Determining the role of the extended amygdala in regulating alcohol consumption in C57BL/6J mice

By

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CERTIFICATE OF APPROVAL

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Acc ---anterior commissure

AP --- anterior posterior

B6 --- C57BL/6J

BEC --- blood ethanol concentration

BLA --- Basolateral Amygdala

BNST --- Bed nucleus of the stria terminalis

BNSTLP --- lateral posterior division of the bed nucleus of the stria terminalis

BNSTMA --- medial anterior bed nucleus of the stria terminalis

BNSTMV --- medioventral portion of the Bed nucleus of the stria terminalis

BNSTDL --- dorsolateral portion of the bed nucleus of the stria terminalis

BNSTOV --- oval nucleus of the bed nucleus of the stria terminalis

cEA --- central extended amygdala

CeA --- central nucleus of the amygdala

CRF --- corticotropin releasing factor

DV --- dorsoventral

EA --- extended amygdala

EW --- edinger westphal

GABA --- gamma aminobutyric acid

GAD --- gamma amino decarboxylase

LH --- lateral hypothalamus

LS --- lateral septum

ML --- mediolateral

MS --- medial septum

NAc shell --- nucleus accumbens shell

PAG --- periaqueductal gray

VTA --- ventral tegmental area

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Abstract

The purpose of the research described in this dissertation was to determine the neural circuits involved with baseline ethanol consumption and increases in ethanol consumption seen in our animal model of ethanol dependency (further described below). The brain region of focus was the central extended amygdala (cEA) since this region has been shown to be involved in baseline consumption and self-administration of ethanol in rats (Hyytia & Koob, 1995; Eiler et al., 2002) and the changes in ethanol consumption induced by chronic intermittent ethanol vapor exposure seen in rats and mice (Funk et al., 2006; Finn et al., 2007). To determine if the cEA is involved in these behavioral phenotypes, the components of the cEA were lesioned separately. These components included the lateral posterior portion of the bed nucleus of the stria terminalis (BNSTLP), the central nucleus of the amygdala (CeA) and the nucleus accumbens shell (NAc shell). Chapter 2 illustrates that lesions of the BNSTLP decreased baseline ethanol consumption in a 2 hr limited access procedure, but not in a continuous access procedure. Chapter 3 and chapter 4 illustrate that the CeA and NAc shell are involved in baseline ethanol consumption in a limited access procedure, since lesions of these nuclei decreased ethanol consumption. To determine if these nuclei were involved in increases in ethanol consumption, a murine model of ethanol dependency was used. In this procedure C57BL/6J (B6) mice are first acclimated to a limited access two-bottle choice preference procedure. The access period begins 3 hrs into the dark-cycle and continues for 2 hrs. Once acclimated, mice undergo chronic exposure to and intermittent withdrawal from ethanol vapor. Results from chapter 4 indicate that intermittent vapor exposure, as opposed to continuous ethanol vapor exposure, optimizes the increased ethanol

consumption response. As indicated in chapter 2, 3, and 4, lesions of these three components of the cEA did not block the intermittent ethanol vapor induced increase in ethanol consumption. In chapter 4, to determine the brain regions that activate in response to increases in ethanol consumption, a c-fos immunoreactivity study was carried out. The results suggest that the NAc shell and NAc core are the two main brain regions that activate as a result of ethanol consumption specifically in the mice that have been exposed to the intermittent ethanol vapor exposure that show the increase in ethanol consumption. Thus the results suggest that while the NAc shell activates in response to heightened levels of ethanol consumption, it is not necessary to see this increase in ethanol consumption. Overall, the results from these three chapters suggest that while the components of the cEA are involved in baseline ethanol consumption, and are responsive to changes in ethanol consumption (as was the case with the NAc shell), they are not necessary to see the ethanol vapor induced increase in ethanol consumption. These results have implications for understanding the neural circuitry involved in ethanol dependence.

Chapter 1: Introduction

Introduction

Alcohol dependency and related disorders remain major public health problems (Kranzler & Rosenthal, 2003). Understanding excessive alcohol consumption, a common characteristic of alcohol dependency, may help to alleviate symptoms of problem drinking. The physiological mechanisms and neural circuits associated with excessive alcohol consumption are poorly understood (Mann, 2004; Mariani & Levin, 2004). Recent studies have focused on brain regions associated with behaviors indicative of positive and negative affective states, since increased alcohol consumption in humans is associated with heightened levels of these states (Kranzler et al., 2004). The central extended amygdala (cEA), a brain region made up of the central nucleus of the amygdala (CeA), the lateral portion of the bed nucleus of the stria terminalis (BNST), primarily the dorsolateral (BNSTDL) and lateral posterior (BNSTLP) portions, and the nucleus accumbens shell (NAc shell), is a region of interest since it is involved with behaviors indicative of affective states such as fear and anxiety (Schulkin et al., 1998; Davis et al., 1997) and depression (Stout et al., 2000). These affective states are hypothesized to be involved in the negatively reinforcing properties of alcohol during alcohol withdrawal (Roberts et al., 2000; Koob, 2003ab). The cEA is also of interest since it is contains neurotransmitters and neuropeptides involved with ethanol consumption and operant ethanol self-administration. These neurotransmitters include dopamine, gamma-aminobutyric acid (GABA), dynorphin and enkephalin, and corticotropin releasing factor (CRF) (Heimer et al., 1997; Alheid & Heimer., 1988). Pharmacological manipulations of

these neurotransmitters in the lateral BNST and CeA have been shown to alter operant ethanol self-administration (Hyytia & Koob; Heyser et al., 1999; Eiler et al., 2003 Funk et al., 2006; Foster et al., 2004; Roberts et al., 1996).

Developing an animal model of alcohol dependence

In certain rodent models of alcohol dependency, the induction of physical dependence and withdrawal can increase subsequent consumption or lever pressing for ethanol. For example, C57BL/6J (B6) mice chronically exposed to and intermittently withdrawn from ethanol vapors showed an increase in their consumption of this drug in a 2-hour limited access procedure (Becker & Lopez, 2004; Lopez and Becker, 2005; Finn et al., 2007). In other studies, rats that received this ethanol vapor treatment increased their operant responding for ethanol (Funk et al., 2006; Roberts et al., 2000). Studies have shown that it is the intermittent aspect of the ethanol vapor exposure that optimizes the increased lever pressing for ethanol, since rats continuously exposed to ethanol showed a slower increase in ethanol self-administration when compared to the intermittently vapor exposed counterparts (O'Dell et al., 2004). Studies done in B6 mice showed that intermittently ethanol vapor exposed mice had a more optimized increased ethanol consumption effect than mice continuously exposed to ethanol (Lopez & Becker, 2005). It has been suggested that the increased consumption was due to an increase in the negatively reinforcing properties of ethanol, as the withdrawal state that follows chronic ethanol exposure is commonly postulated to be negative (Roberts et al., 2000; Koob, 2003ab). In addition to whether the animal learns to associate consumption of alcohol with relief from withdrawal symptoms, other important procedure variables are whether the animal perceives alcohol as a positive reinforcer before becoming physically

dependent, and/or whether the withdrawal symptoms are severe enough to interfere with subsequent drinking behavior (Finn et al., 2007).

The extended amygdala

Conceptualization

The extended amygdala (EA) was first conceptualized by Johnston (1923) who observed a continuum of gray matter linking the CeA to the BNST in several mammalian species as well as in human embryos and nonmammalian vertebrates. The concept of the EA was resurrected by the observation that staining with cupric-silver produced labeling in the CeA and BNST and the continuum between these two nuclei (de Olmos, 1969). The demonstration that lesions of the basolateral amygdala (BLA) caused a continuum of neuronal degeneration from the CeA to the BNST also suggested that these two nuclei share the similarity of receiving afferent projections from the BLA (de Olmos, 1972).

Immunohistochemical features

The CeA and the BNSTDL & BNSTLP nuclei of the cEA have similar immunohistochemical features. All contain tyrosine hydroxylase positive terminals (Freedman & Cassell 1994), D1 dopamine receptors (Eiler et al., 2003; de la Mora, 2005), gamma amino decarboxylase (GAD) and GABA immunoreactivity (Sun & Cassell, 1993), enkephalin, neurotensin and CRF immunoreactivity (Day et al., 1999, Day et al., 2002; Swanson et al., 1983), mu and kappa opioid receptors (Daunais et al., 2002; Mansour et al., 1996), angiotensin II receptors (von Bohlen et al., 1998), vasopressin receptors (Phillips et al., 1988), CRF receptors (Millan et al., 1986; De Souza, 1985), and inositol trisphosphate receptors (Sharpe et al., 1999). The nuclei of the cEA show neuronal activation, as indicated by the immediate early gene c-fos expression,

in response to intraperitonial injection of ethanol (Hitzemann & Hitzemann, 1999; Ryabinin et al., 1997), amphetamine (Day et al., 2001), the atypical antipsychotic clozapine (Pinna, 1999), the tricyclic antidepressants imipramine and desipramine (Duncan et al., 1996; Morelli et al., 1999), the serotonin reuptake inhibitor antidepressant citalopram (Morelli et al., 1999), fluoxetine (Torres et al., 1998), fluvoxamine (Veening et al., 1998), the indirect serotonin agonists, fenfluramine (Lucas et al., 1998), and dexfenfluramine (Rowland et al., 2003; Javed et al., 1999; Laflamme et al., 1997; Li & Rowland, 1996), the 5-HT1A receptor agonist, flesinoxan (Compaan et al., 1997), nicotine (Matta et al., 1997), the opioid antagonists naloxone (Veinante et al., 2003) and naltrexone (Carr et al., 1998), the kappa opioid antagonist nor-binaltorphimine (Carr et al., 1999), morphine (Singh et al., 2004), heroin (Singh et al., 2005), and the endogenous cannabinoid arachidonylethanolamide (Patel et al., 1998). These results show that the nuclei of the cEA share similar immunohistochemistry and are responsive to most drugs of abuse and many psychiatric pharmacotherapies, supporting the concept of the cEA as an area involved in drug dependency and comorbid affective disorders (Heimer et al., 1997; Alheid & Heimer., 1988; Koob, 2003a).

Afferent and efferent projections that contribute to maintaining homeostasis

Refer to figure 1.1 for a diagram of the circuit that is being described in this section.

The lateral BNST and CeA receive and send many of the same afferent and efferent projections to regions involved with interpreting visceral input and maintaining body fluid homeostasis. Such regions include autonomic brain stem nuclei, circumventricular organs and regions of the cerebral cortex involved with visceral

information. The autonomic nuclei that are also involved in visceral input include the pontine parabrachial nucleus (Bernard et al., 1993; Alden et al., 1994), the nucleus of the solitary tract (Ricardo, 1978), and the dorsal motor nucleus of the vagus (Kapp et al., 1989). Both the visceral and gustatory regions of the dysgranular anterior insular cortex send robust projections to the CeA and the BNSTDL & BNSTLP (Mcdonald et al., 1999). The circumventricular organs that project to the CeA (Ku & Li, 2003) and the BNSTDL & BNSTLP (Sunn et al., 2003) include the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT), neurons that contain angiotensin II receptors that when activated, stimulate water drinking, vasopressin secretion, and increased salt intake. The neurons of the cEA also contain angiotensin II receptors (von Bohlen et al., 1998), and vasopressin receptors (Phillips et al., 1988). Both the CeA and the medial BNST are regions that have been shown to play a role in behaviors motivated to maintain body fluid homeostasis. For example, rats deprived of salt will show an increased consumption of saline when compared to a non-salt deprived rat. This behavior is decreased with a lesion of the CeA or medial BNST (Johnson et al., 1999). One may speculate that a lesion of the BNSTDL & BNSTLP, would have a similar effect as a lesion to the CeA, considering the similarities in afferent and efferent projections to regions involved with maintaining body fluid homeostasis between these two regions.

Dopamine and ethanol consumption

Dopamine in the cEA plays a role in ethanol consumption. The BNSTDL & BNSTLP and CeA receive a dense concentration of dopaminergic projections from the neurons of the ventral tegmental area (VTA) (Freedman & Cassell 1994; Hasue & Shammah-Lagnado 2002), an area believed to be involved in reward (Nestler &

Carlezon, 2006) and ethanol reinforcement (Gonzales et al., 2004). One of the pharmacological effects of ethanol is to stimulate VTA neurons (Brodie et al., 1990; Gessa et al. 1985) to release dopamine into the lateral BNST (Carboni et al. 2000a) and CeA (Yoshimoto et al., 2000). The administration of a D1 dopamine receptor antagonist into the lateral BNST reduced ethanol self-administration at concentrations selective for ethanol, as indicated by the lack of effect of the antagonist at the same concentration to reduce self-administration for sucrose (Eiler et.al., 2003). This finding provides support for the idea that dopamine in the lateral BNST of the cEA is involved in the reinforcing properties of ethanol.

GABA and ethanol consumption

One of the neurotransmitters thought to play a role in positive ethanol reinforcement and reward is GABA, since GABA-A receptor antagonists administered intraperitoneally have been shown to decrease self-administration of and conditioned place preference for ethanol (Chester & Cunningham, 2002). Studies suggest that GABA-A receptors located in the cEA may act as a substrate for ethanol's positive reinforcing effects. For example, administration of the GABA-A receptor antagonist, SR95531, into the cEA (CEA, lateral BNST, and NAc shell) decreased self-administration of ethanol, but not water intake (Hyytia & Koob, 1995). Similarly, microinjections of the GABA-A receptor antagonist, beta-carboline-3-carboxylate-t-butyl ester, into the CeA decreased ethanol responding without affecting sucrose responding (Foster et al., 2004). These findings demonstrate that GABA-A receptor antagonism selectively decreased ethanol consumption. Studies also suggest that GABA in the cEA is involved in the negatively reinforcing effects of ethanol, since the dependency induced

increase in ethanol consumption postulated to relieve a negative affective state is blocked with administration of the GABA-A receptor agonist, muscimol, into the CeA (Roberts et al., 1996). The results suggest that GABAergic modulation in the cEA is involved in both the positive and negative reinforcing effects of ethanol.

The GABAergic system

The lateral portion of the CeA and the lateral BNST contain the highest number of GABA and GAD immunoreactive neurons of all the amygdaloid nuclei and the BNST subdivisions, respectively (Sun & Cassell, 1993; Day et al., 1999). GAD/GABA immunoreactive terminals were found mainly in the lateral CeA and BNSTDL & BNSTLP. GABAergic neurons project from the lateral and medial subdivisions of the CeA to the lateral BNST. There is a significant reduction in GAD immunoreactive terminal staining in the lateral BNST following stria terminalis lesions (Le Gal La Salle et al., 1978), which is likely due to disruption of specific GABAergic projection from rostral CeA (Sun & Cassell, 1993). While the CeA sends major GABAergic projections to the lateral BNST, there are only a small number of GABA-immunoreactive neurons projecting from the lateral BNST to the CeA. Consistent with this, lesions of the stria terminalis cause a slight decrease in GAD terminals and no change in GABA levels (Le Gal La Salle et al., 1978) in the CeA.

The GABAergic system and convulsions

In addition to projection neurons, GABAergic neurons in the CeA are also intrinsic (Sun et al., 1991). GABAergic projections from the lateral CeA project to and inhibit neurons in the medial CeA, an inhibitory response that is mediated, in part, by GABA-A receptors (Nose et al., 1991). Administration of the GABA-A antagonist

bicuculline abolished inhibitory post-synaptic potentials (IPSP) elicited by lateral CeA stimulation (Nose et al., 1991), and increased spontaneous activity of CeA neurons (Veinante et al., 1998). Administration of GABA-A receptor antagonists bicuculline or picrotoxin into the amygdala induced convulsions (Turski et al., 1985). Increases in seizure susceptibility are also seen in rats that receive stimulation to either the lateral or medial CeA (Sitcoske et al., 2000; Mohapel et al., 1996), suggesting that activation of the region with antagonism of the GABA-A receptors or through electric stimulation induces seizures, which is a characteristic of ethanol dependency.

The GABAergic system and ethanol dependency

During withdrawal from chronic intermittent exposure to ethanol vapor in B6 mice, there was an increase in handling-induced convulsions and ethanol consumption (Finn et al., 2007). Since cortical induced seizures are attenuated with administration of γ-vinyl GABA or muscimol into the amygdala (Applegate and Burchfiel, 1988), and increased self-administration of ethanol during withdrawal is decreased with microinjection of muscimol into the CeA (Roberts et al., 1996), it is possible that the GABAergic system in the CeA is involved in ethanol withdrawal-induced seizure activity and increased ethanol consumption. Collectively, these findings suggest that the GABAergic system in the cEA adapts in response to chronic intermittent ethanol vapor exposure, and that it is responsible for some of the signs of ethanol dependency, such as increases in handling induced convulsions and ethanol consumption.

The serotonergic system of the BNST

In the brains of macaque monkeys, serotonin-, and serotonin transporter-immunoreactive fibers were found in highest concentrations in the CeA and the BNSTDL

& BNSTLP (Freedman & Shi, 2001). Data obtained from in vitro whole-cell patch clamp recording in the rat BNST slice show that exogenous application of 5-hydroxytryptamine (5-HT) and the 5-HT_1 agonist, 5-carboxamindotryptamine (5-CT), evoked a membrane hyperpolarization. Infusion of 5-CT into the BNST significantly reduced the acoustic startle response, without affecting the general motor activity of the animals (Levita et al., 2004). The results suggest that enhanced serotonin transmission in the BNST is inhibitory and can impact anxiety-like behaviors, such as the acoustic startle response.

The interaction between the serotonergic and CRF systems of the BNST

A double-label immunocytochemical, light microscopic technique was used to demonstrate axosomatic and axodendritic interactions between 5-HT axons and CRF neurons in the BNST. Both the BNSTDL & BNSTLP and the medioventral BNST (BNSTMV) subpopulations of CRF neurons were targets for the 5-HT afferents. (Phelix et al., 1992). In humans, CRF is increased in cerebrospinal fluid during major depressive episodes, an effect reversed with effective antidepressent therapies (Bissette, 2001). The olfactory bulbectomized rat, used as an animal model of depression, shows increased levels of CRF in the BNST, which then decreased as a result of treatment with the selective serotonin reuptake inhibitor antidepressent, sertraline (Bissette, 2001). The results suggest an interaction between the serotonergic and CRF systems in the BNST.

Role of serotonin and the BNST in contextual learning

Rats exposed to single prolonged stress followed by 14 days of no disturbance showed enhanced freezing upon re-exposure to a context that had been associated with footshock, when compared to rats that received no single prolonged stress. Two-week

treatment with the selective serotonin reuptake inhibitor, paroxetine, returned the freezing response to baseline levels (Takahashi et al., 2006). The selective serotonin reuptake inhibitor, fluoxetine, also reduced freezing that occured in response to an environment chronically associated with footshock (Santos et al., 2006).

Of the two nuclei of the cEA, it is the BNST that is hypothesized to play the major role in responding to contexts that have been chronically associated with stress. Rats exhibit a sensitized acoustic startle response (Gewirtz et al. 1998) and an increased level of plasma corticosterone (Gray et al. 1993) when they are re-exposed to a context that had been chronically associated with footshock. These behavioral and physiological adaptations were blocked from occurring with a lesion of the BNST. Similarly, rats exhibit a freezing behavior upon re-exposure to a context chronically associated with an aversive tone (Schulz & Canbeyli 1999), but rats with BNST lesions showed significantly less of this behavior than sham lesioned animals. The results indicate that both serotonin and the BNST play a role in stress induced contextual conditioning and that they may possibly interact to produce conditioned learning.

Involvement of CRF in the BNST in anxious and depressive behaviors and ethanol withdrawal

Activation of BNST neurons with a site-specific CRF microinjection can induce behaviors indicative of anxiety, such as potentiation of an acoustic startle reflex (Lee and Davis, 1997), avoidance of the open arms of an elevated plus maze (Sahuque et al., 2006), or avoidance of a place associated with CRF injection. Chronic mild stress increased CRF levels in the BNST, which corresponded with an increased threshold for the self-stimulation of VTA neurons (Stout et al. 2000), often interpreted to indicate a

decrease in the salience of reward and the presence of an anhedonic state. Olive and colleagues have shown that animals dependent on ethanol will show an increase in BNST CRF levels during an acute ethanol withdrawal, and that re-consumption of ethanol following this withdrawal period brings CRF levels back to control levels (Olive et al., 2002). Since CRF in the BNST is associated with behaviors indicative of anxiety and depression, it is possible that heightened ethanol consumption during ethanol withdrawal is due to the ability of ethanol to decrease CRF levels in the BNST, and thus, to alleviate the CRF-associated negative affective states.

Role of CRF in the EA on the withdrawal-induced increase in ethanol consumption.

Recent studies have suggested that it is the presence of CRF in the CeA, but not the BNST that is involved in the excessive ethanol consumption induced by chronic intermittent ethanol exposure, since a CRF antagonist injected into the CeA, but not the lateral BNST blocked this increased ethanol consumption (Funk et al., 2006). This null effect of CRF antagonist in the lateral BNST is contrary to what was predicted and validates the further study of the role of this brain region in the change in ethanol consumption.

Chapter Introductions

The following chapters describe work done to determine if the three major nuclei of the cEA, the BNSTLP, the CeA, and the NAc shell are involved and necessary for the maintenance of ethanol consumption and the increases in ethanol consumption behavior that occur in response to chronic intermittent exposure to this drug. The overall hypothesis is that the cEA is involved in and necessary to see the chronic

ethanol vapor induced increase in ethanol consumption, but not in baseline ethanol consumption. The following chapters demonstrate that the cEA is necessary to maintain baseline ethanol consumption (Chapter 2 & Chapter 3), is not necessary to see the ethanol vapor-induced increase in ethanol consumption (Chapter 3 & Chapter 4), but is activated following this increase in consumption (Chapter 4).

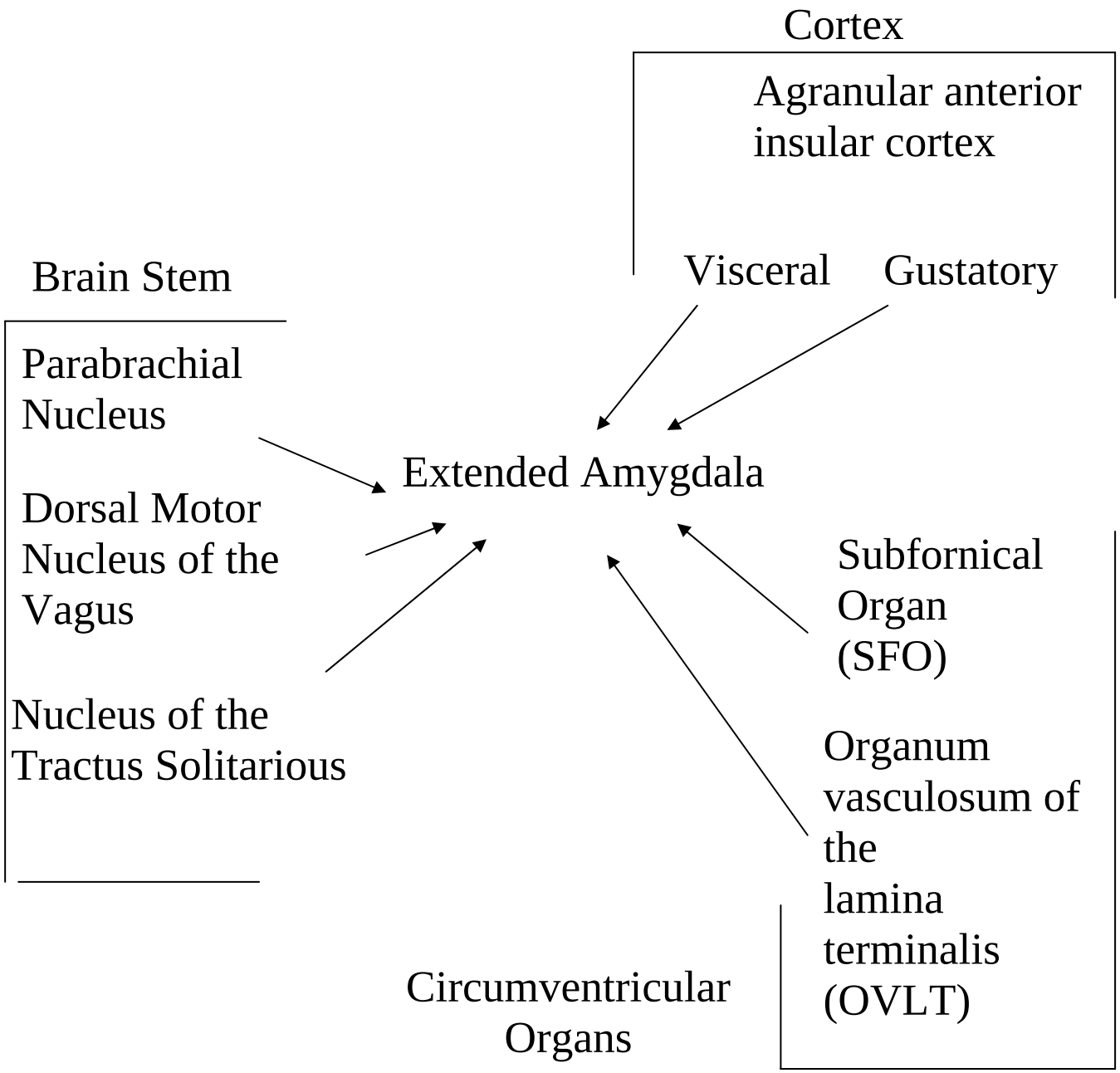


Figure 1.1 The extended amygdala as the center of the neural circuit involved with receiving visceral input to maintain body fluid homeostasis

Chapter 2: Electrolytic lesions of the bed nucleus of the stria terminalis (BNST) decrease ethanol consumption in a limited access, but not a free access procedure

Abstract

The central extended amygdala (cEA) is known to have a key role in the initiation and maintenance of substance abuse. One major nucleus of the cEA, the bed nucleus of the stria terminalis (BNST), mainly the lateral posterior portion (BNSTLP) has been shown to regulate limited access operant self-administration of ethanol; however, the role of the BNSTLP in home-cage ethanol consumption (free access) is not clear. To determine if free access consumption is BNSTLP dependent, we determined the effect of BNSTLP lesions on home-cage drinking in mice, in both a limited access and free access procedure. We found that BNSTLP lesions decreased ethanol consumption in a limited but not a free access procedure. These results suggest that limited access ethanol consumption, which shares some behavioral similarities to limited access operant self-administration, has an underlying neural circuitry that is different from the circuitry associated with continuous free access ethanol consumption.

Introduction

To elucidate the neural circuitry that underlies drug-seeking behavior, many have focused on the central extended amygdala (cEA), which is believed to be involved in both the positive and negative reinforcing effects of drugs of abuse (Koob, 2003). The cEA includes the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), mainly the lateral posterior portion (BNSTLP), and the shell of the nucleus accumbens (NAc shell) (Alheid and Heimer, 1988). The cEA's state of neural plasticity and excitability is highly modulated by numerous neurotransmitters and neuropeptides that affect changes in emotive behaviors (Koob et al., 2004; Heimer et al., 1997). These messengers include, but are not limited to, dopamine, glutamate, gamma-aminobutyric acid (GABA), corticotropin releasing hormone (CRH), dynorphin, and enkephalin (Moga et al., 1989; de Olmos et al., 1985). Pharmacologically manipulating these messengers in the cEA has been shown to affect ethanol self-administration (For review see Koob, 2003).

Of the three main nuclei of the cEA, both the CeA and the NAc shell have been shown to have a key role in ethanol consumption. Lesions of the CeA decreased ethanol consumption in a voluntary free access procedure in rats (Moller et al., 1997), and numerous pharmacological manipulations of the NAc in both rats and mice have induced changes in ethanol consumption (Pandey, 2004). With regard to the BNST, no lesion studies have been carried out to determine its role in ethanol consumption. However, numerous pharmacological studies have implicated the involvement of this region in limited access operant ethanol self-administration. For example, the microinjection of a dopamine D1, but not D2 receptor antagonist into the BNST decreased ethanol

appropriate responses (Eiler et al., 2003) at doses that affected sucrose responding to a smaller degree. The microinjection of the GABA-A receptor antagonist, SR 95531, directly into the BNST also decreased ethanol self-administration at doses that did not affect responding for water (Hyytia & Koob, 1995).

The following studies were carried out to further characterize the role of the BNSTLP (via electrolytic lesion) in ethanol consumption. Research suggests that the neural circuitry that underlies ethanol drinking differs in procedures in which the animal is provided with free access verses limited access to ethanol (McKinzie et al., 1998). For this reason, both a free access and a limited access procedure were used in the current study. As a positive lesion control, the acoustic startle response (ASR) was measured, as this is a behavior that is known to differ between a BNST lesioned animal and a sham control (Gewirtz et al., 1997).

Materials and Methods

Animals and Housing. All studies used male C57BL/6J (B6) mice that were obtained from The Jackson Laboratory (Bar Harbor, ME). Upon arrival, mice were maintained in a temperature controlled colony room (21-23° C) on a 12-h light/dark cycle (lights off at 4:00 PM) and allowed free access to food and water.

Subjects in the free access paradigm. Mice were group housed (5 mice/cage) upon arrival and were approximately 20 weeks of age at time of surgery. Mice were isolate-housed following surgery, and given one week of recovery time prior to any behavioral testing. Total number of mice used for data analysis in this free access procedure were n = 15 for the BNST lesioned mice and n = 19 for the sham lesioned mice. Four mice were

removed from the BNST lesioned group for either damage to the lateral septum or inadequacy of lesion size.

Subjects in the limited access paradigm. Mice were 7-9 weeks of age upon arrival and group housed (4 mice/cage). One week of acclimation was given prior to surgery. Total number of mice used for data analysis in this limited access procedure were n = 15 for the BNST lesioned mice and n = 17 for the sham lesioned mice Approximately 3-6 days of recovery was provided prior to any behavioral testing. Mice were group housed during this recovery period.

Surgery. Animals were anesthetized with ketamine (220mg/kg) /xylazine (44mg/kg) /acepromazine (22mg/kg) and placed on a stereotaxic instrument (Cartesian Research, Inc. Sandy, Oregon). The coordinates (Paxinos and Franklin, 2001) were determined with bregma marking zero for the mediolateral (ML) and anterioposterial (AP) directions and the top of skull marking zero for the dorsoventral (DV) direction. For the BNST, the coordinates were: AP = 0.26 mm, ML =±1.1, DV = -4.20.

Electrolytic lesions. A 50 mm monopolar electrode with a 0.25 mm uninsulated tip (SNE- 300, Rhodes Medical Instruments) was lowered into the stated stereotaxic coordinates. Lesions were produced with a lesion-making device (Ugo Basile, Italy) with a current of 0.7 mA applied for 8 seconds. Sham lesions were produced by simply inserting the electrode into the stated coordinates.

Verification of lesion location. Mice were euthanized by cervical dislocation, and brains were collected and frozen in isopentane chilled with a solution of isopropyl alcohol and dry ice. Brains were stored at -80 °C. Brain slices were cut to a thickness of

40 μm, and stained with thionin on the following day. The extent of tissue damage was examined microscopically.

Acoustic startle. The acoustic startle response was measured using a Coulbourn Instruments test chamber (Allentown, Pa., USA). Methods are as described (McCaughran et al., 2000). Briefly, the startle response was measured 200-ms following the stimulus and was transformed into a digital signal through a strain gauge transducer. Subjects were presented with an orienting 110-db stimulus with 60 ms duration, followed by twelve blocks of four trial types delivered in pseudo-random order. Trial types 1 through 3 included a start pulse of 60 ms duration and amplitude of 95, 105, and 115 db. Trial type 4 was a null trial in which no stimulus was presented. Transducer output on this trial was considered baseline and was used in the calculation of ASR amplitude, which was expressed as a percent of the null trial [ASR (g)/ null trial (g)] x 100.

Free access ethanol consumption. Following measurement of startle response, mice were exposed to the free access ethanol consumption procedure. Fluids were presented in two 25 ml graduated cylinders placed on a stainless steel cage top. Food was placed on the left side and the bottles were placed on the right side. For the first two to four days of the drinking experiment, tap water was available in both tubes for all animals. One water tube was then replaced with a tube containing an ethanol solution. Daily fluid consumption was measured by recording the level of the meniscus on the graduations of the drinking tubes. Tube sides were switched every other day to control for side preference. Mice were weighed once every four days. Mice were allowed to acclimate to the two-bottle choice procedure for 4 days, after which an ethanol bottle replaced one of the water bottles. The initial ethanol solution was 5% v/v (Pharmaco

Products, Brookfield, CT, USA) in tap water. After 4 days, the 5% solution was replaced with a 10% ethanol solution, and following 9 days at this concentration, mice were then withdrawn for three days, followed by re-exposure to the 10% ethanol solution for an additional four days. Then, mice were again withdrawn from the ethanol solution for three days and re-exposed to a 20% ethanol solution for four days.

Limited access ethanol consumption. Following recovery from surgery, mice were isolate housed and for the first two days of this housing, were provided with two water bottles. Then, a two hour limited access to ethanol procedure was begun. One water bottle was replaced by one ethanol bottle three hours after lights off for a two-hour period. The ethanol concentration was 15% v/v. Ethanol consumption was measured over a period of twelve days.

Data Analysis. Analysis was conducted in mice with confirmed BNST lesions. In the free access drinking experiment, data from 4 out of 19 mice were omitted due to insufficient lesion, resulting in a final n = 15 for the BNST lesioned group and n = 19 for the sham control group. In the limited access experiment, data from 1 of 16 mice was omitted due to insufficient lesion, resulting in a final n = 15 for the BNST lesioned group and an n = 17 for the sham control group. For the drinking experiments, the dependent variables were g/kg of ethanol consumed, total volume consumed (i.e. ethanol plus water), and the preference ratio. Preference ratios were calculated by dividing the volume of ethanol consumed by the total volume consumed. Data are expressed as the mean ± standard error. The overall effect of these dependent variables was analyzed with a repeated measures analysis of variance (ANOVA) with lesion status as a between subjects factor and day as a within subjects factor. Mouse weights were also a dependent

variable where a repeated measures ANOVA was carried out over days. Since we were predicting that there would be a significant effect of the lesion on ethanol intake, planned comparisons were conducted in the absence of a significant interaction between main effects. For the acoustic startle experiment, lesion group and decibel were analyzed with a two-way ANOVA. When appropriate, post hoc Neuman Kuels comparisons were made. Significance was set at $p \leq 0.05$.

Results

Verification of lesion location

Figure 2.1 illustrates representative light micrographs of the BNSTLP in intact and lesioned mice. As depicted in figures 1B and 1C, both the BNSTDL & BNSTLP were lesioned, which confirms the selectivity of the lesion. Surrounding structures that were left intact included the anterior commissure (acc), lateral septum (LS), medial striatum, the medial posterior BNST (BNSTMP), and the NAc. Lesion size in the free access group was rated on a scale of 2 to 5 with two as the smallest and 5 as the largest. Average lesion size in these mice was a 3. There was less variability in lesion size in the limited access group with an average size of 4.

Effect of BNSTLP lesion on the acoustic startle response

While mouse weights increased over the time of the experiment, there was no difference between lesion and sham groups at any time. At the beginning of the startle and drinking experiments, mouse weights were at 28.0 ± 0.4 grams for both groups. A repeated

measures analysis of variance (ANOVA) showed an effect of days [$F(1,32) = 2.84$, $p = 0.007$], but no effect of group and no interaction. Figure 2.2 illustrates the acoustic startle response in lesion and sham groups. For this behavior, data was lost from 4 mice in each group due to technical errors. The result was an n=11 for the lesion group and an n=15 for the sham control group. A group by decibel ANOVA demonstrated a significant effect of group [$F(1,24) = 14.68$, $p < 0.001$], and no effect of startle intensity. The lack of significant interaction indicated that the ASR in BNSTLP lesioned mice was significantly lower than values in respective sham animals across all the startle intensities.

No effect of BNSTLP lesion on ethanol consumption in a free access procedure

Figure 2.3 illustrates ethanol consumption over days at three different concentrations. Repeated measures ANOVA, conducted separately for each concentration, demonstrated that there was no effect of lesion on ethanol intake at any concentration. Likewise, there was no difference between sham and lesioned animals in preference for ethanol or in total volume of fluid consumed (data not shown). Preference for the sham and lesioned animals was 49 ± 2 and 52 ± 3 percent at 5% ethanol, 60 ± 3 and 57 ± 3 percent for both groups with the 10% ethanol, and 42 ± 2 and 43 ± 3 for 20% ethanol, respectively. The average amount of 10% ethanol consumed (g/kg) did not correlate with lesion size (data not shown).

Effect of BNSTLP lesion on ethanol consumption in a limited access procedure

Average and daily ethanol consumption: Body weights averaged over the 12 days of the study were 20.9 ± 0.5 g for the BNSTLP lesioned mice, and 22.6 ± 0.5 g for the sham animals. A one-way repeated measures ANOVA indicated that there was a significant effect of group [$F(1,31) = 7.65$, $p < 0.01$], but no effect of day, and no day x group interaction.

As depicted in Figure 2.4A, for both the lesioned animals and the sham controls, 2-hr limited access ethanol intake increased across time. The ANOVA revealed a significant effect for group [$F(1,31) = 15.8$, $p < 0.0001$], and days [$F(11,341) = 4.01$, $p < 0.0001$] but not the group x days interaction. Consistent with the results for daily ethanol intake, averaged ethanol consumption over twelve days showed the BNSTLP lesioned mice at 2.57 ± 0.24 g/kg and the sham group at 3.75 ± 0.13 g/kg (Figure 2.4B).

Average total fluid consumption over twelve days was 0.60 ± 0.05 ml for the lesioned animals and 0.86 ± 0.04 ml for the sham animals. The repeated measures ANOVA revealed a significant effect for group [$F(1,31) = 8.54$, $p < 0.001$] but not days or the days x group interaction.

As depicted in Figure 2.5, water consumption decreased in both sham and lesion groups over days. Focusing on the first 4 days, since water consumption did not differ after the 4th day, the repeated measures ANOVA revealed no significant effect for group, a significant effect of days [$F(3,93) = 13.45$, $p < 0.00001$], and no significant group x days interaction. The post-hoc analysis confirmed that water consumption was higher on the first day, when compared to days 2 to 4 ($p < 0.05$).

Average ethanol preference was 60 ± 5 % for the BNSTLP lesioned group and 70 ± 4 % for the sham animals ($p > 0.05$). The repeated measures ANOVA revealed no effect for group, days or the group x days interaction.

Discussion

The main findings of this research are that lesions of the BNSTLP decreased ethanol consumption in a limited access, but not in a free access procedure. In the latter, no difference was found in the consumption of 5%, 10%, and 20% ethanol solutions. In contrast, the BNSTLP lesion significantly decreased ethanol consumption in a limited access procedure. While 2-hour total fluid intake also was decreased in the lesioned animals, total water consumption during the 2-hour limited access period was not different between the lesion and sham control groups. In addition, there was no significant effect of the lesion on 24-hour total fluid intake. These data suggest that the suppressive effect of the BNSTLP lesion on total fluid intake in the limited access procedure was selective for ethanol and did not have a generally suppressive effect on fluid consumption. The precise specificity of the ethanol effect is unknown; however, previous studies have shown that manipulation of the BNST selectively reduces operant responding for ethanol (Eiler et al. 2003; Hyytia and Koob, 1995)

It is unlikely that BNSTLP lesion size contributed to the differential effect on limited access versus free access ethanol consumption. While the lesions in the free access procedure did not affect ethanol consumption, they were sufficient to decrease the acoustic startle response, indicating that the lesions were sufficient to have an effect on a BNSTLP dependent behavior. Additionally, there was no correlation between lesion size

and amount of ethanol consumed, suggesting that the lack of effect of lesion on ethanol consumption in the free access procedure was not due to an inadequate lesion size. Since the acoustic startle response is often used as a measure of anxiety (Gewirtz et al., 1997), it is possible that the BNSTLP lesioned mice are less anxious than the sham control mice, and this may explain the lower level of ethanol consumption in the 2-hr limited access procedure. Another explanation for the decreased limited access ethanol consumption and decreased acoustic startle response is a decrease in responsivity to stimuli in general. To test this hypothesis, behavioral responses to other stimuli, such as locomotor response to a novel environment or aversion to a quinine solution should be tested.

A potential explanation for the lack of effect of lesion on ethanol consumption in the free access procedure and not the limited access procedure may have been due to the age difference of the mice (20 weeks vs. 7-10 weeks, respectively). Studies have shown that older C57BL/10 mice (30 to 52 weeks), placed in a free access procedure, have a lower preference for a 10 % solution of ethanol than their younger counterparts (7 to 9 weeks) (O'Callaghan et al., 2002). Although using mice of 2 different ages was not an intentional design, it should be noted that animals used in operant procedures and which have demonstrated an effect of BNST manipulation on ethanol consumption are generally several months old (as a result of training for the operant task). Preference in the current study for ten percent ethanol (60%) is somewhat lower than the preference ratio reported elsewhere for B6 mice (e.g. Belknap et al. 1993). Nonetheless, the preference over water was highly significant ($p < 0.001$) and was not substantially different from the preference observed in the limited access procedure.

The present findings with limited access ethanol intake are consistent with other studies in which pharmacological manipulations of the lateral BNST decreased operant reinforced responding for ethanol (Eiler et al., 2003; Hyytia & Koob, 1995). Seeing that BNSTLP lesions decreased ethanol consumption in a limited access procedure suggests that both this procedure and the operant self-administration procedure share a similarity and a common underlying neural circuit. It may well be that it is the limited access characteristic of the operant procedure that makes lever pressing for ethanol BNSTLP dependant. If true, than BNST lesioned mice placed through an operant procedure in which ethanol is provided continuously, would not show a decreased self-administration for ethanol.

The results presented here are consistent with other studies illustrating differences between limited and free access ethanol consumption. The literature on the 5-HT_3 receptor antagonists serves to illustrate this point. 5-HT_3 receptor antagonists have been shown to decrease ethanol consumption in a free access procedure, but only under certain situations in a limited access procedure. As a case in point, the 5-HT_3 receptor antagonist MDL 72222, reduced 24-hour free choice ethanol consumption in the Sardinian alcohol-preferring (sP) line of rats (Fadda et al., 1991); in contrast to free access consumption, the administration of another antagonist, zacopride, did not reduce ethanol intake in a 1 hour limited access procedure (Knapp & Pohorecky, 1991; Svensson et al., 1993). Similarly, other 5-HT_3 receptor antagonists did not reduce 30 minute limited access operant responding for ethanol (Beardsley, 1994). While one study showed that the 5-HT_3 receptor antagonist, ICS 205-930 (ICS), decreased limited access operant responding for ethanol, the doses of ICS that reduced responding were higher than those required to

reduce consumption in a continuous access procedure (Hodge et al., 1993). In addition to these findings, one study (Tomkins et al. 1995) found that a 5-HT_3 receptor antagonist (ondansetron) would reduce ethanol intake in a limited access procedure. Other studies (McKinzie et al., 1998) suggest that this may have to do with a loss of temporal and contextual associations due to variability in the time that the ethanol was presented during the dark cycle (further discussed below).

Contextual and temporal conditioning may differ in procedures involving limited access versus free access ethanol consumption. In a limited access procedure, the presentation of ethanol is associated with contextual and temporal cues. Free access drinking is not necessarily associated with these cues. Consider the following example. McKinzie et al. (1998) observed that the 5-HT_3 receptor antagonist, MDL 72222, did not decrease ethanol consumption when ethanol was presented during a four hour limited access procedure at fixed times in the dark cycle. However, when the time of limited access varied during the dark cycle, MDL 72222 was able to decrease ethanol consumption. Although this effect dissipated over time, McKinzie et al. (1998) interpreted the weakening of the drug effect to the strengthening of conditioning of contextual cues over trials.

From this perspective, it is of interest to note that the BNST is known to have a significant role in contextual conditioning. For example, Sullivan (2004) has shown that BNST lesions attenuate the context – induced freezing behavior and corticosterone release observed in a classical fear conditioning procedure where context was associated with footshock. Similarly, rats with sham surgery, but not those with a BNST lesion, demonstrated an increased freezing behavior upon re-exposure to a context chronically

associated with an aversive tone (Shultz and Canbeyli, 1999). Overall the results suggest that the BNST is involved in affective responses to contextually conditioned stimuli, and may be involved in conditioned stimuli associated with ethanol consumption in a limited access procedure.

The BNST also plays a role in affective responses to changes in contextual stimuli that are unconditioned. Sham lesioned rats placed in an environment that was half light and half dark, preferred the side that was dark. Rats with BNST lesions, however, did not show this spontaneous place preference (Walker and Davis, 1997). On an elevated zero maze, rats with a BNST lesion showed more time on the open arms of the zero maze and a shorter latency to enter the open arms than sham control (Waddell et al., 2006). The effect of lesions on these unconditioned context associated behaviors was interpreted to indicate a decrease in a state of anxiety (Waddell et al., 2006; Walker and Davis, 1995). Rats with a BNST lesion placed in an environment with the odor of a natural predator, displayed a lower freezing response than rats with a sham lesion (Fendt et al., 2003). The collectively described results indicate that affective behavioral responses to contextual cues are BNST dependent.

The BNSTLP is also involved in circadian rhythm, a characteristic that may play a role in ethanol consumption in a limited access procedure. The BNSTLP has been shown to be linked to the suprachiasmatic nucleus of the hypothalamus (SCN) (Leak and Moore, 2001), which is the primary circadian clock regulating daily rhythms in behavior and physiology in mammals (Klein et al., 1991). The BNSTLP and SCN exhibit synchronous rhythms in neural activity (Yamazaki et al., 1998), placing the BNSTLP in a position to influence circadian rhythms. The BNSTLP exhibited a robust daily rhythm in

expression of Period2 genes in rats housed in a light/dark cycle or in constant darkness, in blind rats, and in mice (Amir et al., 2004). The BNSTLP is responsive to corticosterone, and removal of the adrenal glands blunts the rhythmic gene expression of circadian clock period2 protein in the BNSTLP (Amir et al., 2004). Since it has been shown that removal of the period2 gene in mutant mice results in an increase in ethanol consumption (Spanagel et al., 2005), it is possible that it is the period 2 protein expressed in the BNSTLP that regulates the circadian rhythm involved in ethanol consumption. Studies that measure ethanol intake at different limited access periods throughout the dark cycle should be carried out to determine if the BNSTLP lesion had an overall effect of decreasing ethanol consumption independent of the circadian rhythm, or if the lesion simply shifted ethanol consumption to a different period of the dark cycle.

In conclusion, the results of our study indicate that the BNSTLP plays a role in limited access, but not free access ethanol consumption. In this way, the BNSTLP differs from the CeA and NAc, where free access ethanol consumption is affected by manipulations to these regions (Moller et al., 1997; Pandey, 2004). Overall, our results are consistent with a number of studies indicating that the cEA is involved in ethanol consuming behavior. The results have implications for understanding the neural circuitry that underlies ethanol consumption in alcohol dependent people, particularly with regard to differences found between those that drink continuously throughout the day and those that consume alcohol in a large bout in a limited amount of time. Differentiation between these forms of alcohol use and abuse and the underlying neural circuitry will lead to better success in developing pharmacotherapeutic treatments for different forms of alcoholism.

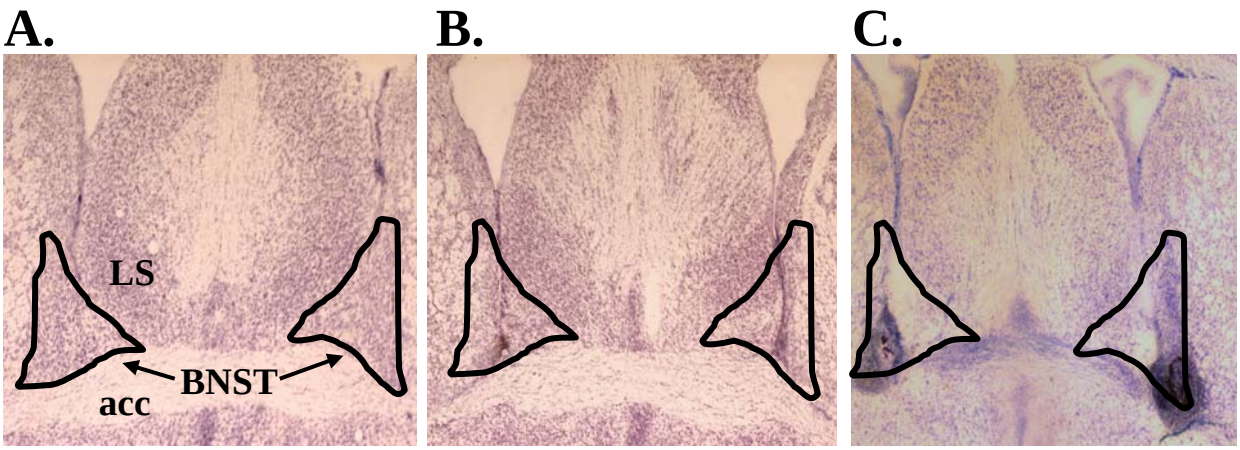


Figure 2.1 Representative slices from the brains of B6 mice.

Slices are from intact mice (A), lesioned mice in the free access procedure (B), and lesioned mice in the limited access procedure (C). Glial scaring can clearly be seen in the lesioned mice. Brains were sectioned at 40 μm intervals and stained with thionin. Lesions were verified under a light microscope. Lesions were limited to the dorsal and lateral posterior portions of the BNST, leaving both the ventral and medial posterior portions intact. (acc) Anterior Commisure (LS), Lateral Septum

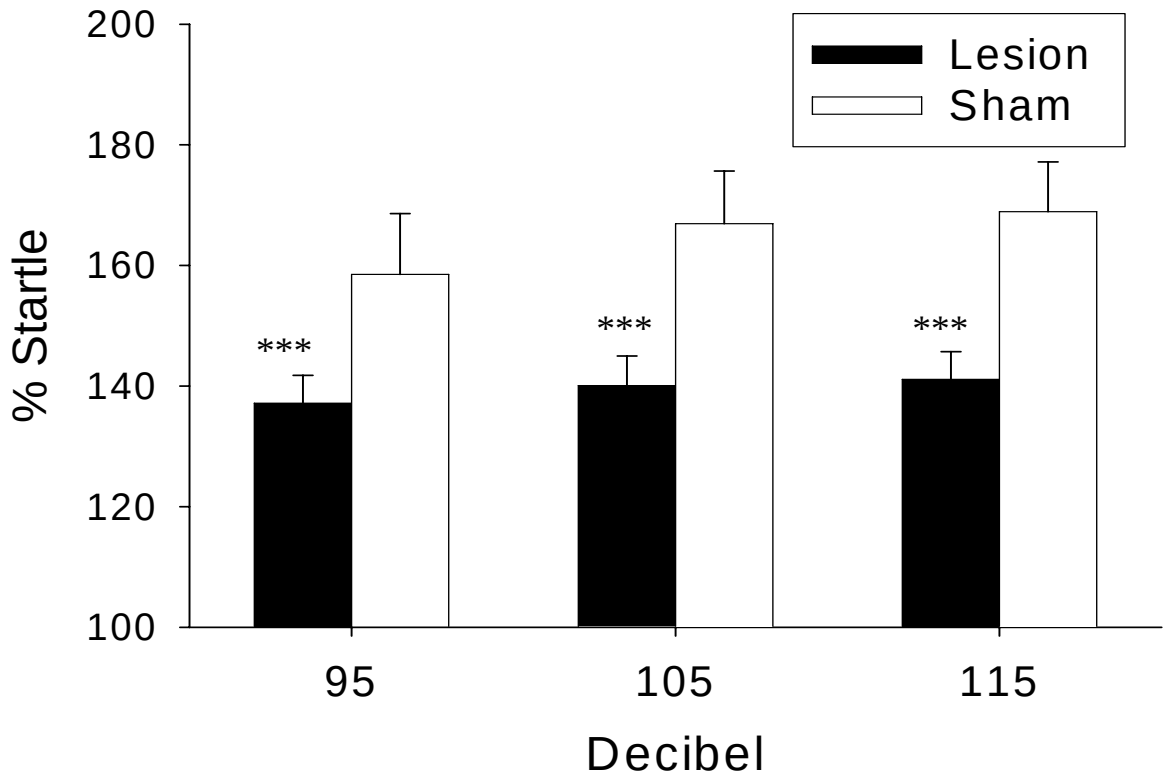


Figure 2.2. The acoustic startle response in B6 mice. The response was measured one week after surgery and before mice were placed in the free access drinking procedure. Mice with BNST lesions (n=11), demonstrated a significantly lower startle level than those in the sham group (n=15). Values are the mean ± SEM. *** $p < 0.001$ versus respective sham

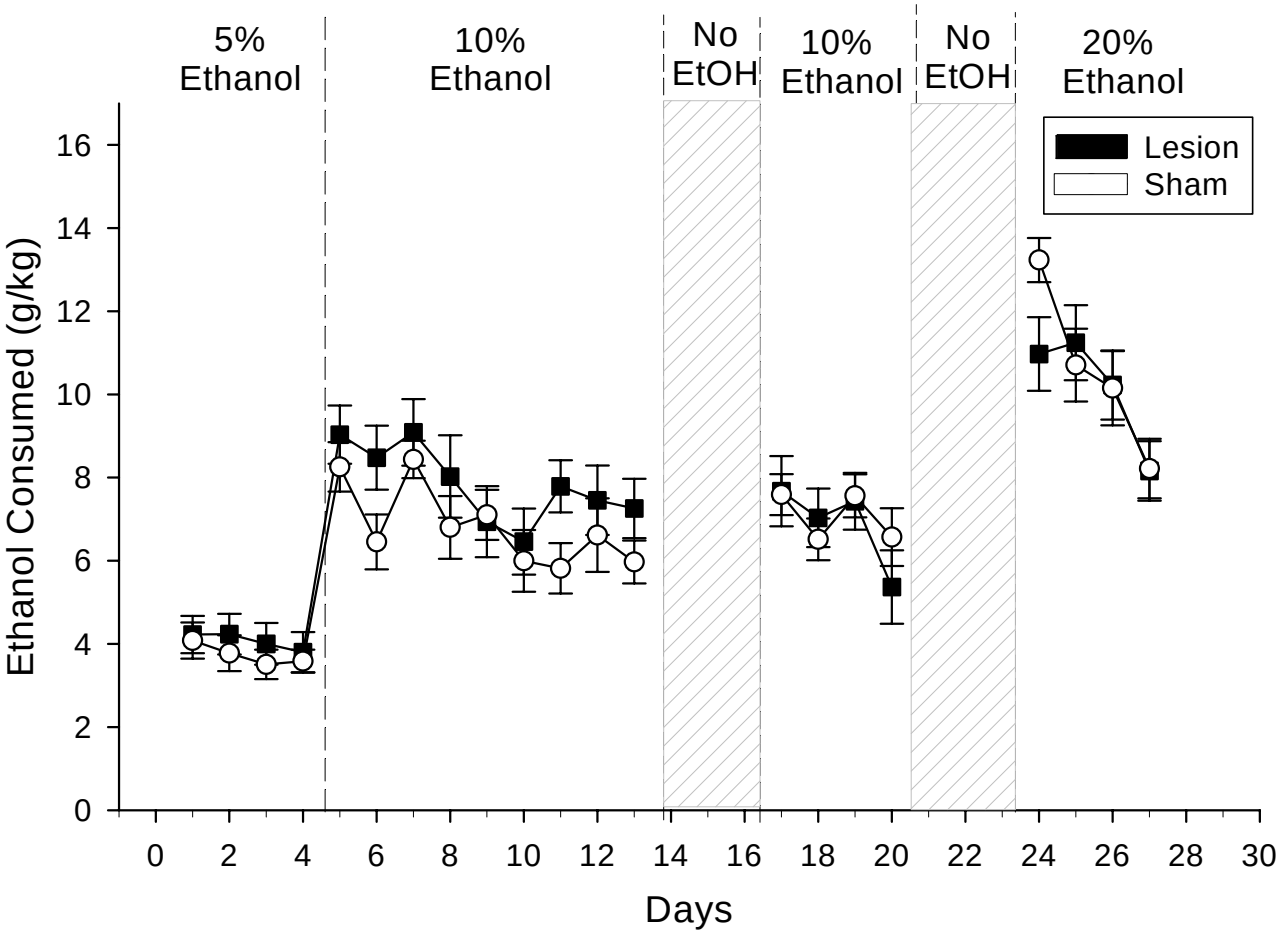
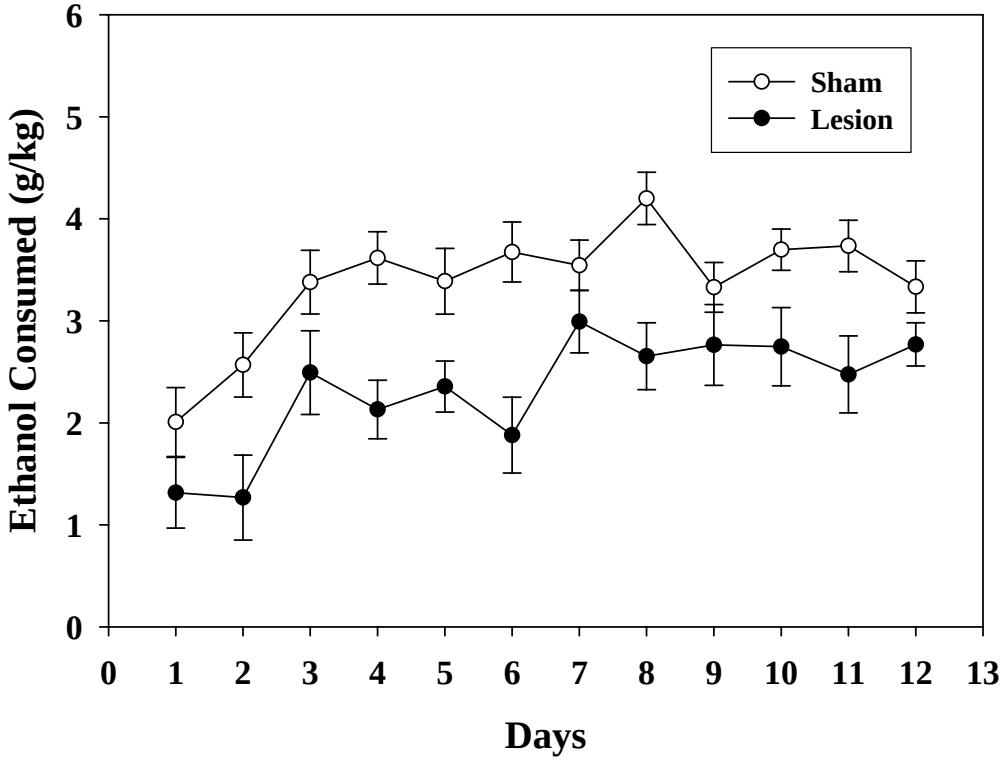


Figure 2.3**.** Ethanol consumption in a free access procedure. Mice were given a choice between water and 5%, 10%, and 20% ethanol concentrations in a two-bottle choice procedure. Mice were also deprived from ethanol twice during the experiment for three days. The lesion (n=15) and sham (n=19) groups did not show a significant difference in ethanol consumption at any concentration either before or after the short periods of ethanol deprivation. Values are the mean ± SEM for the mice depicted in Figure 2.2.

A. Daily Ethanol Intake



B. Averaged Ethanol Intake

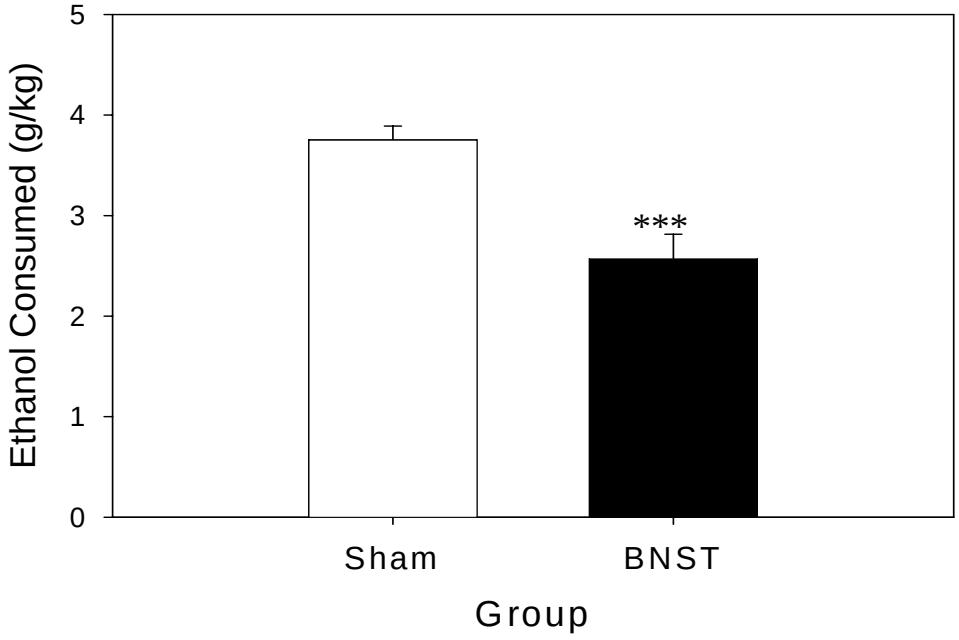


Figure 2.4. Ethanol consumption during a two hour limited access procedure, measured by (A) daily ethanol intake over days and (B) ethanol intake averaged over days. Mice were given a choice between one bottle with a 15% ethanol solution at three to four hours into their dark cycle for a period of two hours. BNSTLP lesioned mice (n = 15) drank significantly less ethanol than the mice that received sham surgery (n = 17). Values represent the mean ± SEM. *** $p < 0.001$ versus respective sham group

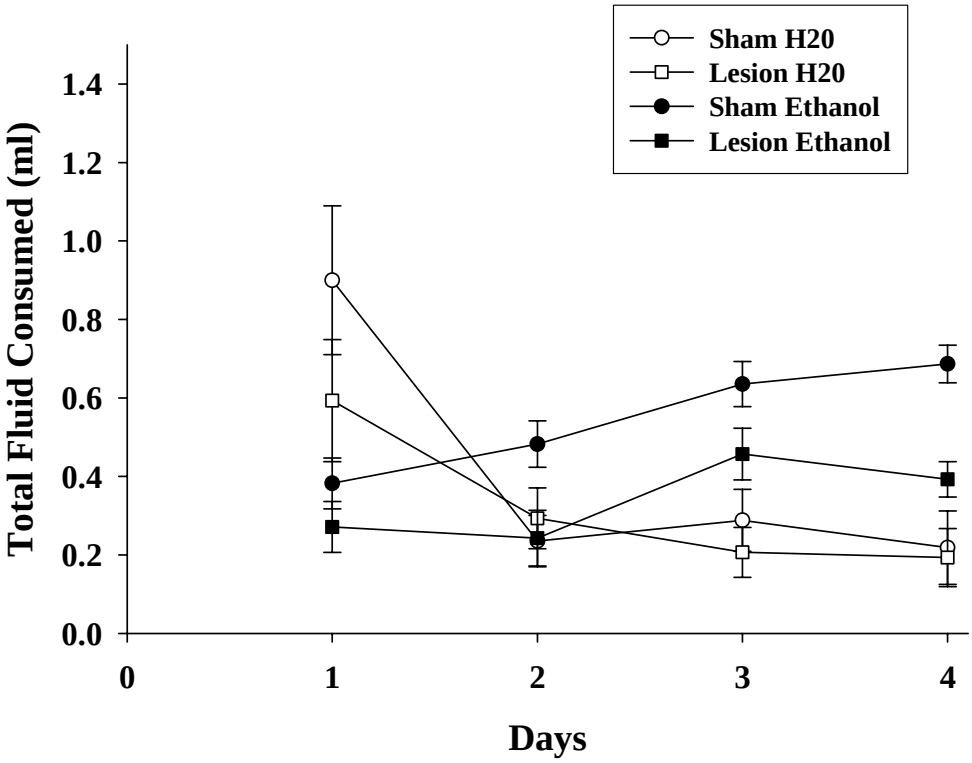


Figure 2.5. Ethanol and water consumption during the first four days of a two hour limited access procedure. Mice were given a choice between one water bottle and one bottle with a 15% ethanol solution at three hours into their dark cycle for a period of two hours. BNSTLP lesioned mice (n = 15) drank significantly less ethanol than the mice that received sham surgery (n = 17). Sham and lesioned mice did not differ in the amount of water consumed. Values represent the mean ± SEM.

Chapter 3: Electrolytic lesions of the central extended amygdala (cEA) decrease ethanol consumption, but do not block the ethanol-vapor induced increase in ethanol consumption

Abstract

The central extended amygdala (cEA), which includes the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), mainly the lateral posterior portion (BNSTLP) and the nucleus accumbens shell (NAc shell) has been proposed to play a role in excessive alcohol consumption. To test this hypothesis we turned to the recently developed murine model of ethanol dependence (Becker and Lopez, 2004). In this procedure C57BL/6J (B6) mice are first acclimated to a limited access two-bottle choice preference procedure. The access period begins 3 hours into the dark-cycle and continues for 2 hours. Once acclimated, mice undergo chronic exposure to and intermittent withdrawal from ethanol vapor. The intermittent withdrawal increases limited access ethanol consumption. To determine if the EA has a role in this ethanol vapor-induced increase in consumption, the CeA and BNSTLP were lesioned. Both lesions reduced pre-exposure ethanol consumption but did not block the increase in consumption induced by the intermittent vapor exposure. The results confirm that while the cEA is involved in limited access ethanol consumption, it is not necessary for the change in ethanol consumption following intermittent alcohol vapor exposure.

Keywords: Electrolytic lesions; Extended amygdala; C57BL/6J; Ethanol consumption; Central nucleus of the amygdala, bed nucleus of the stria terminalis

Introduction

Alcohol related disorders remain major public health problems (Kranzler & Rosenthal, 2003). The physiological mechanisms and neural circuits associated with these disorders are poorly understood (Mann, 2004; Mariani & Levin, 2004). Recent studies have focused on the central extended amygdala (cEA), as it is a brain region full of neurotransmitters and neuropeptides involved with ethanol consumption and operant ethanol self administration. Such neurotransmitters include dopamine, gamma-aminobutyric acid (GABA), dynorphin and enkephalin, and corticotropin releasing factor (CRF) (Heimer et al., 1997; Alheid & Heimer., 1988). The cEA is made up of the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), mainly the lateral posterior portion (BNSTLP), and the nucleus accumbens shell (Nac shell), and pharmacological manipulations of the neurotransmitters in these nuclei have been shown to alter ethanol consumption and operant self-administration of ethanol (Hyytia & Koob; Heyser et al., 1999; Eiler et al., 2003 Funk et al., 2006; Foster et al., 2004; Roberts et al., 1996).

Excessive alcohol consumption is a common characteristic of alcohol dependency, and is believed to occur with heightened levels of positive and negative affective states (Kranzler et al., 2004). In certain rodent models of alcohol dependency, the rodent is placed through a chronic ethanol vapor exposure and withdrawal procedure and the result is increased consumption or lever pressing for ethanol. For example, C57BL/6J (B6) mice chronically exposed to and intermittently withdrawn from ethanol vapors showed an increase in their consumption of this drug in a 2-hour limited access

procedure (Becker & Lopez, 2004; Lopez & Becker, 2005; Finn et al., 2007). In other studies, rats that received this ethanol vapor treatment increased their lever pressing for ethanol (Funk et al., 2006; Roberts et al., 2000). Studies have shown that it is the intermittent aspect of the ethanol vapor exposure that optimizes the increased lever pressing for ethanol, since rats continuously exposed to ethanol vapor showed a slower increase in ethanol self-administration when compared to the intermittently vapor exposed counterparts (O'Dell et al., 2004). Studies done in B6 mice showed that intermittently ethanol vapor exposed mice had a more optimized increase in ethanol consumption than mice continuously exposed to ethanol vapor (Lopez & Becker, 2005). It has been suggested that the increased consumption was due to an increase in the negatively reinforcing properties of ethanol, as the withdrawal state that follows chronic ethanol exposure is commonly postulated to be negative.

Procedures that use dependence to increase ethanol consumption appear to utilize CRF circuits within the cEA. For example, the CRF antagonist, D-Phe-CRF, directly administered into the CEA, attenuated the vapor-induced increase in ethanol self-administration in dependent rats (Funk et al., 2006). In another study, CRF was increased in the lateral BNST in rats withdrawn from chronic ethanol exposure (Olive et al., 2002), and was decreased to baseline levels with the resumption of ethanol consumption. CRF in the cEA is associated with behaviors indicative of negative affective states, such as anxiety (Schulkin et al., 1998) or depression (Stout et al., 2000). These states are thought to drive the excessive lever pressing for ethanol seen in the dependent animals.

The present study was carried out to determine if the increased ethanol consumption seen in B6 mice intermittently exposed to and withdrawn from ethanol is

cEA dependent. To accomplish this goal, we examined the effects of electrolytic lesions of the CeA or the BNSTLP on limited access ethanol intake at baseline (i.e., non-dependent) or following intermittent ethanol vapor exposure and withdrawal (i.e., dependent). The results obtained suggest that while pre-exposure ethanol consumption is cEA –dependent, the ethanol vapor-induced increase in ethanol consumption is not.

Methods and Materials

Animals

Male C57BL/6J (B6) mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and upon arrival, were maintained in a temperature controlled colony room (21-23 ºC) on a 12-h light/dark cycle and allowed free access to food and water. Mice were group housed upon arrival (4 mice /cage) and given at least one week of acclimation prior to surgery. Mice were 7 – 9 weeks of age at the time of surgery, and were given 3-6 days of recovery following surgery and prior to the limited access procedure. All procedures used were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University (OHSU).

Electrolytic Lesions

Animals were anesthetized with ketamine (220 mg/kg) /xylazine (44 mg/kg) /acepromazine (22 mg/kg) and placed on a Cartesian stereotaxic instrument. A 50 mm monopolar electrode with a 0.25 mm uninsulated tip (SNE- 300, Rhodes Medical Instruments) was lowered into the following stereotaxic coordinates with bregma

marking zero for the mediolateral (ML) and anterioposterial (AP) directions and the top of the skull marking zero for the dorsoventral (DV) direction: for the BNST AP = 0.40 mm, ML =±1.1, DV = -4.20; for the CEA, AP = -1.70 mm, ML = ± 3.0, DV = -4.60. For the BNST, the current used was 0.7 mA applied for 8 sec. For the CeA, the current used was 0.5 mA for 8 sec. Sham lesions were produced by inserting the electrode into the stated coordinates without passing current.

Verification of Lesion Location

Mice were euthanized by cervical dislocation, and brains were collected and frozen by placing in an isopentane solution chilled with isopropyl alcohol and dry ice. The brains were stored at -80 °C. Brain slices were cut to a thickness of 40 μm, and stained with thionin. The extent of tissue damage was examined microscopically.

Limited access ethanol consumption.

Fluids were presented in two 25 ml graduated cylinders placed on a stainless steel cage top. Food was placed on the left side and the bottles were placed on the right side. For the first two days of the limited access drinking procedure, tap water was available in both tubes for all animals. Following this acclimation, one water bottle was replaced with one ethanol bottle three hours after lights off for a two-hour period. The ethanol concentration was 15% v/v (Pharmco Products, Brookfield, CT, USA). Fluid consumption was measured by recording the meniscus level. Mice were weighed once every four days.

Chronic and intermittent exposure to ethanol vapors

Mice that received lesions, sham lesions, and those naïve to surgery were exposed to a series of three cycles of 16-hr of ethanol vapor separated by 8-hr withdrawal periods. Ethanol vapor exposure was adjusted to yield target blood ethanol concentrations (BECs) of 1.5 mg/ml.

All groups exposed to ethanol vapor were weighed and injected daily with 1.50 g/kg of ethanol and 68.1 mg/kg of the alcohol dehydrogenase inhibitor, pyrazole. Animals were then placed in groups of three in flow-through cages and placed in vapor exposure chambers (Flare Plastics, Portland, Oregon) for 16 hrs. The chambers were previously brought to equilibrium concentrations of ethanol in air of 7-8 mg/l. Chamber levels were monitored hourly via gas chromatography (Agilent 6890N GC, using a HP-PLOT Q column). Following exposure, the ethanol vapor groups were removed from the Flare chamber and 20 μl tail blood samples were collected and directly diluted into 500 μl of a matrix of 4 mM n-propanol in deionized water for subsequent determination of BEC by head-space gas chromatography (see below). Following three cycles in the vapor chambers, mice were re-housed in their initial cages and 2-hr limited access ethanol drinking measurements were resumed over a period of 6 days. Brains were then collected for lesion verification.

Blood Ethanol Concentration Analysis

The tail blood samples were analyzed immediately after collection. The sealed 2 ml vial containing the blood sample in matrix was vortexed thoroughly before analysis. Analysis was performed via ambient headspace sampling gas chromatography (Agilent 6890N GC, using a DB-ALC1 column, Wilmington, DE) on a 30 μl aliquot. Six pairs of ethanol standards (0.1 – 3.0 mg/ml), which included n-propanol (internal standard), were run before the samples.

Data Analysis.

Analyses were conducted only in mice with confirmed lesions involving at least 50% of the target region; these criteria resulted in the loss of only 1 mouse from each group. Data are presented as the mean ± SEM. Daily outcome measures were ethanol intake (g/kg), preference ratio (ethanol volume / total volume), and total fluid consumed (mL). Based on the preliminary experiments in surgery-naïve animals, for the lesioned animals, average baseline and post-withdrawal values were calculated by averaging across pre- and post-withdrawal days 3-6. Standard analysis of variance (ANOVA) techniques were used to determine the effects of treatment (withdrawal/lesion) on the outcome measures. For the surgery-naïve animals, days of treatment were entered into the analysis as a repeated measure factor. For the post-hoc analyses, the Tukeys test was employed (Tukey, 1953). Based on the *a priori* hypothesis that lesions would differentially alter ethanol intake, lesion groups were analyzed separately with planned comparisons in the absence of any significant interaction. Correlational analysis was conducted between the

ethanol dose consumed and BEC. For all analyses, statistically significance was set at $p \leq 0.05$.

Results

Features of Withdrawal-Induced Drinking (WID).

In preparation for the lesion experiments, various parameters of the WID model were examined. Here we describe data in 140 B6 animals (non-lesioned) that were tested in the standard protocol: 6 days of baseline limited access preference drinking, followed by 3 days of intermittent vapor exposure, followed by an additional 6 days of limited access preference drinking. To the extent possible, baseline consumption was used to balance the animals assigned to the ethanol vapor or the air control groups. The data presented in figures 3.1-3.3 are from five different experiments, with 20 to 40 animals in each experiment. Table 3.1 depicts group sizes for the five experiments. There were a total of 81 animals in the intermittent ethanol vapor treated groups and 59 animals in the air control groups. For the ANOVA, treatment was the between subjects factor, with days of treatment as the repeated measure.

Figure 3.1 illustrates the results for daily ethanol consumption (g/kg). The ANOVA revealed a significant effect for treatment [$F(1,138)=14$, $p<2\text{x}10^{-4}$], days [$F(11,1518)=21$, $p<4.0\text{x}10^{-40}$], and the treatment x days interaction [$F(11,451)=9.2$, $p<3.8\text{x}10^{-16}$]. The post-hoc analysis (Tukey's Unequal N HSD) for the interaction effect revealed no significant difference across days between pre- and post-exposure in the air control group. But there were significant increases in consumption for the group exposed to intermittent ethanol vapor when comparing pre- and post-treatment days 3-6; the

differences were significant at $p<10^{-4}$ or better. Consumption increased across days 3-6 by an average of 37%.

Figure 3.2 illustrates ethanol preference in the air control and ethanol-vapor groups. The ANOVA revealed no significant effect ($p>0.1$) for treatment or the days x treatment interaction. However, there was a significant effect for days [$F(11,1518)=12$, $p<3.0\text{x}10^{-22}$]. Preference increased across days during both the pre- and post-treatment intervals; e.g., the increase from day 1 to day 6 during the pretreatment period in the air control group was 37% ($p < 1.7 \text{ x } 10^{-4}$).

Figure 3.3 illustrates total fluid consumption (mL/2 hours) in the air-control and intermittent ethanol-vapor groups. The ANOVA revealed a significant effect for treatment [$F(1,138)=11$, $p<3.2\text{x}10^{-20}$], days [$F(11,1518)=2.8$, $p<1.0\text{x}10^{-3}$], and the treatment x days interaction [$F(11,1518)=2.9$, $p<0.9\text{x}10^{-4}$]. The post-hoc analysis for the interaction effect revealed a significant difference for fluid consumed in the intermittent ethanol-vapor-exposed group when comparing pre- and post-treatment days 3-6; the differences were significant at $p<10^{-2}$ or better.

In a subset of the air control and ethanol vapor exposed animals, BECs were measured at the end of the limited access period. Consumption in the ethanol vapor exposed group (N=10) was 4.41 +/- 0.24 g/kg and in the air control group (N=11) was 3.58 +/- 0.22 g/kg. This difference in ethanol consumption was significant ($t = 2.33$ $df = 19$, $p < 0.03$). BECs were 1.94 +/- 0.18 mg/ml in the ethanol vapor exposed group and 1.42 +/- 0.21 in the air control group. This group difference in BEC also was significant ($t = 2.09$, $df = 19$, $p = 0.03$; Figure 3.4B). BECs and ethanol consumption were significantly positively correlated ($r = 0.75$, $n= 21$, $p < 0.0001$) (Figure 3.4A).

Lesions of the CeA and BNSTLP.

Since the air control procedures, which was done in a separate group of animals from the lesion experiments, did not significantly alter ethanol intake over baseline values, this group was not included in the lesion experiment. The effect of intermittent ethanol vapor exposure was compared directly to the levels of baseline consumption.

Figure 3.5 illustrates a representative animal with a BNSTLP lesion; data are presented at 3 different A/P levels, comparing the sham controls (left panels, A) and lesioned animals (right panels, B). The section at 0.16 mm anterior to bregma illustrates the extent of damage to the BNSTLP, which we estimate to be in the range of 60 to 70 percent. In some but not all animals the lesion extended anterior and included portions of the medial anterior BNST (BNSTMA) (see section 0.5 mm anterior to bregma). Since there was no overall behavioral change or alterations in consumption associated with damage to the BNSTMA, animals with damage to this region were included in the analysis of the BNSTLP lesioned mice. Damage to the medial posterior BNST (BNSTMP) was not seen (section -0.3 mm from bregma). The NAc shell and NAc core were not damaged (not shown).

Figure 3.6 illustrates a representative CeA lesioned animal; data are presented at 3 different A/P levels, comparing the sham controls (left panels, A) and lesioned animals (right panels, B). Overall, the average extent of damage to the CeA was estimated at > 80%. In all animals, there was some collateral damage to the basolateral amygdala.

Effect of CeA and BNSTLP Lesions on WID.

Across all parameters, no significant differences were detected between the CeA and BNSTLP sham lesioned animals. Thus, these groups were combined as a single sham group (N=17) and were compared to the confirmed CeA (N=13) and BNSTLP (N=12) lesioned animals. In figure 3.7, panel A and B illustrates ethanol consumption (g/kg) during the baseline and post-inhalation limited access periods, respectively, and panel C illustrates averaged intake over days 3-6. Analysis focused on the average data from days 3-6. ANOVA revealed a significant effect of group [$F(2,39) = 14.06$, $p < 0.00001$] and treatment [$F(1,39) = 38.07$, $p << 0.000001$] but not the group x treatment interaction ($p > 0.37$) in averaged ethanol intake. Thus, in all three groups, intermittent vapor exposure and withdrawal significantly increased ethanol intake. Planned comparisons revealed that baseline consumption was significantly higher in the sham (3.56 ± 0.22 g/kg) than in the CeA (2.21 ± 0.30 g/kg) and BNSTLP (2.21 ± 0.29 g/kg) lesioned animals ($p < 0.01$), and that post treatment consumption was significantly higher in the sham (4.90 ± 0.19 g/kg) than in the CeA lesioned (3.33 ± 0.49 g/kg), but not the BNSTLP lesioned mice (4.16 ± 0.24 g/kg).

In figure 3.8, panels A and B illustrate the effect of CeA and BNSTLP lesions on ethanol preference pre-and post-treatment, respectively, and panel C illustrates averaged preference over days 3-6. ANOVA revealed a significant effect of group [$F(2,39) = 14.06$, $p < 0.000001$] and treatment [$F(1,39) = 7.6$, $p < 0.001$] but not the group x treatment interaction ($p = 0.89$). These results indicate that intermittent ethanol vapor exposure and withdrawal significantly increased ethanol preference in all groups.

Planned comparisons revealed that the averaged baseline preference ratio in the CeA (0.49 ± 0.07) lesioned animals was significantly lower than that in the sham group (0.82 ± 0.05) ($p < 0.0001$) and the BNSTLP lesioned (0.72 ± 0.07) animals ($p< 0.0007$). Post treatment preference was significantly higher in the sham (0.90 ± 0.03) lesioned group when compared to the CeA lesioned (0.66 ± 0.08) group ($p< 0.02$), but not the BNSTLP lesioned (0.85 ± 0.03) mice.

In figure 3.9, panels A and B illustrates the effect of CeA and BNSTLP lesions on total fluid consumption (during the limited access period) pre and post-treatment, respectively, and panel C illustrates average total fluid consumption over days 3-6. ANOVA revealed a significant effect of group [$F (2,39) = 9.07$, $p< 0.0001$] and treatment [$F (1,39) = 23$, $p <0.00001$] but not the group x treatment interaction ($p> 0.17$). Thus, exposure to intermittent vapor and withdrawal significantly increased averaged total fluid intake in all three groups. Planned comparisons revealed that averaged baseline fluid consumption was significantly higher in the sham (0.84 ± 0.04 ml) lesioned group than in the BNST (0.55 ± 0.05 ml) lesioned group ($p < 0.01$), but not versus the CeA lesioned (0.78 ± 0.06 ml) group. Post-treatment total fluid consumption was significantly higher in the CeA (1.14 ± 0.07 ml) lesioned group than in the BNSTLP (0.83 ± 0.08 ml) lesioned group ($p < 0.01$); total fluid consumed was 0.97 ± 0.07 ml for the sham lesioned group.

In a subset of the sham and lesioned animals, BECs were measured at the end of the limited access period. Consumption was 3.91 +/- 0.30 g/kg in the sham lesioned group (N=6), 2.60 +/- 0.57 g/kg in the CeA lesioned group (N=4) and 3.88 +/- 0.78 g/kg in the BNSTLP lesioned group (N=4). BECs were 1.64 +/- 0.27 mg/ml in the sham

lesioned group, 1.32 +/- 0.55 mg/ml in the CeA lesioned group, and 1.80 +/- 0.38 mg/ml in the BNSTLP lesioned group (Figure 3.10B). There was no difference in BEC levels between groups which likely was due to the small number of animals that were sampled. There was also no difference in ethanol consumption levels between groups. However, BECs and ethanol consumption were significantly positively correlated (r = 0.68, N=14, p = 0.008) (Figure 3.10A).

Discussion

Results from these studies indicate that withdrawal from chronic intermittent ethanol exposure and withdrawal increases consumption of ethanol in B6 mice. The two major nuclei of the cEA, the BNSTLP and the CeA, are regions that are hypothesized to play a role in behavioral adaptation. Although the BNSTLP and CeA were involved in baseline ethanol consumption, they were not necessary for the intermittent withdrawal-induced increase in ethanol consumption. These results, as well as others (Moller et al., 1997) show that lesions of the cEA have the same effect on ethanol consumption as microinjection of a dopamine receptor 1 antagonist (Eiler et al., 2003) into the lateral BNST, GABA-A receptor antagonist into the NAc shell, lateral BNST, and CeA (Hyytia & Koob, 1995; Foster et al., 2004), and opioid receptor antagonist into the CeA (Heyser et al., 1999; Foster et al., 2004) in that these manipulations decreased baseline self-administration and consumption of ethanol. Since the pharmacological manipulations do not damage fibers of passage, it is likely that the effect of the electrolytic lesions to decrease ethanol consumption is due to damage to the neurons themselves and not fibers of passage. It should be noted that while the electrolytic lesions did not block the intermittent withdrawal-induced increase in ethanol consumption, other studies have shown that pharmacological manipulations of these brain regions do block this change in ethanol consumption. For example, GABA agonists (Roberts et al., 1996) and CRF antagonists (Funk et al., 2006) microinjected into the CeA block the ethanol vapor induced increase in ethanol consumption. One can conclude from this collection of studies that the cEA is involved in the increase in ethanol consumption induced by chronic intermittent exposure to ethanol vapors, but is not necessary to see this increase.

Both regions have been shown to play a role in behaviors motivated to maintain body fluid homeostasis. For example, rats deprived of salt will show an increased consumption of saline when compared to a non-salt deprived rat. This behavior is decreased with a lesion of the CeA or medial BNST (Johnson et al., 1999). The neural connections of the CeA and lateral BNST confirm that the region receives information from the viscera dealing with body fluid homeostasis. From the cerebral cortex, both the visceral and gustatory regions of the dysgranular anterior insular cortex send robust projections to the CeA and lateral BNST (McDonald et al., 1999). Brain stem regions that receive information from the viscera about fluid balance, mainly the pontine parabrachial nucleus (Bernard et al., 1993; Alden et al., 1994), the nucleus of the solitary tract (Ricardo, 1978), and the dorsal motor nucleus of the vagus (Kapp et al., 1989), send major projections to the lateral BNST and CeA. The circumventricular organs, mainly the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT) contain neurons that respond to Angiotensin II by stimulating water drinking, vasopressin secretion, and increased salt intake. These neurons send efferent projection to the CeA (Ku & Li, 2003) and BNST (Sunn et al., 2003). The studies suggest that the effect of the BNST lesions to decrease ethanol consumption and maintain water consumption levels, as in the case of the BNSTLP lesion, or of the CeA lesions to decrease ethanol consumption and increase water consumption levels is related to the role of these brain regions to maintain fluid homeostasis.

It has been argued that the cEA plays a role in maintaining the homeostatic set point for ethanol reinforcement (Koob, 2003a, 2003b). One of the neurotransmitters thought to play a role in positive ethanol reinforcement and reward is GABA, since

GABA-A receptor antagonists administered intra peritoneally have been shown to decrease self-administration of and conditioned place preference for ethanol (Chester & Cunningham, 2002). Studies suggest that GABA-A receptors located in the cEA may act as a substrate for ethanol's positively reinforcing effects. For example, the GABA-A receptor antagonist, SR95531, administered into the cEA (CEA, lateral BNST, and NAc shell) decreased self-administration of ethanol, but not water intake, and the GABA-A receptor antagonist, beta-carboline-3-carboxylate-t-butyl ester, injected into the CeA decreased ethanol responding, but had no effect on sucrose responding (Foster et al., 2004). These findings demonstrate that the effect of the GABA-A antagonists to decrease ethanol consumption was relatively selective for ethanol. Studies also suggest that GABA in the cEA is involved in the negatively reinforcing effects of ethanol. For example, microinjection of GABA-A receptor agonist, muscimol, into the CeA decreased the vapor-induced increase in ethanol consumption, but had no effect in non-vapor exposed (non-dependent) animals (Roberts et al., 1996). The results suggest that GABAergic modulation in the cEA is involved in both the positive and negative reinforcing effects of ethanol.

The dopaminergic and opioid systems in the cEA are also thought to play a role in the rewarding and reinforcing properties of ethanol in non-dependent animals (Koob, 2003a, 2003b; Eiler et al., 2003; Heyser et al., 1999; Foster et al., 2004). With regard to the dopaminergic system, the BNST and CeA receive a dense concentration of dopaminergic projections from the ventral tegmental area (VTA) (Freedman & Cassell 1994; Hasue & Shammah-Lagnado 2002), an area believed to be involved in reward (Nestler & Carlezon, 2006) and ethanol reinforcement (Gonzales et al., 2004). One of the

pharmacological effects of ethanol is to stimulate VTA neurons (Gessa et al. 1985) to release dopamine into the BNST (Carboni et al. 2000) and CeA (Yoshimoto et al., 2000). The administration of a D1 dopamine receptor antagonist into the lateral BNST reduced ethanol self-administration at concentrations that reduced responding for sucrose to a much less degree (Eiler et.al., 2003), demonstrating that dopamine in the BNST is involved in the reinforcing properties of ethanol. With regard to the opioid system, injection of the opiate receptor antagonist methylnaloxonium (Heyser et al., 1999) or naltrexone (Foster et al., 2004) into the CeA also reduced ethanol self-administration, suggesting that both the opioid and dopaminergic systems in the EA play a role in the positively reinforcing properties of ethanol. Thus, the effect that the electrolytic lesions of the BNSTLP and CeA in our study had to decrease ethanol consumption may well be due to the dopamine and opioid neurons in this brain region.

The CRF system in the cEA is thought to play a role in the negatively reinforcing properties of ethanol in ethanol-dependent animals, and the excessive ethanol consumption associated with the dependency (Koob, 2003ab). Funk et al (2006) showed that a CRF antagonist injected into the CeA and lateral BNST had no effect on ethanol self-administration in non-dependent rats, suggesting that CRF in the EA is not involved in the positively reinforcing properties of ethanol. However, intra-CeA microinjection of a CRF antagonist lowered, but did not block the dependence-induced potentiation of operant ethanol self-administration, suggesting that it plays a role in this dependence-induced increase in ethanol self-administration. To further support these findings, Finn et al (2007) found that microinjection of a CRF antagonist into the CeA of B6 mice blocked the intermittent ethanol vapor-induced increase in ethanol consumption while having no

effect on baseline consumption. These results, in conjunction with the result that intra-CeA microinjection of a GABA agonist blocked the vapor-induced increase in ethanol self-administration (Roberts et al., 1996), suggest that inhibition of CRF neurons and/or GABAergic activation of neurons in the CeA blocks the increase in ethanol consumption induced by intermittent ethanol vapor exposure.

Since the CeA is known to be involved in stimulus-reward learning (Knapska et al., 2006; Zhu et al., 2007), it is possible that the CeA lesioned mice had a lower preference for ethanol than the BNSTLP and sham lesioned mice because they were not able to associate the hedonic effect of ethanol with the consumption of ethanol. This interpretation is supported by experiments that have indicated that manipulations made to the CeA have an effect on preference behaviors associated with ethanol, such as an ethanol-induced conditioned place preference (CPP). CeA neurons from rats exhibiting the ethanol-induced CPP behavior showed a significant increase in non-NMDA glutamatergic synaptic transmission. Blockade of this increased synaptic transmission through CeA microinjection abolished the CPP behavior (Zhu et al., 2007). These results provide an explanation for why ethanol preference is low in the CeA lesioned mice.

Overall, our results suggest that the cEA is involved in regulating ethanol consumption, but that it is not necessary to see the increase in ethanol consumption induced by chronic ethanol vapor exposure. These results do not rule out the possibility that the cEA is involved in increases in ethanol consumption since pharmacological manipulations of the GABA and CRF systems of the CeA blocks this increase (Roberts et al., 1996; Funk et al., 2006; Finn et al., 2007). The results have implications for understanding the neural circuitry that underlies excessive alcohol consumption in

alcohol dependent people and will lead to better success in developing pharmacotherapeutic treatments for alcoholism.

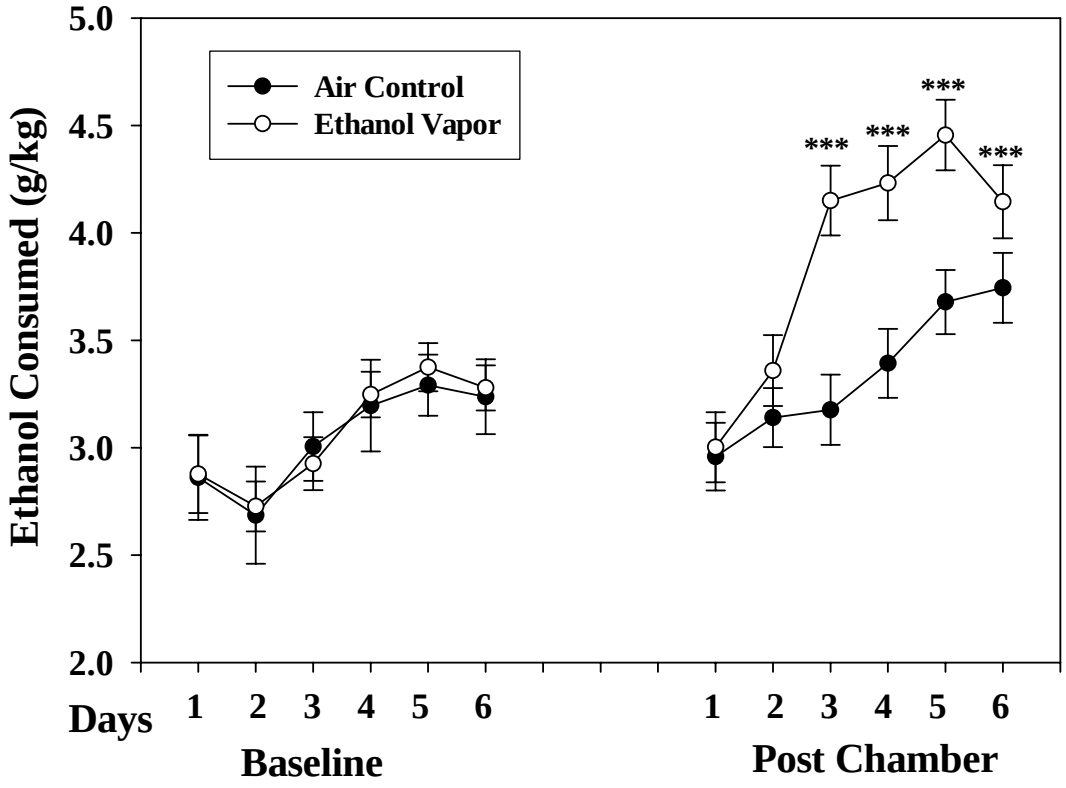


Figure 3.1. Effect of intermittent ethanol-vapor exposure and withdrawal on daily ethanol intake during a 2-hour limited-access procedure. Male B6 mice were assigned to either the air control (n=59) or intermittent ethanol vapor (n=81) groups. The graph illustrates consumption during the baseline and post-treatment periods. Values are the mean g/kg consumed per 2 hours ± SEM. *** $p<0.001$ versus the air control.

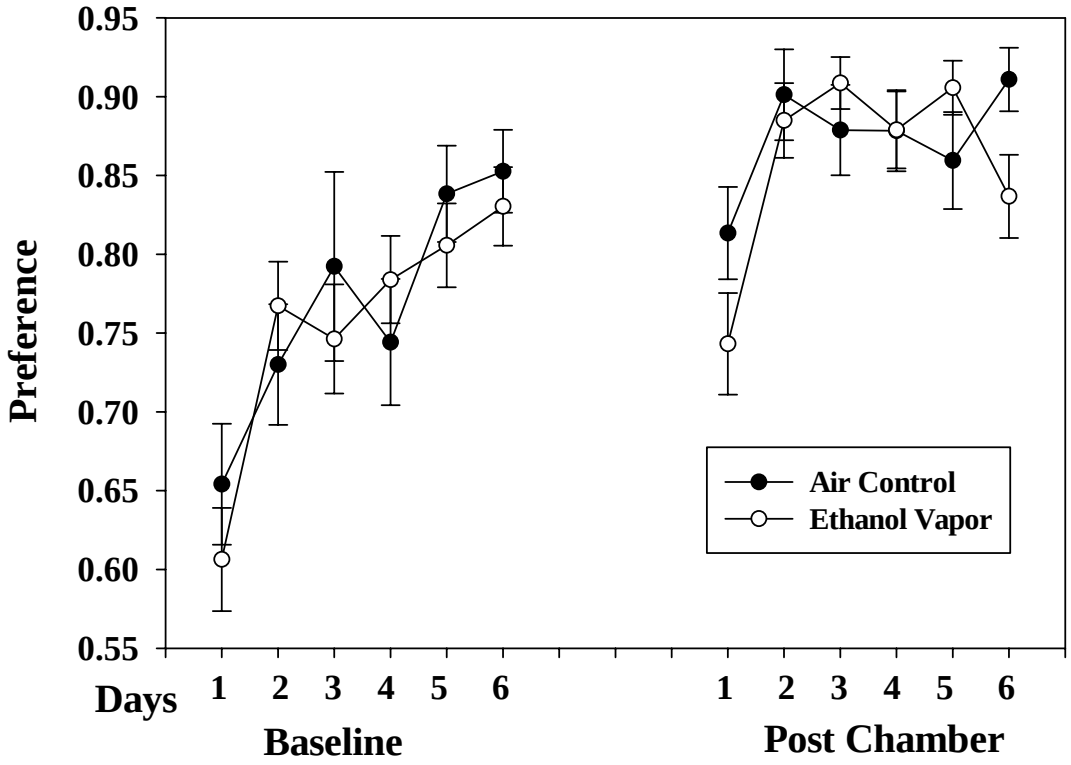


Figure 3.2. Effect of intermittent ethanol-vapor exposure and withdrawal on daily ethanol preference during a 2-hour limited-access procedure. Details are identical to those in the legend to Figure 3.1 except that the graph illustrates ethanol preference (volume of ethanol consumed/total volume consumed).

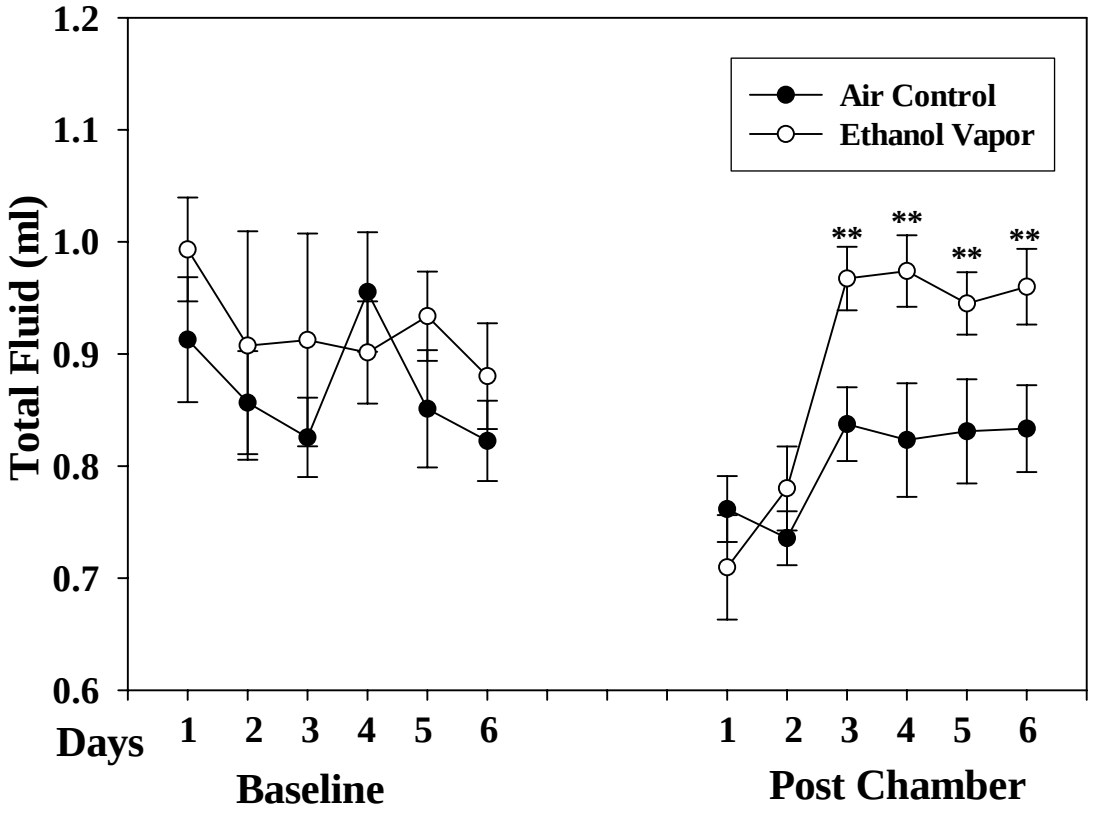
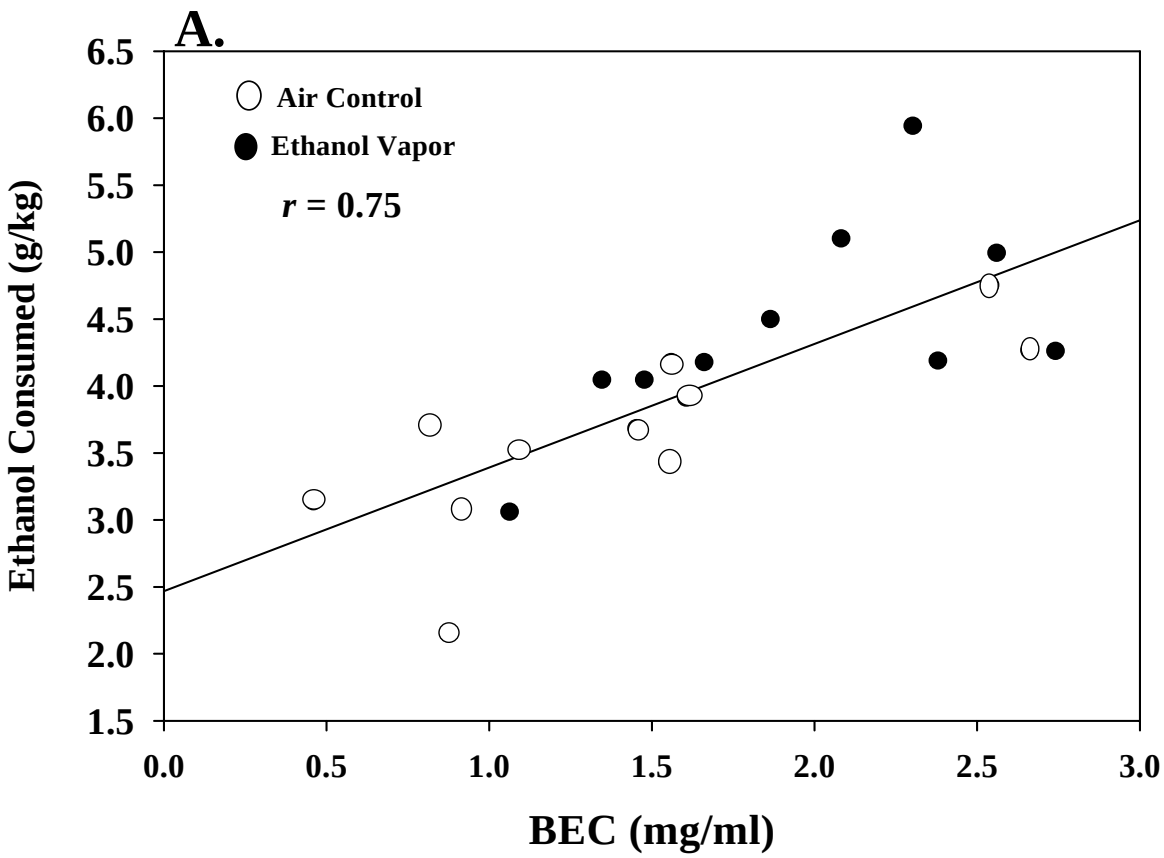


Figure 3.3. Effect of intermittent ethanol-vapor exposure and withdrawal on total fluid consumed during a 2-hour limited-access procedure. Details are identical to those in the legend to Figure 3.1 except that the graph illustrates the total volume of fluid consumed (mL per 2 hours). ** $p<0.01$ versus the air control


A.
Air Control
Ethanol Vapor
r = 0.75
Ethanol Consumed (g/kg)
BEC (mg/ml)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0

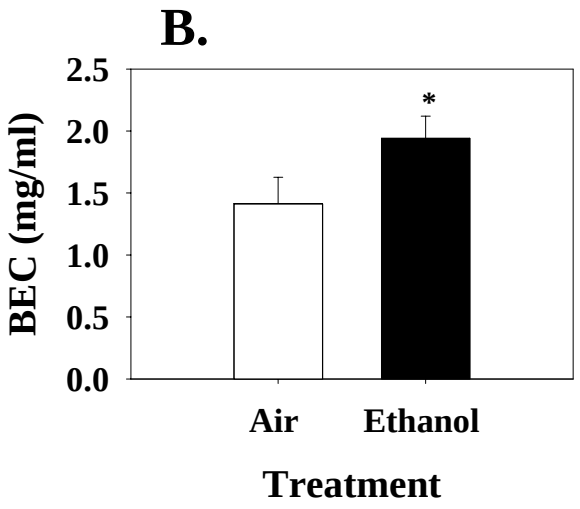

B.
BEC (mg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
*
Air
Ethanol
Treatment

Figure 3.4. Correlation between BECs and ethanol consumption on post-treatment day 6. (**A**) The g/kg of ethanol consumed versus the BECs (mg/mL) on post-treatment day 6 for the intermittent ethanol-vapor-exposed (N=10) and air-control-exposed (N=11) mice. (**B**) Mean ± SEM BECs for the 2 treatment groups on day 6. * $p<0.05$ versus the air control group.

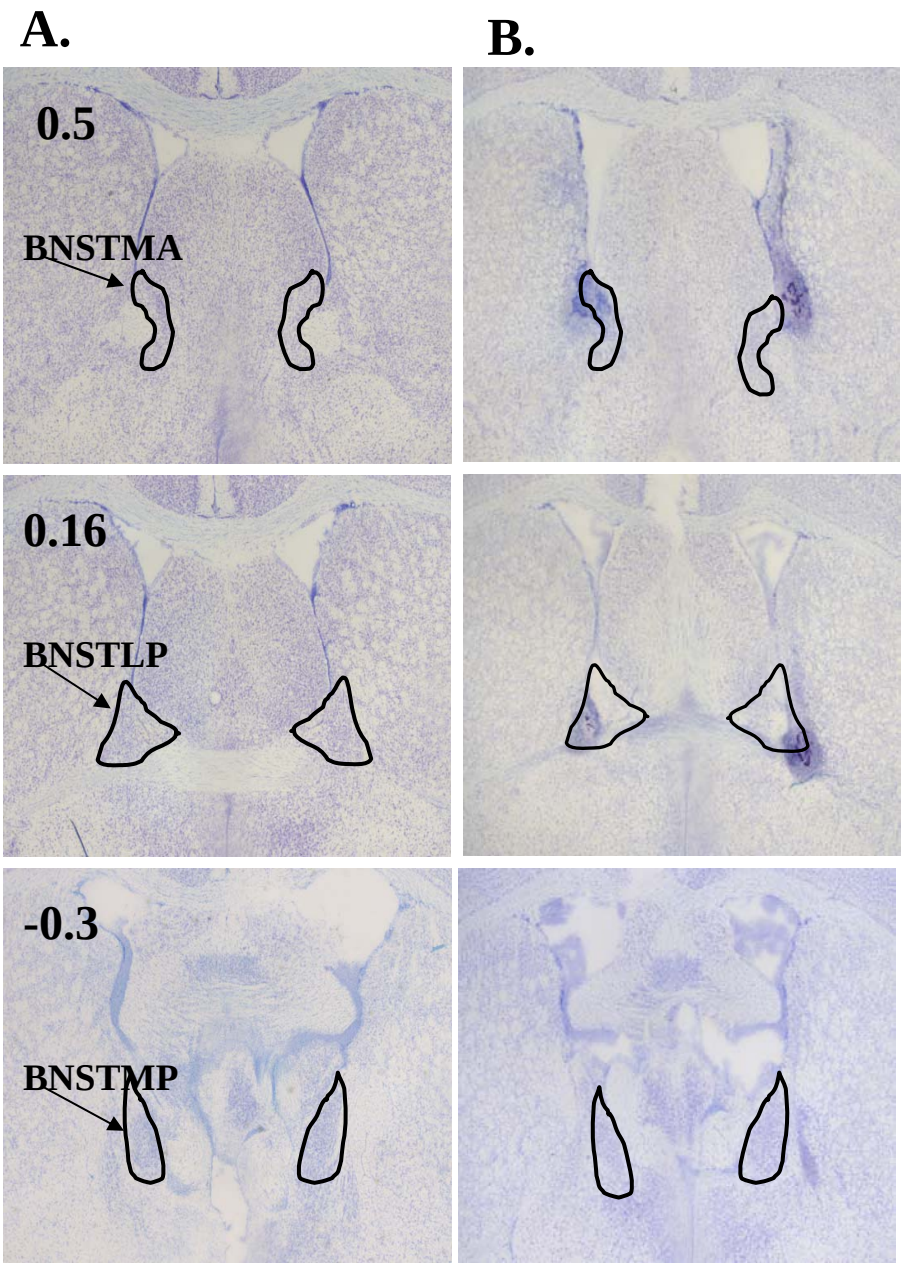


Figure 3.5. Representative photomicrographs of the BNSTLP lesions at 3 different A/P positions. Sham and electrolytic lesions are illustrated in columns A and B, respectively. The A/P positions are relative to bregma.

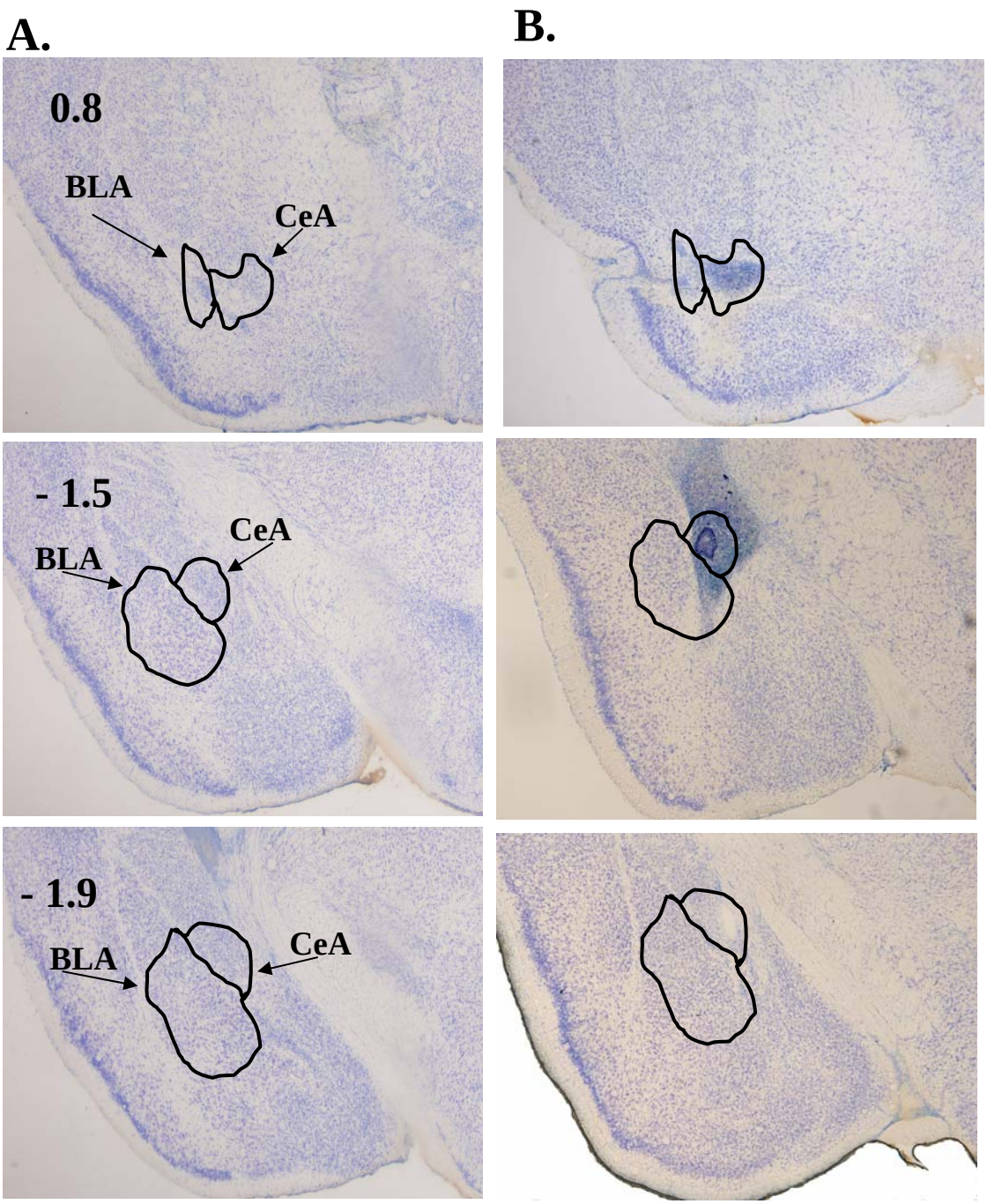
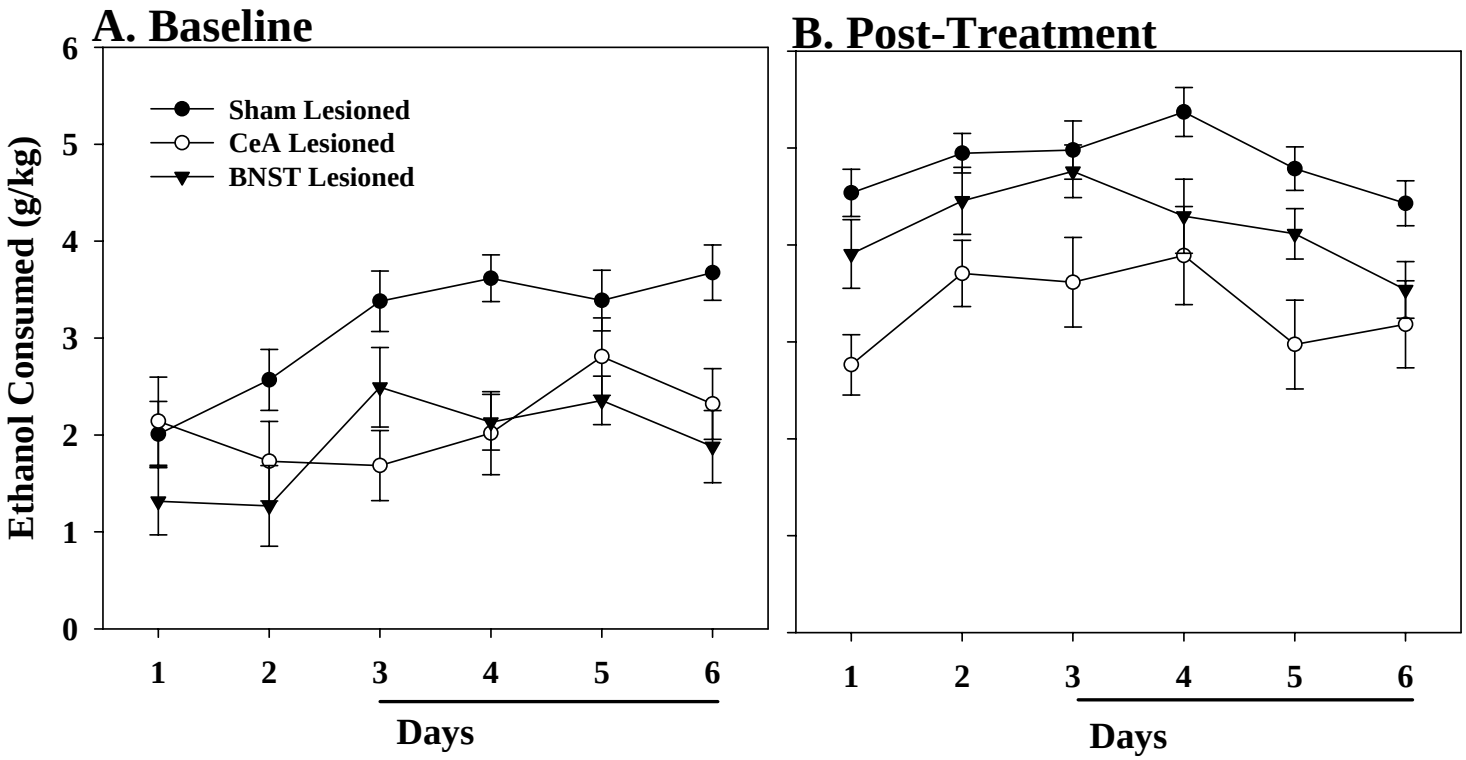


Figure 3.6. Representative photomicrographs of the CeA lesions at 3 different A/P positions. Details are identical to those found in the legend to Figure 3.5.


A. Baseline
B. Post-Treatment
Sham Lesioned
CeA Lesioned
BNST Lesioned
Ethanol Consumed (g/kg)
0
1
2
3
4
5
6
1
2
3
4
5
6
Days
Days

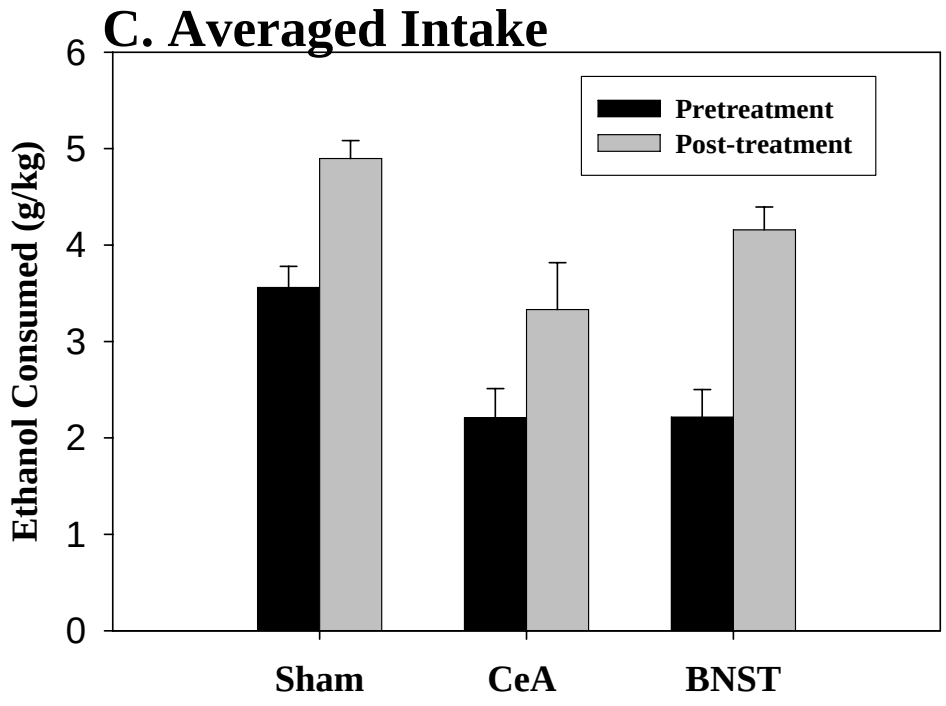
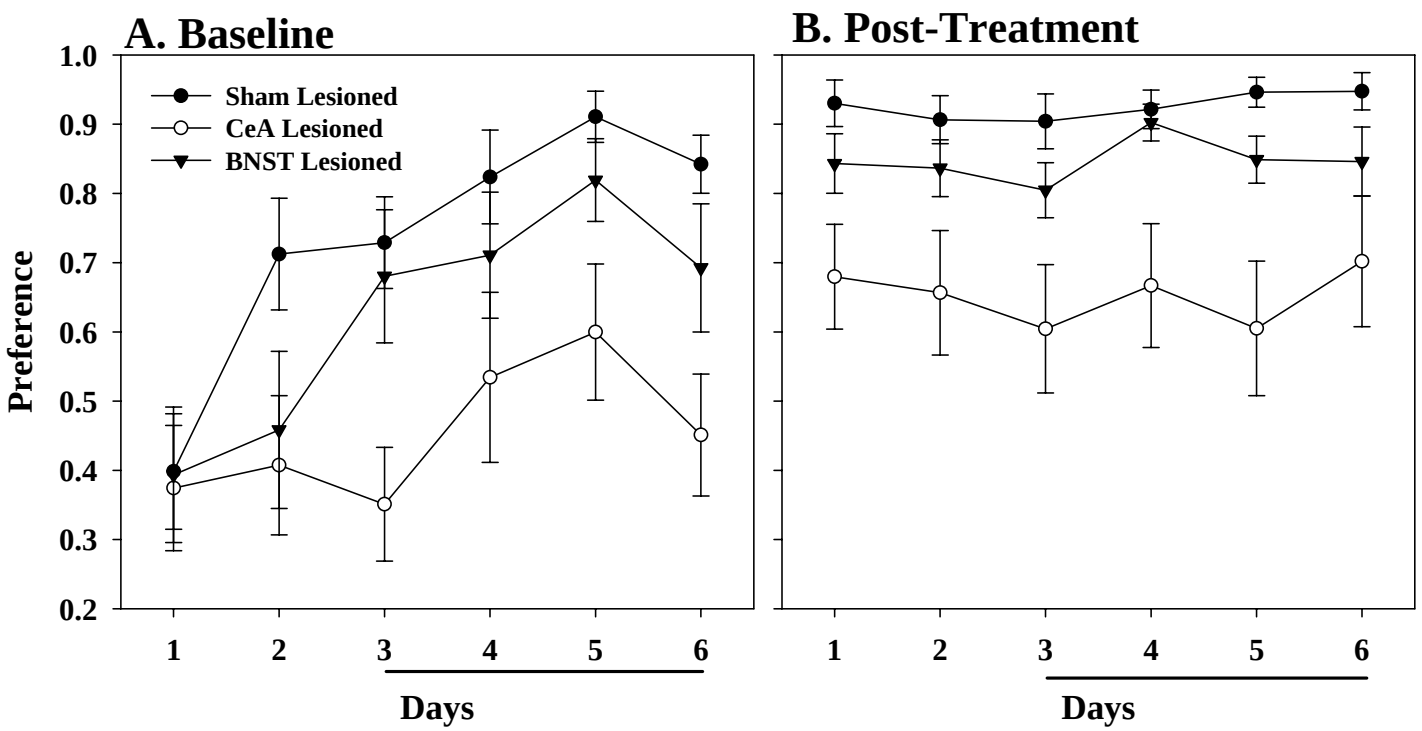

C. Averaged Intake
Pretreatment
Post-treatment
Ethanol Consumed (g/kg)
0
1
2
3
4
5
6
Sham
CeA
BNST

Figure 3.7. Effect of intermittent ethanol-vapor exposure and withdrawal on daily ethanol consumption in sham-lesioned, CeA-lesioned, and BNSTLP-lesioned mice. The mice received sham (N=17), CeA (N=13), or BNSTLP (N= 12) lesions prior to beginning the WID procedure; in addition, there was no air control treatment. (**A**) Baseline ethanol consumption; (**B**) ethanol consumption post-treatment; (**C**) average (days 3 to 6) ethanol consumption pre- and post-treatment. All values are the mean 2 hour ethanol dose consumed (g/kg) ± SEM.


A. Baseline
B. Post-Treatment
Sham Lesioned
CeA Lesioned
BNST Lesioned
Preference
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
1
2
3
4
5
6
Days

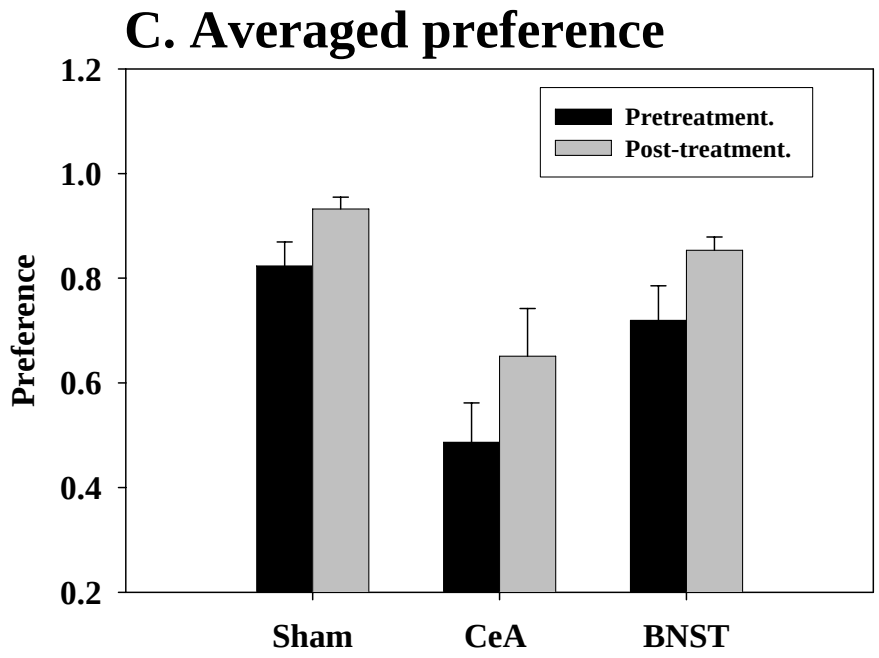
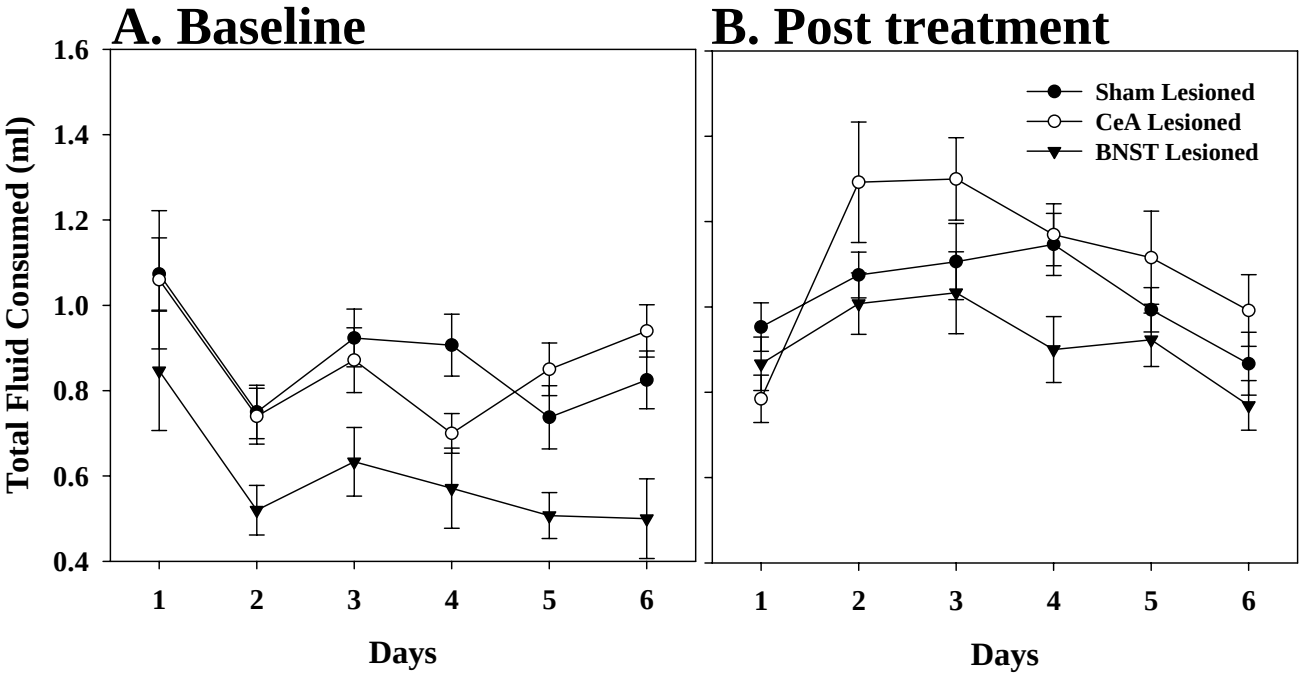

C. Averaged preference
Pretreatment.
Post-treatment.
Preference
1.2
1.0
0.8
0.6
0.4
0.2
Sham
CeA
BNST

Figure 3.8. Effect of intermittent ethanol-vapor exposure and withdrawal on ethanol preference in sham-lesioned, CeA-lesioned, and BNSTLP-lesioned mice. Experimental details are identical to those in the legend to Figure 3.7, except that the graph illustrates ethanol preference (volume of ethanol consumed/total volume consumed).


A. Baseline
B. Post treatment
Total Fluid Consumed (ml)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
1
2
3
4
5
6
Days
Sham Lesioned
CeA Lesioned
BNST Lesioned

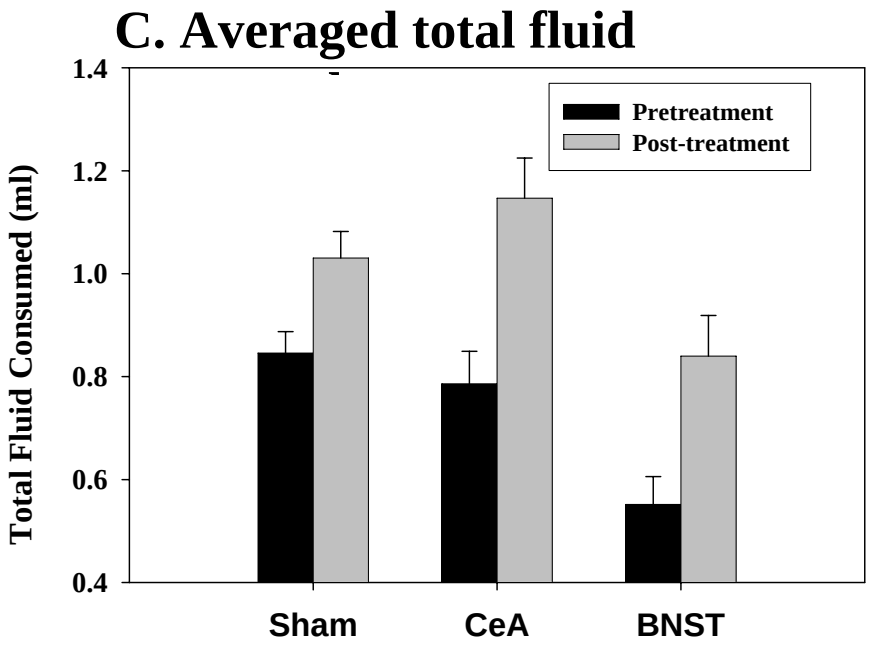
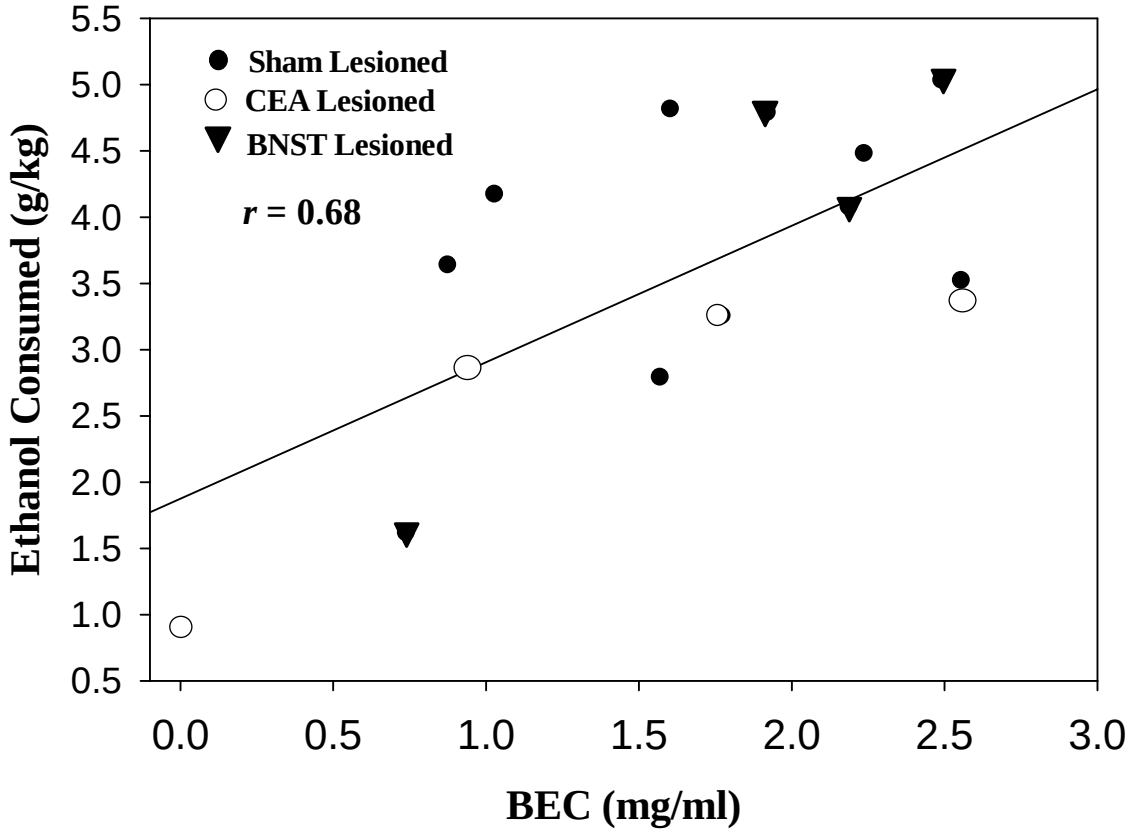

C. Averaged total fluid
Total Fluid Consumed (ml)
1.4
1.2
1.0
0.8
0.6
0.4
Pretreatment
Post-treatment
Sham
CeA
BNST

Figure 3.9. Effect of intermittent ethanol-vapor exposure and withdrawal on total fluid intake in sham-lesioned, CeA-lesioned, and BNSTLP-lesioned mice. Experimental details are identical to those in the legend to Figure 3.7, except that the graph illustrates total volume of fluid consumed (mL per 2 hours).


Sham Lesioned
CEA Lesioned
BNST Lesioned
r = 0.68
Ethanol Consumed (g/kg)
BEC (mg/ml)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0

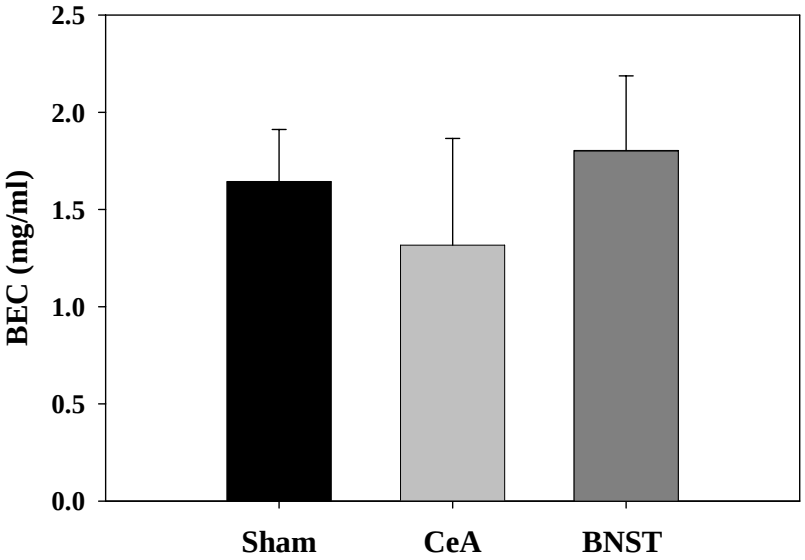

BEC (mg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
Sham
CeA
BNST

Figure 3.10. Correlation between BECs and ethanol consumption on posttreatment day 6 in sham-lesioned, CeA-lesioned, and BNSTLP-lesioned mice. (**A**) The dose of ethanol consumed versus the BEC (mg/mL) on post-treatment day 6 in the CeA-lesioned (N=4), BNST-lesioned (N=4), and sham-lesioned (N=6) groups. (**B**) The day 6 mean ± SEM BECs for the 3 groups. BECs were significantly positively correlated with the ethanol dose consumed (r=0.68, p=0.008).

Replication	Air Control (n)	Ethanol Vapor (n)
1	13	12
2	10	18
3	12	24
4	12	12
5	12	15

Table 3.1 Design table that shows groups and sizes in the 5 experiments pooled

Chapter 4: Increased ethanol consumption following intermittent withdrawal in C57BL/6J mice activates c-fos immunoreactivity in the Nucleus Accumbens shell, but lesions of this brain region do not block the increase in ethanol consumption

Abstract

In Chapter 3, we tested the hypothesis that the two major nuclei of the central extended amygdala (cEA), the central nucleus of the amygdala (CeA) and the lateral posterior portion of the bed nucleus of the stria terminalis (BNSTLP), are involved in the increased ethanol consumption induced with chronic intermittent ethanol vapor exposure (Becker and Lopez, 2004). The results indicated that these nuclei were not necessary to block this increase in ethanol consumption. This chapter will describe c-fos immunoreactivity studies that were carried out to determine the brain regions that were activated as a result of the increased ethanol consumption. C-fos immunoreactivity was studied in two intermittent ethanol vapor groups and an air control group. One vapor group received ethanol intermittently, and the other vapor group received ethanol continuously. The results suggest that both intermittent and continuous ethanol vapor exposure increased ethanol consumption. The results from the c-fos studies suggested that the core (NAc core) and the shell of the nucleus accumbens (NAc shell) were activated as a result of increased ethanol consumption in the intermittent ethanol vapor exposed group. Since the NAc shell is a part of the cEA, the hypothesis was tested that this region was necessary for the increases in ethanol consumption following intermittent ethanol vapor exposure. Results from a lesion study indicate that electrolytic lesions of the NAc shell decreased baseline ethanol consumption, but did not alter the intermittent ethanol vapor-

induced increase in ethanol consumption. The results indicate that while the NAc shell and NAc core were responsive to heightened levels of ethanol consumption, the NAc shell did not appear to be necessary for this increase in ethanol consumption. Overall, the results described in this chapter, taken together with results from chapter 3, confirm that the cEA is not necessary to see the chronic intermittent ethanol vapor-induced increase in ethanol consumption.

Introduction

In the previous chapter the role of the central nucleus of the amygdala (CeA) and the lateral posterior portion of the bed nucleus of the stria terminalis (BNSTLP) in the increased ethanol consumption induced by chronic intermittent exposure to ethanol vapors was examined. Data from several laboratories suggested that the central extended amygdala (cEA) had a role in this increase in ethanol consumption (Funk et al., 2006; Roberts et al., 1996; Finn et al., 2007). However, we observed that lesions of either the BNSTLP or CeA significantly decreased baseline ethanol consumption in a limited access procedure without affecting the increase in consumption seen after the intermittent exposure to ethanol vapor. These data led us to conclude that an intact cEA was not necessary for the expression of the increased ethanol consumption phenotype.

To detect the circuits associated with this intermittent vapor-induced increase in consumption, the experiments described in this chapter examined c-Fos expression. In brain, the expression of immediate early genes, such as *c-fos*, is normally low but can be rapidly increased by a variety of pharmacological, physiological and behavioral manipulations (Morgan et al. 1987, 1991). Immunocytochemical localization of the nuclear Fos protein (or more precisely, Fos immunoreactivity) allows one to detect with some certainty, the neurons being stimulated.

The cEA is one brain region of interest that activates in response to ethanol treatment, as indicated by c-fos immunoreactivity. For example, an intraperitoneal (IP) injection of ethanol increases c-fos immunoreactivity in the CeA, the BNSTLP, and the

NAc shell in both C57BL/6J (B6) and DBA/2J (D2) inbred strains of mice (Hitzemann & Hitzemann, 1997; Bachtell & Ryabinin, 2001) and in rats (Chang et al., 1996). An intracerebroventricular (ICV) injection of ethanol in rats also increased c-fos immunoreactivity in these brain regions (Crankshaw et al., 2003). While IP and ICV injections of ethanol induce c-fos immunoreactivity in the cEA, studies carried out by Ryabinin and colleagues have indicated that c-fos immunoreactivity was not detected in the cEA following self-administration of ethanol in Alco alcohol rats (Weitemier et al., 2001) and following ethanol consumption in B6 mice (Sharpe et al., 2005; Ryabinin et al., 2003; Bachtell et al., 2001).

The studies carried out by Ryabinin and colleagues used a 30 min limited access procedure that resulted in blood ethanol concentrations (BECs) that varied from 0.50 to 2.1 mg/ml (Sharpe et al., 2005). With our 2-hr limited access procedure, BECs in mice naïve to surgery and ethanol vapor treatment ranged from 0.4 to 2.7 mg/ml. Following the intermittent ethanol vapor exposure, BECs after the 2-hr limited access procedure ranged from 1.1 to 2.7 mg/ml. Thus, one purpose of the present study was to use c-fos immunoreactivity to determine whether our procedure for inducing high ethanol intake and BECs would increase c-fos immunoreactivity in the cEA. The hypothesis tested was that the cEA would show activity in response to ethanol consumption that resulted in higher BEC levels than those previously reported in the literature.

To induce increases in ethanol consumption in a 2-hr limited access procedure, B6 mice were intermittently exposed to and withdrawn from ethanol vapor (Becker & Lopez, 2004; Lopez and Becker, 2005; Finn et al., 2007). The use of intermittent rather than continuous vapor exposure builds from operant studies showing that it is intermittent

exposure that optimizes the withdrawal-induced increase in ethanol self-administration (O'Dell et al. 2004). Recent studies in B6 mice show a similar pattern; intermittent exposure and withdrawal was more effective than continuous exposure at increasing subsequent limited access ethanol intake (Lopez & Becker, 2005). Thus, a second purpose was to determine whether continuous versus intermittent vapor exposure produced different patterns of neuronal activity.

The third purpose was to determine if neuronal activity in cEA, as indicated by c-fos immunoreactivity, was differed between ethanol dependent (i.e. ethanol vapor exposed) and non- dependent (i.e. non ethanol vapor exposed) mice, either in the anticipation of ethanol consumption or following ethanol consumption. To address this question, separate groups of ethanol vapor or air exposed mice were euthanized immediately prior to (anticipation) or following consumption of their final 2-hr limited access ethanol session. We hypothesized that (1) neuronal activity in the cEA would increase with heightened levels of ethanol consumption, (2) physically-dependent and non-dependent mice would exhibit different patterns in neuronal activity in the EA either in anticipation of or following ethanol consumption, and (3) the pattern of c-fos induction would be dependent on the method of inducing dependency (i.e., continuous vs. intermittent ethanol vapor exposure).

A final study lesioned the NAc shell, the third major nuclei of the cEA, to determine the effect on ethanol intake. Since c-fos immunoreactivity was increased in this brain region in response to increased ethanol consumption in ethanol dependent mice, we hypothesized that lesioning the NAc shell might block the effect of intermittent ethanol vapor to increase ethanol consumption.

Methods and Materials

Animals

Male C57BL/6J (B6) mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and upon arrival, were maintained in a temperature controlled colony room (21-23 °C) on a 12-h light/dark cycle and allowed free access to food and water. Mice were group housed upon arrival (4 mice /cage) and given at least one week of acclimation prior to the beginning of the limited access ethanol consumption procedure. For the c-fos experiment, mice were 7 – 9 weeks of age at the beginning of the limited access procedure. For the lesion experiment, mice were 7 – 9 weeks of age at the time of surgery, and were given 3-6 days of recovery following surgery before the beginning of the limited access procedure. All procedures used were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University (OHSU).

Limited access ethanol consumption.

Fluids were presented in two 25 ml graduated cylinders placed on a stainless steel cage top. Food was placed on the left side and the bottles were placed on the right side. For the first two days of the limited access drinking procedure, tap water was available in both tubes for all animals. Following this acclimation, one water bottle was replaced with one ethanol bottle three hours after lights off for a 2-hr period. The ethanol concentration was 15% v/v (Pharmco Products, Brookfield, CT, USA). Fluid

consumption was measured by recording the meniscus level on the drinking tubes. Mice were weighed once every four days.

Chronic and intermittent exposure to ethanol vapors

Mice assigned to the intermittent ethanol vapor treatment group were exposed to a series of three cycles of 16-hr of ethanol vapor, separated by 8-hr withdrawal periods. Mice assigned to the continuous ethanol exposure group were exposed to ethanol vapors for 48-hrs with no intermittent withdrawal periods. Ethanol vapor exposure was adjusted to yield target BECs of 1.5 mg/ml.

Mice exposed to ethanol vapor were weighed and injected daily with 1.50 g/kg of ethanol and 68.1 mg/kg of the alcohol dehydrogenase inhibitor, pyrazole. Animals were then placed in groups of 3 in flow-through cages and placed in vapor exposure chambers (Flare Plastics, Portland, Oregon) for 16-hrs. The chambers were previously brought to equilibrium concentrations of ethanol in air of 7-8 mg/l. Chamber levels were monitored hourly via gas chromatography (Agilent 6890N GC, using a HP-PLOT Q column). Following exposure, the ethanol vapor group was removed from the Flare chamber and 20 μl tail blood samples were collected, and directly diluted into 500 μl of a matrix of 4mM n-propanol in deionized water for subsequent determination of BEC by head-space gas chromatography (see below). Following three cycles in the vapor chambers, mice were re-housed in their initial cages and 2-hr limited access ethanol drinking measurements were resumed over a period of six days.

Blood Ethanol Concentration Analysis

The tail blood samples were analyzed immediately after collection. The sealed 2 ml vial containing the blood sample in matrix was vortexed thoroughly before analysis. Analysis was performed via ambient headspace sampling gas chromatography (Agilent 6890N GC, using a DB-ALC1 column, Wilmington, DE) on a 30 μl aliquot. Six pairs of ethanol standards (0.1 – 3.0 mg/ml), which included n-propanol (internal standard), were run before the samples.

Assignment of mice in the c-fos experiment to subgroups

On the final night of the experiment, the ethanol vapor treatment and air control groups were further subdivided into mice that anticipated but did not receive and consume ethanol and mice that did consume ethanol. Brains for the mice that did not consume ethanol were collected 1-hr after presentation of the experimenter (conditioned cue for ethanol) in the drinking room. This time point was picked because expression of c-fos protein takes roughly 1-hr. Brains for the mice that consumed ethanol were collected following the end of the two hour limited access procedure, mainly to make sure that BECs could be accurately measured.

Immunohistochemistry

The immunohistochemical procedure is as described in Hitzemann & Hitzemann (1999). Mice were euthanized by cervical dislocation and brains were removed and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were then transferred to 30% sucrose in PB. 30 μm coronal sections were collected in 10 mM phosphate buffered saline (PBS). The sections were rinsed in PBS and treated with 0.3 %

H_2O_2 in PBS for 15-min to block the endogenous peroxidase activity. The sections were rinsed in PBS to remove the residual H_2O_2. The sections were then blocked for 2-hr in the immunoreaction buffer (10 mM PBS containing 0.25% Triton X-100 and 3% goat serum) without antibody. Antibody (final dilution 1:10,000) was then added and the incubation was continued for 60 to 72 hrs at 4°C. The antibody was obtained from Oncogene Science Inc. (Cambridge, MA). The sections were rinsed in PBS and incubated with 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 2-hrs at room temperature. The sections were then incubated with horseradish peroxidase avidin-biotin complex in 10 mM PBS for 2-hrs at room temperature. The sections were rinsed in PBS and placed in 0.1 M Tris for 5 minutes. The chromatin reaction was completed with diaminobenzidine (50 mg/100 ml of 0.1 M Tris) in the 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. C-fos was counted manually (# of cells), with the experimenter blind to treatment. Data was normalized for analysis by dividing the counts in one brain region by the total number of counts in the brain.

Electrolytic Lesions

Animals were anesthetized with ketamine (220 mg/kg) /xylazine (44 mg/kg) /acepromazine (22 mg/kg) and placed on a Cartesian stereotaxic instrument. A 50 mm monopolar electrode with a 0.25 mm uninsulated tip (SNE- 300, Rhodes Medical Instruments) was lowered into the following stereotaxic coordinates with bregma

marking zero for the mediolateral (ML) and anterioposterial (AP) directions and the top of the skull marking zero for the dorsoventral (DV) direction: for the NAc shell AP = 1.10 mm, ML = ± 0.6, DV = -4.60. The current used was 0.7 mA applied for 8-sec. Sham lesions were produced by inserting the electrode into the stated coordinates without passing current.

Verification of Lesion Location

Mice were euthanized by cervical dislocation, and brains were collected and frozen by placing in an isopentane solution chilled with isopropyl alcohol and dry ice. The brains were stored at -80 °C. Brain slices were cut to a thickness of 40 μm, and stained with thionin. The extent of tissue damage was examined microscopically.

Data analysis for c-fos study

A two-way analysis of variance (ANOVA) was used for each brain region to determine treatment (consumption vs. anticipation) and group effects (air, continuous ethanol vapor, intermittent ethanol vapor) on the number of c-fos positive cells, which was the dependent measure. For the post-hoc analyses, the Tukeys test was employed (Tukey, 1953). For all analyses, statistical significance was set at $p \leq 0.05$

Data Analysis for the ethanol consumption data (c-fos study & lesion study)

For the lesion study, analyses were conducted only in mice with confirmed lesions involving at least 50% of the target region; these criteria resulted in the loss of only 1 mouse from each group. Data are presented as the mean ± SEM. Daily ethanol intake

(g/kg), total fluid consumed (mL), and preference ratio (ethanol volume / total volume) were calculated for each animal. Average baseline intake was calculated from days 3-4 of ethanol consumption before ethanol vapor treatment for the lesion study and days 3-6 for the c-fos study, and from days 3-6 post-inhalation for both studies. The analysis was limited to days 3-6 for pre and post-inhalation ethanol consumption, since previous studies have shown that consumption has stabilized during these days. ANOVA was used to determine the effect of treatment on ethanol consumption, preference ratio and total fluid consumed. Averaged ethanol consumption over baseline days 3-4 for the lesion study and 3-6 for the c-fos study and post-treatment days 3-6 for both studies was analyzed with a two-way ANOVA to determine treatment effects, group effects, and interactions between group and treatment. For the post-hoc analyses, the Tukeys test was employed (Tukey, 1953). Since we were predicting that there would be a significant effect of the lesion on ethanol intake, planned comparisons were conducted in the absence of a significant interaction between main effects. For all analyses, statistical significance was set at $p \leq 0.05$.

Results

The effect of continuous versus intermittent ethanol vapor exposure on ethanol consumption levels in a limited access procedure.

As depicted in Figure 4.1A, there was no difference in baseline ethanol intake between the ethanol vapor groups and the air control group. Following vapor treatment, a repeated measures ANOVA was conducted on the third to the fifth day post treatment. The sixth day was left out of the analysis since half the mice did not consume ethanol on

the sixth day of the experiment. Repeated measures ANOVA indicated that there was a trend towards an effect of group [$F(2,29)=2.85$, $p=0.07$] a significant effect of day [$F(2, 60)=3.78$, $p=0.03$], and no group x days interaction on ethanol intake.

As depicted in Figure 4.1B, baseline ethanol intake averaged over pre-treatment days three through six was 2.77 ± 0.18 g/kg for the intermittent ethanol vapor group, 2.65 ± 0.13 g/kg for the continuous ethanol vapor group, and 2.60 ± 0.18 g/kg for the air control group. After ethanol vapor exposure, ethanol consumption levels over days three through six were at 3.98 ± 0.17 g/kg for the intermittent ethanol vapor group, 3.89 ± 0.22 g/kg for the continuous ethanol vapor group, and 3.26 ± 0.20 g/kg for the air control group. A one-way ANOVA averaged over these post-treatment days indicated a significant effect of group [$F(2, 29) = 3.76$, $p = 0.04$], with post hoc test confirming that the continuous and intermittent ethanol vapor groups had a higher level of ethanol consumption than the air control group ($p = 0.05$).

Average total fluid consumption (data not shown) before ethanol vapor treatment was 0.77 ± 0.07 ml for the intermittent ethanol vapor group, 0.82 ± 0.11 ml for the continuous ethanol vapor group, and 0.72 ± 0.05 ml for the air control group. Repeated measures ANOVA indicated no effect of group, an effect of days [$F(3, 60) = 2.25$, $p = 0.05$], and no group x days interaction. Following ethanol vapor treatment, average total fluid consumed was 0.80 ± 0.04 ml for the intermittent ethanol vapor group, 0.78 ± 0.06 ml for the continuous ethanol vapor group, and 0.67 ± 0.05 ml for the air control group. Repeated measures ANOVA indicated no effect of group or days and no group x days interaction.

With regard to ethanol preference (not shown), average baseline preference was 69 ± 17% for the intermittent ethanol vapor group, 75 ± 8% for the continuous ethanol vapor group, and 70 ± 5% for the air control group. Repeated measures ANOVA indicated no effect of group or days, and no interaction between the two. After ethanol vapor treatment, preference was 90 ± 3% for the intermittent ethanol vapor group, 88 ± 3% for the continuous ethanol vapor group, and 86 ± 6% for the air control group. There was no effect of group or days, and no group x days interaction.

In a subset of the ethanol vapor and air exposed mice, BECs were measured at the end of the limited access period. Ethanol dose consumed on the day that BEC was sampled was 4.06 ± 0.22 g/kg for the intermittent ethanol vapor group (N = 5), 3.70 ± 0.37 g/kg for the continuous ethanol vapor group (N = 6), and 3.29 ± 0.23 g/kg for the air control group (N = 5). The difference between groups was not significant. As depicted in Figure 4.2B, BEC levels were 1.72 ± 0.14 mg/ml for the intermittent ethanol vapor group, 1.58 ± 0.25 mg/ml for the continuous ethanol vapor group, and 1.42 ± 0.22 mg/ml for the air control group. A one-way ANOVA showed no effect of group on BEC, no doubt due to the low n/group. However, BEC was significantly positively correlated with ethanol consumption levels ($r = 0.89$, $N = 16$ $p < 0.001$), as depicted in Figure 4.2A.

The effects of ethanol consumption and ethanol vapor exposure on c-fos immunoreactivity are summarized in Table 4.1. Degrees of freedom are lower in the motor cortex and dorsal raphe due to unavailability of counts in those brain regions. Counts for the basal ganglia of the continuous ethanol vapor group also are not available.

Cortical structures: The cingulate showed a significant effect of group [$F(2,33) = 5.2$, $p<0.01$], but no significant effect of treatment and no group x treatment interaction. The number of c-fos positive cells was significantly lower in the continuous vapor group ($p<0.01$). In the motor cortex, there was an effect of treatment [$F(1,25) = 10.2$, $p<0.004$], no effect of group, and a group x treatment interaction [$F(2,25) = 3.9$, $p < 0.03$]. Ethanol consumption significantly decreased the number of c-fos positive cells in the air control group and the intermittent ethanol vapor group, but not in the continuous ethanol vapor group ($p < 0.05$).

Nucleus Accumbens: Figure 4.3 demonstrates c-fos immunoreactivity in the NAc core and shell of mice in the intermittent ethanol vapor group and the air control group before and after treatment. In the NAc core, there was no effect of treatment, an effect of group [$F(2,37)=12.5$, $p<0.00007$], and a significant group x treatment interaction [$F(2,37)=5.4$, $p<0.008$]. Post hoc analysis confirmed that ethanol consumption increased c-fos immunoreactivity in the intermittent ethanol vapor group ($p<0.02$). In the NAc shell, there was no effect of treatment, an effect of group [$F(2,37)=5.1$, $p<0.01$], and a significant group x treatment interaction [$F(2,37)=4.1$, $p<0.02$]. Post hoc analysis revealed that ethanol consumption increased c-fos immunoreactivity in the intermittent ethanol vapor group ($p<0.02$).

Lateral Septum: In the ventral lateral septum, there was a significant main effect of group on c-fos immunoreactivity [$F(2,37)=4.5$, $p<0.02$], no effect of treatment, and a significant group x treatment interaction [$F(2,37)=5.2$, $p<0.01$]. In the air control group, c-fos immunoreactivity in the ventrolateral septum was higher in the group that anticipated ethanol compared to the group that consumed ethanol ($p<0.02$).

Bed Nucleus: In the BNSTLP, there was no effect of group on c-fos immunoreactivity, a significant effect of treatment [$F(1,37)=4.7$, $p<0.03$], and no group x treatment interaction. Ethanol consumption increased c-fos immunoreactivity. In the BNSTMV, there was no significant effect of treatment on c-fos immunoreactivity, a significant effect of group [$F(2,37)=4.3$, $p<0.03$], and no group x treatment interaction. C-fos immunoreactivity was highest in the continuous ethanol vapor exposure group ($p<0.04$).

Amygdala and Hypothalamus: There were no effects of ethanol consumption or ethanol vapor exposure in the basomedial, basolateral, and central nucleus of the amygdala. In the lateral hypothalamus, there was an effect of group on c-fos immunoreactivity [$F(2,38)=7.3$, $p<0.0002$], but no effect of treatment and no group x treatment interaction. C-fos immunoreactivity was significantly higher in the continuous exposure group ($p<0.01$) than in the other two groups.

Basal Ganglia: The substantia nigra reticulata and the ventral tegmental area showed a significant effect of group [$F(2,38)=4.8$, $p<0.02$], no effect of treatment, and no group x treatment interaction. C-fos immunoreactivity was highest in the intermittent ethanol vapor group ($p<0.05$).

Dorsal Raphe, Periaqueductal gray, and Edinger-Westphal: In the dorsal raphe, there was an effect of group [$F(2,28)=5.82$, $p<0.007$], but no effect of treatment and no group x treatment interaction. C-fos immunoreactivity was lowest in the intermittent ethanol vapor exposure group ($p<0.01$). In the periaqueductal gray, there was an effect of treatment [$F(1,34)=10.6$, $p<0.003$], and an effect of group [$F(2,34)=7.4$, $p<0.002$], but no significant group x treatment interaction. There was a decrease in c-fos immunoreactivity with ethanol consumption ($p<0.01$) and c-fos levels were highest in the

continuous ethanol vapor exposed mice ($p<0.02$). In the Edinger-Westphal, there was an effect of treatment [$F(1,34)=14.5$, $p<0.0006$] , but no significant effect of group or group x treatment interaction. Ethanol consumption increased c-fos immunoreactivity in the Edinger-Westphal ($p<0.0004$) in all three groups. This is as illustrated in figure 4.4.

Figure 4.5. illustrates representative light micrographs of lesions of the Nac shell. Mice in the sham control group (left column, panel A) and lesion group (right column, panel B) are shown. Brain slices at 1.34 mm and 0.86 mm demonstrate that while the lateral portion of the NAc core was left intact, there was damage to the medial septum (MS). Brain slices at 0.26 mm demonstrate that the LS and the BNSTDL and BNSTLP were left intact.

Figure 4.6 illustrates ethanol consumption (g/kg) during the baseline and post-inhalation limited access periods (Panel A). As the analysis of the ethanol vapor studies carried out in naïve mice reported in Chapter 3 and in this chapter confirmed that analysis of daily versus average data yielded similar results, the analysis focused on the average data from days 3-4 pre vapor treatment and days 3-6 post vapor treatment (Figure 4.6B). ANOVA revealed a significant effect of group [$F(1,48) = 35.38$, $p< 0.000001$] (sham>lesion) and treatment [$F(1,48) = 23.91$, $p = 0.00001$] (post>pre), and no significant group x treatment interaction. Baseline consumption was higher in the sham (3.53 ± 0.25 g/kg) than in the NAc shell (2.03 ± 0.33 g/kg) lesioned animals, and post-treatment consumption was also higher in sham (4.90 ± 0.19 g/kg) than in the NAc shell (3.86 ± 0.44 g/kg) lesion group.

Panel B indicates that ethanol consumption increased as a result of intermittent ethanol vapor treatment in both the sham and NAc shell lesioned mice.

Figure 4.7 illustrates total volume of fluid consumed (ml) during baseline and post inhalation limited access periods (Panel A). Data was analyzed in a similar manner as that used to analyze the ethanol consumption data. ANOVA revealed a significant effect of group [$F(1,48) = 26.95$, $p < 0.00001$], a significant effect of treatment [$F(1,48) = 13.93$, $p<0.0001$], and a significant group x treatment interaction [$F(1,48) = 4.30$, $p<0.05$]. Post hoc analysis indicated that during baseline drinking, total volume consumed was lower in the NAc shell lesioned (0.42 ± 0.08 ml) group when compared to the sham (0.90 ± 0.05 ml) control group ($p< 0.0001$). There was no significant difference between groups post treatment. Total volume consumed was 0.81 ± 0.10 ml for the NAc shell lesioned group, and 1.03 ± 0.05 ml for the sham control group. The significant interaction was due to the fact that total volume was increased to a greater extent post treatment in the NAc shell lesioned animals (Figure 4.7B)

Figure 4.8 illustrates the preference ratio (total volume of ethanol consumed / total volume of fluid consumed) during baseline and post inhalation limited access periods (Panel A). Data was analyzed in a similar manner as that used to analyze the ethanol consumption data. ANOVA revealed no significant effect of group, a significant effect of treatment [$F(1,48) = 8.36$, $p<0.001$], and no significant group x treatment interaction. Before treatment, preference ratio was 0.82 ± 0.07 for the NAc shell lesioned mice, and 0.82 ± 0.04 for the sham control group. Following treatment, preference ratio was $0.94 \pm$

0.01 for the NAc shell lesioned group, and 0.93 ± 0.02 for the sham control group. Panel B illustrates that the preference ratio increased following intermittent alcohol vapor exposure in both groups.

Discussion

The main findings of this research are that the NAc shell and NAc core activate, as indicated by c-fos immunoreactivity, in response to consumption of ethanol. This effect occurred in mice intermittently exposed to ethanol vapor that showed increased ethanol consumption, but not in the mice exposed to continuous exposure that also showed an increase in ethanol consumption, or in the air control mice that did not show increased ethanol consumption. While the c-fos study indicated that the NAc shell and NAc core activated in response to ethanol consumption in mice made ethanol dependent through intermittent ethanol vapor exposure, lesions of the NAc shell did not block the intermittent withdrawal-induced increase in ethanol consumption. The finding of the lesion study suggests that while the region has adapted in response to ethanol vapor exposure to respond differently to ethanol consumption, this adaptation was not necessary to see the intermittent ethanol vapor-induced increase in ethanol consumption. Thus this study, in addition to the studies carried out in Chapter 3, indicate that the cEA as a whole (BNSTLP, CeA, and NAc shell) is not necessary to see the increase in ethanol consuming behavior that occurs in response to intermittent ethanol vapor exposure.

One interesting finding of the research outlined in this chapter is that both intermittent and continuous ethanol vapor exposure produced an increase in ethanol consumption. These findings differ from other studies that have compared the effect of intermittent vs. continuous ethanol vapor exposure on ethanol consumption. For example, the change in operant ethanol self-administration in rats that received continuous exposure to ethanol vapor (2 weeks) was indistinguishable from air-exposed rats (O'Dell et al., 2004). Rats given intermittent exposure to ethanol vapors over a

period of four weeks, showed an increase in ethanol consumption when compared to air control. When the rats that were exposed to continuous ethanol vapors were given a four-day period without ethanol, this group began to diverge from the air control group with regard to an increase in ethanol consumption. Similar results were obtained in B6 mice continuously exposed to ethanol vapors (Lopez & Becker, 2005). Limited access ethanol intake in these mice did not differ from that in air control mice until a second bout of continuous ethanol vapor exposure that followed a period of limited access ethanol consumption was given. Studies from our lab differ from these other studies by showing that mice exposed to continuous ethanol vapor show a higher level of ethanol consumption than the air controls.

C-fos immunoreactivity studies indicated that mice that received continuous exposure to ethanol had a difference in neural activity in response to ethanol consumption compared to the intermittent ethanol vapor exposed group and the air control group. For example, in the motor cortex the intermittent exposure and air control groups showed a decrease in c-fos immunoreactivity with ethanol consumption, whereas the continuous vapor group did not show this decrease. In the medioventral BNST (BNSTMV), lateral hypothalamus (LH), and periaqueductal gray (PAG), the continuous vapor group showed the highest level of c-fos immunoreactivity. Seeing that these latter three brain regions play a role in autonomic output, these findings may suggest that there is a baseline increase in autonomic activation in the mice that have been continuously exposed to ethanol vapors.

One hypothesis tested in this chapter was that neuronal activity in the cEA, as indicated by c-fos immunoreactivity, would be induced with the heightened level of BEC

achieved in our 2-hour limited access procedure when compared to the 30-min limited access procedure carried out by Ryabinin and colleagues. While the CeA did not activate in response to ethanol consumption in any of the three groups, ethanol consumption increased c-fos immunoreactivity in all three groups in the BNSTLP, and ethanol increased c-fos immunoreactivity in the NAc shell in the highest drinking (intermittent ethanol vapor) group. The results suggest that certain nuclei of the cEA (e.g., BNSTLP & NAc shell) activate in response to ethanol consumption, but that not all key regions of the cEA (e.g., CeA) are activated by ethanol consumption. This is the first study to date that indicated that ethanol consumption increased neural activity in the BNSTLP and the NAc shell.

The other major nucleus that showed an increase in c-fos immunoreactivity in response to ethanol consumption in all three groups was the Edinger-Westphal (EW) nucleus. In fact, this effect of ethanol consumption to increase c-fos immunoreactivity in the EW was the biggest effect (smallest p value) from all the brain regions counted ($p<0.0006$). This result is consistent with findings from studies done by Ryabinin and colleagues (Weitemier et al., 2001; Sharpe et al., 2005; Ryabinin et al., 2003; Bachtell et al., 2001), who have consistently shown that ethanol increases c-fos immunoreactivity in this brain region. Lesions of the EW decrease ethanol consumption in a continuous access procedure (Bachtell et al., 2004), by decreasing preference for ethanol. EW lesioned mice also consumed less of a sucrose solution; however preference for sucrose did not decrease. The results indicate that regions that are activated by ethanol consumption, as indicated by c-fos immunoreactivity, such as the EW, are involved in ethanol consumption. The BNSTLP, which also expresses c-fos in response to ethanol

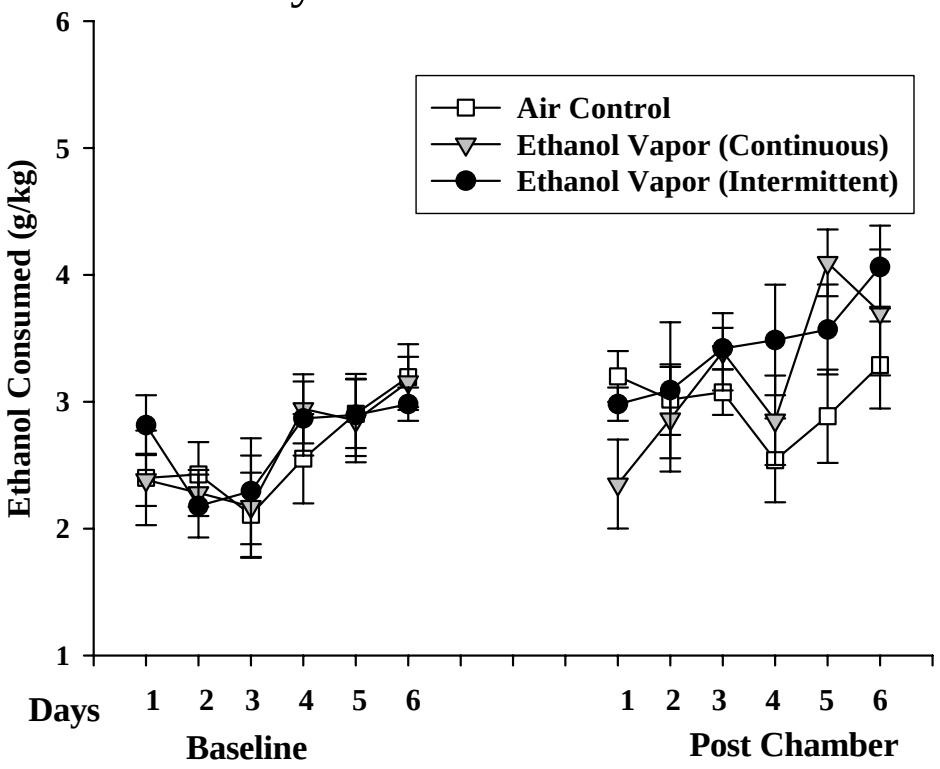
consumption, also decreased baseline ethanol consumption when lesioned. However, it should be noted that regions of the cEA that did not show expression of c-fos in response to ethanol consumption in air control non-dependent mice, such as the CeA and the NAc shell, also decreased baseline ethanol consumption when lesioned. Collectively, these findings suggest that a particular brain region does not have to exhibit a change in neural activity, as indicated by c-fos immunoreactivity, to be involved in baseline ethanol consumption.

One major finding reported in this chapter is that electrolytic lesions of the NAc shell decreased ethanol consumption in a limited access procedure. This is an important finding since others have shown that the effect of NAc lesions on ethanol consumption is dependent on the type of lesion being used. For example, neurotoxin 6 hydroxydopamine (6-OHDA) lesions, which are selective for damaging dopaminergic neurons, did not affect lever pressing for ethanol in the Wistar rat (Rassnick et al., 1993). While 6-OHDA lesions decreased the acquisition of ethanol consumption in ethanol naïve female Alcohol Preferring rats, it did not block the maintenance of ethanol consumption seen in these rats (i.e., no effect once animals had experience with ethanol; Ikemoto et al., 1997). These results suggest that the dopaminergic system was not necessary to see lever pressing for ethanol or for the maintenance of ethanol consumption. However, other studies have shown that 6-OHDA lesions of the NAc increased ethanol consumption in Sprague-Dawley rats (Quarfordt et al., 1991). This latter result is counterintuitive, since injection of dopamine agonists, such as d-amphetamine or quinpirole, into the NAc have resulted in increased ethanol consumption in High Alcohol Drinking rats and Long Evans rats, respectively (Hodge et al. 1992; Samson et al. 1993). The discrepancy between the

results may have to do with the use of Sprague Dawley rats as opposed to other types of rats. Interestingly, others have shown that ibotenic acid lesions of the NAc increased ethanol consumption by increasing total volume of fluid consumed (Johansson & Hansen, 2000). Total volume of water consumed was not different between the sham and NAc lesioned rats, suggesting the specificity of the effect of the lesion for ethanol consumption. Overall, while the lesion studies seem to show contradictory results, the results suggest an involvement of the NAc in ethanol consumption.

In conclusion, c-fos immunoreactivity in the NAc shell increased in response to ethanol consumption specifically in the intermittent ethanol vapor-exposed mice. This indicated that the brain region adapted in response to the intermittent ethanol vapor exposure to become active in response to ethanol consumption. Thus, the main hypothesis that arose from this finding was that the NAc shell was involved in and necessary to see the intermittent ethanol vapor-induced increase in ethanol consumption. The results from the study that lesioned the NAc shell indicated that this brain region was not necessary to see the intermittent ethanol vapor-induced increase in ethanol consumption. Thus the overall finding from the studies reported in Chapter 3 and this chapter are that while certain aspects of the cEA activate in response to ethanol consumption and the intermittent ethanol vapor-induced increase in ethanol consumption, the cEA is not necessary to see this increase in ethanol consumption. The results have implications for understanding the neural circuits involved in alcohol dependence.

A. Daily Ethanol Intake.



B. Average Ethanol Intake.
Days 3-6 pre- and post-treatment

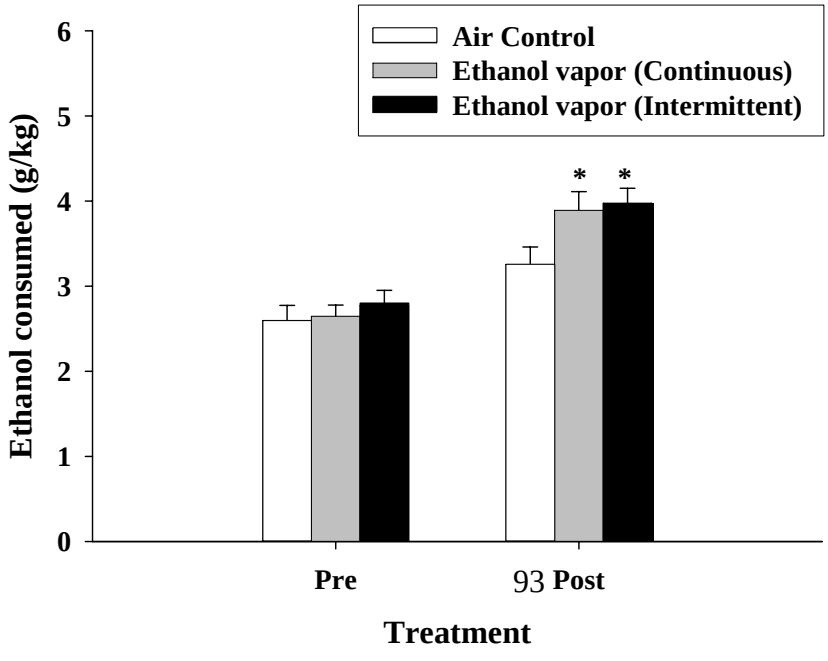
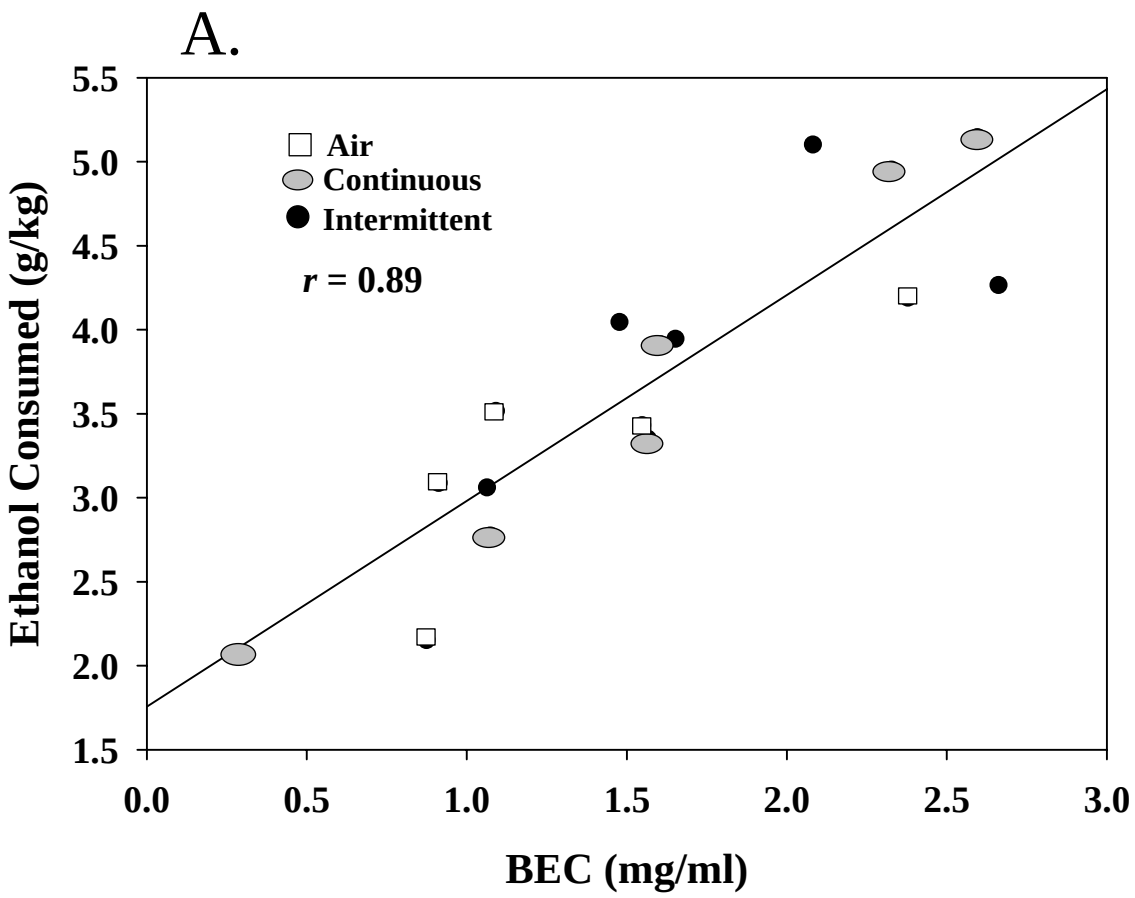


Fig. 4.1. Effect of chronic ethanol vapor exposure with and without intermittent withdrawal on ethanol consumption during a two hour limited access procedure. Panel (A) shows daily ethanol intake prior to and following ethanol vapor treatment and Panel (B) shows ethanol intake averaged over days before and after treatment. Prior to ethanol vapor treatment, there was no difference between the continuous ethanol vapor group, (n = 11), the intermittent ethanol vapor group (n = 11), and the air control group (n = 11) in daily baseline intake or in the averaged baseline intake. Following ethanol vapor treatment, both the continuous and intermittent ethanol vapor groups showed a higher level of ethanol consumption averaged over days than the air control group. Values represent the mean ± SEM. * $p \leq 0.05$ versus the respective air control group.


A.
Air
Continuous
Intermittent
r = 0.89
Ethanol Consumed (g/kg)
BEC (mg/ml)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0

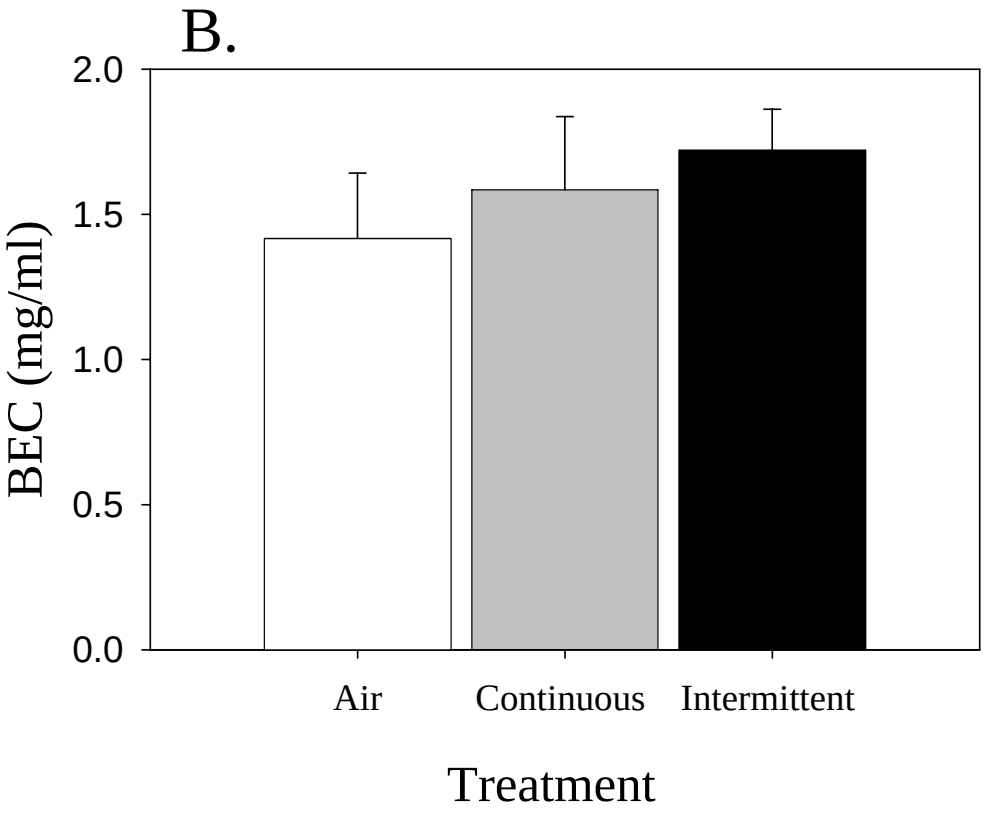
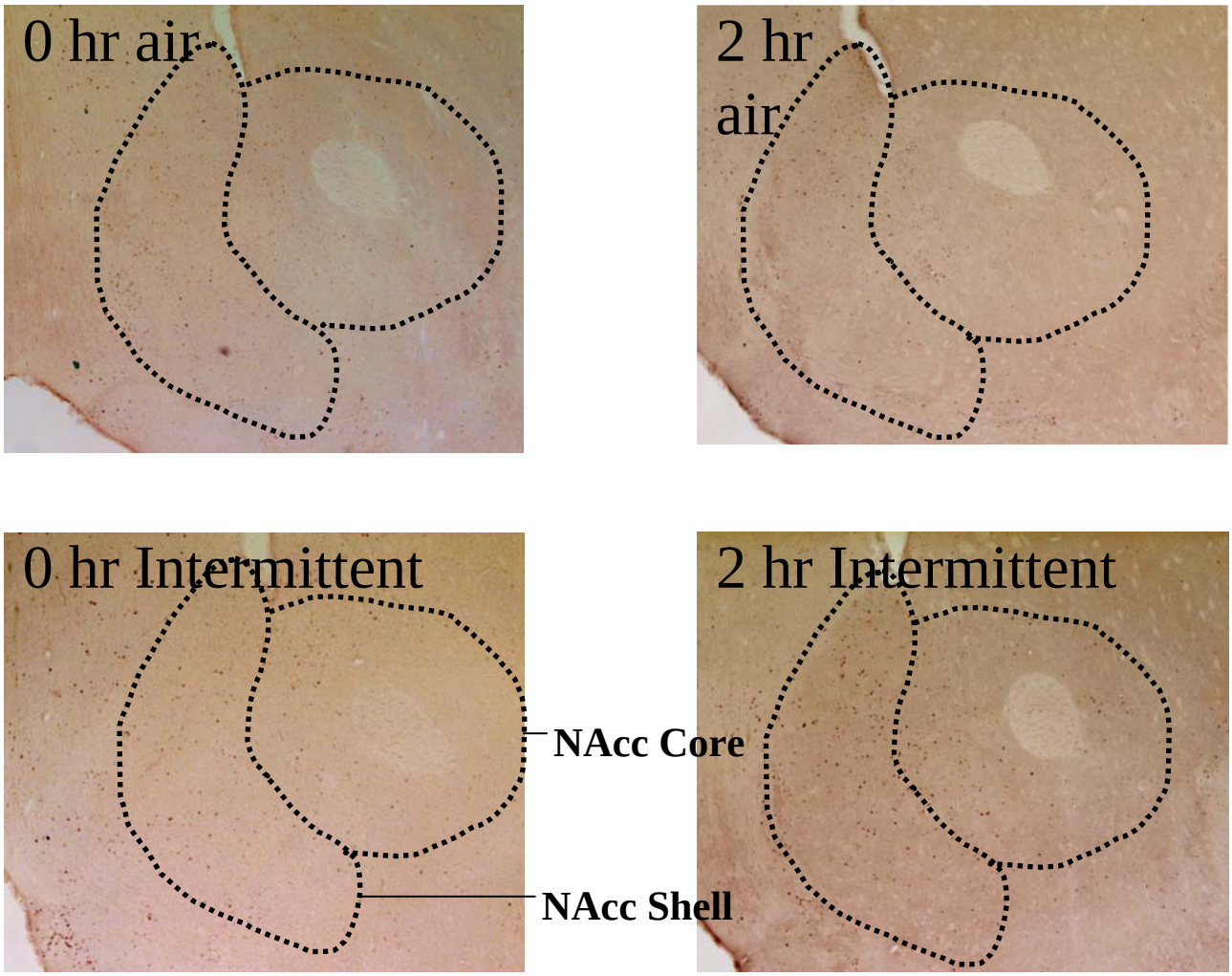

B.
BEC (mg/ml)
2.0
1.5
1.0
0.5
0.0
Air
Continuous
Intermittent
Treatment

Fig. 4.2. Relationship between BEC and ethanol consumption levels during the final session of a 2-hour limited access procedure. Panel (A) shows the correlation between BEC and ethanol consumption in the continuous ethanol vapor group, (gray ellipses, n = 6), the intermittent ethanol vapor group (black circles, n = 5), and the air control group (white squares, n = 5). Panel (B) shows the mean ± SEM BEC levels for the three treatment groups. BEC levels were significantly positively correlated with the ethanol dose consumed ($r=0.89$, $n=16$, $p<0.001$), but average BEC levels did not differ between groups.



1.18 mm anterior to bregma

Figure 4.3: c-fos in NAc shell and core

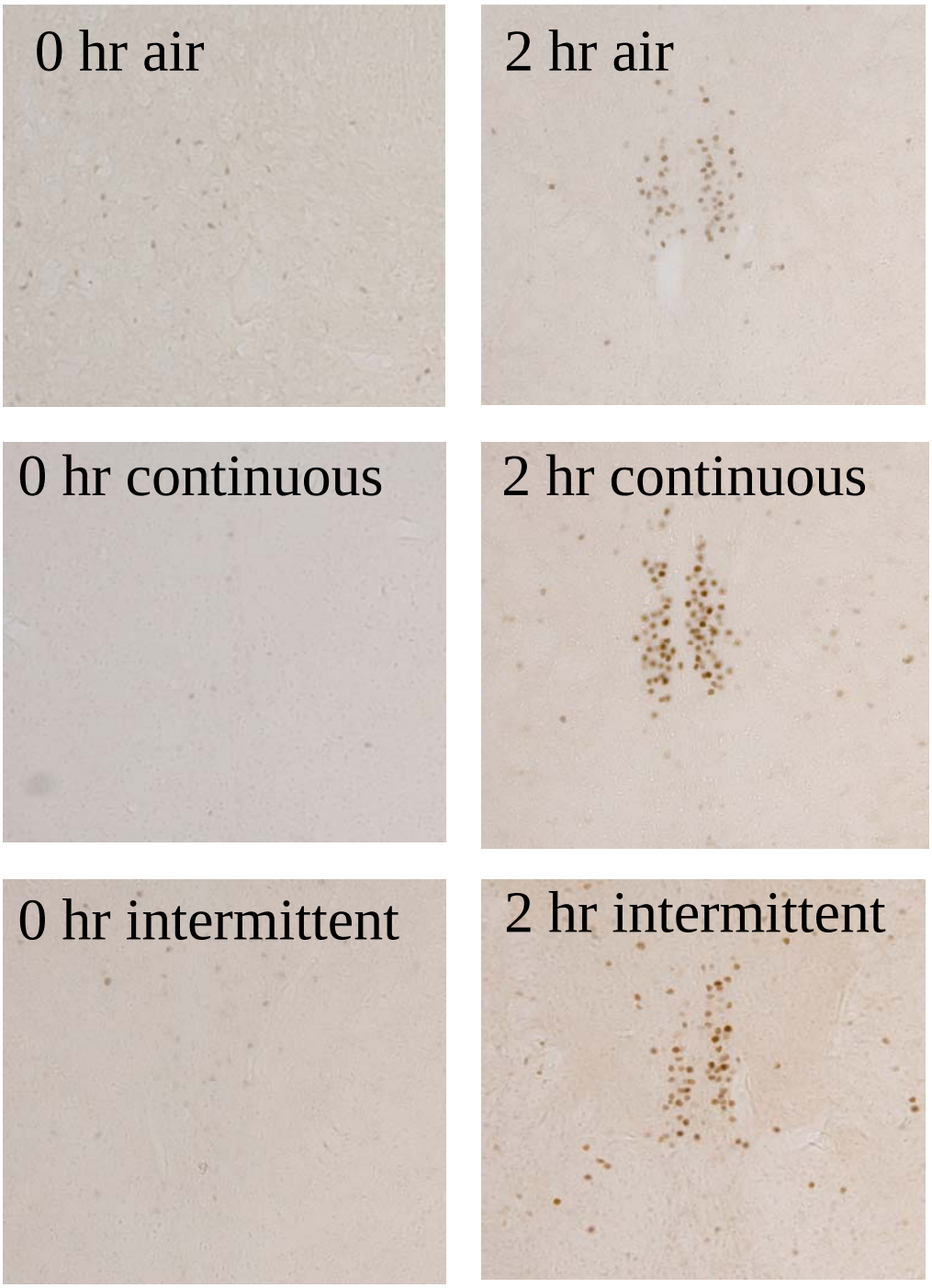


Figure 3.4. C-fos in EW

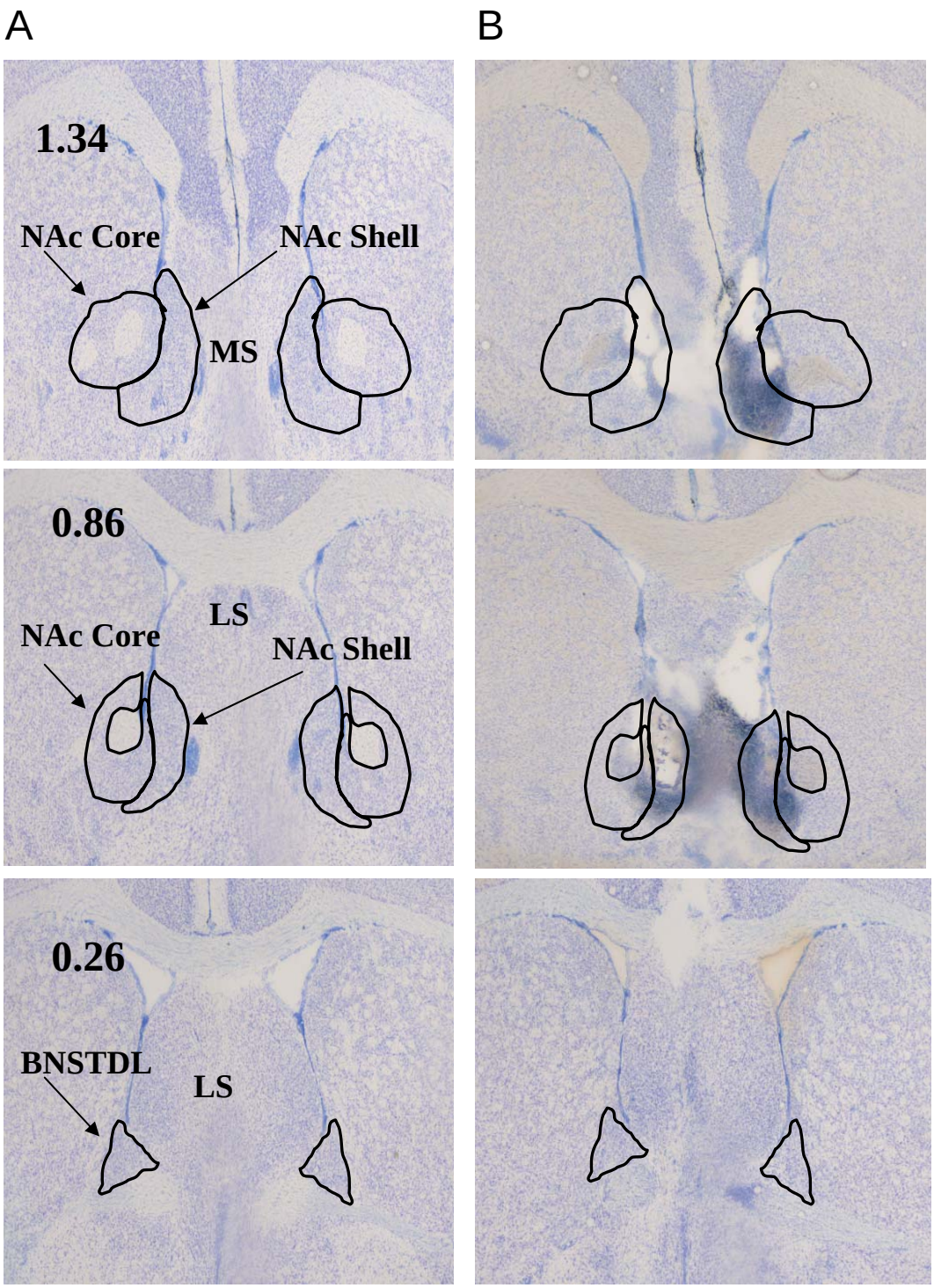
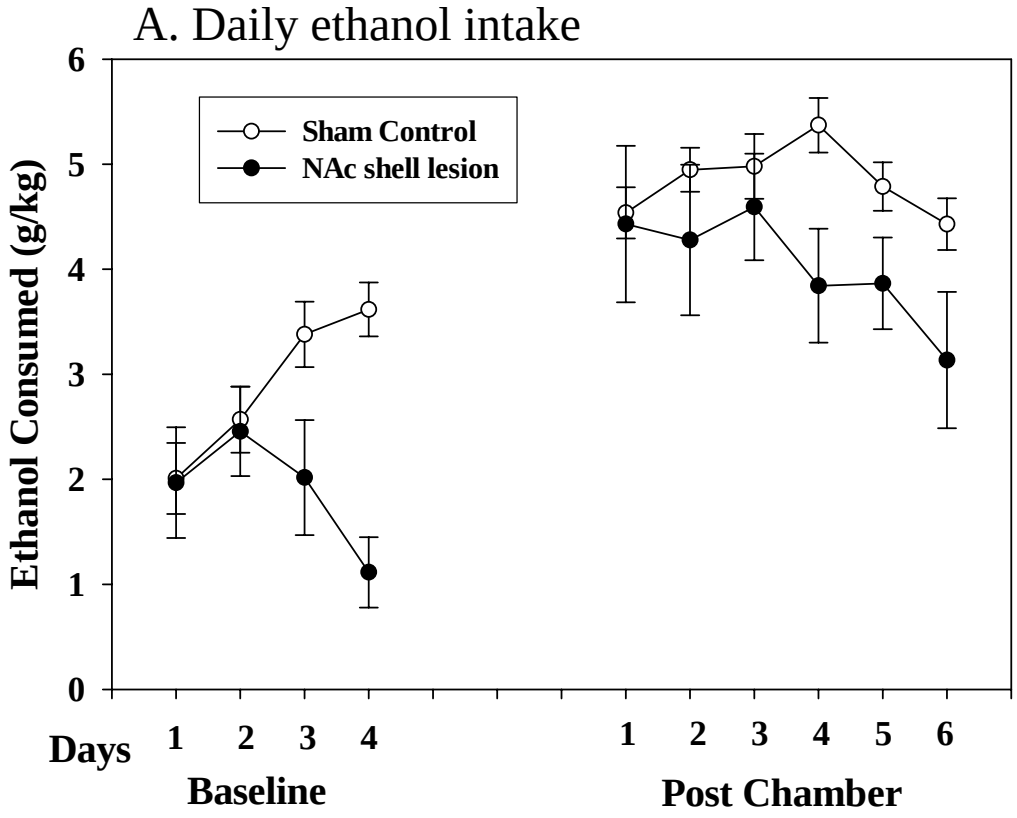

A
B
1.34
NAc Core
NAc Shell
MS
0.86
LS
NAc Core
NAc Shell
0.26
BNSTDL
LS

Fig. 4.5. Representative slices of the NAc shell lesions. Intact mice are located to the left in column A and lesioned mice are located to the right in column B. Glial scaring can clearly be seen in the lesioned mice. Brains were sectioned at 40 μm intervals and stained with thionin. Lesions were verified under a light microscope. Brain slices at 1.34 and 0.86 mm anterior to bregma demonstrate that lesions were limited to the NAc shell and medial septum, leaving the NAc core intact. Brain slices at 0.26 mm indicate that the BNSTDL and LS were left intact.


A. Daily ethanol intake
Sham Control
NAc shell lesion
Ethanol Consumed (g/kg)
0
1
2
3
4
5
6
Days
1
2
3
4
1
2
3
4
5
6
Baseline
Post Chamber

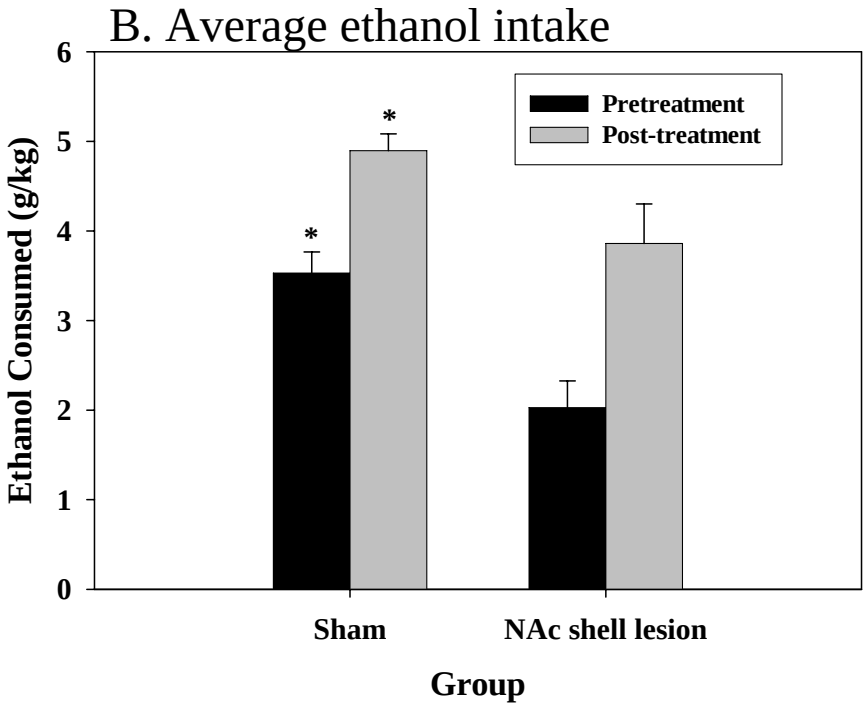
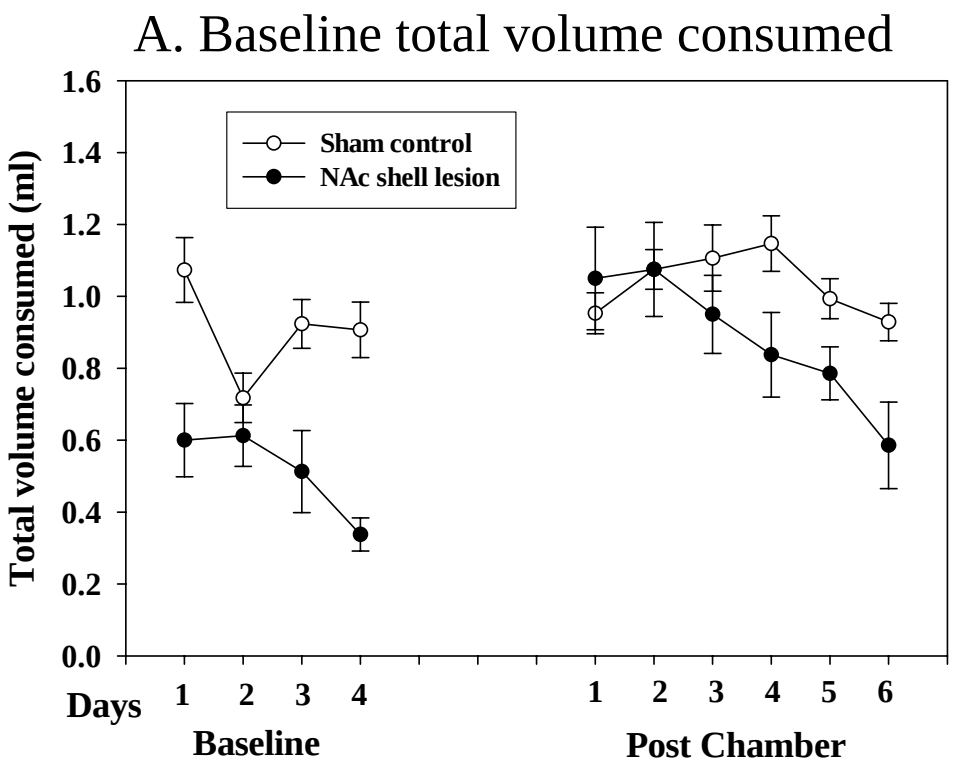

B. Average ethanol intake
Pretreatment
Post-treatment
Ethanol Consumed (g/kg)
0
1
2
3
4
5
6
*
*
Sham
NAc shell lesion
Group

Fig. 4.6. Effect of intermittent vapor exposure and withdrawal on daily ethanol intake in sham and NAc shell lesioned mice during a two-hour limited access procedure. Panel (A) indicates daily ethanol consumption prior to and following vapor treatment, and panel (B) illustrates average ethanol consumption over days three through four pre vapor treatment and three through six post vapor treatment. The dose of ethanol consumed (g/kg), bot pretreatment and post-treatment, was significantly lower in the NAc shell lesioned group (N=7) compared to the sham lesioned group (N=17). * $p<0.05$ vs respective lesion group. Values represent the mean ± SEM.


A. Baseline total volume consumed
Total volume consumed (ml)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham control
NAc shell lesion
Days
1 2 3 4
1 2 3 4 5 6
Baseline
Post Chamber

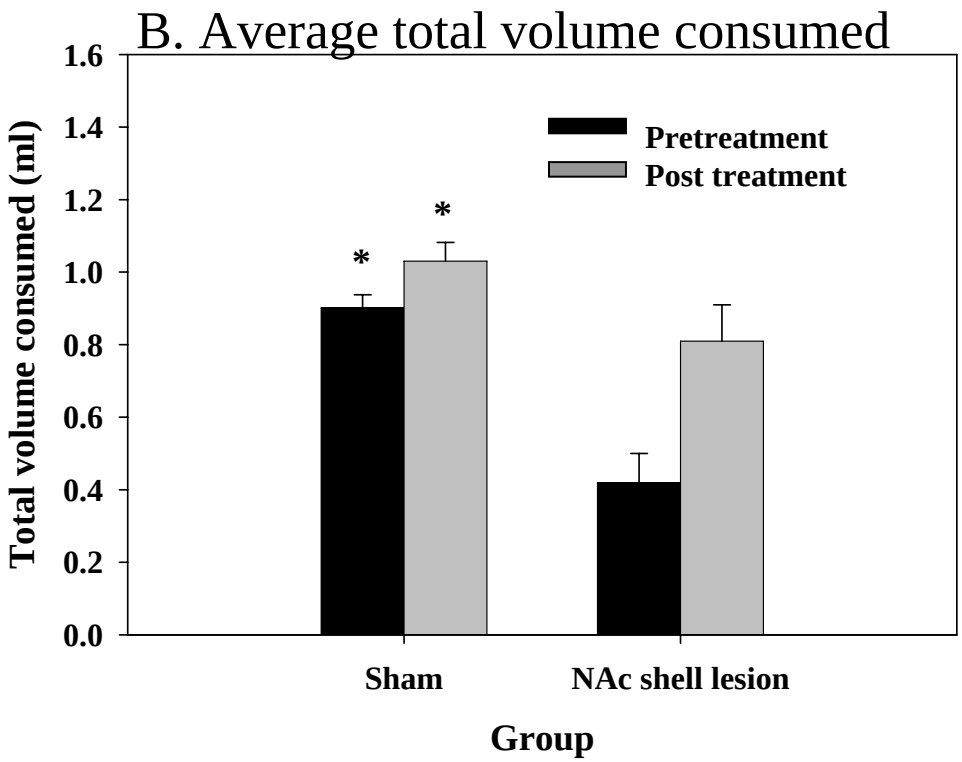
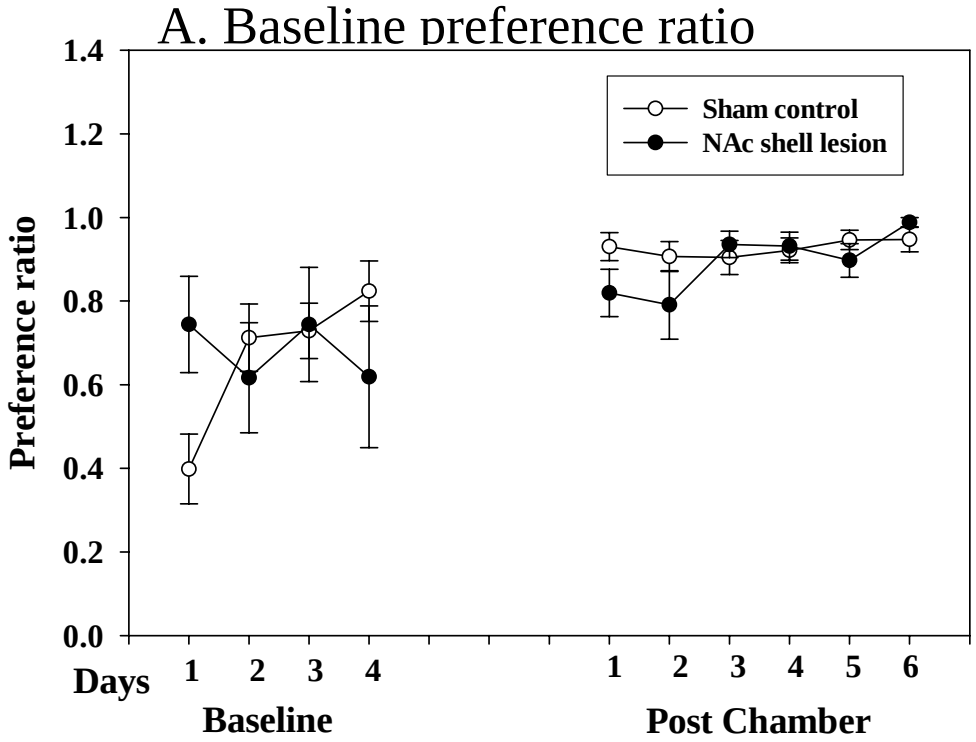

B. Average total volume consumed
Total volume consumed (ml)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pretreatment
Post treatment
*
*
Sham
NAc shell lesion
Group

Fig. 4.7. Effect of intermittent vapor exposure and withdrawal on daily total fluid volume intake in sham and NAc shell lesioned mice during a two-hour limited access procedure. Details are identical to those in the legend to Figure 4.6, except that the graph illustrates the total volume of fluid consumed (mL per 2 hours). Total volume consumed was significantly lower in the NAc shell lesioned group compared to the sham surgery control group. * $p<0.05$ versus the respective lesion group. Values represent the mean ± SEM.


A. Baseline preference ratio
Sham control
NAc shell lesion
Preference ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Days
1
2
3
4
1
2
3
4
5
6
Baseline
Post Chamber

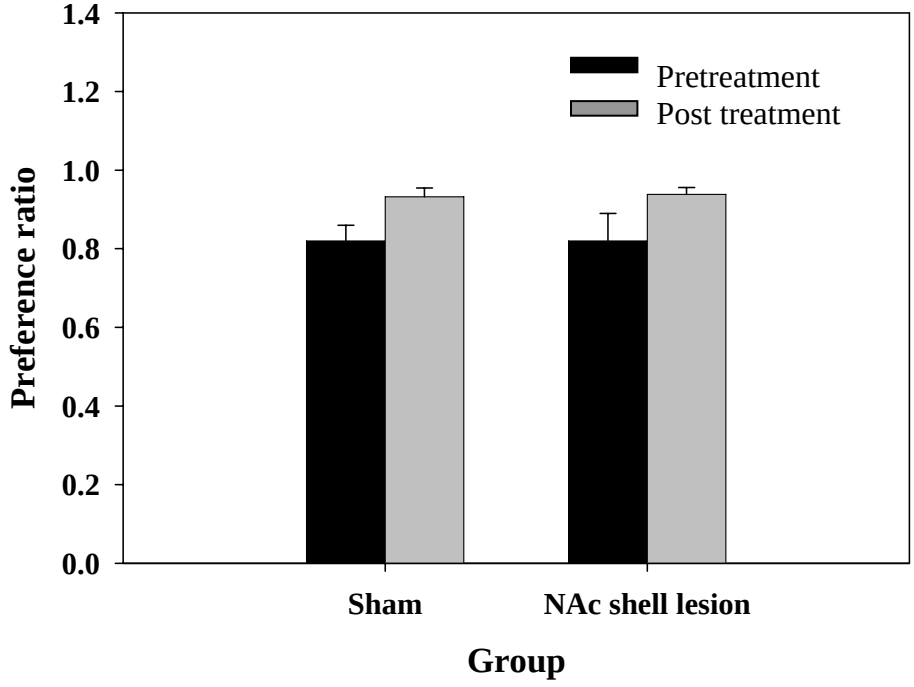

Pretreatment
Post treatment
Preference ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
NAc shell lesion
Group

Fig. 4.8. Effect of intermittent vapor exposure on ethanol preference in sham and NAc shell lesioned mice during a two-hour limited access procedure. Details are identical to those in the legend to Figure 4.6, except that the graph illustrates preference (volume of ethanol consumed/total volume consumed). Values represent the mean ± SEM.

	Air Control		Continuous Withdrawal		Intermittent Withdrawal		
	0 hour	2 hours	0 hour	2 hours	0 hour	2 hours	
Cortex			Mean ± SE				Effect
Cingulate	8.96 ± 1.93	9.31 ± 3.16	6.50 ± 0.95	2.46 ± 0.66	11.74 ± 1.99	8.44 ± 0.55	G
Piriform	7.55 ± 1.74	13.43 ± 2.33	10.93 ± 1.28	10.52 ± 4.66	10.88 ± 0.93	9.86 ± 1.48	
Motor	9.62 ± 2.33	1.82 ± 0.36*	4.33 ± 0.57	4.87 ± 1.45	6.23 ± 1.19	0.58 ± 0.098*	T,GXT
Nucleus Accumbens							
Shell	7.59 ± 1.77	10.08 ± 1.59	6.78 ± 2.07	5.54 ± 0.95	7.54 ± 1.44	15.28 ± 2.01*	G, GXT
Core	4.05 ± 0.68	3.52 ± 0.78	2.34 ± 0.71	2.17 ± 0.29	3.88 ± 0.73	7.01 ± 0.51*	G, GXT
Lateral Septum							
Dorsal	1.68 ± 0.34	1.91 ± 0.56	1.43 ± 0.17	1.35 ± 0.49	2.17 ± 0.42	2.22 ± 0.58	
Intermediate	10.88 ± 3.18	3.00 ± 0.39	6.99 ± 1.39	7.98 ± 1.65	5.52 ± 1.41	4.50 ± 0.87	
Ventral	8.18 ± 0.80	3.91 ± 0.90*	2.70 ± 0.38	4.02 ± 0.80	4.95 ± 0.89	4.78 ± 0.87	G, GXT
Bed Nucleus							
Lateral Posterior	2.22 ± 0.53	3.27 ± 0.50	1.28 ± 0.28	3.19 ± 0.57	3.50 ± 0.78	4.29 ± 1.06	T
Medial Anterior	1.38 ± 0.29	1.38 ± 0.17	0.64 ± 0.06	1.45 ± 0.34	1.36 ± 0.33	0.96 ± 0.26	
Medial Ventral	3.53 ± 0.64	2.11 ± 0.45	5.06 ± 1.08	3.82 ± 0.77	2.78 ± 0.50	2.40 ± 0.50	G
IntraAmygloid	1.80 ± 0.37	1.72 ± 0.53	0.88 ± 0.20	1.37 ± 0.30	1.46 ± 0.27	1.48 ± 0.19	
Amygdala							
Basomedial	3.65 ± 0.93	4.39 ± 0.54	5.00 ± 0.63	4.71 ± 0.86	4.23 ± 0.42	4.41 ± 0.45	
Basolateral	5.45 ± 1.13	4.92 ± 0.82	5.22 ± 0.95	9.00 ± 3.81	4.89 ± 0.74	5.20 ± 1.08	
Central	2.13 ± 0.66	4.44 ± 0.47	1.82 ± 0.56	2.95 ± 0.72	2.49 ± 0.81	2.54 ± 0.67	
Hypothalamus							
Lateral	12.44 ± 1.51	9.33 ± 1.42	15.75 ± 3.04	15.52 ± 1.70	10.41 ± 0.82	8.64 ± 1.50	G
Dorsolateral	4.83 ± 1.12	6.46 ± 1.53	2.41 ± 0.13	4.86 ± 0.98	4.15 ± 0.79	3.94 ± 0.71	
Basal Ganglia							
Sub.Nigra - compacta	1.00 ± 0.86	0.46 ± 0.38	N/A	N/A	0.75 ± 0.28	1.09 ± 0.54	
Sub.Nigra - reticulata	1.48 ± 1.19	0.91 ± 0.69	N/A	N/A	2.57 ± 1.48	3.68 ± 1.78	G
Ventral Tegmental	0.52 ± 0.34	0.29 ± 0.19	N/A	N/A	0.64 ± 0.27	0.55 ± 0.26	G
Other							
Dorsal Raphe	2.74 ± 1.10	6.44 ± 1.21	3.38 ± 0.94	3.93 ± 1.10	0.88 ± 0.21	1.10 ± 0.39	G
Periaqueductal Gray	6.75 ± 1.11	4.34 ± 0.71	10.20 ± 1.31	6.40 ± 1.13	5.93 ± 0.82	3.30 ± 0.90	G,T
Edinger-Westphal	1.75 ± 0.23	3.65 ± 0.84	1.91 ± 0.27	2.81 ± 0.41	*1.06 ± 0.22*	*2.33 ± 0.21*	T

Table 4.1 Effect of limited access ethanol consumption to alter brain activity as indicated by c-fos immunoreactivity in mice exposed to intermittent ethanol vapor, continuous ethanol vapor, or air. Mice assigned to the intermittent ethanol vapor treatment group were exposed to a series of three cycles of 16-hr of ethanol vapor, separated by 8-hr withdrawal periods. Mice assigned to the continuous ethanol exposure group were exposed to ethanol vapors for 48 hours with no intermittent withdrawal periods. Ethanol vapor exposure was adjusted to yield target BECs of 1.5 mg/ml. On the final night of the experiment, the ethanol vapor treatment and air control groups were further subdivided into mice that anticipated but did not receive and consume ethanol (0 hour) and mice that did consume ethanol (2 hour). Brains from 0 hour mice were collected one hour after presentation of the experimenter (conditioned cue for ethanol) in the drinking room. Brains from the 2-hour group were collected following the end of the two hour limited access procedure. Values represent the mean ± SEM number of Fos positive neurons per slice. Effect refers to the result from the ANOVA for group (G), treatment (T), and the G x T interaction. *$p<0.05$ vs respective 0 hr group, post-hoc tests following significant interaction with ANOVA. N/A stands for not available.

Chapter 5: Discussion

The intent of the research outlined in this dissertation was to determine basic scientific knowledge of the neural circuitry that underlies increases in alcohol consumption, so as to lead to more effective pharmacotherapies to treat alcoholism. Alcohol dependency was modeled in C57BL/6J (B6) mice, as this genotype increased ethanol consumption upon chronic intermittent exposure to ethanol vapors. This finding was demonstrated in the ethanol vapor chamber studies carried out in chapter 3, chapter 4, and the Appendix. Lesion and c-fos immunoreactivity studies were carried out to determine the brain regions involved in this increase in ethanol consumption. The research has focused on the central extended amygdala (cEA) as a brain region of interest, since this intermittent vapor-induced increase in ethanol consumption was blocked from occurring with site-specific pharmacological manipulations to this region (Roberts et al., 1996; Funk et al., 2006; Finn et al., 2007) in both rats and mice. These same pharmacological manipulations had no effect on ethanol consumption in animals not exposed to ethanol vapors.

Results from chapter 2 demonstrated that the lateral posterior portion of the bed nucleus of the stria terminalis (BNSTLP), a major nucleus of the cEA, is involved in the homeostatic set point for ethanol consumption in a limited access, but not a free access procedure. This conclusion was based on the finding that lesions of the BNSTLP significantly decreased ethanol consumption only in animals on a schedule of limited access to ethanol. Chapter 3 and chapter 4 confirmed and extended this result by demonstrating that the BNSTLP as well as the central nucleus of the amygdala (CeA) and

the nucleus accumbens shell (NAc shell), two additional major nuclei of the cEA, are involved in baseline ethanol consumption in a limited access procedure (Table 5.1). Since lesions of these nuclei did not significantly alter ethanol intake following intermittent vapor exposure, they may not be necessary to see a chronic intermittent ethanol vapor induced increase in ethanol consumption. Results from chapter 4 indicated that the NAc shell is the main area of the cEA that adapts and activates, as indicated by the presence of c-fos immunoreactivity, in response to chronic intermittent ethanol vapor exposure.

The hypothesis of the lesion and c-fos immunoreactivity experiments carried out in this dissertation was that the cEA was not involved in the baseline ethanol consumption seen in our non-dependent air exposed mice, but that it would be involved in the increase in ethanol consumption seen in our dependent ethanol vapor exposed mice. In general, the overall findings were the opposite of our initial prediction. The results suggest that the cEA was necessary for maintaining baseline ethanol consumption and that neural activity in discrete cEA nuclei (NAc shell) had changed in response to ethanol dependency. However, as a whole, the three major nuclei of the cEA did not appear to be necessary for the increase in ethanol consumption seen upon chronic intermittent exposure to ethanol.

Chapter 2 demonstrated that the BNST was involved in ethanol and total fluid consumption during a limited access, but not a free access procedure. The main differences between free access and limited access ethanol consumption pointed out in chapter 2 were that: (1) Blood ethanol concentration (BEC) was higher per unit time when access to ethanol was limited, as opposed to when access was free, and (2)

Contextual and temporal cues could influence limited access ethanol intake. In other words, if ethanol consumption was partially driven by the contextual and temporal cues that had been associated with limited access to ethanol, then lesions of the BNSTLP could decrease ethanol consumption by blocking the association of these context and temporal cues with limited access to ethanol.

Other possible reasons for the involvement of the BNSTLP in limited access versus free access ethanol consumption are worth noting. First, the lesion size was smaller on average in the mice that went through the free access procedure than in the mice that went through the limited access procedure. On a scale of one to five, lesion size in the mice in the limited access procedure was a four versus a three in the mice in the free access procedure. Second, the mice in the free access procedure were given a longer time to recover from surgery and a longer time isolate housed before being placed in the ethanol consumption procedure than the mice in the limited access procedure (Surgery: 9 days vs 2 days; Isolate Housing: 9 days vs 4 days, respectively). Finally, mice in the free access procedure were given three different concentrations of ethanol while mice in the limited access procedure were given only one concentration.

It is unlikely that BNSTLP lesion size contributed to the differential effect on limited access versus free access ethanol consumption. While the lesions in the free access procedure did not affect ethanol consumption, they decreased the acoustic startle response, indicating that the lesions were sufficient to have an effect on a BNSTLP-dependent behavior. Additionally, there was no correlation between lesion size and amount of ethanol consumed, suggesting that the lack of effect of lesion on ethanol consumption in the free access procedure was not due to an inadequate lesion size.

It is unlikely that the difference in recovery time from surgery between the free access and limited access procedures was responsible for the difference in the ability of the BNSTLP lesion to affect one ethanol consumption behavior and not the other. In the limited access procedure, where recovery time was shorter, there was no difference in ethanol consumption levels between mice that received sham surgery and mice that received no surgery at all (data not shown). This indicates that recovery time from surgery was adequate and would argue that differences in recovery time did not impact ethanol intake. With respect to the effect of isolate housing time on ethanol consumption, it does not seem plausible that the five additional days of isolate housing in the free access procedure contributed to the lack of effect of lesion on ethanol intake, since the effect of the lesion to decrease ethanol consumption in the limited access procedure did not change over the time that the animals were isolate housed. This indicates that the lack of effect of lesion in a free access procedure was not due to a longer time isolate housed.

Chapter 3 and chapter 4 demonstrated that the three major nuclei of the cEA (BNSTLP, CeA, and NAc shell) are involved in baseline ethanol consumption in a limited access procedure, but that they are not necessary to see an increase in ethanol consumption that occurred upon chronic exposure to intermittent ethanol vapors. This complements findings in other laboratories that show that lesions to and pharmacological manipulations of the region decreased self-administration for and consumption of ethanol (Moller et al., 1997; Hyytia et al., 1995; Foster et al., 2004; Eiler et al., 2003; Heyser et al. 1999)

The finding that lesions of the CeA do not block the intermittent ethanol vapor-induced increase in ethanol consumption is most interesting, when considering the

findings from all the pharmacological manipulations to this region that show a blockade in this ethanol dependency-induced change in ethanol intake. For example, GABA agonists (Roberts et al., 1996) and CRF antagonists (Funk et al., 2006) microinjected into the CeA block the ethanol vapor induced increase in self-administration for ethanol in rats, and microinjection of a CRF antagonist into the CeA of B6 mice blocked the ethanol vapor induced increase in ethanol consumption in a limited access procedure (Finn et al., 2007). Unlike the lesion studies, where only baseline ethanol consumption was affected, the pharmacological manipulations of the GABA and CRF systems had no effect on baseline ethanol intake, but did block the ethanol vapor-induced change in ethanol intake. One can conclude from the collection of background pharmacological and lesion studies that the cEA is involved in changes in ethanol consumption, as indicated by blockade of this change with pharmacological manipulations, but that it is not necessary to see this change, as indicated by the lack of effect of these lesions to block increased ethanol consumption.

Some possible reasons for the lack of an effect of lesions of nuclei of the cEA to block the intermittent ethanol vapor-induced increase in ethanol consumption are that 1) There are redundant circuits for the withdrawal effect and/or 2) In the absence of one nucleus of the cEA, the other nuclei may take on the role of engendering the withdrawal effect.

In chapter 4, c-fos immunoreactivity studies were carried out to determine the neural circuits that activate in response to heightened levels of ethanol consumption seen in our ethanol dependent mice. The results indicate that in the intermittently ethanol vapor exposed mice (the mice that drink the most), ethanol consumption increased c-fos

immunoreactivity in the NAc shell and NAc core. While lesions of the NAc shell indicated that the region was not necessary to see an ethanol vapor induced increase in ethanol consumption, the NAc core was not lesioned. It is possible that both the NAc shell and the NAc core must be lesioned to block the intermittent ethanol vapor-induced increase in ethanol consumption.

In our study, c-fos immunoreactivity was measured in mice with ethanol consumption experience that were anticipating ethanol administration upon the presence of the experimenter. The brains were collected one hour after the mice were exposed to the experimenter to determine the brain regions involved in anticipation to ethanol. Unfortunately, there was no group of mice that did not have experience with ethanol. Thus, the difference between brain activity of mice that anticipated ethanol but did not drink ethanol with mice that had no experience with ethanol could not be determined.

Ryabinin and colleagues have helped to map brain regions that differ between B6 mice that consume ethanol during a thirty-minute limited access procedure with mice that did not consume ethanol and that had no ethanol consuming experience. The results indicated that ethanol consumption produced a higher expression of c-fos immunoreactivity in the edinger-westphal (EW), the medioposteroventral portion of the CeA, and the NAc core, when compared to the mice with no ethanol consuming experience. (Bachtell et al., 1999). Other studies in the same lab have shown that the EW is the only nucleus that activates in response to ethanol consumption (Sharpe et al., 2005, Weitemier et al., 2001; Ryabinin et al., 2003). While the reason for the discrepancy between the results is not clear, the authors of Sharpe et al (2005) postulate that activation

of the CeA might be due to an unexpected increase in alcohol intake by the mice during the last drinking session in the study by Bachtell et al (1999).

In our study, consistent with the study by Sharpe et al (2005), ethanol consumption also did not increase c-fos immunoreactivity in the CeA. Also consistent with all the studies carried out by Ryabinin and colleagues, ethanol consumption increased c-fos immunoreactivity in the EW. An interesting finding in our study, which was not present in the studies by Ryabinin and colleagues, is that ethanol consumption induced c-fos immunoreactivity in the BNSTLP. The reason for the difference in results may well be due to the fact that in our studies c-fos immunoreactivity as being compared between mice that have experience with ethanol, as opposed to the studies by Ryabinin and colleagues where comparisons were being made between mice with ethanol experience versus no experience with ethanol.

Overall, the results from chapters 2, 3, and 4 suggest that while the components of the cEA are involved in baseline ethanol consumption, and are responsive to changes in ethanol consumption (as was the case with the NAc shell), they are not necessary to see the ethanol vapor-induced increase in ethanol consumption. This is an important finding considering that the cEA is highly postulated to be involved in alcohol dependence (Funk et al., 2006; Finn et al., 2007 Roberts et al., 2000; Koob, 2003ab, Roberts et al., 1996). Taken in conjunction with the numerous pharmacological studies that have shown that manipulations made to the CeA block the intermittent ethanol vapor-induced increase in lever pressing for ethanol in rats (Roberts et al., 1996; Funk et al., 2006) and increase in ethanol consumption in B6 mice (Finn et al., 2007), it is counterintuitive that a lesion of the CeA would not block this increase in ethanol consumption. Nonetheless, these results

have implications for understanding the neural circuitry involved in excessive alcohol consumption, a common characteristic of alcohol dependency, and may help to alleviate symptoms of problem drinking.

Lesion	Baseline Ethanol Consumption	Post intermittent ethanol vapor Ethanol Consumption
Sham	No change	Increased
CeA	Decreased	Increased
BNSTLP	Decreased	Increased
NAc shell	Decreased	Increased

Table 5.1 Effect of lesion and withdrawal from intermittent ethanol vapor exposure on ethanol consumption

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Appendix

The ethanol vapor chamber study reported in this Appendix was carried out to determine the impact of varying blood ethanol concentrations (BECs) during intermittent ethanol vapor exposure on subsequent limited access ethanol intake in male C57BL/6J (B6) mice. Separate groups of B6 mice were exposed to ethanol vapor concentrations that were adjusted to yield target BECs of 1.25 or 2.0 mg/ml

Housing of subjects upon arrival and home cage drinking are as described in Chapter 3 and 4. Briefly, mice were 7-9 weeks of age upon arrival, housed 4 per cage and given one week to acclimate before isolate housing, and given food and water ad libitum. Mice were given two days of isolate housing before the limited access ethanol consumption procedure was begun. During this procedure, one water bottle was replaced by one ethanol bottle at three hours after lights off for a two-hour period. The ethanol concentration was 15% v/v. Ethanol consumption was measured over a period of five (post-inhalation) to seven (baseline) days.

Intermittent exposure to ethanol vapors

Mice in the intermittent ethanol vapor group were exposed to a series of three cycles of 16-hr of ethanol vapor separated by 8-hr withdrawal periods. Ethanol vapor exposure was in inhalation chambers and was adjusted to yield target blood ethanol concentrations (BECs). An air control group received the same treatment as the ethanol vapor exposed groups, but with air, rather than ethanol vapor. BEC analysis is as described in Chapters 3 and 4.

Following three cycles in the vapor chambers, mice were re-housed in their initial cages and 2-hr limited access ethanol drinking measurements were resumed over a period of five days.

Data Analysis

For the drinking study, the dependent variables were g/kg of ethanol consumed, total volume consumed (i.e. ethanol plus water), and preference ratio. Preference ratios were calculated by dividing the volume of ethanol consumed by the total volume consumed. Since we were predicting that there would be a significant effect of ethanol vapor exposure on ethanol intake, planned comparisons were conducted in the absence of a significant interaction between main effects. When appropriate, post hoc comparisons were used to determine significant effects of ethanol vapor exposure and days. Significance was set at $p \leq 0.05$.

An effect of ethanol vapor exposure that achieves BEC levels of 1.25 and 2.0 mg/ml on ethanol consumption levels in a limited access procedure.

As depicted in Figure A.1A, baseline ethanol intake did not differ between the ethanol vapor and air control groups. Following vapor treatment, the repeated measures ANOVA conducted on days three through five (since these are the days when a vapor effect is normally seen; See Chapter 3), indicated no effect of group or days and no group x day interaction.

As depicted in Figure A.1B, ethanol intake averaged over the third through fifth days post-treatment was 3.5 ± 0.33 g/kg for the 2.0 mg/ml ethanol vapor group, 3.8 ±

0.28 g/kg for the 1.25 mg/ml ethanol vapor group, and 2.7 ± 0.23 g/kg for the air control group. A one-way ANOVA indicated that these group differences in averaged ethanol intake were significantly different [F (2,29) = 5.0, p = 0.01].

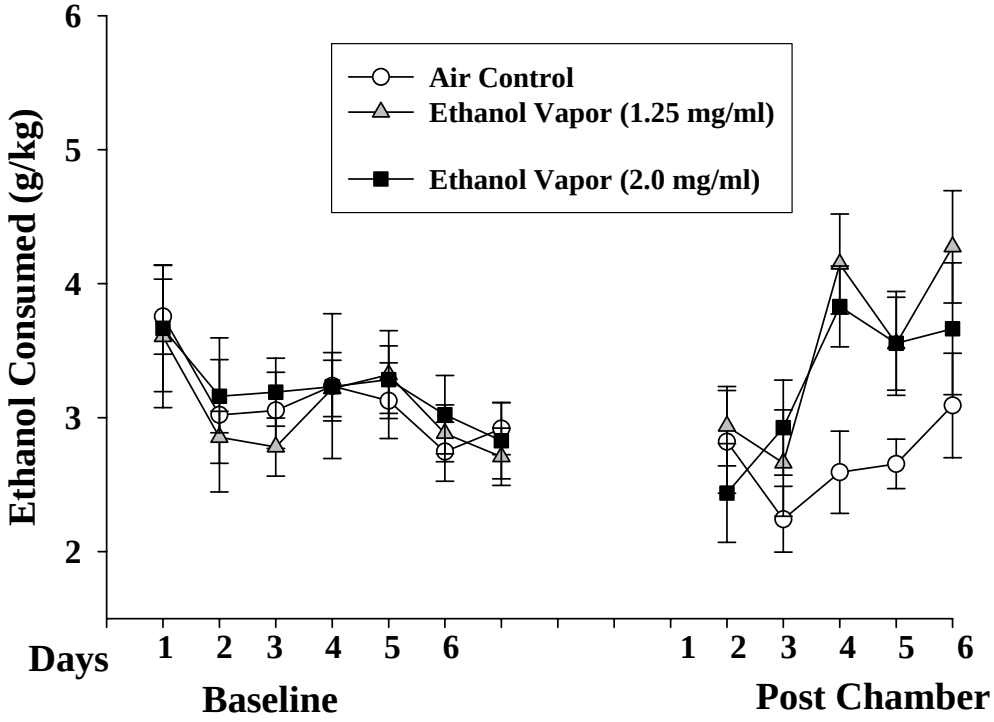
Average total fluid consumption (not shown) before ethanol vapor treatment was 0.94 ± 0.05 ml for the 2.0 mg/ml ethanol vapor group, 1.05 ± 0.05 ml for the 1.25 mg/ml ethanol vapor group, and 0.97 ± 0.06 ml for the air control group. Repeated measures ANOVA indicated no significant effect of group, a significant effect of days [F (2,93) = 2.54, p = 0.02], and no group x days interaction. Following ethanol vapor treatment average total fluid consumed over the third through fifth days was 0.90 ± 0.07 ml for the 2.0 mg/ml ethanol vapor group, 0.93 ± 0.08 ml for the 1.25 mg/ml ethanol vapor group, and 0.75 ± 0.09 for the air control group. Repeated measures ANOVA indicated no effect of group or days and no interaction between the two.

With regard to ethanol preference (not shown), average baseline preference ratio was 72 ± 6% for the 1.25 mg/ml ethanol vapor group, and 80 ± 2% for the 2 mg/ml ethanol vapor group and 75 ± 6% for the air control group. Repeated measures ANOVA indicated no effect of group, an effect of days [F (2,93) = 4.18, p = 0.0005], and no interaction between group and days. After ethanol vapor treatment, mean ± SEM ethanol preference ratio (from the third to fifth days post-treatment) was 89 ± 7 % for the 2.0 mg/ml ethanol vapor group, 95 ± 6% for the 1.25 mg/ml ethanol vapor group, and 87 ± 7% for the air control group. There was no effect of group or days, and no interaction between the two.

Conclusion

The overall goal of this study was to determine the range of effective BECs during ethanol vapor exposure for increasing ethanol intake post inhalation. In our experience, BECs below 1.0 mg/ml were marginally effective, whereas BECs that markedly exceeded 1.0 mg/ml tended to decrease ethanol intake (Dhaher & Finn, unpublished). While optimizing the method for the intermittent vapor-induced increase in limited access ethanol consumption, our preliminary data suggested that a target BEC of 1.5 mg/ml would be best. However, prior to conducting the studies described in chapters 3 & 4, the present study was conducted to examine the effect of 2 different BEC levels on subsequent ethanol intake. The two target BECs were chosen to be at the extremes of our presumed "optimal range" of BEC levels that would produce the subsequent increase in ethanol intake. Since the results indicated that the 1.25 and 2.0 mg/ml BEC groups produced a similar increase in ethanol intake, we concluded that our target BEC of 1.5 mg/ml (Chapters 3 & 4) was appropriate. Importantly these findings (ethanol intake, total fluid intake, and preference ratio) are comparable to those in the non-lesioned mice described in chapters 3 & 4.

A. Daily Ethanol Intake.



B. Average Ethanol Intake.

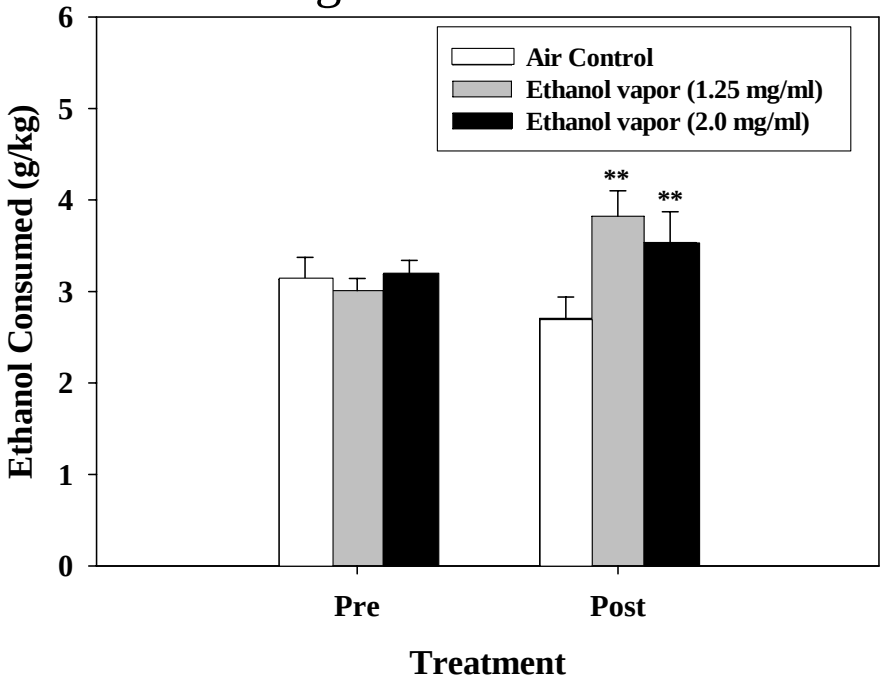


Fig. A.1. An effect of ethanol vapor exposure that achieved BEC levels of 1.25 and 2.0 mg/ml with intermittent withdrawal on ethanol consumption levels in a two hour limited access procedure. Panel (A) shows daily ethanol intake prior to and following ethanol vapor treatment and Panel (B) shows ethanol intake averaged over days 3-5, before and after treatment. Mice were given a choice between one bottle containing tap water and one bottle with a 15% ethanol solution at three to four hours into their dark cycle for a period of two hours. Prior to ethanol vapor treatment, there was no difference in baseline ethanol intake between the 1.25 mg/ml ethanol vapor group (n = 9), the 2.0 mg/ml ethanol vapor group (n = 11), and the air control group (n = 12). Following intermittent ethanol vapor treatment and withdrawal, the ethanol vapor groups showed a higher level of ethanol consumption than the air control group. Values represent the mean ± SEM. ** $p \leq 0.01$ versus the respective air control group.