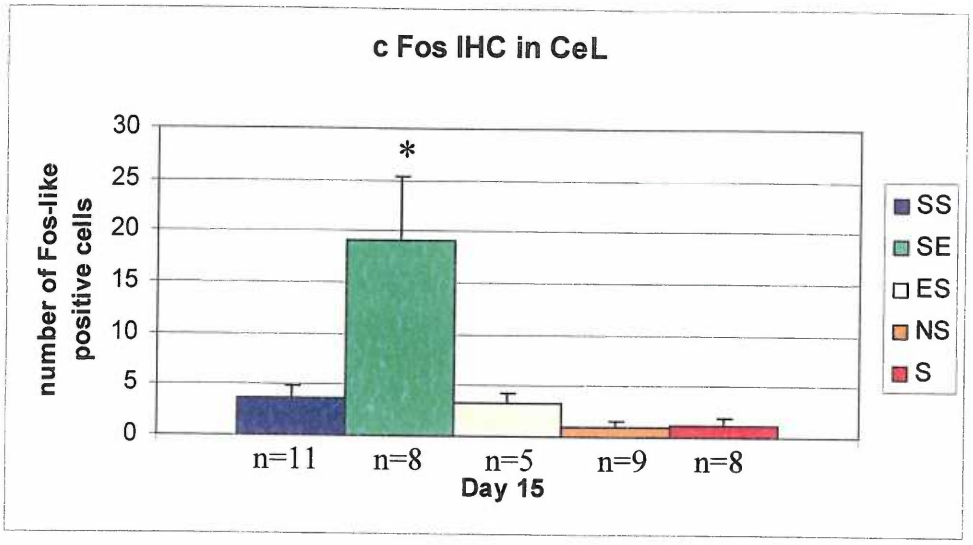


Figure 3.3. c-Fos immunohistochemistry in the lateral portion of the central nucleus of the amygdala for all groups of mice on day 15. Depicted is the mean $\pm$ SEM number of c-Fos positive cells within the CeL for the number of animals listed under each group. SE mice had significantly greater c-Fos staining in this portion, as well as the capsular and medial portions of the amygdala, compared to all other groups. Asterisk represents significance at $p<0.05$ with Neuman-Keuls post-hoc testing.

Figure 3.4 Diagram of tissue used for RNA analysis.

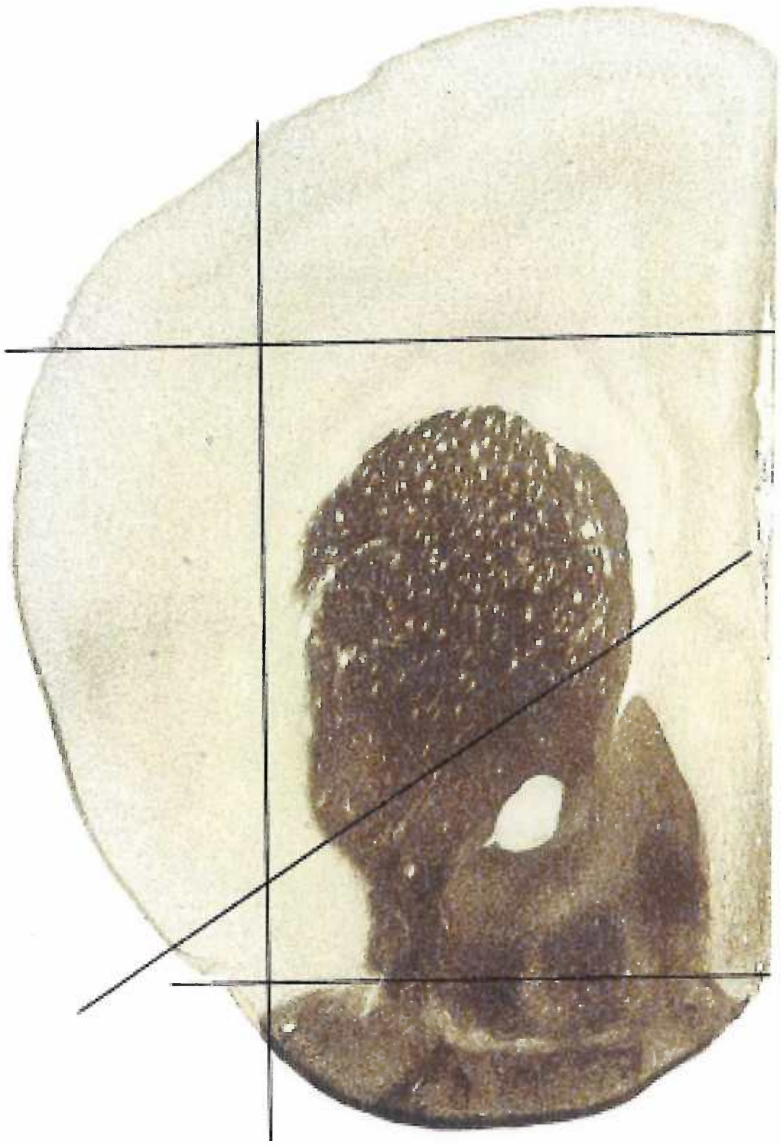


Figure 3.4 Diagram of tissue used for RNA analysis.

When a 1mm accumbens/caudate slice was extracted from the brain, a vertical cut was made to remove somatosensory cortex and piriform cortex. Two horizontal cuts were then made to remove the dorsal motor corticies and neurons in the olfactory tubercule. A 45° diagonal cut was then made to separate caudate putamen from nucleus accumbens.

Table 3.2 Genes increased in expression in the accumbens of sensitized mice (relative to non-sensitized mice).

Affy Gene Id	Gene Symbol	Percent Increase	Chromosomal Location (bases or cM when available)
Transcription			
1417084_at	eukaryotic translation initiation factor 4E binding protein 2 Eif4e2	61.14	10: 63316222-63316669; 32 cM
1418469_at	negative regulation of transcription from Pol II promoter Nrip1	96.30	16: 77445569-77445965
1422264_s_at	basic transcription element binding protein 1 Bteb1	51.38	19: 23311250-23311479
1424598_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 Ddx6	215.63	9: 46405070-46405471; 26 cM
1426777_a_at	Wiskott-Aldrich syndrome-like (human) Was1	134.70	6: 24505332-24536688
1436790_a_at	SRY-box containing gene 11 Sox11	248.92	12: 21781127-21781633 approximately 14.52 cM*
1436898_at	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated) Sfpq	436.14	4: bases 125940981-125941446; 57.6 cM
Channels			
1417416_at	potassium voltage-gated channel, shaker-related subfamily, member 1 Kcna1	447.74	6: 128569822-128570368; 61 cM
1423180_at	potassium voltage gated channel, Shab-related subfamily, member 1 Kcnb1	109.08	2: 167715850-167716311 ; 97 cM
1424848_at	potassium large conductance calcium-activated channel, subfamily M, alpha member 1 Kcnma1	506.66	14: 18093823-18094344; 11.2 cM
Cytoskeletal			
1427564_at	diaphanous homolog 2 (Drosophila) Diap2	165.47	X: 118328079-118499409

1422945_a_at	kinesin family member 5C Kif5c	255.35	2: 50026675-50033660; 32.5 cM
1418429_at	kinesin family member 5B Kif5b	93.53	18: 6415066-6415441; 1.0 cM
1428820_at	microtubule-associated protein, RP/EB family, member 1; Mapre1	48.14	2: 154396575-154397154 approximately 9.76 cM
Signal Transduction			
1419038_a_at	casein kinase II, alpha 1 polypeptide Csnk2a1	250.25	1: 159988978-159989183 approximately 10.6 cM
1422553_at	phosphatase and tensin homolog; Pten	483.39	19: 32617554-32622075; 24.5 cM
1424398_at	DEAH (Asp-Glu-Ala-His) box polypeptide 36 Dhx36	233.21	3: 64034301-64038748
1424922_a_at	bromodomain containing 4; Brd4	356.72	17: 31875829-31882403; 20 cM
1434177_at	endothelin converting enzyme 1; LOC230857	126.54	4: 136744902-136745392
ESTs			
1426389_at	RIKEN cDNA E030025C11 gene E030025C11Rik	130.12	2: 5214675-5215125 approximately 3.47 cM

Table 3.3 Genes increased in expression in the caudate putamen of sensitized mice (relative to non-sensitized mice).

Affy Gene Id	Gene Name & Symbol	Percent Increase	Chromosomal Location (bases or cM when available)
Transcription			
1417084_at	eukaryotic translation initiation factor 4E binding protein 2 Eif4e 2	45.16	10: 63316222-63316669; 32 cM
1418469_at	negative regulation of transcription from Pol II promoter Nrip1	81.54	16: 77445569-77445965
1422264_s_at	basic transcription element binding protein 1 Bteb1	71.73	19: 23311250-23311479
1424598_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 Ddx6	89.98	9: 46405070-46405471; 26 cM
1426777_a_at	Wiskott-Aldrich syndrome-like (human) Was1	65.20	6: 24505332-24536688
1436790_a_at	SRY-box containing gene 11 Sox11	90.33	12: 21781127-21781633 approximately 14.52 cM
1436898_at	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated) Sfpq	145.28	4: 125940981-125941446; 57.6 cM
Channels			
1417416_at	potassium voltage-gated channel, shaker-related subfamily, member 1 Kcna1	89.47	6: 128569822-128570368; 61 cM
1423180_at	potassium voltage gated channel, Shab-related subfamily, member 1; Kcnb1	132.32	2: 167715850-167716311; 97 cM
1424848_at	potassium large conductance calcium-activated channel, subfamily M, alpha member 1 Kcnma1	103.02	14: 18093823-18094344; 11.2 cM
Cytoskeletal			
1427564_at	diaphanous homolog 2 (Drosophila) Diap2	71.73	X: 118328079-118499409

1422945_a_at	kinesin family member 5C Kif5c	74.34	2: 50026675-50033660; 32.5 cM
1418431_at	kinesin family member 5B Kif5b	81.30	18: 6415066-6415441; 1.0 cM
1428820_at	microtubule-associated protein, RP/EB family, member 1; Mapre1	73.84	2: 154396575-154397154 approximately 9.76 cM
Signal Transduction			
1419038_a_at	casein kinase II, alpha 1 polypeptide Csnk2a1	59.74	1: 159988978-159989183 approximately 10.6 cM
1422553_at	phosphatase and tensin homolog; Pten	92.68	19: 32617554-32622075; 24.5 cM
1424398_at	DEAH (Asp-Glu-Ala-His) box polypeptide 36 Dhx36	65.76	3: 64034301-64038748
1424922_a_at	bromodomain containing 4; Brd4	57.57	17: 31875829-31882403 ; 20 cM
1434177_at	endothelin converting enzyme 1; LOC230857	71.38	4: 136744902-136745392
ESTs			
1426389_at	E030025C11Rik	89.20	2: 5214675-5215125 approximately 3.47 cM

Tables 3.2 and 3.3. Genes increased in expression in the NAc (3.2) and caudate putamen (3.3) of sensitized mice as compared to non-sensitized mice (determined by Affymetrix chip MOE 430A and the Bioconductor/R package). For simplicity, percent increases are given (as determined by RMA). Estimates for the chromosomal location of genes (in cM; obtained from Lu et al. (unpublished data)) were performed for those chromosomes associated with an ethanol-sensitized response (chromosomes 1, 8, 10; Phillips et al. (1995); chromosomes 2, 12, and 15; Cunningham (1995)).

Table 3.4. Genes increased in expression in the accumbens of non-sensitized mice (relative to sensitized mice).

Affy Gene Id	Gene Symbol	Percent Increase	Chromosomal Location (in bases or cM when available)
Transcription			
1416814_at	cytotoxic granule-associated RNA binding protein 1 Tia1	46.22	6: 87848155-87848435
1417210_at	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked Eif2s3y	59.08	Y: 3025559-3025689; 2.12 cM
1424252_at	heterogeneous nuclear ribonucleoprotein D-like Hnrpdl	47.64	5: 99139016-99139321; 54 cM
1424641_a_at	THO complex 1 Thoc1	44.93	18: 10196922-10197094
Metabolism			
1417765_a_at	amylase 1, salivary Amy1	60.65	3: 116866114-116871861; 50.0 cM
Signal Transduction			
1426124_a_at	CDC-like kinase Clk	54.95	1: 59945736-59947859; 30.0 cM
1426823_s_at	proteasome (prosome, macropain) activator subunit 4 Psme4	46.53	11: 30778342-30778596
ESTs			
1434962_x_at		41.96	
1420048_at		55.72	8: 95611029-95611385 approximately 6.06 cM
1426369_at	3732409C05Rik	50.16	7: 105423115-105423515
1426832_at	6330505F04Rik	53.61	X: 46151520-46151924
1435315_s_at	3732409C05Rik	52.19	7: 105422366-105422669
1417001_a_at	DNA segment, Chr 4, Wayne State University 53, expressed D4Wsu53e	44.13	4: 133782220-133782788; 65.4 cM

Table 3.5 Genes increased in expression in the caudate putamen of non-sensitized mice (relative to sensitized mice).

Affy Gene Id	Gene Symbol	Percent Increase	Chromosomal Location (in bases/cM when available)
Transcription			
1416814_at	cytotoxic granule-associated RNA binding protein 1 Tia1	50.78	6: 87848155-87848435
1417210_at	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked Eif2s3y	54.28	Y: 3025559-3025689; 2.12 cM
1424252_at	heterogeneous nuclear ribonucleoprotein D-like Hnrpdl	49.60	5: 99139016-99139321; 54 cM
1424641_a_at	THO complex 1 Thoc1	47.65	18: 10196922-10197094
Metabolism			
1417765_a_at	amylase 1, salivary Amy1	44.43	3: 116866114-116871861; 50.0 cM
Signal Transduction			
1426124_a_at	CDC-like kinase Clk	56.48	1: 59945736-59947859; 30.0 cM
1426823_s_at	proteasome (prosome, macropain) activator subunit 4 Psme4	41.55	11: 30778342-30778596
ESTs			
1434962_x_at		57.80	4: 41818528-41820892
1420048_at		43.27	8: 95611029-95611385 approximately 6.06 cM
1426369_at	3732409C05Rik	51.23	7: 105423115-105423515
1426832_at	6330505F04Rik	55.78	X: 46151520-46151924
1435315_s_at	3732409C05Rik	48.93	7: 105422366-105422669
1417001_a_at	DNA segment, Chr 4, Wayne State University 53, expressed D4Wsu53e	64.80	4: 133782220-133782788; 65.4 cM

Tables 3.4 and 3.5. Genes increased in expression in the NAc (3.4) and caudate putamen (3.5) of non-sensitized mice as compared to sensitized mice (determined by Affymetrix chip MOE 430A and the Bioconductor/R package). For simplicity, percent increases are given (as determined by RMA).

Chapter 4: Global Discussion.

In the set of experiments described in this document, changes in motor behavior were dissociated from the observed immunohistochemical changes. To summarize, D2 mice repeatedly treated with ethanol did not show a sensitized motor response but did show a significant increase in both c-Fos and Fos B expression in NAc core and BLA, when compared to mice acutely treated with ethanol. HS mice that expressed a sensitized motor response did not show significant changes in Fos B expression, when compared to non-sensitized mice and conditioning control mice (groups that were also treated repeatedly with ethanol). Furthermore, HS mice acutely treated with ethanol did not show an increased stimulant response in comparison to saline controls, but did show a significant increase in c-Fos expression in the CeA, when compared to all other groups of mice. The paragraphs below detail possible reasons for the lack of sensitized behavior in D2 mice as well as potential substrates in the NAc core and BLA that may affected by repeated ethanol administration. Stress sensitivity is also discussed with respect to HS and D2 mice as a possible explanation for the differences in the development of locomotor sensitization.

It has been postulated that tolerance to a specific behavior must develop in order for horizontal locomotor sensitization to be expressed (Meyer and Phillips, 2003; Hitzemann and Hitzemann, 1999; Cunningham et al. 2002). For example, during an ethanol locomotor sensitization paradigm, increases in both rearing behavior and horizontal locomotion occur (Phillips et al. 1994). It was formerly thought that the two types of behavior were mutually exclusive. However Meyer and Phillips (2003) found that increases in rearing behavior throughout the ethanol sensitization paradigm did not

inhibit the development of within-groups horizontal locomotor sensitization (2 g/kg ethanol to male D2 mice for 13 consecutive days). Thus, tolerance to rearing behavior is not necessary for the expression of locomotor sensitization. Hitzemann and Hitzemann (1999) proposed that tolerance to the freezing behavior would be necessary in order for the acute stimulatory effect of ethanol to occur, however this theory has not been investigated with respect to either acute or chronic administration of ethanol. Furthermore, the authors proposed that ethanol inhibited GABA-ergic interneurons within CeL allowing for GABA-ergic projection neurons to prevent competing (i.e. freezing) behaviors. Grahame et al. (2000) sensitized high and low alcohol-preferring (HAP and LAP, respectively) mice by administering one dose of ethanol during the development phase (0, 1.25, 2.0, 2.75, or 3.5 g/kg every other day for eight days to subsets of mice within the HAP and LAP phenotypic groups), and a lower drug dose on challenge/testing day (2 g/kg). It is thought that by using a medium dose ($\geq$ 3 g/kg ethanol) of alcohol during the development phase of a sensitization paradigm, tolerance to the ataxic effects will develop allowing for the expression of sensitization upon drug challenge. However, static dowel testing showed that both lines of mice developed similar amounts of tolerance, while only HAP mice showed ethanol-induced locomotor sensitization with repeated injections of 2.75 or 3.5 g/kg ethanol. Thus, sensitization expression was not correlated with tolerance development on the static dowel. In contrast, Ponomarev and Crabbe (2002) found that acute functional tolerance (AFT) to the loss of righting reflex (LORR) with a dose of 3 g/kg ethanol was *correlated* with acute ethanol-induced stimulation (activity was monitored in a grid test for 10 minutes following acute ethanol [2 g/kg] administration) in a panel of nine inbred strains. This same correlation

held for FAST mice such that they developed greater AFT and were more stimulated with acute ethanol than SLOW mice. However, the authors did not investigate correlations between LORR and ethanol-induced locomotor sensitization. It may be that tolerance to LORR coincides with the expression of ethanol-induced locomotor sensitization and that the two behaviors are related.

Modifying the sensitization paradigm by including a time-off period before challenge could have helped to express sensitization in D2 mice. Itzhak and Martin (2000) administered the same dose of ethanol to male D2 mice for seven consecutive days (1.5 g/kg) but waited one week to challenge and test for sensitization expression with the same dose of ethanol. The concept of a time-off period has been used when examining sensitization to psychostimulants. Reith et al. (1987) found that sensitization was expressed following intermittent cocaine exposure over an 18-day period (20 mg/kg; a total of 430 mg/kg per subject). When rats were implanted with osmotic mini-pumps and administered cocaine via continuous release, experimenters noted tolerance to the locomotor effect (25 mg/kg/day; a total of 450 mg/kg per subject). Differences in behavior were not attributable to pharmacokinetics as brain concentrations of cocaine following an i.p. cocaine challenge to both groups yielded similar concentrations of drug. It is postulated that repeated application of the drug induces tolerance, while abrupt drug removal renders the neuronal system(s) involved in sensitization, hyperexcitable (Crabbe and Metten, 1996). This hyperexcitable neuronal system would remain in an active, excited state but would eventually fall back to a new baseline instituting neuroadaptive changes (Grace, 2000). For instance, acute ethanol application has been shown to increase dopamine cell spike activity, inducing a phasic response in the VTA neurons

(burst activity within the cell body of dopamine neurons; Grace, 2000). With repeated drug application, dopamine accumulates in the post-synaptic space, but at concentrations too low to stimulate postsynaptic receptors. These concentrations however, are thought to be enough to stimulate dopamine autoreceptors to clear dopamine from the synapse (Grace, 2000). The activity of dopamine neurons is interpreted as tonic (i.e. non-bursting) following repeated drug application and is influenced by glutamate input into the accumbens (West et al.2003). Investigators have postulated that the tonic condition in dopamine neruons increases the probability of drug-seeking behaviors to achieve the phasic dopamine response (Grace, 2000; West et al. 2003). Thus, using different drug doses during the development and expression phases of a sensitization paradigm or a time-off period could have instituted both the development and the expression of ethanol-induced locomotor sensitization in D2 mice.

Experimenters have also investigated the types of alterations that are present within the nucleus accumbens following repeated drug treatment. For example, He et al. (2004) have observed changes in levels of stimulated DA release in the accumbens core ten days after administration of morphine. Rats received systemic morphine (final dose: 50 mg/ml at 12.51 ml/kg/day) via infusion for a week. On day 10, investigators administered amphetamine (10 uM) via a microdialysis probe inserted in the accumbens core and measured extracellular dopamine levels. Morphine-treated subjects showed a 50-60% decrease in stimulated dopamine release as compared to saline-infused/amphetamine treated rats. There were no significant differences between groups with regards to basal dopamine levels. Following the microdialysis on day 11, investigators trained subjects in a biased conditioned place preference paradigm (dose of

morphine used, 5 mg/kg). Subjects were tested on day 12 for the expression of preference or avoidance. Both groups showed significant place preference for the morphine-conditioned chamber. However, the morphine-infused subjects showed significantly more place preference than saline-infused controls. The authors therefore concluded that low dopamine levels in the accumbens core following morphine withdrawal might mediate drug-seeking behaviors. The sensitized levels of c-Fos and increased Fos B expression in the accumbens core that was observed in the present set of experiments in D2 mice repeatedly treated with ethanol might be representative of a decreased level of a neuronal substrate. Double IHC for the IEG and dopamine may confirm the above findings with regards to morphine. However, the differences between the two studies should be noted. First, different drugs were used, and secondly, a different time course was used to observe changes in dopamine in the accumbens. The He et al. (2004) study waited at least three days into morphine withdrawal before examining core levels of DA, while our studies examined IEG differences one hour post-ethanol injection.

The observation that the basolateral amygdala and lateral portion of the central nucleus of the amygdala showed an enhanced Fos B response in control groups (SS and SE D2 mice) as well as experimental groups (EE and ES) was unexpected. The significantly increased Fos B levels on day 15 compared to day 3 in the control groups within these brain regions may be indicative of 1. an association between environment and paradigm that is not behaviorally manifested as a conditioned response (BLA) or 2. stress (CeA). Both the CeA and BLA are involved in different aspects of conditioned learning, especially with regard to fear paradigms. The CeA is thought to be involved in the acquisition and elicitation of fear-conditioned behaviors (Kim and Davis, 1993;

Hitchcock and Davis, 1986) while the BLA is thought to be a brain region important for the storage of fear-related memories (LaLumiere et al. 2003). With respect to cocaine, reversible inactivation of the BLA has been shown to inhibit non-reinforced reinstatement responding for drug when subjects are presented with stimuli (both visual and auditory) previously paired with delivery of drug (McLaughlin and See, 2003). Glucose injections into BLA following extinction training for amphetamine-induced CPP in rats facilitated extinction behavior compared to subjects that received intra-BLA vehicle injections (Schroeder and Packard, 2003). Thus, the statistically significant increase in Fos B expression in BLA of D2 SS and SE mice on day 15 (compared to day 3) could be representative of contextual influence on the sensitization paradigm that failed to manifest itself behaviorally (i.e. lack of conditioned activation in group ES on day 15). Furthermore, activation of this brain region may inhibit the development of ethanol-induced sensitization.

Fos expression within the CeA has also been shown to be sensitive to stress. Significant increases in c-Fos expression were observed in the CeA of rats that had undergone repeated restraint stress (60 minutes/day for 10 days) and challenged once with a social stressor (social defeat; Chung et al. 2000). This increase in c-Fos expression was significant compared to protein levels in rats that underwent repeated restraint alone. Using a similar paradigm, investigators noted increased Fos B expression in BLA and the medial amygdala following nine days of repeated restraint stress (60 minutes/day; Stamp and Herbert, 2001). The increased Fos B expression was significant compared to protein levels following a single restraint session. Finally, a study by Nikulina et al. (2004) showed that in rats exposed to a social defeat paradigm three times (once every third day

for ten days) and challenged with amphetamine (1 mg/kg) sixty days later, Fos immunoreactivity was increased above those levels observed under each condition alone (social defeat or amphetamine) in the CeA and VTA. Thus, these studies show that robust increases in Fos expression can be observed in CeA following repeated exposure to a psychological stressor, a physical stressor, or some combination of both. As stated earlier (see section 2.5; discussion), repeated handling and injection could have stressed D2 SS and SE mice inducing a statistically significant Fos B response on day 15 (compared to day 3) in the BLA and CeA of these mice. Thus, the increase in Fos B in CeA could represent a stressful reaction to repeated handling and injection, instead of an actual involvement in the ethanol-sensitization circuit.

Because HS mice contain alleles from the B6 strain (in addition to alleles from seven other inbred strains), the genetic make-up may render them less sensitive to stress than D2 mice. This reduced sensitivity to stress may also help them to express sensitization more easily. For instance, in our sensitization paradigm HS chronic saline (SS) mice had no significant variability in their motor activity across the fifteen-day procedure. This was in contrast to what was observed in D2 SS mice; these mice had significantly increased motor scores on days 6 and 9 compared to their motor scores on habituation day (day 2). Furthermore, a paper by Roberts et al. (1992) showed that at approximately six hours after an acute 4g/kg ethanol injection, D2 mice demonstrated a significant increase in corticosterone compared to B6 mice. D2 mice also exhibit significantly higher extracellular dopamine levels in the nucleus accumbens in response to forced swim (an acute stress) as compared to B6 mice (Ventura et al. 2002). B6 mice

are therefore generally believed to be less sensitive to stress-induced increases in corticosterone than D2 mice.

There is not much data concerning corticosterone, glucocorticoid receptor expression, or stress for the HS strain or even the other inbred strains composing HS mice. One report by Patacchioli et al. (1990) compared corticosterone binding sites in the hippocampus of BALB/c and B6 mice and found that B6 mice had a significantly lower number of binding sites in addition to having lower morning corticosterone concentrations than BALB/c mice. Unfortunately, other inbred strains were not evaluated in this study. Roberts et al. (1995) demonstrated that plasma corticosterone concentrations were bimodally distributed in BXD RI mice following an acute 4 g/kg ethanol injection. It is possible that our selection of subjects from seventeen of the fifty breeding pairs maintained in our colony may be biased. Even though similar ethanol-sensitization paradigms were employed, genetic factors may have rendered HS mice less susceptible to stress effects as compared to D2 mice.

Our preliminary microarray study showed that specific subunits of two distinct types of potassium channels were increased in expression in sensitized HS mice compared to non-sensitized HS mice. The expression level of the shaker subunit, Kcna1, was four times higher in sensitized mice while the shab subunit, Kcnma1, was increased by one-fold in sensitized mice as compared to non-sensitized mice. These two types of potassium channels differ subtly in their function and may differentially serve to regulate sensitized behavior. The shaker family of potassium channels is highly sensitive to voltage and can induce a large but transient potassium current. An additional function of this subunit is to contribute to distal spike propagation (Baro et al. 2000). The shab

family of potassium channels has a slightly different role, where members of this family function as delayed rectifiers, contributing to late-onset neuronal repolarization (Wicher et al. 2001). Thus, it is possible that the presence of these two receptors serve to maintain a phasic response in the Nac medium-spiny GABA-ergic neurons. However, at this time it is unknown whether the two types of receptors are located on the same nucleus accumbens neurons.

WebQTL is a neuroinformatics program that attempts to correlate the expression of known genes with specific phenotypes (both types of data are stored in a database managed by the University of Tennessee; for further details, please see www.webqtl.org). A cursory analysis showed correlations between several genes and within-groups sensitized behavior in BXD mice repeatedly treated with ethanol (Phillips et al. 1995). The gene expression chips used by the WebQTL program are U74Av2 chips, or older versions of the MOE 430 A chip used in chapter 3. There are two major differences between these two types of chips. The first is that the probe sequences encoding some of the genes have been changed on the MOE 430 A chip in order to be more specific for their respective message. The second difference is that MOE 430 A has more EST sequences spotted on it compared to the U74 chip. As with the microarray data in chapter 3, the U74Av2 chip data has been analyzed using the RMA as well as the MAS 5.0 algorithms. RMA analysis showed that Eif4a1 ($p=0.002$, $r=0.59$, chromosome 11, 69.2 Mb) was positively correlated with a sensitized phenotype in the BXD strain. In our own microarray analysis in chapter 3, different forms of this gene were increased in both sensitized and non-sensitized HS mice. A quick search of the WebQTL database showed that this protein was not associated with a habituated, saline phenotype in BXD RI mice.

The phenotype for the saline response used corrected day 2 saline scores (corrected for saline/novel environmental activity on day 1 in a grid test) in BXD mice (Phillips et al. 1996). Additionally, a search was conducted for genes associated with the acute ethanol phenotype (Phillips et al. 1996). Eif(4a1) was also absent in this analysis. Thus, various forms of Eif (eukaryotic translation initiation factor) seem to be affected by repeated ethanol treatment. Both RMA and MAS 5.0 analyses on the WebQTL site found that different subunits of the cholinergic nicotinic receptor (alpha polypeptide 7, r = -0.47; gamma polypeptide, r = -0.61 respectively) were negatively correlated with a sensitized phenotype. Cholinergic interneurons are located within the nucleus accumbens, and thus may affect output from the accumbens to the VP (part of the motor circuitry) and to the VTA. Because the WebQTL program uses the BXD phenotype, it can provide investigators with a global picture of the types of genes associated with a specific phenotype.

Although sensitization behavior was not observed in D2 mice, increases in transcription factors c-Fos and Fos B were present in the NAc core of repeated ethanol-treated mice. Furthermore, microarray analyses in HS mice demonstrated the association between other transcription factors, potassium channels, ESTs, and intracellular signaling components and the sensitized phenotype. These substrates within the NAc represent a starting point for a bottom-up approach for the analysis for the molecular basis of sensitization.

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