CHARACTERIZATION OF THE EDINGER-WESTPHAL RESPONSE TO ETHANOL

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ABSTRACT

The study of the neurobiology of alcoholism has primarily focused on the mesocorticolimbic dopamine system, the extended amygdala and associated peptide systems. Additional systems contributing to the development of alcoholism are likely to exist. Recent studies using inducible transcription factors as a tool for brain mapping suggest that the Edinger-Westphal nucleus may represent a novel site of action for ethanol in the brain. The aim of this dissertation was to characterize the Edinger-Westphal nucleus and its sensitivity to ethanol, which was accomplished using several strategies.

First, the mechanisms of ethanol-induced c-Fos in the Edinger-Westphal nucleus were analyzed using several agonists and antagonists of neurotransmitter systems thought to be involved in regulating the activity of the Edinger-Westphal nucleus. The results of these studies demonstrated that the Edinger-Westphal nucleus is influenced by a complex set of neurotransmitter systems including the γ-aminobutyric acid, noradrenaline, dopamine and opioid systems. In particular, the γ-aminobutyric acid system was both necessary and sufficient for ethanol-induced c-Fos expression, while the noradrenaline and dopamine systems were necessary, but not sufficient for ethanol-induced c-Fos expression. The opioid system was unique in that agonists of opioid receptors significantly increased c-Fos expression, but had no effect on ethanol-induced c-Fos expression suggesting a separate mechanism. In addition to the pharmacological mechanisms, the signal transduction mechanisms were analyzed. The results of these experiments revealed that signal transducer and

activator of transcription-3 and extracellular regulated kinase 1/2 were elevated 15 min following ethanol administration compared with saline-treated animals. In addition, a pharmacological manipulation of MEK resulted in complete blockade of ethanol-induced c-Fos expression in the Edinger-Westphal nucleus. These results suggest that a unique signaling pathway exists in Edinger-Westphal nucleus neurons.

The ethanol responsiveness of the Edinger-Westphal nucleus was also characterized on the cellular level by analyzing the cell types that express c-Fos upon ethanol administration. In addition to this, genetic relationships were established to identify potential responses that are mediated through the Edinger-Westphal nucleus during ethanol intoxication. The results showed that ethanol-sensitive neurons in the Edinger-Westphal nucleus contain the neuropeptide urocortin. A comparison between the C57BL/6J and DBA/2J mouse strains revealed a marked difference in Urocortin expression in the Edinger-Westphal nucleus. Due to the urocortin differences and the dramatic behavioral differences between these two inbred strains, an F2 intercross of these strains was created and analyzed using correlational analyses between urocortin expression in the Edinger-Westphal and ethanol-mediated responses. The results revealed that Urocortin expression in the Edinger-Westphal nucleus correlated with ethanol-induced hypothermia as well as ethanol consumption.

The final portion of this dissertation utilized an electrolytic lesion strategy to extend the validity of the behavioral correlations observed in the F2 cross. Thus, ethanol responses were measured following electrolytic lesions of the

Edinger-Westphal nucleus in male C57BL/6J mice. Both lesioned and sham-operated animals were tested for several ethanol sensitivity measures and ethanol consumption in a 2-bottle choice test. The results showed that lesions of the Edinger-Westphal nucleus significantly disrupted ethanol-induced hypothermia, while having no effect on pupil dilation, locomotor activity or ethanol-induced sedation. In addition, lesioned animals showed significantly lower ethanol preference and total ethanol dose consumed in the 2-bottle choice test. These data support the genetic correlations between Edinger-Westphal nucleus urocortin expression and both ethanol-induced hypothermia and ethanol consumption that were observed in the F2 cross. Overall, this dissertation presents a sequence of experiments describing pharmacological and intracellular mechanisms that influence urocortin neurons in the Edinger-Westphal nucleus during ethanol exposure. Further experimentation identified the relationship between urocortin cells in the Edinger-Westphal nucleus and both hypothermic responses to ethanol and willingness to consume ethanol. The results of these experiments were strengthened using an electrolytic lesion strategy, which revealed a similar relationship.

GENERAL INTRODUCTION

Drug and alcohol abuse are complex disorders that can be characterized by changes occurring in distinct neural systems. Within these neural systems, abused substances alter specific cellular and molecular targets over the course of the disorder (White, 2002). These changes may be reflected in the many behaviors that coincide with substance abuse including uncontrollable drug intake, and the development of tolerance, sensitization, dependence, and withdrawal syndromes.

The study of the neurobiology of drug abuse has primarily revolved around the study of reward circuitry. Interest in this area began nearly 50 years ago when Olds and Milner (Olds and Milner, 1954) discovered that animals vigorously respond for intracranial self-stimulation delivered to the septal region. This led to the identification of an endogenous motivational circuit involving the mesocorticolimbic system and the neurotransmitter dopamine that were deemed important for initiating and directing various motivated behaviors including food reward, sexual reward, and drug reward. The study of the neurobiology of drug abuse, and alcoholism in particular, has since evolved from an approach focused on the mesocorticolimbic dopamine system to one that integrates many different brain regions, neurotransmitters, and neuropeptides. These different systems and the complex interaction between them have become a major focus in neurobiological studies of drug abuse disorders.

The importance of identifying and characterizing novel drug-related neurocircuitry is paramount to the understanding of the neurobiology of drug

abuse. The following paragraphs briefly review the literature associated with traditional views of the neurobiology of drugs of abuse, which becomes focused on the neurobiology of alcoholism and the techniques associated with identifying ethanol-sensitive targets. The discussion concludes with a description of the Edinger-Westphal nucleus, which is thought to be a novel neural target sensitive to ethanol administration and the primary topic of this dissertation.

Neurobiology of Drug Abuse: A common neural system

Drugs of abuse are thought to target several neural systems, and while some appear to be drug-specific, the mesocorticolimbic dopamine system is the most common neural target for all drugs of abuse. This system is considered the major substrate mediating the acute rewarding and reinforcing actions of most drugs. Anatomically, the mesocorticolimbic system originates in the ventral tegmental area (VTA), which is one of two primary sources of dopamine in the midbrain. Neurons from the VTA project to terminal regions in the nucleus accumbens and prefrontal cortex. In the absence of drug administration, this system acts to gate, modulate and filter signals arising from limbic regions. It is through this system that an organism is thought to control basic biological drives and motivational variables, which are artificially tapped into upon administration of abused drugs (Koob, 1992).

Psychostimulants such as cocaine and d-amphetamine directly impact the mesocorticolimbic dopamine system by either inhibiting reuptake of dopamine through the dopamine transporter or by promoting reverse transport of dopamine,

respectively. Through these actions, self-administered cocaine elevates extracellular dopamine in the nucleus accumbens as measured by in vivo microdialysis (Pettit and Justice, 1989). The selective elimination of mesocorticolimbic dopamine neurons in the VTA or dopamine fibers in the nucleus accumbens using the neurotoxin 6-hydroxydopamine causes a severe disruption in cocaine self-administration behavior (Roberts et al., 1980). In addition, animals will self-administer dopamine, amphetamine, and the selective dopamine reuptake inhibitor, nomifensine directly into the nucleus accumbens (Hoebel et al., 1983; Dworkin et al., 1986; Carlezon et al., 1995). Furthermore, animals learn to approach environments previously paired with microinjections of amphetamine into the nucleus accumbens, which is indicative of a rewarding experience (Carr and White, 1983). Psychostimulants also influence the cortical component of the mesocorticolimbic dopamine system. This is evidenced by animals that lever-press for microinjections of cocaine directly into the medial prefrontal cortex (Goeders and Smith, 1993). In addition, cocaine injections into the medial prefrontal cortex increases dopamine turnover in the nucleus accumbens (Goeders and Smith, 1993).

Like psychostimulants, opiate drugs also appear to influence the mesocorticolimbic dopamine system. While opiate drugs possess the ability to increase the release of dopamine in the nucleus accumbens, it appears that the reinforcing effects of opiates may occur independently of the rise in nucleus accumbens dopamine (Pontieri et al., 1995). Thus, Pettit and colleagues (1984) showed that selective disruption of dopamine fibers in the nucleus accumbens

had no effect on responding for heroine. Based on these findings, it has been proposed that opiate reward is driven by a dual mechanism involving direct interactions with opioid receptors in both the VTA and nucleus accumbens (Wise, 1998). This hypothesis is supported by data showing that opiate drugs are self-administered directly into the VTA, which creates enhanced dopamine output to the nucleus accumbens (Bozarth and Wise, 1981; Devine et al., 1993). These effects are thought to result from the disinhibition of VTA dopamine neurons. The VTA dopamine neurons are tonically inhibited by γ-aminobutyric acid (GABA) neurons expressing μ-opioid receptors. Activation of these receptors releases the inhibition on VTA dopamine neurons thus causing an increase in dopamine output to the nucleus accumbens (Johnson and North, 1992). Likewise, opiate drugs also act on GABAergic neurons expressing μ-opioid receptors in the nucleus accumbens (Jiang and North, 1992; Gracy et al., 1997). Thereby, opiate injections into the nucleus accumbens also produce reward-related behavior (van der Kooy et al., 1982).

Phencyclidine (PCP) is another abused substance that also taps into the mesocorticolimbic dopamine system. PCP, which acts by antagonizing the glutamate *N*-methyl-D-aspartate (NMDA) receptor, is capable of increasing extracellular dopamine in the nucleus accumbens when administered systemically (Carboni et al., 1996). In operant paradigms, animals will lever press for microinjections of PCP, and other NMDA antagonists (e.g. MK-801 and CPP), into the primary dopamine projection regions, the medial prefrontal cortex and nucleus accumbens (Carlezon and Wise, 1996). PCP appears to act locally in

each of these regions in that dopamine increases in nucleus accumbens are noted when PCP is administered locally to either the medial prefrontal cortex or nucleus accumbens (Hernandez et al., 1988; Hondo et al., 1994). The influence of MK-801, another NMDA antagonist, on the mesocorticolimbic dopamine release is less clear. It was reported that systemic and intra-VTA administration of MK-801 were not sufficient to increase nucleus accumbens dopamine levels (Druhan et al., 1996; Westerink et al., 1996), however, a subsequent report demonstrated that intra-accumbens administration and systemic administration produced elevated dopamine levels in the nucleus accumbens (Mathe et al., 1999).

Another widely abused substance, nicotine, also influences the mesocorticolimbic dopamine system, which is evidenced by increases in dopamine release in the nucleus accumbens (Imperato et al., 1986). This increase is thought to occur through nicotine acting at presynaptic nicotinic acetylcholine receptors in the nucleus accumbens (Westfall et al., 1983). Further evidence shows that self-administration of intravenous nicotine is blocked by administration of dopamine antagonists and dopamine-selective disruptions of the nucleus accumbens (Corrigall et al., 1992; Dani and Heinemann, 1996).

Neurobiology of Alcoholism

The sedative-hypnotics are another class of drugs with high abuse potential. This class of drugs includes benzodiazepines, barbiturates, and ethanol. While this class of drugs recruits a number of neurobiological systems

for its behavioral effects, there are several clear links to the mesocorticolimbic dopamine system for acute ethanol actions. First, ethanol increases VTA dopamine neuron firing (Gessa et al., 1985; Brodie et al., 1990; Brodie et al., 1999). Animals will also self-administer ethanol into the VTA suggesting that ethanol has direct actions in the VTA (Gatto et al., 1994). Third, consumption and systemic injection of ethanol increases extracellular concentrations of dopamine in the nucleus accumbens (Di Chiara and Imperato, 1988; Blanchard et al., 1993). Finally, microinjections of dopamine receptor antagonists injected into the nucleus accumbens and VTA disrupt ethanol-reinforced responding (Nowak et al., 2000; Czachowski et al., 2001).

Adaptive changes in dopamine signaling also occur during chronic ethanol exposure such that the mesocorticolimbic system falls into a hypoactive dopamine state. For example, activity of dopaminergic VTA neurons is decreased following withdrawal of chronic ethanol (Diana et al., 1992; Shen and Chiodo, 1993). Likewise, extracellular dopamine levels in the nucleus accumbens are also diminished following chronic exposure to ethanol (Rossetti et al., 1992; Weiss et al., 1996). The dopamine hypoactivity resulting from chronic ethanol exposure likely results from both enhanced synaptic dopamine clearance, which is mediated by increased dopamine transporter levels, and decreased dopamine production resulting from decreased expression of tyrosine hydroxylase, the primary biosynthetic enzyme in dopamine production (Rothblat et al., 2001). This hypoactive state may also result from adaptive changes occurring in other systems that modulate the mesocorticolimbic dopamine system such as the

glutamatergic and GABAergic systems. Regardless of the mechanisms, the decreased dopaminergic functioning likely contributes to the withdrawal-related anhedonia and perhaps, the motivation to resume ethanol consumption (Schulteis et al., 1995; Roberts et al., 2000).

While these studies show that the mesocorticolimbic dopamine system is associated with ethanol reinforcement, other evidence calls this notion into question. For example, depletion of dopamine inputs in the nucleus accumbens with 6-hydroxydopamine fails to affect voluntary responding for ethanol (Ikemoto et al., 1997). In addition, ethanol self-administration is unaltered by elevations in nucleus accumbens dopamine caused by GBR12909, a selective dopamine reuptake inhibitor (Engleman et al., 2000). These data suggest that ethanol action may occur through interacting systems, which extends beyond the mesocorticolimbic dopamine system and includes GABA, serotonin, glutamate, and various neuropeptide systems (Koob et al., 1998).

One of the most sensitive sites for the acute and chronic actions of ethanol, and other sedative-hypnotics, is the $GABA_A$ receptor. Ethanol has been shown to interact with the $GABA_A$ receptor to augment GABA-activated chloride influx (Suzdak et al., 1986). Through this action, $GABA_A$ receptors have been implicated in the acute behavioral actions of ethanol, the development of tolerance and dependence, and ethanol reinforcement (Grobin et al., 1998). Thereby, antagonists at $GABA_A$ receptors are capable of reducing or reversing many behavioral effects of ethanol (Liljequist and Engel, 1982; Samson and Harris, 1992). The most notable GABAergic compound used for the alleviation of

ethanol-mediated behaviors is RO 15-4513. This compound is a benzodiazepine inverse agonist that acts at the benzodiazepine site to inhibit GABA-activated chloride channels (Turner et al., 1991). Administration of RO 15-4513 reverses some behavioral effects of ethanol, and dose-dependently reduces oral ethanol self-administration in rats (Samson and Harris, 1992).

The use of microinjections of GABA antagonists has helped in the identification of the extended amygdala as a set of interconnected neural substrates involved in ethanol self-administration (Hyytia and Koob, 1995). The extended amygdala includes such forebrain areas as the nucleus accumbens shell, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and the sublenticular substantia innominata (Alheid and Heimer, 1988). Structures within the extended amygdala are noted as having similarities in both cellular morphology and neural connectivity (Alheid and Heimer, 1988). The identification of this system has also brought several other neurotransmitter and neuropeptide systems into light.

The central nucleus of the amygdala (CeA) is a major component of the extended amygdala, which when lesioned reduces voluntary ethanol consumption (Moller et al., 1997). The CeA is also influenced by several neurotransmitter systems that can influence ethanol self-administration. For example, the CeA receives dopaminergic input from the VTA (Asan, 1998). Extracellular levels of dopamine are dose-dependently elevated following an acute injection of ethanol suggesting that the CeA is intricately tied to the activation of the mesocorticolimbic dopamine system (Yoshimoto et al., 2000). In

addition, opioid peptides in the CeA have also been implicated in the consumption of ethanol. Heyser and colleagues (1999) demonstrated that microinjections of opioid antagonists were most effective in reducing ethanol self-administration in the CeA over the nucleus accumbens. The CeA is also known to contain serotonin input (Li et al., 1990). Like dopamine, a dose-dependent increase in serotonin was observed following an injection of ethanol (Yoshimoto et al., 2000). Manipulation of this system by microinjecting serotonin 5-HT_3 receptor antagonists into the amygdala reduces ethanol drinking during a 2-hour session (Dyr and Kostowski, 1995). These data combined with the evidence that microinjections of the $GABA_A$ antagonist, SR 95531, significantly decreased ethanol reinforced responding suggest that the CeA is a major component of the ethanol self-administration neurocircuitry (Hyytia and Koob, 1995).

Until now, this discussion has revolved around the neuroanatomy associated with the positive aspects of ethanol that influence self-administration. The development of alcoholism, however, is also characterized by negative withdrawal symptoms that contribute to persistent alcohol use. The alleviation of these negative aspects is referred to as negative reinforcement and has been suggested to involve separate neural systems (Koob et al., 1998). The study of these systems has only recently begun. One system that has been relatively well characterized with regard to these negative affects is the corticotropin-releasing factor (CRF) system. This system is primarily associated with hormonal responses to stress, which are initiated by actions of CRF in the hypothalamic pituitary adrenal axis. The actions of CRF, however, are more widespread in the

brain and are known to influence other behavior responses such as anxiety, food intake, and sexual behavior (Dautzenberg and Hauger, 2002).

CRF has been associated with the physical or psychological aspects of ethanol withdrawal, which was highlighted by elevated levels of CRF in the cerebrospinal fluid of alcohol-dependent patients who were undergoing acute ethanol withdrawal (Adinoff et al., 1996). In animals given 2-3 weeks of an ethanol-containing diet, ethanol withdrawal produced a time-dependent increase of CRF in the amygdala. The peak of CRF levels occurred at 10-12 hours, the time at which withdrawal symptoms peak. Manipulation of the CRF system with both intracerebroventricular or intra-amygdala administration of a CRF antagonist decreases the heightened anxiety-like behaviors associated with ethanol withdrawal in animals (Baldwin et al., 1991; Rassnick et al., 1993). These findings suggest not only that the CRF system plays a major role in the negative affective states associated with withdrawal, but that the amygdala is an important neural structure that mediates these effects. Work in this area is ongoing to identify and understand the neurobiology of this and other components of alcoholism.

Genetic animal models for studying neurobiological correlates of alcoholism

It is well accepted that alcoholism is heavily influenced by genetic factors dictating whether humans are susceptible or resistant to alcoholic-like tendencies. Not only do genetic animal models provide insight into the genes conferring risk and protective traits, the identification and study of many

neurobiological substrates of alcoholism is also aided. Genetic animal models do not generally provide a model of the entire disease, but provide valuable representations of individual traits that likely contribute to alcoholism.

Panels of genetically divergent inbred mouse strains are classic models that have been useful in studying the genetic and neurobiological correlates of alcohol-related behaviors. Through breeding of close genetic relatives, each mouse of an inbred strain is rendered a monozygotic twin, or genetically identical with other mice in that strain. Comparisons between different inbred mouse strains takes advantage of the preexisting genetic differences that exist between various inbred mouse strains. While more than 100 mouse strains are available from The Jackson Laboratory, two strains, the C57BL/6J (B6) and DBA/2J (D2) strains, have been the traditional models for alcohol-related behaviors given their near dichotomous relationship on many traits. For example, McClearn and Rodgers (1959) demonstrated that B6 mice highly preferred ethanol-containing solutions when a water alternative was available, while D2 mice avidly avoided the ethanol-containing bottle and drank primarily from the water bottle. These two strains also differ on several other ethanol-related behaviors including taste conditioning, conditioned place preference, ethanol-induced locomotor activation and ethanol withdrawal severity (Crawley et al., 1997).

An extension of the use of inbred mouse strains is the use of an F2 cross, which provides a population of genetically segregating mice. An F2 cross is derived from two inbred mouse strains and is a heterogeneous population of mice with alleles derived from one or both of the progenitor strains. This can be a

statistically powerful model because many mice can be tested for phenotypic variations, which can then be used for correlational analyses using genetic variation or neurobiological variates as the correlate. Recombinant inbred strains are another powerful model. These strains are fully inbred strains that are derived by mating close genetic relatives of an F2 population of mice. Utilization of this model can also be useful in demonstrating genetic linkage or correlations with neurobiological substrates.

Another model that is extremely powerful for determining neurobiological substrates of behavior is selectively bred lines. Selective breeding is accomplished through the selection of animals possessing extreme values on a heritable trait and mating them with animals possessing similarly extreme values on that trait. If performed bidirectionally, after breeding for many generations using this selection procedure, two lines may diverge suggesting that the genes favoring the extreme high and low values have increased in frequency over the selection process. Several groups have used this strategy to develop lines of rats and mice, either preferring or avoiding ethanol-containing solutions (Eriksson, 1971; Lumeng et al., 1977; Mardones and Segovia-Riquelme, 1983).

A combination of these genetic animal models has been used to establish a relationship between activation of the central nucleus of the amygdala and the locomotor stimulant response to ethanol. Using c-Fos immunohistochemistry, a brain mapping method (detailed in the following section), it was demonstrated that, following locomotor-activating doses of ethanol, mice from the D2 strain possessed substantial increases in c-Fos expression in the central nucleus of the

amygdala compared with the B6 strain (Hitzemann and Hitzemann, 1997). This response essentially mirrored the behavioral response to ethanol, where D2 mice display large increases in locomotor activity while B6 mice are relatively insensitive to locomotor changes following acute ethanol. This initial analysis was followed by an analysis of a B6D2 F2 cross that was tested for ethanol-induced locomotor activity (Demarest et al., 1998). A comparison of the mice displaying very high and very low locomotor activation demonstrated a similar result, where high-responders possessed robust elevations in c-Fos expression in the central nucleus of the amygdala, while low responders did not. Finally, a third report utilized the FAST and SLOW selectively bred mouse lines, which were selected based on their locomotor response to ethanol (Demarest et al., 1999). Confirmation of the previous analyses was obtained as the high-responding FAST mice possessed large c-Fos elevations in the central nucleus of the amygdala, while the low-responding SLOW mice had no significant elevations of c-Fos. Thus, the combined use of these and other genetic animal models possesses incredible potential for furthering the understanding of neurobiological substrates of ethanol.

Alcohol and Brain Mapping

While many studies have targeted traditional neural systems associated with alcohol's rewarding effects (i.e. mesocorticolimbic dopamine system), it is likely that ethanol also targets neural systems beyond this conventionally studied system. Actions in these systems may represent modes through which ethanol

produces behavioral effects, in part, by contributing to excessive alcohol intake such as initial drug sensitivity or the development of tolerance and sensitization to drug effects. While it is possible that an overlap in neural systems exists between behaviors directly associated with drug reward and other behaviors directly associated with the development of excessive alcohol intake, a complete overlap is unlikely and additional neural systems are likely involved. Therefore, the identification of such neural target sites for ethanol is critical to understand the neural mechanisms of all contributors to alcoholism.

Identification of neural targets has been accomplished using a number of brain mapping strategies. In humans, the use of positron emission tomography (PET), single photon emission computed tomography (SPECT) and functional magnetic resonance imaging (fMRI) has proven very effective in providing useful brain mapping data. In animals, the use of immunohistochemistry, autoradiography, and several functional imaging techniques has been fruitful in elucidating the neural targets of ethanol. While each of the different strategies possesses certain advantages and disadvantages, the synthesis of information gained from each of them, helps in elucidating additional neural targets for ethanol.

Brain imaging in humans

The two most widely used tools for human brain mapping, as a whole, have been PET and SPECT. In general, these strategies utilize radiolabeled tracers that can be used in a multitude of ways to answer a number of questions. First, the drug can be radiolabeled to establish regional distributions throughout

the brain, which infers where the drug is acting. Second, tracers can be used to identify local concentrations of drug binding sites and estimate receptor occupancies. Third, radiolabeling of distinct neurochemical substrates allows the effects of a drug on specific systems to be evaluated. Finally, the most widely used strategy for PET and SPECT, is to identify blood flow and metabolism of glucose, which can serve as indices of both overall neural activity and regionally specific neural activity (Gatley and Volkow, 1998). This last approach will serve as the primary approach for the descriptions presented here with regard to the neural targets of ethanol in the human brain.

Volkow and colleagues (1988) were the first to use PET technology to measure cerebral blood flow during acute ethanol intoxication. The results revealed that the cerebellum was an extremely sensitive brain site following administration of ethanol. A significant suppression of blood flow in the cerebellum at the highest dose (1 g/kg) was proposed to relate to the motor incoordinating effects of ethanol. In contrast to cerebellar suppression, cerebral blood flow was increased in both the prefrontal cortex and the right temporal cortex. The responsiveness of these regions may be related to elevations in mood or arousal, which are reported with ethanol intoxication (Tiihonen et al., 1994; Volkow et al., 1995; Ingvar et al., 1998). In addition, the basal ganglia were shown to have regional activations during ethanol intoxication (Wang et al., 2000). The basal ganglia are a target for the midbrain dopamine systems, which are implicated in the rewarding aspects, and may suggest a reward-related role during ethanol intoxication. In addition, prominent activation in blood flow was

observed in midline brain stem structures (Ingvar et al., 1998). These areas may represent part of an ascending pathway that modulates excitability in the neocortex during ethanol intoxication (Steriade, 1996).

The development of fMRI has aided human brain mapping because of its better temporal and spatial resolution capabilities over PET and SPECT technologies. Additionally, fMRI does not use radiation, which allows multiple analyses to be performed in the same individual without the potential adverse effects of radiation. This technology has just recently begun to be applied to the study of the neural substrates of ethanol. While direct mapping of ethanol-sensitive targets has not been performed using fMRI, as it was for cocaine (Breiter et al., 1997), several studies have used fMRI strategies to identify other aspects of neural sites responsive to ethanol. For example, the effect of ethanol on acoustically and visually stimulated blood oxygenation level-dependent (BOLD) signal responses has been reported (Levin et al., 1998; Seifritz et al., 2000). These reports demonstrate cortical suppression of the acoustic- and photic-stimulated BOLD signal, which were limited to the auditory and primary visual cortex, respectively. Other reports have utilized fMRI to identify neural targets of alcohol-associated cues in abstinent alcoholic patients (George et al., 2001; Wrase et al., 2002). In these studies, presentation of alcohol cues was found to increase activity in the prefrontal cortex, ventral striatum, anterior thalamus and extrastriate visual areas. The continued use of this strategy is certain to produce important information regarding the neuroanatomical sensitivity to ethanol.

Metabolic mapping in animals

While human mapping procedures provide a tremendous amount of information directly relevant for the human condition, some factors, such as polydrug use and adequate subject matching, limit the questions that can be addressed with these strategies. In such cases, it may be more advantageous to perform mapping experiments in animals. One of the primary techniques used to identify ethanol-responsive neural structures is deoxyglucose mapping in rodents (Lyons et al., 1995). This technique utilizes radiolabeled 2-deoxyglucose, which substitutes for the metabolic fuel, glucose, in neural tissue. Brain region-specific glucose utilization is thought to correlate with changes in functional activity (Sokoloff, 1981; Theurich et al., 1984).

A number of reports have used the 2-DG mapping strategy coupled with various dose manipulations and route of administration variations. Williams-Hemby & Porrino (Williams-Hemby and Porrino, 1994) observed that differential glucose utilization in rat brain occurred in discrete brain regions, which were highly dependent on the ethanol dose. For example, the lowest dose (0.25 g/kg) resulted in a distribution of regions that, for the most part, included components of the mesocorticolimbic dopamine system such as the nucleus accumbens, prefrontal cortex, and VTA. The highest dose (1.0 g/kg) produced widespread decreases in glucose utilization that included several thalamic and cortical structures as well as the dentate gyrus and CA3 regions of the hippocampus. In addition to this initial report, the 2-DG strategy has been performed in animals self-administering ethanol (Williams-Hemby et al., 1996; Porrino et al., 1998a;

Porrino et al., 1998b). The results of these reports generally reveal that self-administration of 10% ethanol, whether it be sweetened with sucrose or not, produces a discrete pattern of glucose utilization in the brain that primarily includes components of the mesocorticolimbic dopamine system.

Inducible transcription factor mapping in animals

While the aforementioned human and animal techniques provide valuable information about large-scale changes in populations of neurons, they provide little information about changes occurring on the cellular level. Another method that allows brain mapping with cellular resolution is the detection of inducible transcription factors (ITFs) by immunohistochemistry. ITFs are encoded by immediate early genes and can be used as cellular markers for small populations of neurons. ITFs used in mapping procedures generally include transcription factors of the Fos family (c-Fos, FosB, Fra-1 and Fra-2), Jun family (c-Jun, JunB and JunD), and Egr family (Egr1, Egr2 and Egr3) (Herdegen and Leah, 1998). ITFs from these families provide ideal markers because basal expression is typically low and it can be assumed that increased levels following a particular treatment are a result of increased neural activation associated with the treatment (Morgan et al., 1987; Sagar et al., 1988).

ITF brain mapping following ethanol administration has been performed by a number of groups (Zoeller and Fletcher, 1994; Chang et al., 1995; Thiele et al., 1996; Ryabinin et al., 1997). Studies using c-Fos as a marker for brain mapping have identified several brain regions that are activated after involuntary administration of ethanol. These areas include the lateral portion of central

nucleus of the amygdala, bed nucleus of stria terminalis, lateral septum ventrale, paraventricular nucleus of hypothalamus, paraventricular nucleus of the thalamus and the Edinger-Westphal nucleus (Zoeller and Fletcher, 1994; Chang et al., 1995; Thiele et al., 1996; Ryabinin et al., 1997). Topple et al. (Topple et al., 1998) demonstrated an increase in c-Fos expression in the Edinger-Westphal nucleus (EW) of rats self-administering ethanol-containing beer. Using the selectively bred Alko alcohol-preferring rats trained to operantly respond for 10% ethanol or 0.2% saccharin/10% ethanol, a dose-dependent increase in c-Fos expression was observed in the EW (Weitemier et al., 2001). In addition to these two reports, it was shown that the EW was also preferentially sensitive to voluntary ethanol drinking in mice (Bachtell et al., 1999; Ryabinin et al., 2001; Ryabinin et al., 2003).

The route of administration is extremely important for measuring the induction of c-Fos following ethanol exposure. This is highlighted by the vastly different patterns of c-Fos expression following experimenter-administered ethanol and voluntarily consumed ethanol. Thereby, voluntary ethanol consumption results in fewer changes in ITFs than experimenter-administered ethanol, such that, no induction of ITFs was observed in several brain regions showing strong reactivity to injections of ethanol including the central nucleus of the amygdala, bed nucleus of the stria terminalis, paraventricular nucleus of the thalamus, and the paraventricular nucleus of the hypothalamus. In addition, it is important to note that regions of the mesocorticolimbic dopamine pathway do not show elevations in c-Fos expression following ethanol that is voluntarily

administered (Bachtell et al., 1999; Ryabinin et al., 2001; Ryabinin et al., 2003). One region that proved to be preferentially sensitive to ethanol exposure by both experimenter-administered and self-administered ethanol was the EW. The mechanisms through which the EW is sensitive to ethanol, and the functional significance of the EW's sensitivity to ethanol is yet unknown and is the primary focus of this dissertation.

The Edinger-Westphal nucleus: An historical perspective

The EW was first identified in the fetal brain by Edinger (Edinger, 1885) where he observed two pairs of nuclei lying just dorsal and ventral to the main oculomotor nucleus on both sides of the midline and contained spindle-shaped cells. In 1887, Westphal made observations in the brain of an adult man (Westphal, 1887). The patient died with tertiary syphilis, which was associated with complete ophthalmoplegia but partially spared pupil responses to light and accommodation. While there was severe atrophy of the oculomotor, trochlear and abducens nuclei, which contributed to the ophthalmoplegia, two pairs of nuclei lying dorsal to the oculomotor nucleus were spared. Westphal referred to these pairs of nuclei as the medial and lateral cell groups. It was noted that the cells in these areas sent small myelinated axons into the fibers of the oculomotor fasiculi. Based on his observations, Westphal proposed that these fibers were responsible for the eye's ability to accommodate to changes in distance and intensity of light. Despite the fact that Edinger had described two pairs of nuclei lying dorsal and ventral to the oculomotor nucleus, and Westphal described two

pairs of nuclei lying medial and lateral to the oculomotor nucleus, the midbrain cluster of cells became known as the Edinger-Westphal nucleus.

Today, the EW is regarded as part of the oculomotor complex that functions to support ocular accommodation (Roste and Dietrichs, 1988; Burde and Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). The definition and precise identity of the EW, however, is less than clear. A schematic of the EW in the mouse brain is depicted in Figure 1, however, the EW is defined in a variety of ways. Some investigators define the EW as the medial visceral cell column and the lateral visceral cell column plus the anterior median nucleus (Warwick, 1954; Pierson and Carpenter, 1974; Benevento et al., 1977). In this case, it appears that the lateral visceral cell column is the most clearly associated with pupil constriction (Kourouyan and Horton, 1997). In other cases, the EW is simply defined as the cluster of cells supplying preganglionic parasympathetic input to the ciliary ganglion (Akert et al., 1980). However, this distinction is less clear because retrograde tracers injected into the ciliary ganglion produce labeling in several midbrain nuclei of the oculomotor complex, but does not label the morphologically-identified EW (Ishikawa et al., 1990; Erichsen and May, 2002).

The Edinger-Westphal nucleus: Composition and circuitry

The cellular composition of the EW is quite diverse. The most documented cell type in the EW is the cholinergic neuron that projects to autonomic ganglia and spinal cord (Strassman et al., 1987; Lauterborn et al., 1993). Colocalization

studies have shown that these neurons also contain Substance P and cholecystokinin (Innis and Aghajanian, 1986; Schiffmann and Vanderhaeghen, 1991; Smith et al., 1994). An early report suggested that CRF was present in the EW (Chung et al., 1987), however, recent studies have contradicted this finding by showing that the CRF-related 40-amino acid peptide, urocortin (Ucn) is preferentially expressed in the EW (Vaughan et al., 1995; Yamamoto et al., 1998; Bittencourt et al., 1999; Weninger et al., 2000).

It has repeatedly been suggested that the connectivity of EW neurons is more complex than previously thought (Bittencourt et al., 1999). This is evidenced by the number of neural targets projecting to and from the EW. The olivary pretectal nucleus sends projections to the EW in a circuitry that supports the traditional mydriadic functions (Klooster et al., 1995; Klooster and Vrensen, 1998; Klooster et al., 2000). Other regions, however, may represent more diverse functioning. For example, input from several hypothalamic nuclei transcend caudally (Saper et al., 1976; Koss, 1986; Zheng et al., 1995), while the ventral noradrenergic bundle relays noradrenergic input from the locus coeruleus (Loewy et al., 1973; Breen et al., 1983). Both sets of projections support EW involvement in autonomic functions, but do not agree with the neurocircuitry associated with pupillary function.

The EW also has a diverse set of sites to which it projects. The lateral septum is the most prominent forebrain target of the EW where Ucn projections coexist with a high density of CRH-R2 receptors (Bittencourt et al., 1999). Additionally, Ucn-containing cells of the EW project to the spinal cord. Other

prominent target sites include the trigeminal nucleus, inferior olive, periaqueductal gray, parabrachial nucleus, reticular formation and cerebellum (Sugimoto et al., 1978; Roste and Dietrichs, 1988; Klooster et al., 1993).

The diverse nature of the EW cellular composition, projections, and afferents lend support to the notion that the EW is more functionally diverse than originally portrayed. Traditionally, the EW's function was reduced to oculomotor functions where activation, through disinhibitory mechanisms, produces mydriatic responses to light (Westphal, 1887; Warwick, 1954; Roste and Dietrichs, 1988; Burde and Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). However, the preferential expression of Ucn and its forebrain projection to the lateral septum suggest that additional functions may be linked to EW activation. Neuropeptide activity at CRH-R1 and CRH-R2 receptors is known to mediate stressful- and anxiety-related behaviors (Koob and Heinrichs, 1999; Weninger et al., 1999; Coste et al., 2000; Weninger et al., 2000). It has recently been shown Ucn expression in EW is upregulated by restraint stress suggesting the involvement of the EW in mediating anxiety-like responses (Weninger et al., 2000).
The EW has also been shown to play a role in mediating the choroidal vasculature that is responsible for temperature modulation in the eye (Parver, 1991). In support of this function, a recent report identifying neural circuits involved in heat loss showed retrograde labeling in EW neurons (Smith et al., 1998). These studies lead to the idea that EW activation may play a role in thermoregulatory responses. The EW may also be involved in the mediation of motor behaviors. This evidence stems from reports showing that the EW is a

target site of vestibular apparatus projections and other studies showing that descending EW neurons project to precerebellar and cerebellar areas, and the spinal cord (Roste and Dietrichs, 1988; Spence and Saint-Cyr, 1988a; Bittencourt and Sawchenko, 2000). The potential involvement of the EW in these responses makes it an interesting target because of the significant overlap with several responses that are altered during ethanol intoxication such as ethanol's anxiolytic response, effects on thermoregulation and motor incoordinating effects.

Summary and Dissertation Goals

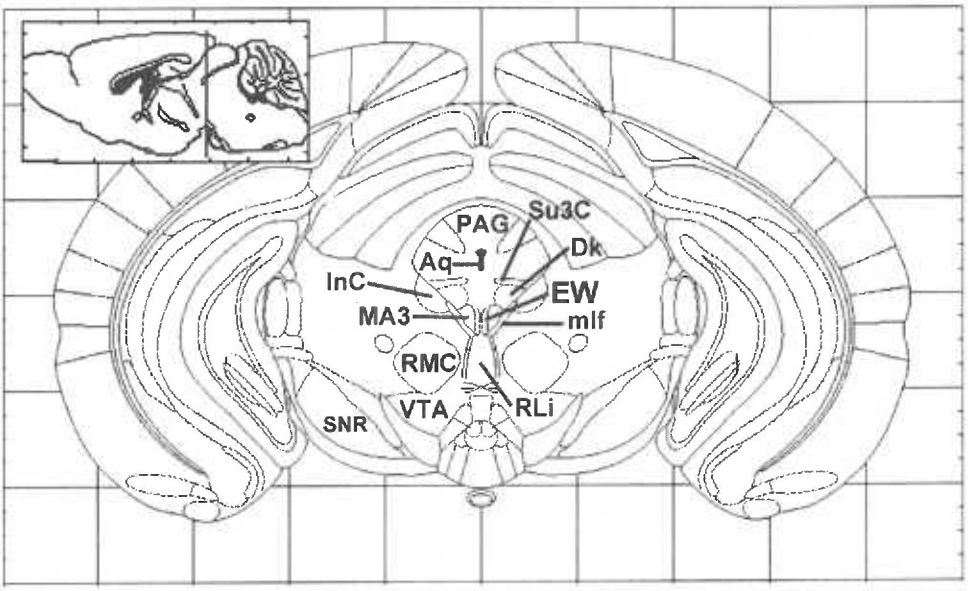
The study of the neurobiology of drug abuse has provided a great amount of information concerning the neural systems and the unique, yet functionally congruent mechanisms associated with individual drugs of abuse. These studies have traditionally focused on the mechanisms associated with drug reward and reinforcement. While these mechanisms are important to the understanding of drug and alcohol abuse, it is likely that other systems and mechanisms exist that also contribute indirectly to their abuse potential. The identification and characterization of such systems is important to establish a more complete understanding of the neurobiology of drug and alcohol abuse.

The use of brain mapping tools is one approach that allows the identification of additional neural systems. PET studies in humans and 2-DG studies in rodents have provided a great deal of information regarding the regional sensitivity to ethanol. In addition, the use of ITFs, such as c-Fos has proven extremely useful in isolating populations of cells showing changes in

expression patterns. Using this brain mapping strategy, the EW was identified as the only structure being strongly implicated following both voluntary and involuntary ethanol administration. This suggests that EW neurons are activated during ethanol exposure. The EW as a target for ethanol is poorly understood and therefore is the object of this dissertation.

The primary goal of this dissertation project was to characterize the responsiveness of the EW to ethanol intoxication including the mechanisms involved and the functional significance of EW activation. This broad goal was broken into three aims. The first aim sought to identify the mechanisms involved in the activation of EW neurons upon ethanol intoxication. The experiments within this aim detail the pharmacological and signal transduction mechanisms responsible for alcohol-induced elevations in c-Fos expression in the EW. The second aim sought to identify the neurochemical cell types of the EW and to identify the type of cells expressing c-Fos following alcohol administration. The second aim also relied heavily on establishing relationships between EW neurochemical cell types and ethanol-related behaviors using genetic animal models. Finally, the third aim determined the functional significance of the EW for ethanol intoxication by creating electrolytic lesions of the EW and measuring ethanol-mediated behavioral responses.

Figure 1. A coronal schematic of the Edinger-Westphal nucleus (EW) and several regions in close proximity of the EW including the periaqueductal gray (PAG), cerebral aqueduct (Ag), supraoculomotor capsule (Su3C), Interstitial nucleus of Cajal (InC), nucleus of Darkschewitsch (Dk), medial accessory oculomotor nucleus (MA3), medial longitudinal fasciculus (mlf) red nucleus magnocellular (RMC), rostral linear nucleus of raphe (Rli), ventral tegmental area (VTA), and the substantia nigra reticular (SNR). The vertical line in the inset corresponds to approximately –3.4 mm from Bregma in the mouse brain, which is a central coordinate of the EW in the saggital plane and that which is shown in the coronal brain schematic (adapted from Franklin and Paxinos, 1997).


PAG
Su3C
Dk
Aq
InC
EW
MA3
mlf
RMC
VTA
RLi
SNR

CHAPTER 1: Pharmacological characterization of the Edinger-Westphal nucleus' response to ethanol

While most brain regions do not exhibit elevated c-Fos expression following all routes of ethanol administration, c-Fos expression in the EW is equally robust following both voluntary and involuntary ethanol administration (Bachtell et al., 1999; Ryabinin et al., 2001; Ryabinin et al., 2003). This indicates that it may be an important nucleus in regulating ethanol's effects on the central nervous system. It is therefore essential to characterize the mechanisms of ethanol-induced c-Fos expression in EW neurons. To this aim, the set of experiments outlined below sought to identify the pharmacological and signal transduction mechanisms of ethanol-induced c-Fos expression in the EW.

There has been little data generated on the localization of receptors on EW neurons. However, behavioral data and other manipulations provide some insight into likely candidates for neurotransmitter targets on EW neurons. For example, post-synaptic α_2-adrenoceptors in the EW appear to play a large role in the oculomotor response (i.e. mydriasis) (Heal et al., 1995). In addition, pharmacological studies using benzodiazepines suggest the involvement of $GABA_A$ receptors in activating EW neurons (Cutrera et al., 1993; Skelton et al., 2000). Other evidence shows that microinjections of opioid agonists (e.g. morphine and fentanyl) into the EW produce behavioral effects without significantly modulating mydriasis (Kamenetsky et al., 1997). Thus, opioid systems may play a role in non-mydriatic behaviors stemming from EW neurons. In addition to these receptor systems, tyrosine hydroxylase-containing

projections stemming from the dopamine-rich ventral tegmental area suggest that the dopaminergic system may also play a role. Therefore, these four transmitter systems were tested for their involvement in ethanol-induced c-Fos expression in EW neurons.

The second part of this study was aimed at examining the signal transduction mechanisms in the EW during ethanol intoxication. Previous reports showed that ethanol administration elevates expression of c-Fos, but does not elevate expression of the inducible transcription factor, Egr1 (also known as NGFI-A, Zif268 and Krox-24) in the EW (Bachtell et al., 1999; Bachtell and Ryabinin, 2001). The promoter regions of *c-fos* and *egr1* genes contain several common regulatory elements, including the serum response element (SRE) and the calcium/cyclic-adenosine monophosphate response element (Ca/CRE). On the other hand, the *c-fos* promoter contains the c-Sis inducible element (SIE), whereas the *egr1* promoter does not (Herdegen and Leah, 1998). We hypothesized therefore, that ethanol induces c-Fos mainly via an element that is unique to the *c-fos* promoter. To address this hypothesis we used immunohistochemistry to analyze the signal transduction machinery not only acting through the SRE and Ca/CRE pathway (the CREB and Elk1 transcription factors), but also acting through an SIE mechanism, which involves the signal transducer and activator of transcription (Stat) family of proteins .

MATERIALS AND METHODS

Animals

Male C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) were placed four per cage for a minimum of 1 week. All animals were 7-9 weeks of age at the beginning of the experimental procedure. While other strains were considered, C57BL/6J mice were selected for this analysis because of the well-characterized ethanol-induced c-Fos expression in our previous experiments in the EW nucleus (Bachtell et al., 1999; Ryabinin et al., 2001; Ryabinin et al., 2003). Animals were maintained on a 12 h light-dark cycle with lights on beginning at 6 a.m. Water and food were available ad libitum throughout the experiment in the home cage. All animal procedures were in accordance with National Institutes of Health guidelines.

General Procedures.

All animals were habituated to handling and injections on the four days prior to the drug treatment day. Our preliminary experiments have shown this procedure to be satisfactory in reducing basal c-Fos expression to minimal levels. During the habituation procedures animals were exposed to two injections. All injections in all experiments were intraperitoneal. The second injection was made contralateral to the first injection. On the test day, animals were injected with the pretreatment drug and placed back into the home cage. Animals were injected again at precise intervals (depending on the drug properties), on the contralateral side, with the second injection (see specific experiments). Animals were then

placed back into the home cage. Animals were sacrificed 100 min after the second injection by CO_2 inhalation. Animals were not perfused, however, brains were postfixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) following removal. Brains were then immersed in 20% sucrose in PBS containing 0.1% NaN_3 for 24 hours followed by two 24 hour incubations in 30% sucrose in PBS containing 0.1% NaN_3.

Experiment #1: Tests of GABAergic involvement

Two negative modulators of the $GABA_A$ receptor were used to identify its impact on ethanol-induced c-Fos expression in the EW. First, the direct competitive $GABA_A$ antagonist, (-) bicuculline (BIC, Sigma Biochemicals, St. Louis MO, USA), was dissolved in DMSO and diluted twice in sterile water. Animals were pretreated with 4.0 mg/kg (-) bicuculline prior to either 2.4 g/kg ethanol (n=4, 20% v/v in 0.9% saline) or saline (n=4). Control animals were pretreated with the DMSO vehicle prior to either 2.4 g/kg ethanol (n=4) or saline (n=3). The negative modulator of $GABA_A$, pentylenetetrazole (PTZ, Sigma), which acts at the picrotoxin site to block chloride influx, was dissolved in saline. Animals were administered 45 mg/kg PTZ as a pretreatment to either 2.4 g/kg ethanol (n=4) or saline (n=4). Control animals were pretreated with the saline vehicle prior to either 2.4 g/kg ethanol (n=4) or saline (n=3). Both negative modulators of the $GABA_A$ receptor were administered ten minutes prior to the ethanol or saline injection. The 2.4 g/kg dose of ethanol was previously shown to produce maximal c-Fos expression in EW neurons of C57BL/6J mice (Bachtell et al., 2002).

Assessment of ability of the $GABA_A$ receptor to increase EW c-Fos expression was performed using two positive modulators at the $GABA_A$ receptor. 3α-hydroxy-5α-pregnan-20-one (3α5α-P, from Dr. Deborah Finn, Oregon Health & Science University (OHSU), which targets the steroid site on the $GABA_A$ receptor, and chlordiazepoxide (CDP, from Dr. Robert Hitzemann, OHSU), targeting the benzodiazepine site, were used. Actions of both compounds at the $GABA_A$ receptor act to enhance GABA-activated chloride influx. Drugs were dissolved in 2-hydroxypropyl-β-cyclodextrin and saline, respectively. In all cases, a saline pretreatment was administered 10 minutes prior to the vehicle, 3α5α-P or CDP injection. 3α5α-P was tested at 10mg/kg (n=6) and 20 mg/kg (n=5) doses, while CDP was tested at 10 mg/kg (n=4), 20 mg/kg (n=4) and 30 mg/kg (n=4) doses. An additional group of animals (n=4) treated with 2.4 g/kg ethanol was analyzed to allow comparison of the effects of 3α5α-P and CDP with those produced by ethanol. In all cases, animals were sacrificed 100 minutes following the second injection and were analyzed for c-Fos expression using the immunohistochemistry protocol described below.

Experiment #2: Tests of opioid involvement

The nonselective opioid receptor antagonist, naltrexone (Sigma) was used to evaluate the involvement of the opioid receptors on ethanol-induced c-Fos expression in the EW. Animals were pretreated with 30 mg/kg naltrexone (dissolved in saline) prior to either 2.4 g/kg ethanol (n=4, 20% v/v in 0.9% saline) or saline (n=4). Control animals were pretreated with the saline vehicle prior to

either 2.4 g/kg ethanol (n=4) or saline (n=4). The effects of morphine, a μ-opioid agonist, were analyzed for its effects on c-Fos expression in EW. 10 mg/kg and 100 mg/kg morphine sulfate (RBI/Sigma Biochemicals, St. Louis MO, USA, dissolved in saline) was administered 10 minutes following a saline vehicle (n=4) or naltrexone injection (n=4). Animals were sacrificed 100 minutes following the second injection and analyzed for c-Fos immunohistochemistry as described below.

Experiment #3: Tests of noradrenergic involvement

The involvement of the noradrenergic system in ethanol-induced c-Fos expression in the EW was first tested with pretreatment with either saline vehicle or nonselective antagonists for either the α_2- or the β-adrenoceptor. These included yohimbine (Tocris Cookson Inc., Ballwin, MO, USA), and propranolol (PRP, Tocris Cookson Inc.), respectively, which were dissolved in saline. Animals were pretreated with 5 mg/kg (n=12) and 10 mg/kg (n=12) yohimbine (dissolved in saline) prior to either 2.4 g/kg ethanol (n=16) or saline (n=8). Control animals were pretreated with the saline vehicle prior to either 2.4 g/kg ethanol (n=4) or saline (n=3). Another set of animals were pretreated with 20 mg/kg propranolol (n=8) prior to either 2.4 g/kg ethanol (n=8) or saline (n=8). Control animals were pretreated with the saline vehicle prior to either 2.4 g/kg ethanol (n=4) or saline (n=4). These experiments were followed up by tests of selective α_2-adrenoceptor antagonists on ethanol-induced c-Fos expression. Therefore, ARC 239 ($\alpha_{2B/C}$-selective, Tocris Cookson Inc) or RX 821002 ($\alpha_{2A/D}$

selective, Tocris Cookson Inc) were administered prior to ethanol treatment (Trendelenburg et al., 1996a; Callado and Stamford, 1999). Animals were pretreated with 10 mg/kg ARC 239 prior to either 2.4 g/kg ethanol (n=6) or saline (n=4). Control animals were pretreated with the saline vehicle prior to either 2.4 g/kg ethanol (n=8) or saline (n=4). Animals were pretreated with 5 mg/kg (n=12) and 10 mg/kg (n=12) RX821002 (dissolved in saline) prior to either 2.4 g/kg ethanol (n=16) or saline (n=8). Control animals were pretreated with the saline vehicle prior to either 2.4 g/kg ethanol (n=4) or saline (n=3). In order to identify the involvement of the noradrenergic system in CDP-induced and morphine-induced EW c-Fos expression, these compounds were administered following yohimbine and RX 821002 (n=4/group), respectively.

Assessment of the ability the noradrenergic receptors to increase EW c-Fos expression was performed using the α_2-adrenoceptor agonist, clonidine (Tocris Cookson, Inc.), and the α_1-adrenoceptor agonist, cirazoline (Tocris Cookson, Inc.) Each compound was administered to animals (n=4/group) following a saline pretreatment. Both compounds were dissolved in saline. In all cases, animals were sacrificed 100 minutes following the second injection and analyzed for c-Fos immunohistochemistry as described below.

Experiment #4: Tests of dopaminergic involvement

The involvement of the dopaminergic system in ethanol-induced c-Fos expression in the EW was tested with the dopamine receptor antagonist, haloperidol (Sigma, dissolved in DMSO). Haloperidol has higher specificity for D2

dopamine receptors compared with D1 receptors. Twenty minutes following the pretreatment with haloperidol or DMSO, 2.4 g/kg ethanol or saline vehicle was administered (n=5/group). In addition to the antagonist, the nonselective D1/D2 dopamine receptor agonist, apomorphine (from Dr. Charles Meshul, OHSU), was administered following pretreatment with saline (n=5/group).

Experiment #5: Identification of the signal transduction cascades

The following sequence of experiments was performed in animals that were habituated to saline injections (n=4-8/group). On the final day, animals were injected with either saline or 2.4 g/kg ethanol. Unlike the previous experiments, animals were sacrificed by cervical dislocation at the following time points after the injection: 5, 15, or 30 minutes. However, the 30 min time point was excluded from analyses due to the absence of positive signals indicating a return to baseline expression levels. Due to the use of antibodies detecting phosphorylated proteins, 1 mM NaF was added to all buffers and 0.1 mM NaF was introduced to all incubation solutions in an effort to enhance signal strength by inhibiting phosphatase activity. With this exception, all immunohistochemical steps were identical to that presented for c-Fos immunohistochemistry. Final dilutions of the primary antibodies were determined through preliminary experiments. We first tested antibodies detecting the primary substrate of SRE phospho-(Ser 383)-Elk1 (1:100, Cell Signaling Technology, Beverly, MA, USA). We next targeted substrates of Ca/CRE including CREB (1:500) and phospho-CREB (1:500, Cell Signaling Technology). Finally, we tested substrates acting on

SIE. These included phospho-(Tyr701)-Stat1 (1:250), Stat3 (1:500), phospho-(Tyr705)-Stat3 (1:250), and phospho-(Ser727)-Stat3 (1:300). All were purchased from Cell Signaling Technology. Targeted upstream regulators of many of these substrates were also detected including phospho-p90 RSK (1:500), phospho-ERK 1/2 (1:500), which were also purchased from Cell Signaling Technology. A number of other antibodies were tested, but were excluded due to lack of antibody detection specificity.

Experiment #6: Tests of MEK 1/2 inhibition

Inhibition of MEK 1/2 activity was accomplished with the MEK 1/2 inhibitor, SL327 (from Dr. James Trzaskos, DuPont Pharmaceuticals), which has the primary advantage of being active via systemic injections (Selcher et al., 1999). SL327 was dissolved in DMSO and given to animals 40 minutes prior to a saline or 2.4 g/kg ethanol injection (n=5/group). Animals were sacrificed 100 minutes following the second injection and immunohistochemistry was performed for c-Fos as described below.

Blood Ethanol Measurement

Trunk blood was collected in microcentrifuge tubes upon sacrifice and placed on ice. All samples were immediately centrifuged at 14,000 rpm for 20 minutes. Serum was collected and used to assess NAD-ADH reactivity from measured in 3 µl blood samples from individual animals using the spectrophotometric NAD-ADH Detection System (Sigma Diagnostics, St. Louis, MO, USA). A 10 set of

standards ranging from 10-3000 mg/dl was run periodically to assess the accuracy of the NAD-ADH Detection system.

c-Fos Immunohistochemistry

Immunohistochemistry was performed on 40 µm floating slices according to previously published protocols (Ryabinin and Wang, 1998). Immunohistochemistry for c-Fos was initiated by inhibiting endogenous peroxidase activity with a pretreatment of 0.3% hydrogen peroxide. Blocking was performed with 4% goat serum. Rabbit polyclonal antibodies against amino acids 210-335 of human c-Fos protein that are not cross-reactive with FosB, Fra-1 and Fra-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used in a dilution of 1:10,000. The immunoreaction was detected with Vectastain ABC kit (Vector Laboratory, Burlingame, CA, USA). Enzymatic development was performed with the Metal Enhanced DAB kit (Pierce, Rockford, IL, USA).

Data Analysis

Quantitative image analysis for c-Fos immunohistochemistry was performed using a system consisting of an Olympus microscope BX40 and Sony CCD IRIS/RGB video camera connected to a Power PC. Each digitized video image was analyzed using NIH image 1.62 software. Two slices containing the EW were selected from each animal at approximately –3.00 to –3.40 from Bregma as described by Franklin and Paxinos (Franklin and Paxinos, 1997). Our preliminary experiments have determined that sections between –3.00 and –3.40 from

Bregma produce the most consistent results with c-Fos immunohistochemistry. Nuclear c-Fos labeling was detected using a threshold normalization procedure, where neighboring areas with no immunoreactivity were adjusted to contain no positive signals. Remaining grains in a size range from 7 to 50 pixels were automatically counted. All counting was performed by an individual, blind to the experimental groups. To control for variability in DAB intensity, all statistical analyses were performed on sections processed simultaneously. Values from two sections from each animal were averaged and used as a single data point for statistical analysis using analysis of variance (ANOVA). When antagonists were administered prior to an ethanol injection, 2-way ANOVAs were performed with Pretreatment and Ethanol as the two factors. However, tests with the administration of agonists with saline pretreatment were analyzed with a one-way ANOVA with Dose/Group as the factor. Significant main effects were analyzed with the Fisher's LSD pair-wise post-hoc test and interactive effects were further analyzed with a Simple Main Effects analysis followed by the Fisher's LSD pair-wise post-hoc test, where applicable.

RESULTS

Experiment #1:GABAergic involvement in ethanol-induced c-Fos expression in EW neurons.

Administration of $GABA_A$ receptor antagonists produced significant changes in ethanol-induced c-Fos expression in the EW (Figure 2). Specifically, pretreatment with 4 mg/kg bicuculline significantly attenuated ethanol-induced c-

Fos expression ($F(1,19) = 100.31$, $p < 0.001$). Like bicuculline, pretreatment with 45 mg/kg PTZ significantly attenuated ethanol-induced c-Fos expression ($F(1,20) = 28.76$, $p < 0.001$). These doses of bicuculline and PTZ approach doses known to produce seizures (Johansson et al., 1996), however we observed no convulsive effects in our study.

Administration of positive modulators of the $GABA_A$ receptor also produced significant effects. The neurosteroid, 3α5α-P, produced a dose-dependent increase in EW c-Fos expression (Figure 3). In particular, 10 mg/kg 3α5α-P produced a modest, statistically insignificant increase in c-Fos expression while the 20 mg/kg 3α5α-P showed a significant increase in EW c-Fos expression. This dose of 3α5α-P in mice is expected to produce a similar locomotor response to that produced by 2.4 g/kg of ethanol (Palmer et al., 2002). Therefore, higher doses of 3α5α-P were not included. The benzodiazepine, CDP, showed a uniform, but significant, increase in c-Fos expression across all administered doses (Figure 3). These elevations in c-Fos expression following both GABAergic agonists are significantly below that shown after ethanol administration (Figure 3).

Experiment #2: Opioid involvement in ethanol-induced c-Fos expression in EW neurons

Pretreatment with 30 mg/kg naltrexone prior to 2.4 g/kg ethanol had no effect on ethanol-induced c-Fos expression (Figure 4). When morphine was administered at 10 mg/kg and 100 mg/kg, however, a significant increase in EW

c-Fos was detected (Figure 4). As was evident with the positive modulators of the $GABA_A$ complex, morphine-induced c-Fos expression was significantly below c-Fos levels observed following 2.4 g/kg ethanol. While 30 mg/kg naltrexone did not affect ethanol-induced c-Fos expression in the EW, 30 mg/kg naltrexone significantly blocked the effect of both the 10 and 100 mg/kg doses of morphine (Figure 4).

Experiment #3: Noradrenergic involvement in ethanol-induced c-Fos expression in EW neurons

The non-selective α_2-adrenergic receptor antagonist, yohimbine, produced a dose-dependent blockade of ethanol-induced c-Fos expression (Figure 5a). Ethanol-induced c-Fos remained elevated when 20 mg/kg propranolol, a non-selective β-adrenergic receptor antagonist, was administered prior to ethanol (Figure 5b). Although only one dose of propranolol was administered, sedative effects were observed, indicating behavioral activity of the drug. In order to further characterize the α_2-adrenergic receptor antagonist effects, two compounds were used to elucidate α_2-adrenoceptor subtype-specific mechanisms of ethanol-induced c-Fos expression in EW (Figure 5e and 5f). First, 10 mg/kg of ARC 239, a $\alpha_{2B/C}$-selective antagonist, showed no statistically significant changes in ethanol-induced c-Fos expression (Figure 5c). Behaviorally, this dose of ARC 239 was extremely sedative. Second, we used 5 and 10 mg/kg RX 821002, which has been shown to be a $\alpha_{2A/D}$-selective antagonist (Trendelenburg et al., 1996b). When administered prior to ethanol, RX

821002 dose-dependently diminished ethanol-induced c-Fos expression in EW neurons (Figure 5d).

To further characterize the pharmacological mechanisms associated with c-Fos expression in EW neurons, we evaluated the effects of the noradrenergic system on CDP-induced and morphine-induced c-Fos expression in the EW (Figure 6). This was tested by pretreating a group of animals with 5 mg/kg yohimbine and subsequently injecting them with 20 mg/kg CDP. This resulted in a significant decrease in CDP-induced c-Fos in EW neurons suggesting an adrenergic mechanism in GABA-induced c-Fos expression. We also tested the ability of 10 mg/kg RX821002 to disrupt EW c-Fos expression resulting from administration of 10 mg/kg morphine. Indeed, the α_2-adrenoceptor antagonist, RX821002, significantly blocked morphine-induced c-Fos expression, also suggesting a noradrenergic mechanism with morphine-induced c-Fos expression in EW neurons.

Administration of adrenergic receptor agonists produced no effects on c-Fos expression in EW neurons (data not shown). Specifically, 0.1 mg/kg and 0.5 mg/kg of the non-selective α_2 adrenergic agonist, clonidine, produced no changes in c-Fos expression. Interestingly, these same doses are capable of producing mydriatic changes in mice, which is thought to involve EW activity (Heal et al., 1989). Furthermore, the non-selective α_1-adrenergic agonist, cirazoline, also showed no alterations in c-Fos expression at 0.5 mg/kg and 1.0 mg/kg.

Experiment #4: Dopaminergic involvement in ethanol-induced c-Fos expression in EW neurons

Pretreatment with 0.5 mg/kg of the dopamine receptor antagonist haloperidol prior to ethanol administration resulted in a complete blockade of ethanol-induced c-Fos expression (Figure 7). Administration of both 1 mg/kg and 10 mg/kg apomorphine resulted in no significant elevation in c-Fos expression (data not shown).

Experiment #5: Signal transduction cascades following acute ethanol administration

Overall, relatively few changes were observed in the targeted upstream modulators of the c-Fos promotor at 5, 15, or 30 minutes after ethanol injection (Figure 8). In particular, no changes were observed in phospho-Elk1 levels, which is one of the primary regulators of the SRE site thus lending support to the hypothesis that the SRE is not involved in ethanol-induced c-Fos expression (Figure 8a and 8b). As a positive control for immunohistochemistry sensitivity, changes in phospho-Elk 1 levels were observed in the cingulate cortex. EW neurons were also absent of changes in phospho-CREB in EW neurons suggesting that the pathway targeting the Ca/CREB element is not involved in ethanol-induced c-Fos expression in the EW (Figure 8c and 8d). Changes in phospho-CREB were observed in the paraventricular nucleus of hypothalamus suggesting that lack of signal in the EW is not due to compromised immunohistochemistry sensitivity.

Interestingly, however, ethanol produced significant elevations in serine-727 phosphorylated Stat3 in the nuclei of EW neurons only at the 15-minute time point (Figure 9). Tyrosine-705 phosphorylated Stat3, however, was not elevated at any time points following ethanol or saline treatment. EW neurons did not show any changes in other tested members of the Stat family, suggesting specificity for Stat3, with preferential phosphorylation activity at the serine 727-phosphorylation site. In addition to the changes in serine-727 phosphorylated Stat3, we observed changes in the upstream regulator of many substrates, phospho-ERK1/2. Specifically, significantly higher levels of phospho-ERK 1/2 were observed in EW neurons in animals receiving ethanol (Figure 9). As with the elevations noted with phospho-Stat3, these changes were apparent at 15-minutes post-injection. No changes in other upstream regulators including p90RSK, phospho-Stat1, and phospho-MAPK 38 were observed (data not shown).

Experiment #6: Expression of c-Fos in EW following pretreatment of the MEK 1/2 inhibitor, SL 327

In an attempt to evaluate the role of the MEK/ERK pathway in ethanol-induced c-Fos in EW neurons, we blocked MEK 1/2 activity pharmacologically with SL327. Administration of SL 327 40 minutes prior to ethanol or saline treatment showed a dose-dependent blockade of c-Fos expression in EW neurons (Figure 10). Specifically, 50 mg/kg SL327 produced relatively no effect on ethanol-induced c-Fos expression. Pretreatment with 100 mg/kg SL327,

however, completely blocked ethanol-induced c-Fos expression in EW neurons, suggesting a MEK/ERK dependent pathway.

DISCUSSION

The findings presented suggest that, upon involuntary ethanol exposure, EW neurons are influenced by several neurotransmitter receptors. The $GABA_A$ receptor appears necessary for ethanol-induced c-Fos expression and sufficient for enhanced basal expression of c-Fos in EW neurons suggesting that ethanol is acting directly upon $GABA_A$ receptors. This is supported by data showing that positive modulators of the $GABA_A$ receptor (e.g. CDP and 3α5α-P) lead to elevated c-Fos in EW neurons as well as data showing attenuations of ethanol-induced c-Fos upon $GABA_A$ receptor antagonist pretreatment. In agreement with our findings are reports showing elevated c-Fos expression and increased urocortin mRNA in EW neurons upon treatment with benzodiazepines (Cutrera et al., 1993; Skelton et al., 2000).

Ethanol actions at $GABA_A$ receptors do not, however appear to be the only transmitter system involved. This is evidenced by the inability of either CDP or 3α5α-P to induce c-Fos expression in EW to levels comparable to that of ethanol alone. Furthermore, complete blockade of ethanol-induced c-Fos expression was observed by pretreatment of $\alpha_{2A/D}$-adrenoceptor and dopaminergic receptor antagonists. These receptors are therefore necessary for ethanol-induced c-Fos expression. Administration of direct agonists at these sites, however suggests that these receptors are not sufficient for increased c-Fos expression in EW

neurons. Thereby, administration of the α_2-adrenoceptor agonist, clonidine, did not produce significant elevations of EW c-Fos even when doses capable of inducing mydriasis were administered. Likewise, administration of apomorphine had no effect on c-Fos expression in the EW. The latter supports previous data showing no increased EW c-Fos expression following cocaine administration, which potently increases extracellular concentrations of dopamine (Ryabinin et al., 2000).

The dopaminergic effects demonstrated in this report may represent non-specific effects in that we only note dopaminergic modification with haloperidol. While haloperidol binds potently to the D2 dopamine receptor, it also displays affinity for D3 and D4, which are other D2-like dopamine receptors, and D1 and D5 receptors. Recently, the specificity for neuroleptic treatment for the dopamine system has been questioned and haloperidol was demonstrated to have actions at serotonin receptors as well ((Millan, 2000). In particular, haloperidol displays the highest affinity for 5-HT_{2b}, 5-HT_{2c}, 5-HT_3 serotonin receptors. Thus, the use of haloperidol as a dopamine antagonist is less than ideal. Follow-up experiments using more specific dopamine and serotonin antagonists should be performed to ascertain the effects observed with haloperidol. Regardless, it is believed that ethanol is acting directly at $GABA_A$ receptors, and this activity is modified by actions at $\alpha_{2A/D}$ and possibly dopaminergic receptors. Support for this claim stems from data in which CDP-induced c-Fos expression in EW is blocked by the non-selective α_2-adrenoceptor antagonist, yohimbine.

The μ-opioid system is not necessary for ethanol-induced c-Fos

expression as was evidenced by naltrexone's inability to modulate ethanol-induced c-Fos in the EW. Interestingly, the opioid system does appear sufficient for c-Fos expression in EW neurons since morphine significantly elevates c-Fos levels. As was shown with CDP- and 3α5α-P- induced c-Fos expression, morphine-induced elevations in c-Fos are significantly lower than that produced by ethanol alone. Morphine-induced c-Fos expression may be influenced by $\alpha_{2A/D}$ receptors in a similar manner to that of ethanol and CDP. This notion is supported by the data showing that the $\alpha_{2A/D}$ antagonist, RX821002, blocks morphine-induced c-Fos expression in EW neurons. This finding is intriguing given data suggesting that morphine and fentanyl are acting directly on EW neurons to produce nociception and catalepsy, but not mydriasis, which is thought to be primarily mediated by post-synaptic $\alpha_{2A/D}$ receptors in EW (Kamenetsky et al., 1997).

Taken together, it seems that the receptor systems impacting EW neurons are quite complex and most likely involve a number of interactions occurring through both pre- and post-synaptic actions. Furthermore, these data implicate the involvement of other brain nuclei upstream from the EW nucleus. In order to aid the interpretation of such complex inputs, this set of experiments also sought to identify the intracellular events that are initiated upon ethanol administration.

Interestingly, all of the receptors suspected to modulate ethanol-induced c-Fos expression in EW neurons act to downregulate adenylate cyclase activity. This alone is suggestive of a unique signal transduction cascade culminating in ethanol-induced c-Fos expression. Further suggesting that a unique cascade is

involved is the observation that c-Fos, but not Egr1 is induced following ethanol administration (Bachtell et al., 1999; Bachtell and Ryabinin, 2001). Indeed, a unique sequence of intracellular events was observed in our experiments. Thus, we did not find phospho-CREB to be increased at any of the time points analyzed. This indicates that adenylate cyclase activation of protein kinase A, and subsequent phosphorylation of CREB is not involved with ethanol-induced c-Fos expression in EW neurons. Thereby, we do not believe that the Ca/CRE site is responsible for ethanol-induced c-Fos expression in EW neurons. We also detected that Elk1 phosphorylation was not upregulated at any of the time points suggesting that SRE actions are not responsible for ethanol-induced c-Fos expression.

Rather, we observed that serine-727 phosphorylated, but not tyrosine-705 phosphorylated Stat3 was enhanced following ethanol administration. This enhancement occurred at a similar time point to the ethanol-induced elevations in phosphorylation of ERK 1/2. The similar time course in elevations suggests that these may be causally related, however more precise time intervals need to be analyzed to make definite conclusions on the relationship of these two proteins. It does appear, however that ERK 1/2 phosphorylation is necessary for ethanol-induced c-Fos induction in the EW. This is evidenced by significant blockade of ethanol-induced c-Fos expression when the MEK 1/2 inhibitor, SL327, was administered prior to ethanol. Taken together, the data implicate the activation of a MEK 1/2-ERK 1/2 pathway that may phosphorylate Stat3 upon ethanol exposure which culminates in c-Fos expression. Interestingly, a similar pathway

has been observed in tissue culture experiments where it was shown that MEK 1/2 phosphorylation of ERK 1/2 occurs simultaneously with serine-727 phosphorylation, but not tyrosine 705 phosphorylation, of Stat3 (Ceresa et al., 1997).

SUMMARY AND CONCLUSIONS

These findings demonstrate that numerous pharmacological systems are involved in ethanol-induced c-Fos expression in the EW. In addition, we showed that intracellular mechanisms of ethanol-induced c-Fos expression in the EW involve a MEK/ERK/Stat3 pathway. These data provide important information for the development of research tools enabling the functional significance of ethanol-induced c-Fos expression in EW neurons to be further investigated. Specifically, the data gathered in these experiments provide a first glance at the pharmacological and intracellular mechanisms affected by ethanol in EW neurons and lend suggestions of how to directly manipulate the ethanol-induced EW activity. Direct manipulation combined with behavioral analyses of ethanol sensitivity measures (e.g. ethanol-induced hypothermia and loss of righting reflex) and addiction models (e.g. ethanol drinking and conditioned place preference) will allow the determination of the role of EW during ethanol intoxication.

Figure 2. Expression of c-Fos following: (A) Saline/Saline, (B) Saline/2.4 g/kg alcohol, (C) 4 mg/kg bicuculline/2.4 g/kg alcohol, (D) 45 mg/kg pentylenetetrazole/2.4 g/kg alcohol. Quantification of c-Fos expression data from $GABA_A$ antagonist pretreatment of (E) bicuculline and (F) PTZ (n = 4-8/group). Note the robust elevation in c-Fos expression following alcohol administration alone (B), and significant decreases produced by pretreatment of 4 mg/kg bicuculline (C) and 45 mg/kg pentylenetetrazole (D). * significant post-hoc difference from saline ($p < .05$), ** significant post-hoc difference from saline ($p < .01$), *** significant post-hoc difference from all groups ($p < .001$)

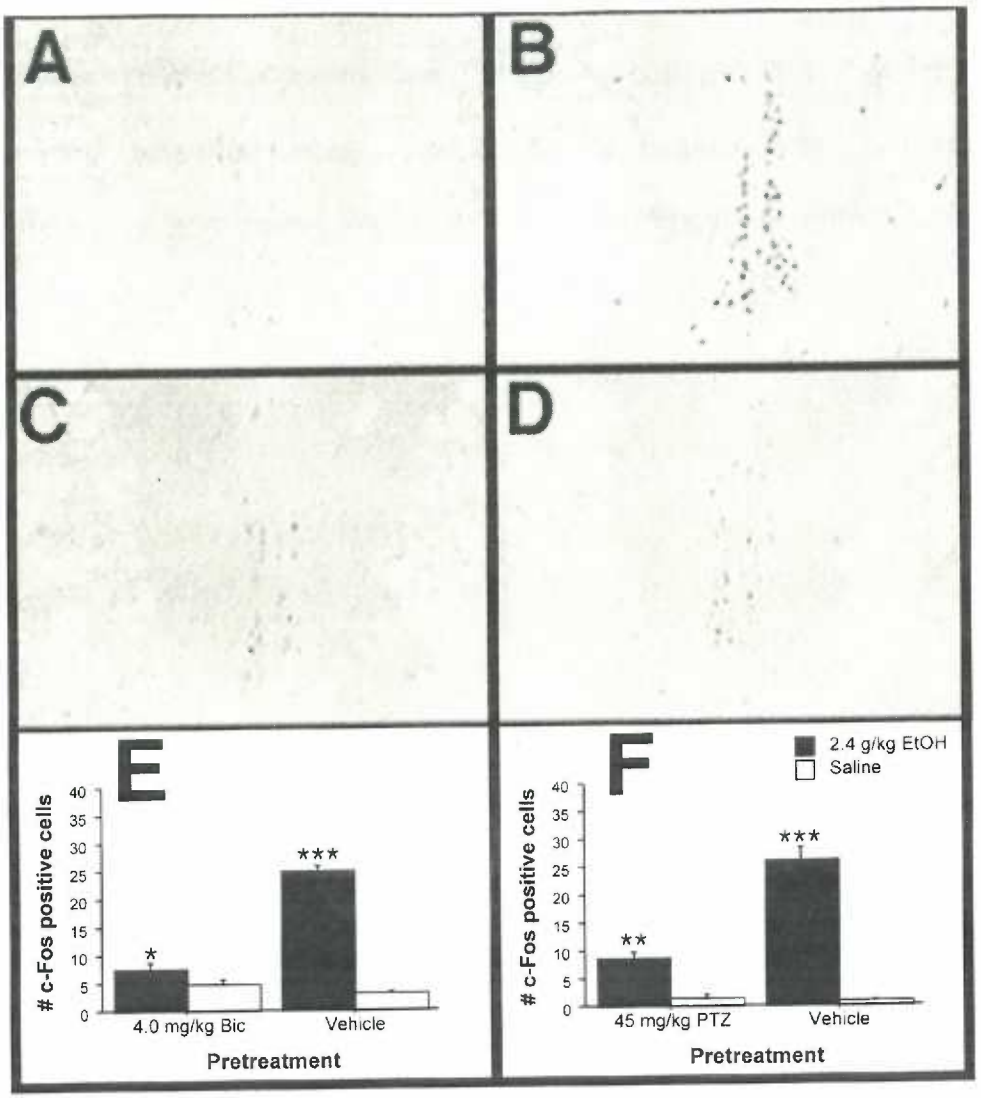

A
B
C
D
E
c-Fos positive cells
40
35
30
25
20
15
10
5
0
*
***
4.0 mg/kg Bic
Vehicle
Pretreatment
F
2.4 g/kg EtOH
Saline
c-Fos positive cells
**
***
45 mg/kg PTZ
Vehicle
Pretreatment

Figure 3. Expression of c-Fos following the administration of positive modulators of the $GABA_A$ receptor. (A) 3α5α-P produced significant increases in c-Fos expression following the 20 mg/kg dose, but not the 10 mg/kg dose ($F(3,16) = 12.764$, $p < .001$). This increase, however, was significantly below elevations produced by 2.4 g/kg alcohol. (B) Similarly, chlordiazepoxide produced significant increases in EW c-Fos expression ($F(4,20) = 32.66$, $p < .001$) that were significantly less than that produced by 2.4 g/kg alcohol. Note: ** significant post-hoc difference from saline ($p < .01$), *** significant post-hoc difference from all groups ($p < .001$)

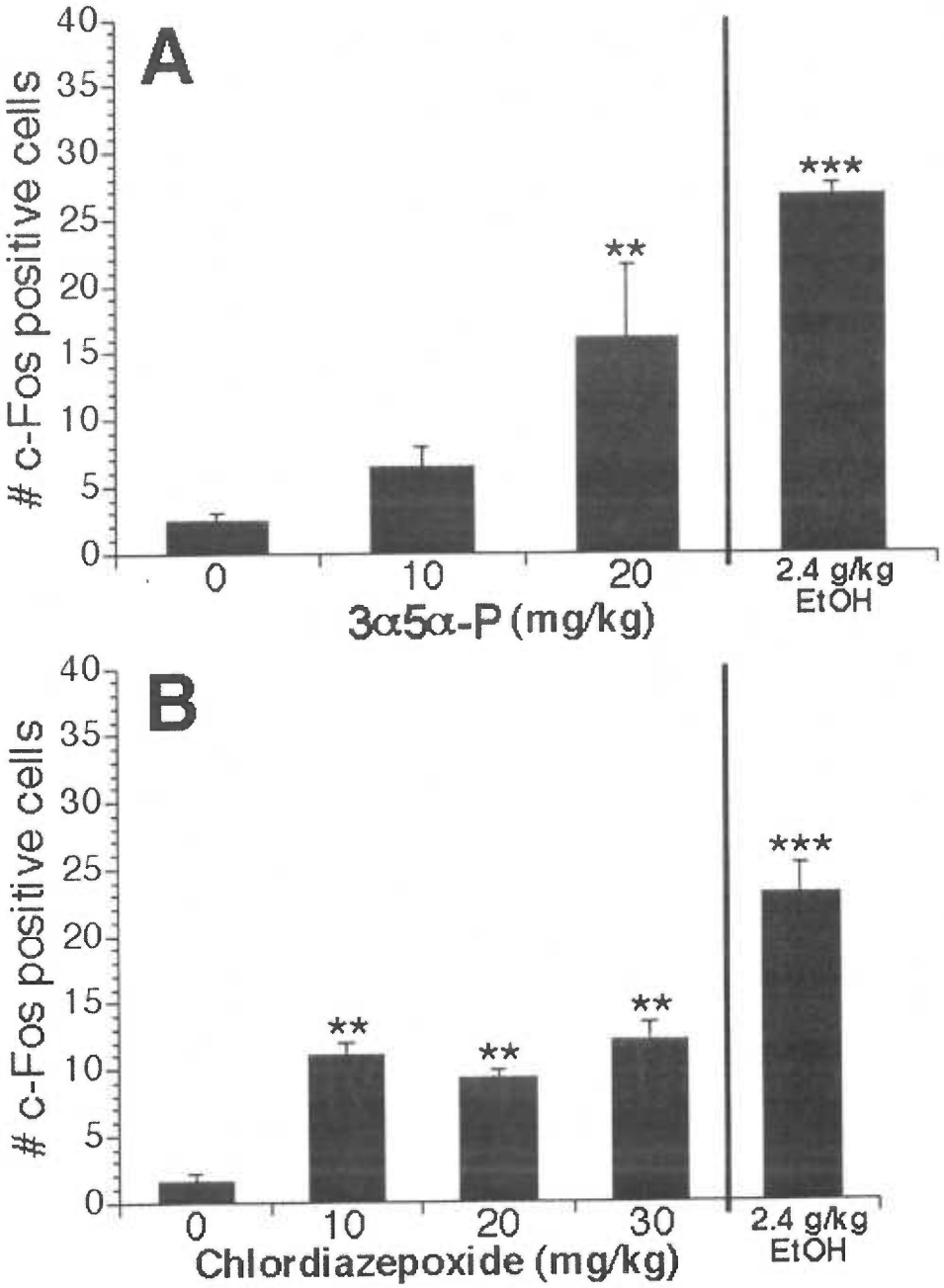

A
c-Fos positive cells
40
35
30
25
20
15
10
5
0
**
***
0
10
20
2.4 g/kg EtOH
3α5α-P (mg/kg)
B
c-Fos positive cells
40
35
30
25
20
15
10
5
0
**
**
**
***
0
10
20
30
2.4 g/kg EtOH
Chlordiazepoxide (mg/kg)

Figure 4. Expression of c-Fos following administration of morphine and alcohol after pretreatment with saline vehicle or 30 mg/kg naltrexone. There was a main effect of morphine administration on EW c-Fos expression ($F(2,18) = 3.84$, $p < .05$), which was significantly blocked with the pretreatment of naltrexone ($F(2,18) = 3.84$, $p < .05$). c-Fos expression in EW resulting from morphine administration was below c-Fos expression levels following 2.4 g/kg ethanol. This effect was not compared statistically because the animals were treated in separate experiments. Notably, however, is that ethanol-induced c-Fos in the EW was not affected by pretreatment of naltrexone. Fisher's PLSD post hoc comparisons: ** significant difference from saline and naltrexone pretreated ($p < .01$)

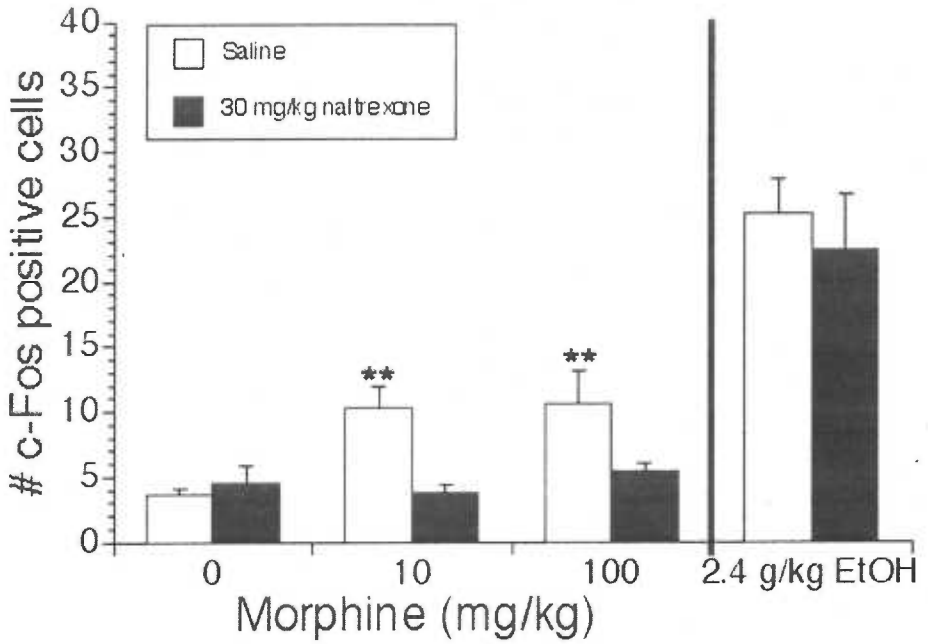

40
35
30
25
20
15
10
5
0
c-Fos positive cells
Saline
30 mg/kg naltrexone
**
**
0
10
100
2.4 g/kg EtOH
Morphine (mg/kg)

Figure 5. Alcohol-induced expression of c-Fos in EW following the pretreatment of adrenoceptors antagonists. (A) Yohimbine significantly blocked alcohol induced c-Fos expression in EW neurons as revealed by a significant interaction between ethanol treatment and yohimbine pretreatment ($F(2,26) = 11.6$, $p < .001$). (B) Propranolol had no significant effect on alcohol-induced c-Fos expression ($F < 1.0$). (C) ARC 2239 had no significant effect on alcohol-induced c-Fos expression ($F < 1.0$). (D) RX821002 significantly blocked alcohol-induced c-Fos expression in EW neurons as noted by a significant interaction between ethanol treatment and RX821002 pretreatment ($F(2,24) = 20.99$, $p < .001$). Expression of c-Fos following 2.4g/kg ethanol and pretreatment with (E) 10 mg/kg ARC 239 and (F) 10 mg/kg RX 821002. Fisher's PLSD post hoc comparisons: *** significant difference from all groups ($p < .001$)

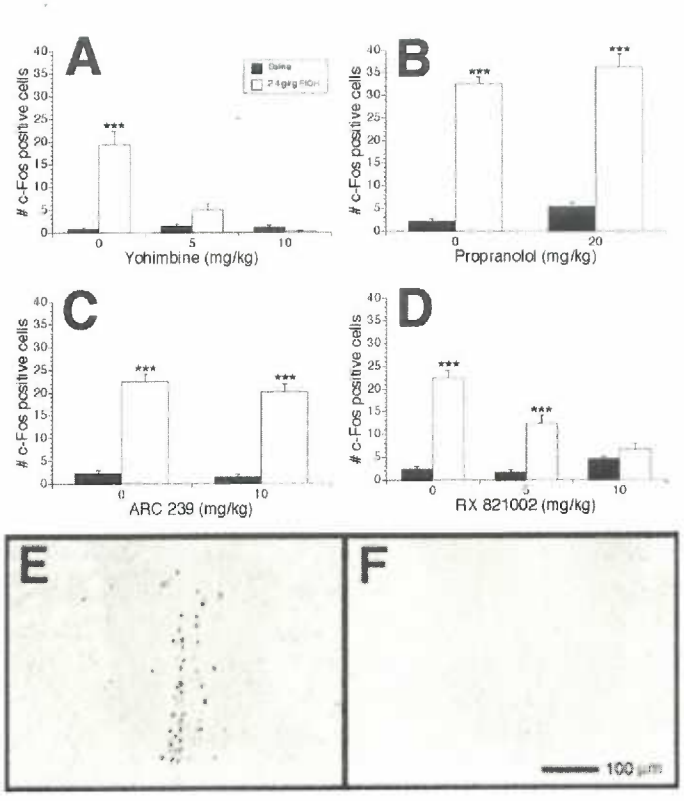

A
B
C
D
E
F
c-Fos positive cells
Yohimbine (mg/kg)
Propranolol (mg/kg)
ARC 239 (mg/kg)
RX 821002 (mg/kg)
***
100 µm

Figure 6. Expression of c-Fos in EW following administration with chlordiazepoxide and morphine is modulated by α_2-adrenoceptors. (A) A significant interaction between chlordiazepoxide treatment and yohimbine pretreatment ($F(1,12) = 7.11$, $p < .05$) demonstrates that chlordiazepoxide-induced c-Fos expression is significantly blocked by pretreatment with 5 mg/kg yohimbine. (B) A significant interaction between morphine treatment and RX821002 pretreatment ($F(1,12) = 96.542$, $p < .001$) indicates that morphine-induced c-Fos expression is significantly blocked by pretreatment with 10 mg/kg RX821002. Fisher's PLSD post hoc comparisons: ** significant difference from all groups ($p < .01$)

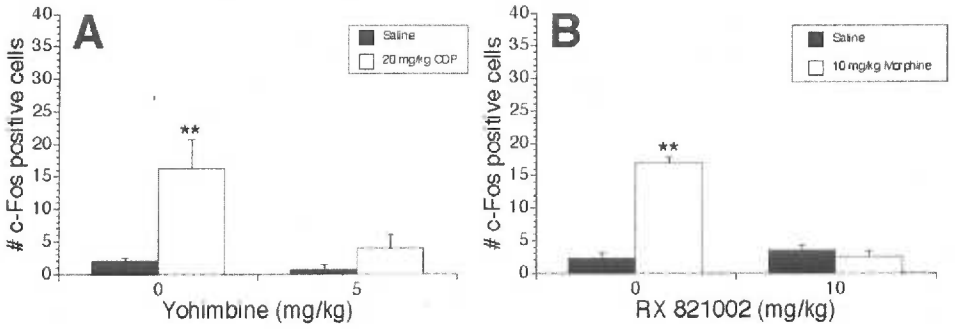

A
Saline
20 mg/kg CDP
c-Fos positive cells
40
35
30
25
20
15
10
5
0
**
0
5
Yohimbine (mg/kg)
B
Saline
10 mg/kg Morphine
c-Fos positive cells
**
0
10
RX 821002 (mg/kg)

Figure 7. A significant interaction between ethanol treatment and haloperidol pretreatment ($F(1,16) = 64.4$, $p < .001$) indicates that haloperidol significantly blocks ethanol-induced c-Fos expression in the Edinger-Westphal nucleus. Fisher's PLSD post hoc comparisons:: *** significant post-hoc difference from all groups ($p < .001$)

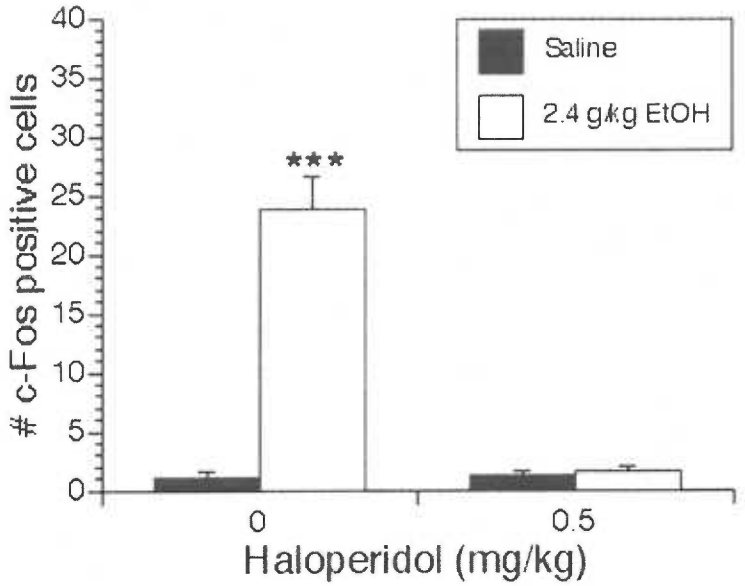

40
35
30
25
20
15
10
5
0
c-Fos positive cells
***
Saline
2.4 g/kg EtOH
0
0.5
Haloperidol (mg/kg)

Figure 8. Administration of 2.4 g/kg alcohol produced robust expression of phosphorylated Elk1 in the cingulate cortex (A) at 15 minutes post-injection. Relatively low levels of expression, however, were observed in Edinger-Westphal neurons at this same time point following 2.4 g/kg alcohol (B). Likewise, robust levels of phosphorylated CREB were detected in the paraventricular nucleus of the hypothalamus (C) 15 minutes after 2.4 g/kg alcohol, while no changes were detected in Edinger-Westphal neurons (D).

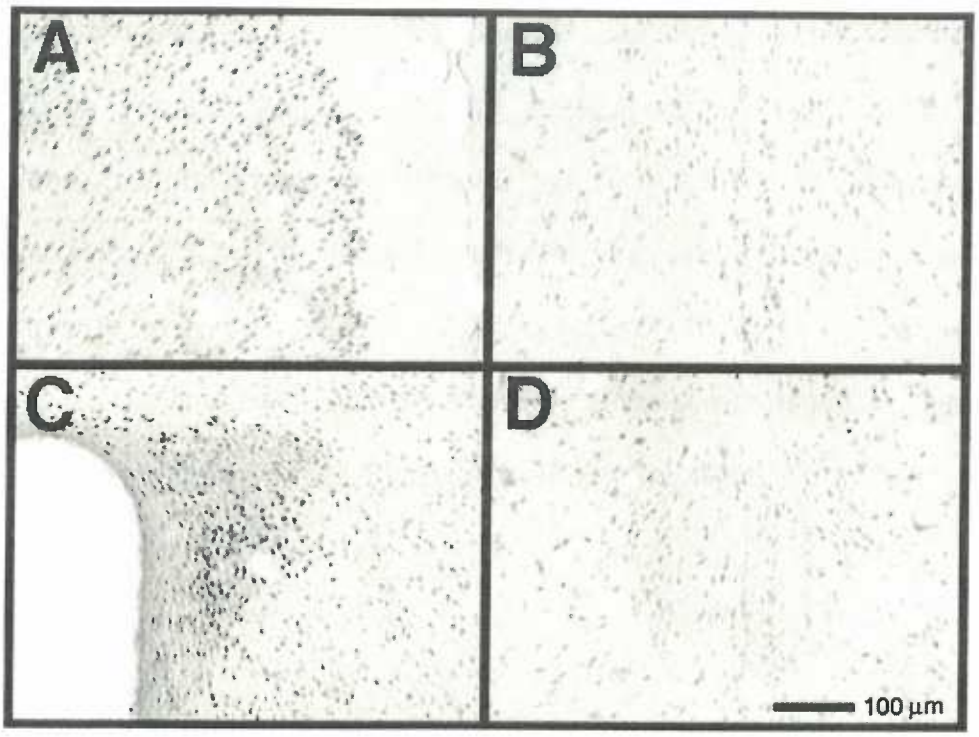

A
B
C
D
100 µm

Figure 9. Significant changes were observed in the expression of serine 727 phosphorylated Stat3 in the Edinger-Westphal as indicated by the significant Time X Ethanol treatment interaction ($F(1,27) = 4.6$, $p < .05$, A). Significant expression changes at the 15-minute time point are shown to the right for the saline (C) and 2.4 g/kg ethanol (D) treated animals. Importantly, no changes were observed in the tyrosine 705 phosphorylated form of Stat3. Small punctate staining (arrows) was observed in the medial amygdaloid nucleus (E) and other regions, however the Edinger-Westphal nucleus was absent of staining (F). A significant Time X Ethanol treatment interaction was also observed in the expression of phosphorylated ERK 1/2 ($F(1,20) = 4.6$, $p < .05$, B) where at 15 minutes post-ethanol, significant elevations were seen. These expression changes between saline (G) and 2.4 g/kg alcohol (H) are shown to the right for the 15-minute time point. Fisher's PLSD post hoc comparisons:: ** significant post-hoc difference from all groups ($p < .01$), *** significant post-hoc difference from all groups ($p < .001$)

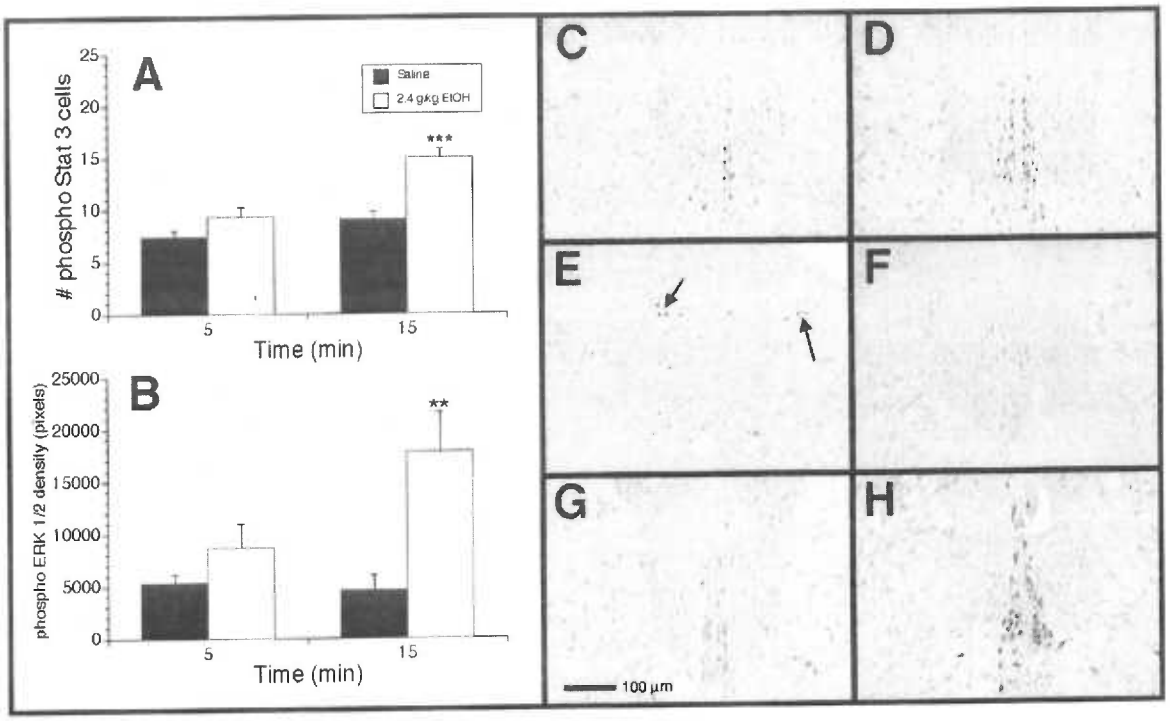
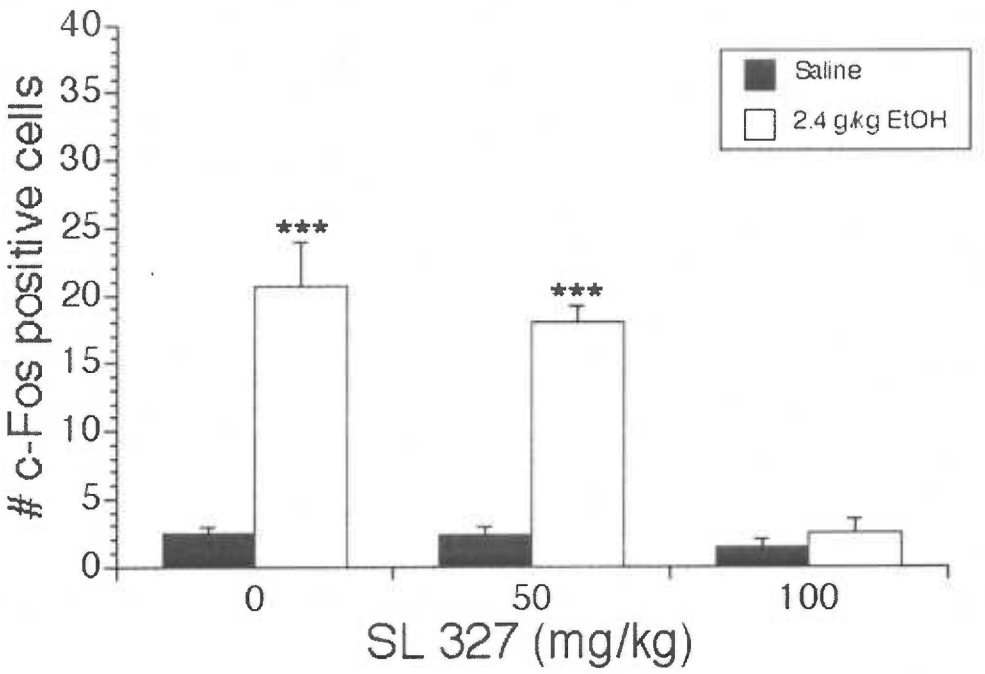

A
Saline
2.4 g/kg EtOH
phospho Stat 3 cells
25
20
15
10
5
0
***
5
15
Time (min)
B
phospho ERK 1/2 density (pixels)
25000
20000
15000
10000
5000
0
**
5
15
Time (min)
C
D
E
F
G
H
100 µm

Figure 10. A significant interaction between ethanol treatment and the MEK 1/2 inhibitor, SL 327, pretreatment ($F(2,18) = 19.21$, $p < .001$) indicates that ethanol-induced c-Fos expression is significantly blocked by pretreatment with 100 mg/kg SL 327. Fisher's PLSD post hoc comparisons: *** significant post-hoc difference from all groups ($p < .001$)


40
35
30
25
20
15
10
5
0
c-Fos positive cells
Saline
2.4 g/kg EtOH
***
***
0
50
100
SL 327 (mg/kg)

CHAPTER 2: Genetic relationship between neurochemical Edinger-Westphal nucleus phenotypes and ethanol-related behavioral measures

The EW is a compact cholinergic brain structure primarily characterized through its involvement in oculomotor adaptation (Westphal, 1887; Warwick, 1954; Roste and Dietrichs, 1988; Burde and Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). However, projections of the EW appear to be too complex to be associated exclusively with oculomotor functioning (Loewy and Saper, 1978; Sugimoto et al., 1982; Vaughan et al., 1995; Bittencourt et al., 1999). Therefore, the EW may be involved in other functions, including thermoregulation, nociception and anxiolysis (Innis and Aghajanian, 1986; Smith et al., 1998; Weninger et al., 2000). This idea is supported by preferential expression of the CRF family member, urocortin (Ucn), in the EW of rodents (Vaughan et al., 1995; Yamamoto et al., 1998; Bittencourt et al., 1999; Morin et al., 1999; Weninger et al., 2000).

The CRF neuropeptide family plays major roles in generating endocrine, physiological and behavioral responses to stressful environmental and internal events through stimulation of the hypothalamic-pituitary-adrenal (HPA) axis. CRF is a 41-amino acid polypeptide that was first isolated from ovine hypothalamus by Vale and colleagues (1981). CRF exists in numerous peripheral organs and throughout the central nervous system. Its distribution in the brain is quite extensive with major sites of expression in the paraventricular nucleus of the hypothalamus, amygdala, the bed nucleus of the stria terminalis, and numerous autonomic brainstem regions (Sawchenko et al., 1993). CRF acts as a

neurotransmitter in the brain where it integrates autonomic, behavioral and immunological responses to form a tightly regulated and unified stress response (Menzaghi et al., 1993; Sawchenko et al., 1993; Koob et al., 1994; Arborelius et al., 1999). In the hypothalamus, for example, CRF initiates activation of the HPA axis by stimulating release of adrenocorticotropin hormone in the anterior pituitary gland (Turnbull and Rivier, 1997). It is becoming more apparent, however, that CRF is not the only regulator of autonomic processes in the stress response and it only represents a portion of a complex system.

The structurally related CRF peptides, urotensin and sauvagine, which were identified in fish and amphibians, were originally thought to be non-mammalian forms of CRF (Montecucchi et al., 1980; Lederis et al., 1982). However, the identification of CRF analogues in fish and amphibians suggested that there were additional members of this family (Okawara et al., 1988; Stenzel-Poore et al., 1992). In 1995, the 40-amino acid peptide, Ucn, was isolated from the rat EW. This peptide has a 63% identity to fish urotensin I, 45% sequence identity to rat CRF and 35% identity to sauvagine (Vaughan et al., 1995). In 2001, the search for additional members of the CRF family resulted in the cloning of two additional members. Thus, stresscopin related peptide and stresscopin were isolated from human cDNA, while structurally similar peptides Ucn II and Ucn III, respectively, were isolated from mouse cDNA (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001).

Actions of the CRF/Ucn family are mediated by corticotropin-releasing hormone receptor I (CRH-R1) and corticotropin-releasing hormone receptor II

(CRH-R2) G-protein coupled receptors (Chang et al., 1993; Chen et al., 1993; Perrin et al., 1993; Vita et al., 1993; Kishimoto et al., 1995; Lovenberg et al., 1995; Perrin et al., 1995; Stenzel et al., 1995). These receptors are both positively coupled to adenylate cyclase and are distinguished functionally by their differential expression in the brain (Chalmers et al., 1996). The presence of a CRF binding protein, which binds CRF ligands with higher affinity than the CRF receptors, acts as an inducible inhibitor of CRF ligand activity at CRF receptors (Potter et al., 1991; Lowry, 1993; Petraglia et al., 1993; Behan et al., 1995; Kemp et al., 1998). Interestingly, the urocortins possess the highest affinities for CRF receptors. Characterization of binding affinities *in vitro* shows that Ucn > CRF >> Ucn II = Ucn III for CRH-R1 while Ucn = Ucn II > Ucn III > CRF for CRH-R2 (Vaughan et al., 1995; Lewis et al., 2001; Reyes et al., 2001).

More important than receptor affinity for functional activity, however is the coexistence of receptors and ligand in similar regions. CRH-R1 is primarily expressed in the cerebral cortex, medial septum, cerebellar and sensory relay centers, while CRH-R2 is localized to the lateral septum, olfactory bulb, ventromedial hypothalamus, the bed nucleus of the stria terminalis and brain stem nuclei (Chalmers et al., 1996; Van Pett et al., 2000). CRF, Ucn II and Ucn III are expressed throughout the brain with abundant expression in the hypothalamic and amygdalar structures (Sawchenko et al., 1993; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001). Ucn, on the other hand, is very discretely expressed in the brain, with a preferential expression in the EW and lower levels of expression occurring in the lateral superior olive and

numerous brain stem nuclei (Vaughan et al., 1995; Wong et al., 1996; Kozicz et al., 1998; Yamamoto et al., 1998). Both ascending and descending Ucn projections from the EW represent targets bearing CRH-R2 receptors, including the primary forebrain projection to the lateral septum and the descending projections to the brain stem and spinal cord (Vaughan et al., 1995; Bittencourt et al., 1999). The significance of preferential Ucn expression in EW neurons and the limited projection regions is unknown.

C57BL/6 (B6) and DBA/2 (D2) inbred strains of mice have been compared for their HPA axis response to ethanol administration (Roberts et al., 1992). It was shown in this report that release of adrenocorticotropin hormone and CRF upon acute ethanol administration were higher in D2 mice compared with B6 mice. These two strains of mice are also known to differ in many aspects of normal and drug-induced behavior, including sensitivity to ethanol, cocaine, anxiolytics and other drugs, which is inferred to represent genetic differences between the strains (Crawley et al., 1997). Identification of neuroanatomical and neurochemical differences between these inbred strains is important for understanding differences in their behavior and further understanding the role of the EW in ethanol-mediated responses. The B6 and D2 strains of mice differ in several neuroanatomical features, but their systematic comparison has only recently begun (Rosen and Williams, 2001). A strain comparison of the EW has never been performed.

The differences between B6 and D2 inbred strains are vast and they have therefore been the favored strains to study traits related to ethanol

reinforcement/aversion and ethanol sensitivity. It is well documented that B6 mice willingly ingest large quantities of ethanol compared with D2 mice, even when given a water alternative suggesting that these inbred strains may possess inherent differences in the reinforcing effects of ethanol (McClearn and Rodgers, 1959). The Pavlovian model of drug reward, conditioned place preference, however, has provided evidence contrary to this hypothesis. Using a place-conditioning model, D2 mice display preferences for environments previously paired with ethanol, while B6 mice display no preference for the ethanol-paired environment (Cunnigham et al., 1992). Other data also calls the notion of a B6/D2 distinction surrounding the rewarding properties of ethanol into question. Graham and Cunningham (Grahame and Cunningham, 1997) demonstrated that while B6 mice self-administered slightly more ethanol during early training, both strains achieved equivalent response levels and preference for the side resulting in ethanol infusion during the later stages of the experiment. Analysis of the aversive properties of ethanol coincides closer with the dichotomous relationship between the B6 and D2 strains of mice on oral ethanol consumption. Thus, using a conditioned taste aversion procedure, D2 mice developed aversions to ethanol-paired flavors at lower doses than B6 mice, suggesting that D2 mice are more sensitive to the aversive properties of ethanol (Risinger and Cunningham, 1995).

A comparison of the two strains on ethanol sensitivity measures also reveals their different responsiveness on ethanol traits. Low doses of ethanol administered acutely possess locomotor activating effects in D2 mice, but not in B6 mice under most test conditions (Crabbe et al., 1982). Higher doses of

ethanol administered acutely produce motor impairments in mice. A comparison of the B6 and D2 strains reveals that B6 mice tend to develop greater motor impairment compared with D2 mice when using the grid test, which is a measure of locomotor ataxia (Belknap and Deutsch, 1982; Phillips et al., 1996). Ethanol-induced hypothermia and sedation are two responses seen following even higher doses of ethanol. Comparisons between B6 and D2 mice have been made for these responses, however the results prove to be quite inconsistent. In some cases, B6 and D2 mice possess similar durations of the loss of righting reflex (Alkana et al., 1988; Dudek and Phillips, 1990), while in other instances the strains differed with D2 mice being more sensitive (Belknap et al., 1972; Spuhler et al., 1982; Phillips and Dudek, 1989). Ethanol-induced hypothermic responses are similarly inconsistent. No strain differences in temperature loss have been observed (Alkana et al., 1988), while other reports demonstrate that the B6 strain is more sensitive to ethanol-induced hypothermia compared to the D2 strain (Crabbe et al., 1994b; Broadbent et al., 2002).

Given the differences between the B6 and D2 strains using several measures of ethanol sensitivity we hypothesized that the structure and ethanol-sensitivity of EW neurons would also differ between these strains. To address this hypothesis, Ucn expression and c-Fos induction in the EW were compared in both ethanol-treated and ethanol-naïve B6 and D2 mice. Results of these experiments led to the hypothesis that differences in the EW between B6 and D2 mice are manifest in behaviors related to ethanol intoxication. This hypothesis was addressed by generating heterogeneous populations of B6D2 F2 mice and

subsequent correlational analyses relating differences in EW Urocortin with several measures of ethanol sensitivity and ethanol drinking.

MATERIALS AND METHODS

Animals and General Procedures.

B6, D2 and B6D2 F1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ethanol sensitivity measures were made in a population of B6D2 F2 mice generated from B6D2 F1 mice. Eight litters consisting of 30 male and 40 female B6D2 F2 mice were used for these experiments. A separate population of B6D2 F2 intercross mice was used for the ethanol drinking procedures. These mice were generously provided by J.K. Belknap (Oregon Health & Science University, Portland, OR). Ten B6D2 F2 litters (n=85 mice, male=42, female=43) were generated from 10 pairs of B6D2 F1 breeders. All animals were 8-10 weeks of age at the beginning of each experimental procedure. Animals were maintained on a 12 h light-dark cycle with lights on beginning at 6 a.m. Water and food were available ad libitum throughout the experiment in the home cage. All animal procedures were in accordance with National Institutes of Health guidelines.

Acute ethanol procedures.

In order to identify the optimal doses for c-Fos expression in EW, animals from both the B6 and D2 strains were administered several doses of ethanol (saline, 0.6, 1.2, 1.8, 2.4, 3.6, and 4.8 g/kg, i.p.). Ethanol was prepared as a 20% v/v

solution in 0.9% saline. Four to eight animals were included in each dose group. All animals were habituated to handling and injections for four days prior to the test day. During the habituation procedures animals were exposed to one saline injection per day (approx. 0.4 ml/injection). On the final day, animals were injected with the appropriate dose of ethanol, placed back into the home cage and sacrificed 100 min later by CO_2 inhalation for brain removal. This time point was chosen as a peak time for c-Fos expression in neurons after different types of stimulation (Morgan and Curran, 1989). In the double-labeling experiment, identical habituation and treatments were performed except only saline and 2.4 g/kg doses of ethanol were used.

Repeated ethanol procedures.

Twelve mice of each strain were divided into three groups (n = 4/group). The first group received 14 days of saline injections. The second group received 14 days of 2.4 g/kg i.p. ethanol. The third group received 13 days of saline injections followed by an acute injection of 2.4 g/kg i.p. ethanol on day 14. On the final day, animals were injected with either ethanol or saline, placed back into the home cage and sacrificed 100 min later by CO_2 inhalation for brain removal.

Blood Ethanol Measurement

Trunk blood was collected in microcentrifuge tubes upon sacrifice and placed on ice. All samples were immediately centrifuged at 14,000 rpm for 20 minutes. Serum was collected and stored overnight at $-20^{O}C$. Blood samples (3 µl) from

individual animals were used to assess NAD-ADH reactivity with the spectrophotometric NAD-ADH Detection System (Sigma Diagnostics, St. Louis, MO, USA). A set of 10 standards ranging from 10-3000 mg/dl was run periodically to assess the accuracy of the NAD-ADH Detection system.

Sensitivity measurements in B6D2 F2 mice.

All B6D2 F2 offspring were tested for various ethanol related phenotypes. Mice were first tested for ethanol-induced locomotion and anxiolysis using a five-day procedure. During the procedure, animals were habituated to the saline injections and activity chambers during the first four days. On day 5, all animals were injected with 1.8 g/kg ethanol (i.p.) dissolved in saline (20% v/v). This dose of ethanol has previously been shown to increase locomotor activity when administered acutely. On all days, animals were placed into dimly lit activity chambers enclosed in sound-attenuated boxes two minutes following the injection. Activity was assessed by an automated photobeam system that included a 10 X 12 array of photocells, situated 1 cm off the floor, surrounding a 21 X 25 X 18 cm Plexiglas arena. Activity was assessed in five-minute intervals for a total of 15 min. The distance traveled during each interval was used as a measure of activity. For a more detailed analysis, the arena was separated into 60 equal zones, 48 of which were located next to the arena wall and defined as the peripheral region. Thus, activity was divided into both peripheral and central activity. Central activity includes consecutive beam breaks across both horizontal dimensions in one of the 12 central zones. Based on this division of activity, a

ratio of central to total activity was created and used as a generic model to assess anxiety differences. This model is based on the observation that during an animal's first exposure to a novel environment a majority of their activity occurs in the peripheral regions of the arena (i.e. close to the wall), which is referred to as thigmotaxic behavior. While this measure does not provide the best measure of anxiety because the test environment was not designed to be maximally anxiogenic, it does allow for efficient modeling using minimal behavioral measurement.

Five days following locomotor activity testing, all animals were tested for the sedative and hypothermic effects of ethanol. To evaluate these effects, animals were first assessed for baseline rectal temperatures immediately followed by an injection of 3.6 g/kg ethanol (i.p.). The time of loss of righting reflex (LORR) onset and the duration of LORR was recorded for each animal. Upon return of righting reflex, defined as two rightings within 30 sec, each animal's rectal temperature was again measured (Finn et al., 1990). Thus, depending on the duration of the LORR, rectal temperatures of each animal were measured at different intervals following the injection of ethanol. Mice were sacrificed by cervical dislocation and brains extracted 30-32 hours following the LORR/hypothermia assessment. Trunk blood was collected during sacrifice for BEC measurements as previously described.

Several dependent variables resulted from these behavioral assessments. Locomotor activity testing was analyzed using a difference score calculated by subtracting the baseline (averaged activity during habituation on Day 3 and 4)

from ethanol-induced activity (Day 5). Additionally, locomotor activity testing allowed us to assess initial anxiety (Day 1) and the degree of anxiolysis produced by ethanol (Day 5) by calculating a ratio of central activity to total activity. The distribution of this measure was strongly positively skewed. That is, measures generally congregated in the low percentages suggesting that very little activity occurred in the center regions compared to the peripheral regions. This measure was logarithmically transformed to comply with assumptions of parametric statistics. Behavioral responses following the high dose of ethanol include: baseline rectal T (°C), time until LORR (min), duration of LORR (min), ethanol-induced rectal T (°C) and ethanol-induced T change from baseline (°C). As an additional variable, body weight (g) used to calculate dosages were also included in the analysis.

Ethanol drinking in B6D2 F2 mice

A separate population of ethanol-naive B6D2 F2 mice was used in these analyses. Data in this population were collected from two cohorts of mice (Cohort 1 n=37, Cohort 2 n=48). Both cohorts were exposed to a two-bottle drinking procedure followed by a limited-access drinking procedure. To control for possible Ucn expression changes produced by exposure to ethanol, 10 control subjects were included in Cohort 2 that received no ethanol at any time during the two procedures. The experiment was initiated when animals were individually housed in metal hanging racks. On the first through the fourth day of the experiment, animals were given continuous access (24-h) to two 25 ml cylinders

(one containing tap water and one containing 3% ethanol in tap water). On the fifth through eighth and ninth through twelfth day of the experiment, the ethanol concentration was 6% and 10%, respectively. Control animals from Cohort 2 received two bottles of tap water throughout the experiment. Body weights and fluid consumption (tenths of ml) from both cylinders were recorded daily at 8 AM (one hour after light onset). Bottle positions were alternated daily to avoid development of a position preference. Both preference measures (ml ethanol consumed/ ml total fluid consumed) and consumption measures (g/kg/day) were calculated and used as dependent variables. Mice were then given a four-day break from ethanol, at which time only one bottle of tap water was provided 24 hours a day. On the fifth day following the end of the two-bottle procedure, animals were exposed to a limited access ethanol/sucrose drinking procedure. Mice were initiated into the procedure with a 22 hour-water deprivation phase. They were then allowed access to a 20% sucrose solution for 2 hours. This was designated as day 1. During the next 3 days, access to the sucrose solution was gradually decreased from 2 hours to 30 minutes, and access to water was increased from 0 to 20 hours. On the fifth day, the sucrose solution was changed to 10%. Starting on this day, the schedule of access to drinking solution and procedures was maintained as follows: access to tap water was from 10:00 AM to 8:00 AM; from 8:00-9:00 AM, bottles removed and body weight measured; from 9:00-9:30 AM, mice had access to the test solutions. During the next 9 days, ethanol was then gradually added to the 10% sucrose solution at increasing concentrations (3%, 6%, and 10%) for 3 days each. The control

animals in Cohort 2 were maintained on a 10% sucrose solution during the 30-minute access session. The inclusion of sucrose in the 30-minute limited access procedure was necessary to provide additional incentive for animals to consume the solution in a non-deprived state. Mice were sacrificed by cervical dislocation and brains extracted 30-32 hours following the final drinking session. Mice in this experiment were not euthanized by CO_2 overdose because of the necessity to process many animals in a short amount of time and the potential for CO_2 exposure to alter Ucn levels. Ucn immunohistochemistry was performed as outlined below.

Histology and Immunohistochemistry.

Dissected brains were postfixed overnight in 2% paraformaldehyde in 10 mM phosphate-buffered saline at pH 7.4 (PBS), cryoprotected with 30% sucrose in PBS and sectioned coronally on a cryostat. Histological thionine staining was performed on mounted 20 µm slices, while Neutral Red staining was performed on 40 µm slices (McManus and Mowry, 1960). Immunohistochemistry was performed on 40 µm floating slices according to previously published protocols (Ryabinin and Wang, 1998). Immunohistochemistry for c-Fos was initiated by inhibiting endogenous peroxidase activity with a pretreatment of 0.3% hydrogen peroxide. Blocking was performed with 4% goat serum. Rabbit polyclonal antibodies against amino acids 210-335 of human c-Fos protein that are not cross-reactive with FosB, Fra-1 and Fra-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used in a dilution of 1:10,000. The immunoreaction was

detected with the Vectastain ABC kit (Vector Laboratory, Burlingame, CA, USA). Enzymatic development was performed with the Metal Enhanced DAB kit (Pierce, Rockford, IL, USA). Similarly, Ucn immunohistochemistry was initiated with the inhibition of endogenous peroxidase activity by 0.3% peroxide in PBS. Blocking was performed with 4% horse serum. A goat polyclonal antibody against an epitope at the carboxy terminus of rat Ucn (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:10,000. Biotinylated anti-goat secondary antibody was used to detect the primary antibody (Vector Laboratory Inc., Burlingame, CA). The immunoreaction was detected with the Vectastain ABC kit (Vector Laboratory Inc.) and enzymatic development was accomplished with the Metal Enhanced DAB kit (Pierce, Rockford, IL). Ucn-positive cells were topographically identified as EW according to the brain atlas (Franklin and Paxinos, 1997). However, the identity of peptidergic neurons in the EW proper with cells projecting to the ciliary ganglion in non-primate mammals remains to be determined (Erichsen and May, 2002). Identical procedures were used for acetylcholinesterase (AChE, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), substance P (1:1000, Sigma), neuropeptide Y (1:1000, AbCam), tyrosine hydroxylase (1:1000, Chemicon, Temecula, CA), glutamate aldehyde dehydrogenase 67 (GAD 67, 1:1000, Sigma), and GABA (1:1000, Sigma) immunohistochemistry. Dilutions of antibodies were determined in preliminary experiments.

Double Immunohistochemistry.

Double immunohistochemistry was initiated as described above for c-Fos immunohistochemistry with the exception of 4% horse serum being used for blocking. Following the first reaction and DAB staining, unbound Avidin and Biotin resulting from initial reactions were blocked using the Avidin/Biotin Blocking kit (Vector Laboratories, Burlingame, CA). Sections were then incubated for 4 hours in 4% horse serum (Vector Laboratories, Burlingame, CA), followed by incubation with the goat anti-Ucn primary antibody at a dilution of 1:100,000. All remaining steps are identical to that identified above except that staining of the Ucn immunoreactivity was performed with Vector VIP staining kit (Vector Laboratories, Burlingame, CA). To confirm specificity of the staining parallel sections were taken through the identical procedure with omission of either the c-Fos- or the Ucn-specific antibodies.

Data Analysis.

Quantitative image analysis for c-Fos and Ucn immunohistochemistry was performed using a system consisting of an Olympus microscope BX40 and Sony CCD IRIS/RGB video camera connected to a Power PC. Each digitized video image was analyzed using NIH image 1.62 software. Nuclear c-Fos labeling was detected using a threshold normalization procedure, where neighboring areas with no immunoreactivity were adjusted to contain no positive signals. Remaining grains in a size range from 7 to 50 pixels were automatically counted. All counting was performed by the same individual, blind to the experimental group

of the analyzed animal. The value for a single matched region was summed and used as a single data point for statistical analysis using a one-way ANOVA. Control for variations in brain area size were made based on careful slice selection with strong consideration of their location detailed by Franklin and Paxinos (Franklin and Paxinos, 1997). It is believed that this methodology controls for issues in cell counting and is thus an unbiased method (Saper, 1996). For analysis of some brain regions, we were not able to match sections from all thirty animals to the same location. In such situations, counts in this brain region for this animal were not performed. Therefore, the actual number of animals per group analyzed for c-Fos expression was 7-10.

Ucn-positive cells in EW (-3.20 to –3.60 mm from Bregma) were counted manually by an experimenter blind to the experimental conditions, due to the software's inability to present accurate counts of overlapping cells. Colocalization was also assessed manually by counting c-Fos positive nuclei, Ucn-containing cells and the occurrence of c-Fos positive nuclei localized within Ucn-containing cells. Ucn expression measures were performed using an automated system on the NIH Image 1.62 software. Each digitized image was adjusted for equal background by subtracting out noise signals. Each image was calibrated according to a standard optical density curve. The average optical density was then generated and recorded. Measurements of the number of Ucn cells per brain slice and optical density of Ucn in EW were performed on 3-8 sections per animal and averaged for each animal providing a single data point per animal used in the ANOVA. Due to differences in staining intensity among

immunohistochemical reactions, exact measures of immunoreactivity were not compared between experiments. Therefore, all statistical comparisons were made only on sections processed simultaneously. Differences between individual groups were evaluated post-hoc using the Fisher test. Effects with $p<0.05$ were considered statistically significant.

RESULTS

Neurochemical characterization of the Edinger-Westphal nucleus in B6 mice

The localization of several neurotransmitters and neuropeptides was performed using immunohistochemistry (Table 1). It was observed that the primary cell type within the EW, as indicated by the highest intensity of immunoreactivity, was the neuropeptide, Ucn. The immunoreactivity for Ucn appeared as two columns of large cell bodies lying laterally to midline (Figure 11). Using AChE as a marker for acetylcholine-containing cells it was observed that a moderate number of AChE-positive large cell bodies were contained within the EW. Further characterization revealed that tyrosine hydroxylase, which is a marker for both noradrenaline and dopamine, was contained within many processes and few cell bodies surrounding the EW (Figure 11). Fewer numbers of processes were observed with antibodies for Neuropeptide Y and Substance P. Using an antibody for GAD 67, which can be a marker for GABAergic cells, no immunoreactivity was detected. Likewise, an antibody for GABA also revealed no immunoreactivity.

Comparison of Ucn immunoreactivity in the Edinger-Westphal nucleus of B6 and D2 mice

Because the preliminary neurochemical characterization was performed only in B6 mice and because the goal of this dissertation was to ascertain the role of ethanol's activity in the EW, a comparison of the EW was made between the B6 and D2 strains of mice, two strains known to differ markedly on ethanol-related phenotypes. Analysis of Ucn immunoreactivity in brain sections encompassing Bregma levels –0.87 to –4.05 mm in D2 and B6 mice (n=4 per strain) confirmed preferential expression of Ucn in the EW of both strains. Specific immunoreaction of the sera was confirmed by lack of immunoreactivity in CRF-rich regions of paraventricular nucleus of hypothalamus and the central nucleus of amygdala (data not shown). Immunoreactivities in other brain regions were also on the level of background. Omission of primary antibody led to lack of immunohistochemical staining in the EW. Importantly, Ucn expression appeared substantially lower in the EW of D2 as compared to B6 mice based on the number of immuno-positive cells (Figure 12). As can be seen, the number of Ucn-positive cells was significantly different between the two strains ($F(1,14)=97.0$, $p<0.001$).

Neuroanatomical characterization of the Edinger-Westphal nucleus in B6 and D2 mice

To confirm that the lower number of Ucn immunoreactive cells in EW of D2 mice is not only due to lower expression of this protein but is indeed

characterized by lower number of cells, four mice per strain were analyzed for EW cellular structure using Thionine staining. Cells were quantified based on cell size (μm^2). Thus, cells were separated into one of six bins (Table 2). Each bin consisted of 50 μm^2 cell sizes and strain comparisons were made both within and between each bin. Cell sizes equal to or below 50 μm^2 contained fragments of cells and were therefore not included in the analysis. Cell sizes greater than 300 μm^2 represented clusters of cells and were included in the analysis. Statistical comparisons show that D2 mice generally have lower cell numbers ($F(1, 36)=11.7$, $p=0.0016$). However, this effect was masked by the interaction of cell size and strain ($F(5, 36)=7.5$, $p<0.0001$). This interaction reflected that D2 mice had higher numbers of small cells while B6 mice had higher numbers of larger cells and clusters. Visual confirmation of the structural differences of EW between the strains was obtained using Neutral Red staining and immunohistochemical analysis of AChE expression as a marker of cholinergic neurons (Figure 13).

Dose-response relationship of c-Fos expression in the Edinger-Westphal nucleus of B6 and D2 mice

To investigate whether differential structure of the EW in B6 and D2 mice is reflected in differential sensitivity to ethanol, a range of doses from 0.6 to 4.8 g/kg (N=4-8 per dose per strain) was tested for their ability to induce c-Fos immunoreactivity in both strains. Surprisingly, strains were comparable in the expression of c-Fos across all doses (Figures 14 and 15). Results revealed that

c-Fos expression in EW neurons is dependent on the administered dose of ethanol ($F(6,65) = 34.46$, $p < .001$) with no significant effects of strain or strain by dose interaction ($p>0.05$). Although elevations noted at the 0.6 g/kg dose of ethanol did not reach statistical significance, significant elevations in c-Fos expression were seen at 1.2 g/kg. Expression continued to increase until an asymptotic level was reached for both strains at 2.4 g/kg.

Colocalization of c-Fos and Ucn in Edinger-Westphal nucleus neurons

Differential number of Ucn-positive, but not c-Fos-positive cells, in B6 and D2 mice suggests that c-Fos expression is expressed in a different population of cells in EW of the two inbred strains. We confirmed this finding using immunohistochemical double-labeling studies. Analysis of colocalization results revealed that in B6 mice 95 ± 2.8% of c-Fos-positive nuclei were contained within Ucn cells. In contrast, because of the lower number of Ucn-positive cells in D2 mice, only 36 ± 7.8% of c-Fos-positive cells in D2 mice was colocalized with Ucn cells (Figure 16 and 17). Statistical analyses revealed significant differences between the strains in number of Ucn-expressing cells and in the percentage of cells with c-Fos and Ucn showing colocalization ($F(1,6) = 20.69$, $p < 0.004$ and $F(1,6) = 51.04$, $p < 0.001$, respectively). As noted with the dose response curve, strain differences were not seen in the number of c-Fos positive cells ($p > 0.05$)

Effects of acute and repeated ethanol administration on c-Fos and Ucn expression in the Edinger-Westphal nucleus

Based on previous data demonstrating that c-Fos expression remains elevated during extended ethanol self-administration (Topple et al., 1998; Bachtell et al., 1999; Ryabinin et al., 2001; Weitemier et al., 2001), we tested whether repeated injections would produce similar persistent c-Fos expression in EW neurons. Overall, there was a significant effect of treatment ($F(2,18) = 172.82$, $p < .001$) on c-Fos expression in the EW, but no effect of strain or treatment by strain interaction ($p > 0.05$). As was expected, c-Fos expression in the repeated ethanol group was significantly elevated after 14 days of ethanol administration. However, this expression was significantly decreased from acutely administered ethanol (Figure 18). Blood ethanol levels at time of sacrifice were significantly lower in the group receiving repeated ethanol (147 ± 11.69 mg/dl) compared with acutely treated animals (186.34 ± 9.27 mg/dl, $F(1,12) = 7.09$, $p < .03$), which may have contributed to the lower levels of c-Fos expression. To investigate whether acute or repeated ethanol administration could change the number of Ucn cells, parallel sections were quantitatively analyzed for Ucn expression (Figure 19). Once again, D2 mice had significantly lower numbers of Ucn cells than B6 mice ($F(1,17) = 56.50$, $p < .001$). The main effect of treatment and the interactive effect of treatment and strain were not statistically significant ($p > 0.05$) arguing against regulation of Ucn by ethanol. Sections from two animals per strain from the repeated ethanol group were analyzed for c-Fos/Ucn colocalization with similar results after acute ethanol administration (i.e. c-Fos was expressed primarily in Ucn-expressing cells in EW of B6 and in a mixed population of cells in EW of D2 mice – data not shown).

Ethanol sensitivity measures in the B6D2 F2 intercross mice and the relationship to Ucn in the Edinger-Westphal nucleus

B6D2 F2 mice were used as a heterogeneous population of mice predicted to vary on several ethanol-related phenotypes and Ucn-expression cells in EW. We obtained several distributions of data representing various ethanol sensitivity phenotypes in these mice (Figure 20). For the most part, performance on the various measures was consistent with previous studies using B6F2 populations. Thereby, varying degrees of hypothermic responses, LORR, and ethanol-stimulated activity were observed. The central/total activity, which was used to measure anxiety-like behavior, was positively skewed and was therefore logarithmically transformed for correlation analyses. In addition, the measure of central/total activity following ethanol administration was not included in the analyses given that habituated animals likely perceive the environment as familiar and not anxiety provoking.

Using these data sets, we correlated all behavioral phenotypes with the level of Ucn-expressing cells in EW (Table 3). The results of these analyses present suggestive evidence to support a behavioral manifestation for the differing levels of Ucn-expressing cells in B6 and D2 mice as well as the expression of ethanol-induced c-Fos in different populations of cells between the strains. In particular, the hypothermic response to ethanol was significantly correlated with the number of Ucn-expressing cells in the EW. In addition, baseline temperatures were also significantly correlated with numbers of Ucn

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cells in the EW. Both of these correlations were positive indicating that higher numbers of Ucn cells in EW are related to higher baseline temperatures and higher sensitivity to ethanol-induced hypothermia.

Ethanol consumption patterns in the B6D2 F2 intercross mice and the relationship to Ucn in the Edinger-Westphal nucleus

Based on the highly dichotomous patterns of drinking observed between the B6 and D2 strains and their dichotomous expression of Ucn in the EW, it could be hypothesized that parallel differences between Ucn measures and ethanol consumption in a heterogeneous population of mice would exist. To this aim, we obtained several distributions of data representing consumption and preference measures of B6D2 F2 mice (Figure 21). Using these data sets, we generated correlation coefficients for consumption and preference and the number and density of Ucn-expressing cells in EW (Table 4). There were no significant correlations with consumption or preference from the continuous access study, which likely resulted from the positively skewed distribution of scores. Ucn expression and number of Ucn-positive cells in EW were significantly correlated with consumption of 10% ethanol/sucrose in the 30-minute limited access drinking procedure. The results of these analyses present suggestive evidence for a relationship between high ethanol consumption and high EW Ucn cell number and density in a situation where a high dose of ethanol is consumed in a short time period. They may also provide a physiological

explanation for the differential ethanol consumption levels seen in B6 and D2 mice.

The implementation of the control group, which consumed no ethanol throughout the continuous access and limited-access procedures, enabled us to reveal potential regulatory effects of Ucn in the EW following long-term ethanol consumption in the B6D2 F2 mice (Figure 21). Analysis of ethanol drinking mice and control mice of Cohort 2 showed no regulation in the number of EW Ucn cells or density by ethanol consumption ($F(1,35) < 1$, NS and $F(1,35) < 1$, NS, respectively).

DISCUSSION

These findings demonstrate that the EW has a diverse neurochemical content, with Ucn being the prevalent cell type in the EW of B6 mice. Comparisons between the B6 and D2 mice reveal differences in both the structure and content of the EW. Histological staining revealed that EW neurons of B6 mice appear large and are densely packed, while the EW of D2 mice contains few large cells, which are loosely distributed. Immunohistochemistry for AChE confirmed major differences in the structure of the EW between these strains. Additionally, lower numbers of Ucn-expressing cells were detected in the EW of D2 compared to B6 mice. These strains are not different in the Ucn peptide since they are encoded by identical DNA sequences, which suggests that the Ucn differences between the strains are not a result of differential immunoreactivity to the peptide resulting from coding differences (Bachtell et al.,

2002). Quite surprisingly, the structural difference in the EW between the strains does not dictate a difference in the sensitivity of ethanol-induced c-Fos expression. It is apparent however, that while ethanol-induced c-Fos expression is occurring primarily in Ucn cells in the B6 strain, c-Fos expression in the D2 strain incorporates another cell type. Taken together, these findings suggest that differential regulation of EW Ucn may be manifest in behavioral differences between the strains. Indeed, correlation analyses of B6D2 F2 mice provide some indications that Ucn expression in the EW contributes to ethanol-induced hypothermia and ethanol consumption.

The CRF/Ucn system has been linked to the development of drug addiction and alcoholism (Menzaghi et al., 1994; Koob, 1999; Koob and Heinrichs, 1999; Le et al., 2000). Thereby, this system is quite sensitive to ethanol administration. Acute ethanol administration elevates CRH (Rivier et al., 1984; Rivier et al., 1990), c-Fos and CRF-R1 levels in hypothalamus (Rivier and Lee, 1996; Lee and Rivier, 1997). Importantly, genetic differences in this system may play a role in the development of alcoholism. Strain dependent effects are seen in the release of adrenocorticotropin hormone and CRF upon acute ethanol administration where D2 mice show significantly higher elevations in both compared with B6 mice (Roberts et al., 1992). These data support our findings suggesting that D2 and B6 mice differ in both Ucn cell levels and Ucn cell activation upon ethanol exposure.

George and colleagues (1990) demonstrated that Wistar rats showing high ethanol preferences possess elevated CRF-like immunoreactivity in the

hypothalamus. Comparisons in CRF levels and electroencephalographic activity between alcohol preferring and non-preferring selected lines of rats show that preferring rats have depressed CRF levels in the hypothalamus, amygdala, and cortex while also having enhanced electroencephalographic responses to exogenous CRF (Ehlers et al., 1992). It is conceivable that differences in other components of this system (e.g. Ucn) could exist in animals predisposed to high and low ethanol sensitivity and/or high and low alcohol consumption.

Correlations in the B6D2 F2 intercross mice suggest that one consequence of strain differences in EW neurochemistry and structure is ethanol-induced hypothermia. This lies in opposition to the traditional view of EW function that incorporates the EW into the neurocircuitry of oculomotor adaptations (Roste and Dietrichs, 1988; Burde and Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). Others have also supported a role apart from this traditional view of EW including nociception and anxiolysis as possible functions (Innis and Aghajanian, 1986; Lanteri-Minet et al., 1993 Weninger, 1999 #2432; Weninger et al., 2000). Supporting our results is the idea that the EW is also involved with thermoregulation (Parver, 1991; Smith et al., 1998). Our data also support notions that unidentified midbrain autonomic nuclei are important in the underlying neurocircuitry of thermoregulation (Nagashima et al., 2000).

The correlations of ethanol-induced hypothermia with distribution of Ucn cells in EW of B6D2 F2 intercross provide only a preliminary analysis of behavioral manifestations of Ucn differences between the B6 and D2 strains because of several confounding factors. First, as previously mentioned, B6 and

D2 strains do not consistently show differing degrees of ethanol-induced hypothermia (Alkana et al., 1988). Second, the measurement of rectal temperatures at varying intervals following the ethanol injection introduces imprecision in the measurement and obscures the relationship between Ucn and ethanol-induced hypothermia. Furthermore, the differences observed in baseline body temperature likely played a large role in the hypothermic responses to ethanol. Indeed, further statistical analyses revealed a predictive relationship between baseline temperature and ethanol-induced hypothermia. Specifically, higher baseline temperatures reflect a greater susceptibility for hypothermic responses to ethanol. Ucn is known to play a role in energy balance and it is quite possible that animals with increased energy expenditures and increased EW Ucn may possess higher basal temperatures and larger hypothermic responses to ethanol (Asakawa et al., 2001).

However, the use of the HOT/COLD selected lines of mice, which were selectively bred for their susceptibility or resistance to ethanol-induced hypothermia, provide support for the EW's involvement in ethanol-induced hypothermia without the confound of differing baseline temperatures (Crabbe et al., 1987; O'Connor et al., 1993). Mice from replicate 1 of the HOT line show substantially lower numbers of Ucn cells in the EW compared with mice from replicate 1 of the COLD line (Bachtell et al., 2002). A direct manipulation of the EW Ucn system would aid in elucidating the role of the EW in temperature regulation.

In addition to the genetic differences seen in the sensitivity and tolerance to hypothermic effects of ethanol in B6 and D2 mice (Crabbe et al., 1994a Crabbe, 1994 #2643; Crawley et al., 1997), they are also known to differ markedly in behavioral responses associated with the hedonic effects of ethanol (Plomin and McClearn, 1993; Phillips et al., 1994; Cunningham, 1995). For example, B6 mice willingly ingest ethanol-containing solutions, while D2 mice avoid such solutions (McClearn and Rodgers, 1959; Belknap et al., 1993). Interestingly, ethanol-induced hypothermia has been shown to genetically correlate with some of ethanol's hedonic properties. Cunningham and colleagues (1991) showed that the HOT/COLD selected lines of mice differ on several measures of ethanol's hedonic effects including conditioned place preference, ethanol drinking and conditioned taste aversion. Specifically, replicate 1 COLD mice ingested ethanol solutions at high concentrations to a greater extent than replicate 1 HOT mice. Such effects were not observed in the replicate 2 HOT/COLD mice, where there were also no Ucn differences. Thus, a relationship may exist in the HOT/COLD mice of replicate 1 such that high levels of Ucn in the EW predicts high ethanol consumption.

Not only is this relationship supported by Ucn differences in the ethanol-consuming B6 and ethanol-avoiding D2 inbred strains, but in a separate population of B6D2 F2 mice. In this population, a positive correlation between ethanol consumption and measures of Ucn in the EW exists. This positive relationship has also been validated in several other genetic models of ethanol consumption including the congenic line B6.D2 *Alcp1* 2.2 and the high alcohol

preferring and low alcohol preferring selected lines of mice (Grahame et al., 1999; Whatley et al., 1999; Bachtell et al., 2003b). In addition, characterization of ethanol drinking behaviors in mice with a targeted deletion of the CRF prohormone (Muglia et al., 1995) reveals a similar positive relationship between high Ucn in the EW and high ethanol consumption. Thereby, while possessing no CRF, a compensatory enhancement of Ucn expression exists in the EW of these mice (Weninger et al., 2000). Homozygous CRF KO mice show enhanced ethanol drinking in the two-bottle choice test, while having no apparent differences in the total fluid consumption, taste preferences, or ethanol clearance (Olive et al., 2002). Taken together, data from several genetic models provides sound evidence to support a relationship between high Ucn expression in the EW and high ethanol consumption.

SUMMARY AND CONCLUSIONS

This series of experiments highlights phenotypic differences in both the structure of the EW and the neurochemical phenotypes of EW neurons between the B6 and D2 inbred strains of mice, which may result from the genetic divergence of the two strains. While the differences do not alter the sensitivity of the EW to ethanol, the results do reveal that a qualitative difference exists in the response of the EW to ethanol. This qualitative difference is represented by separate populations of neurons responding to ethanol between the B6 and D2 mouse strains. As evidenced in the B6D2 F2 intercross mice, this differential response to ethanol between the B6 and D2 strains may be related to differences

in ethanol-induced hypothermia and ethanol drinking. Further elucidation of these behavioral effects would benefit from direct manipulations of the EW and subsequent behavioral assays.

Table 1. Neurochemical characterization in the C57BL/6J mouse using immunohistochemical detection of several markers for neurotransmitters and neuropeptides

Transmitter/Peptide	Immunohistochemical Marker	Representation in the EW
Urocortin	Urocortin	Large Cell Bodies
Acetylcholine	AChE	Large Cell Bodies
Noradrenaline/ Dopamine	TH	Processes
Neuropeptide Y	NPY	Processes
Substance P	SP	Processes
GABA	GAD 67	None
GABA	GABA	None

Table 2. Number of Thionine-stained cells in EW per slice in B6 and D2 mice

Strain	Cell size (mm^2)					
	50-99	100-149	150-199	200-249	250-299	>300
B6	13.8±1.8	11.3±1.4	5.0±0.9	3.5±1.4	2.0±0.4	6.8±0.9
D2	19.5±1.2↑	6.3±1.3↓	1.3±0.8↓	0.8±0.5	0.5±0.3↓	1.5±0.5↓

Arrows indicate statistically significant difference between B6 and D2 mice ($p<0.05$).

Table 3. Correlation coefficients of B6D2 F2 ethanol sensitivity phenotypes

Phenotype	Total EW Ucn Cells	EW Ucn Density
Body Weight	-0.224	-0.222
Locomotor Difference	0.129	0.035
Central/Total	-0.198	0.009
Baseline T	0.286*	0.225
EtOH T	-0.138	-0.074
T Difference	0.278*	0.194
Time until LORR	0.180	0.156
LORR Duration	0.093	0.148

* denotes statistically significant correlation coefficient ($p < .05$)

Table 4. Correlation coefficients of B6D2 F2 consumption measures and EW Ucn parameters.

	Consumption Measure	*Total EW Ucn Cells*	EW Ucn Density
Two-Bottle Choice Paradigm	3% EtOH Preference	0.027	0.087
	6% EtOH Preference	0.029	0.076
	10% EtOH Preference	0.029	0.070
	3% EtOH Dose	0.090	0.211
	6% EtOH Dose	0.066	0.184
	10% EtOH Dose	-0.016	0.071
Limited-Access Paradigm	10% Sucrose Consumption	0.061	0.021
	10% Sucrose/3% EtOH Dose	0.213	0.214
	10% Sucrose/6% EtOH Dose	0.180	0.227
	10% Sucrose/10% EtOH Dose	0.366**	0.409**

** denotes statistically significant correlation coefficient ($p < 0.005$)

Figure 11. Immunohistochemical staining for urocortin (A and B) and tyrosine-hydroxylase (C and D) in the Edinger-Westphal nucleus. Cellular expression of urocortin in the EW confirms that it is the prominent cell type and is characterized by two dorsoventral columns parallel to the saggital midline. Lower magnification (C) shows TH-positive fibers extending to EW from the ventral tegmental area. Higher magnification (D) shows presence of few TH-positive cells within EW and many processes.

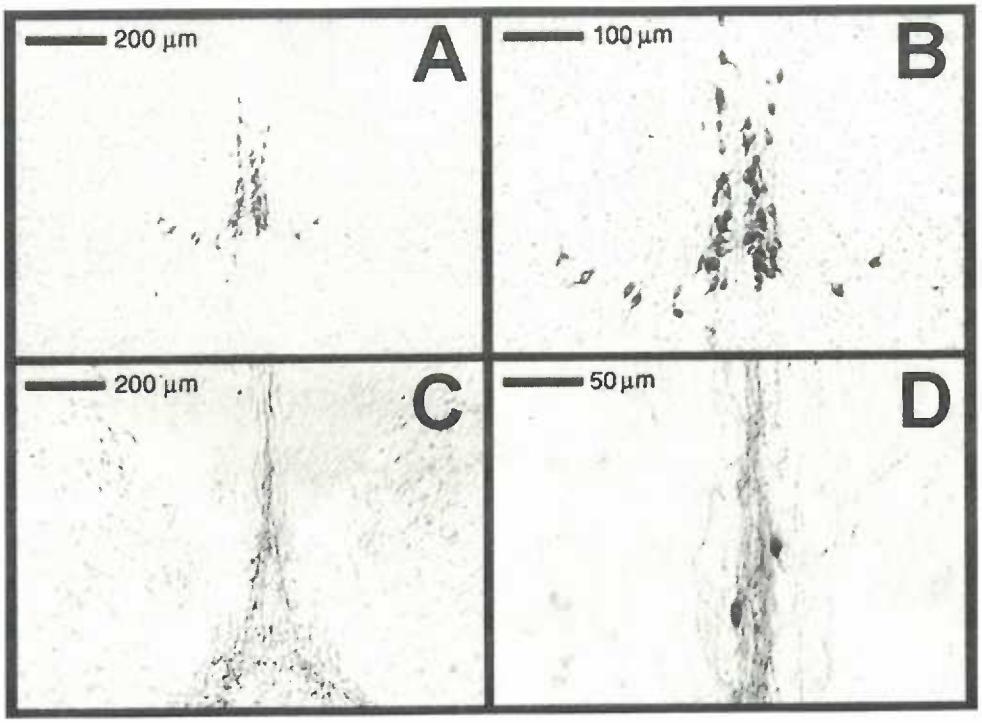

200 µm
A
100 µm
B
200 µm
C
50 µm
D

Figure 12. Expression of urocortin cells in B6 and D2 mice. Left, representative coronal sections. Right, quantitative data reflecting significant differences ($p < .0001$) between B6 and D2 mice in number of Ucn-expressing cells in EW.

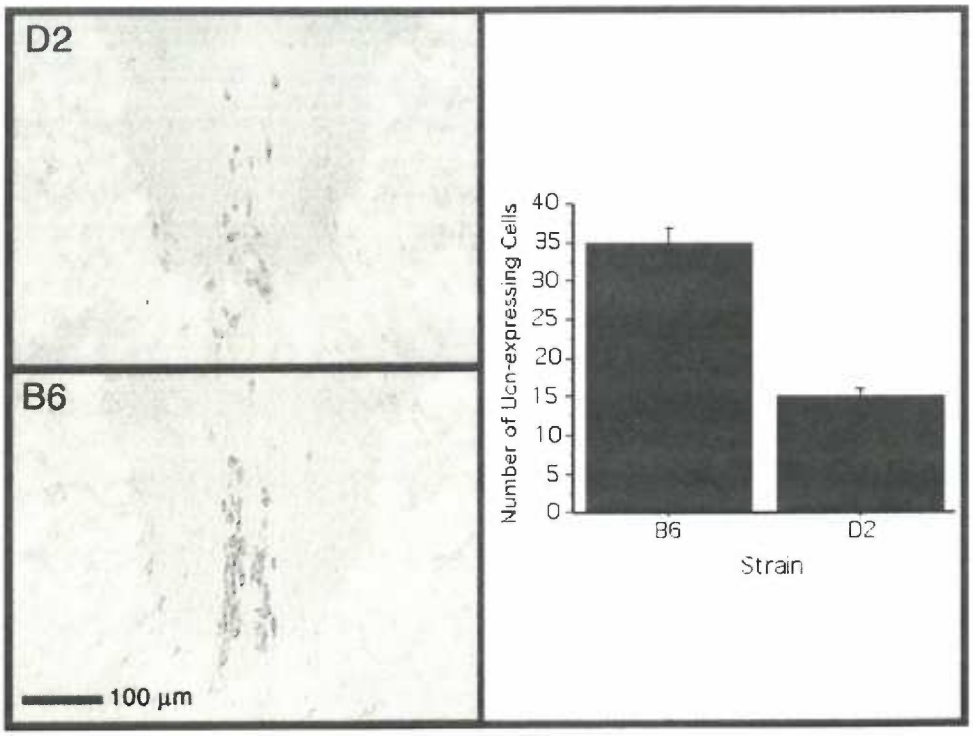

D2
B6
100 µm
Number of Ucn-expressing Cells
40
35
30
25
20
15
10
5
0
B6
D2
Strain

Figure 13. Histology of the EW in D2 and B6 mice illustrated with Thionine staining, Neutral Red staining, and AChE immunohistochemistry. Note differences between the strains in cellular structure and organization in Thionine and Neutral Red staining, and similar strain differences in expression of AChE.

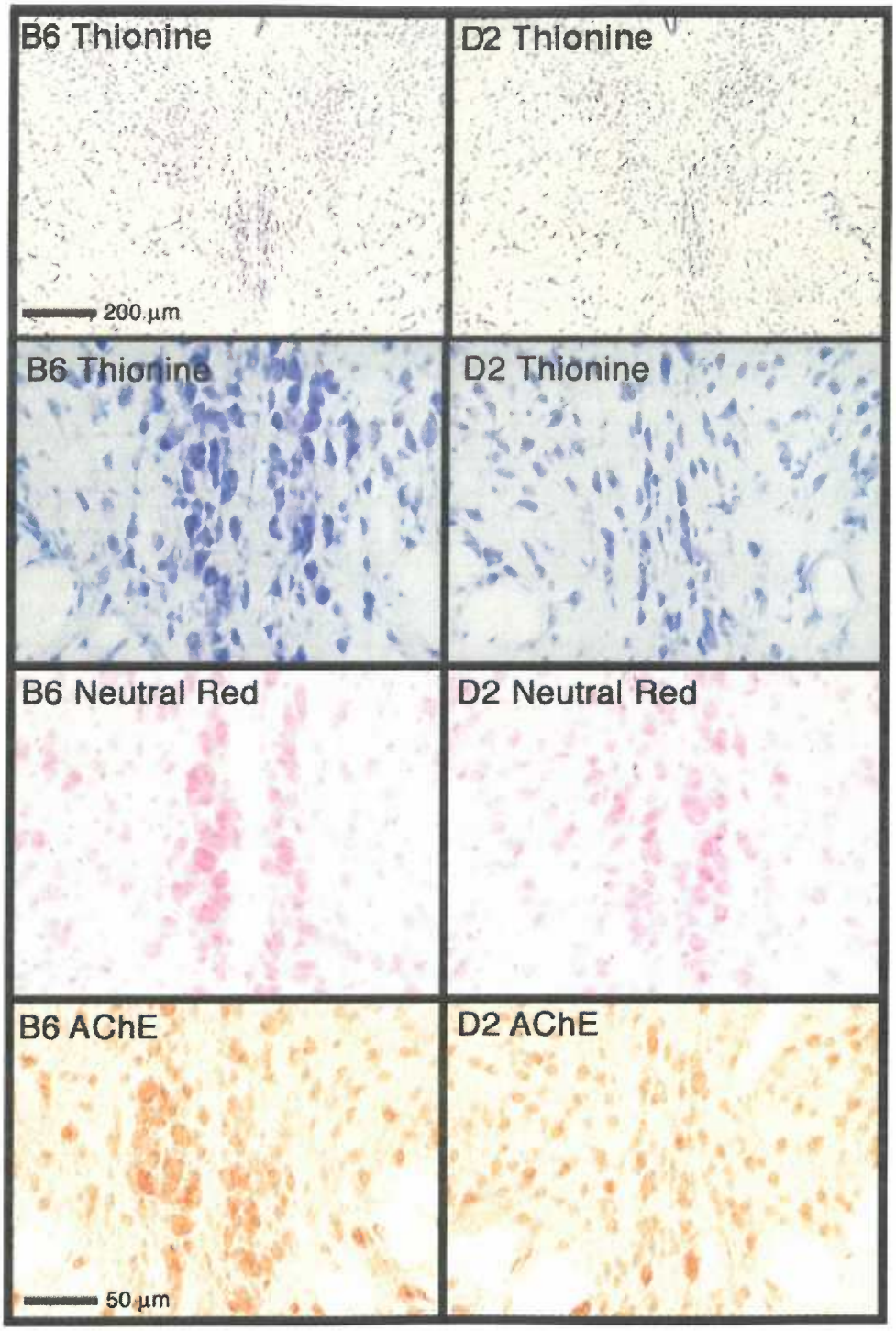

B6 Thionine
D2 Thionine
200 µm
B6 Thionine
D2 Thionine
B6 Neutral Red
D2 Neutral Red
B6 AChE
D2 AChE
50 µm

Figure 14. c-Fos expression in the EW following ethanol administration in both B6 and D2 strains. Note equivalent expression between the strains, but differences between the saline, 1.2 g/kg and 2.4 g/kg panels.

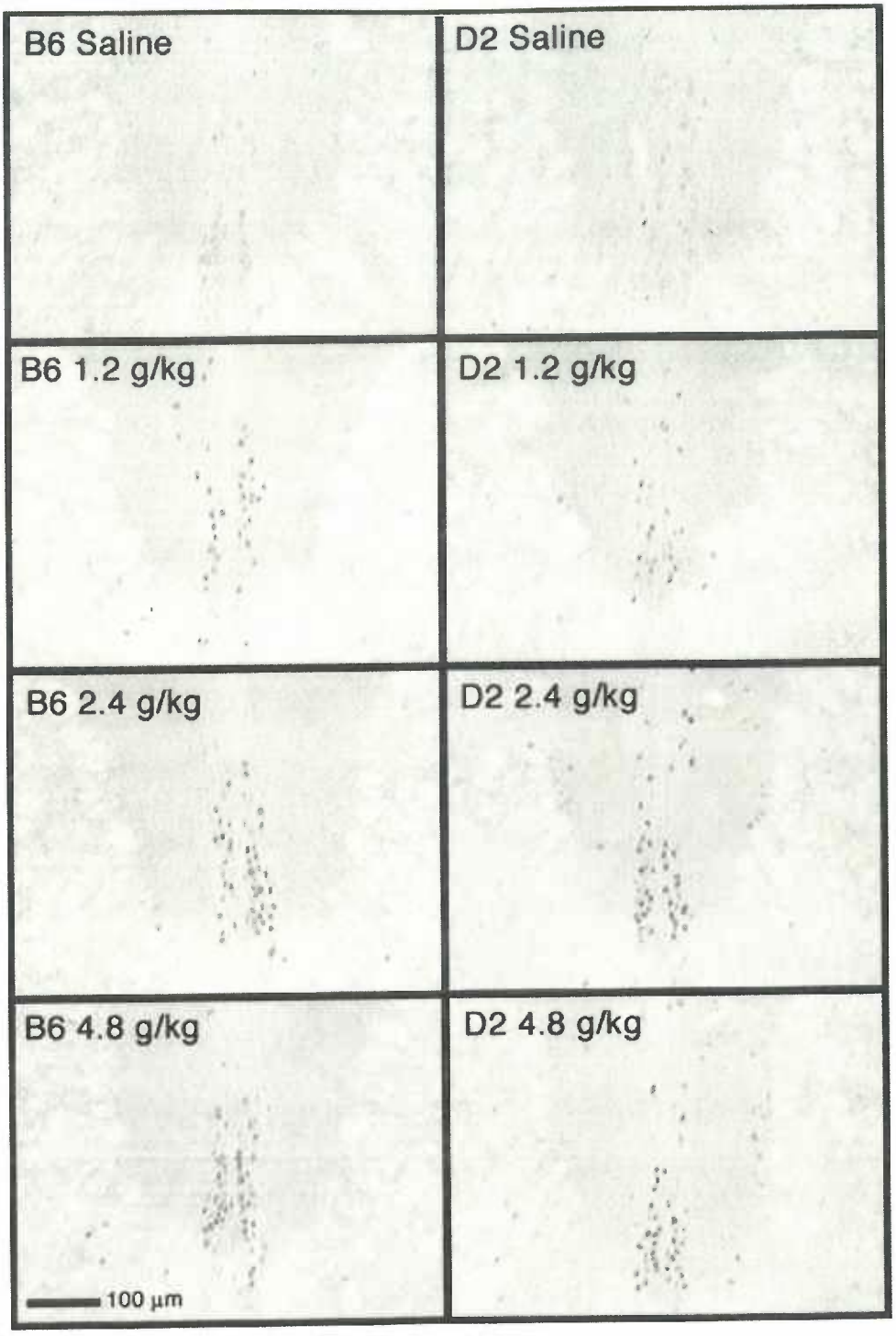

B6 Saline
D2 Saline
B6 1.2 g/kg
D2 1.2 g/kg
B6 2.4 g/kg
D2 2.4 g/kg
B6 4.8 g/kg
D2 4.8 g/kg
100 µm

Figure 15. Dose-responsiveness of c-Fos expression in EW of B6 and D2 mice following ethanol administration. No statistical strain differences were found (n = 4-8/group).

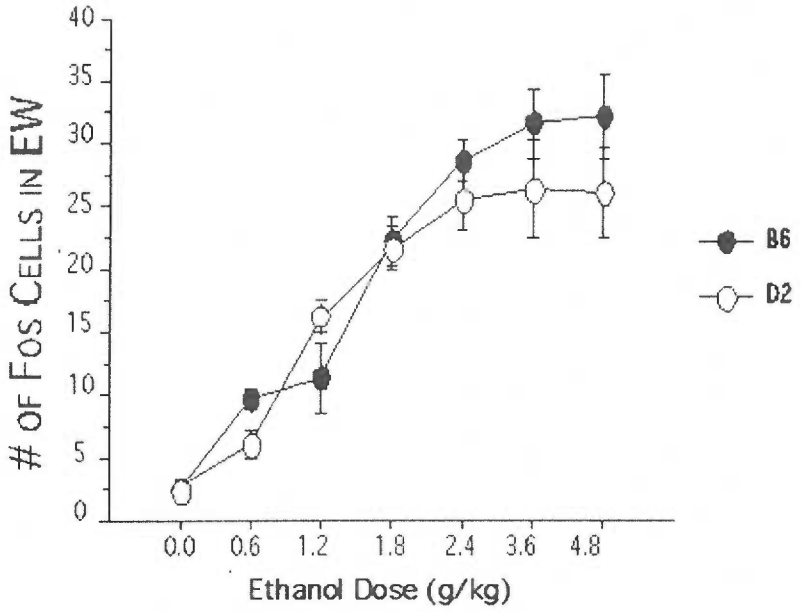

# of Fos Cells in EW
40
35
30
25
20
15
10
5
0
0.0
0.6
1.2
1.8
2.4
3.6
4.8
Ethanol Dose (g/kg)
B6
D2

Figure 16. Colocalization of urocortin and c-Fos in EW of B6 and D2. Upper panel: double-labeling; middle panel: same procedure without anti-c-Fos primary; lower panel: same procedure without anti-Ucn primary. Note all c-Fos positive nuclei (brown DAB staining) in urocortin-positive cytoplasm (purple VIP staining) in B6 mice. Arrows indicate c-Fos positive nuclei that exist outside of urocortin-positive cells in D2 mice.

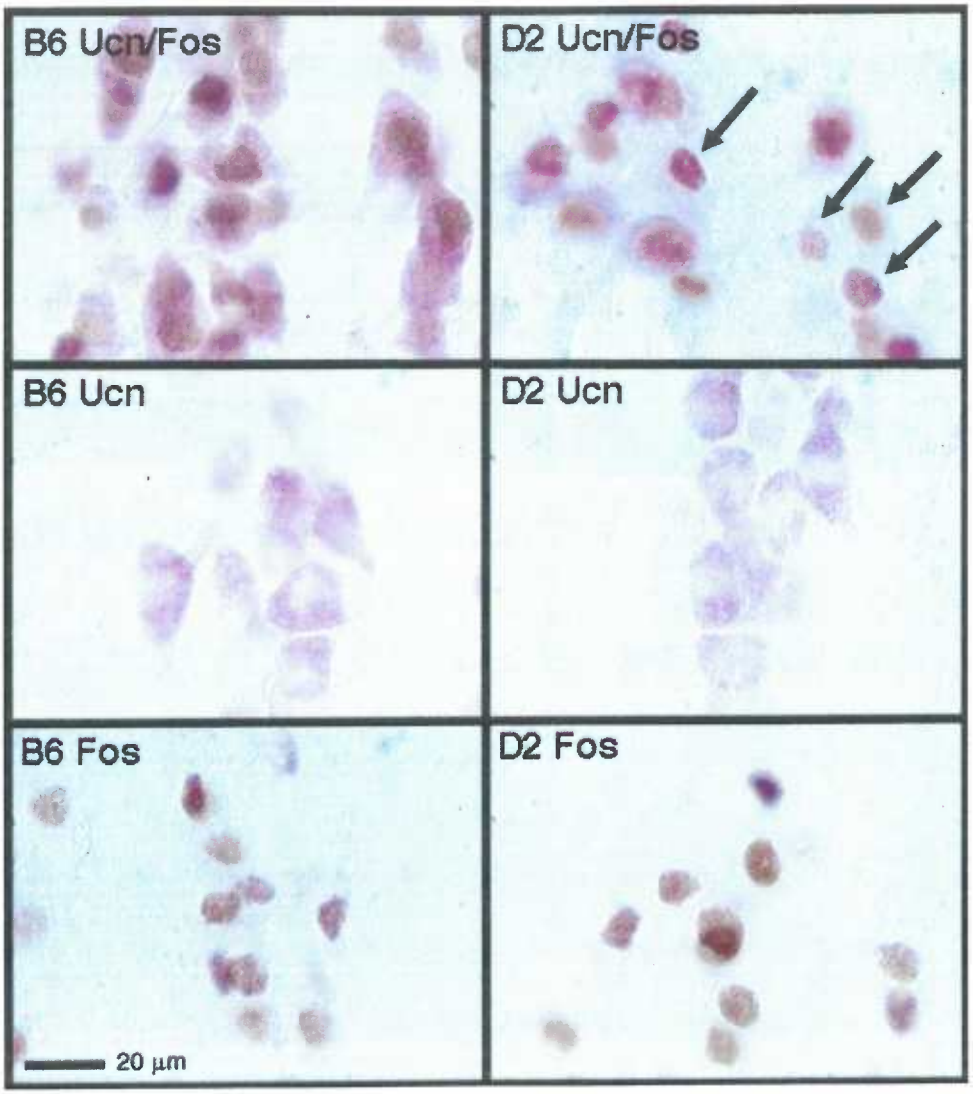

B6 Ucn/Fos
D2 Ucn/Fos
B6 Ucn
D2 Ucn
B6 Fos
D2 Fos
20 µm

Figure 17. Quantification of colocalization double immunohistochemistry. A) Number of c-Fos positive cells between the two strains. B) Percentage of c-Fos positive nuclei observed within Ucn-expressing cells.

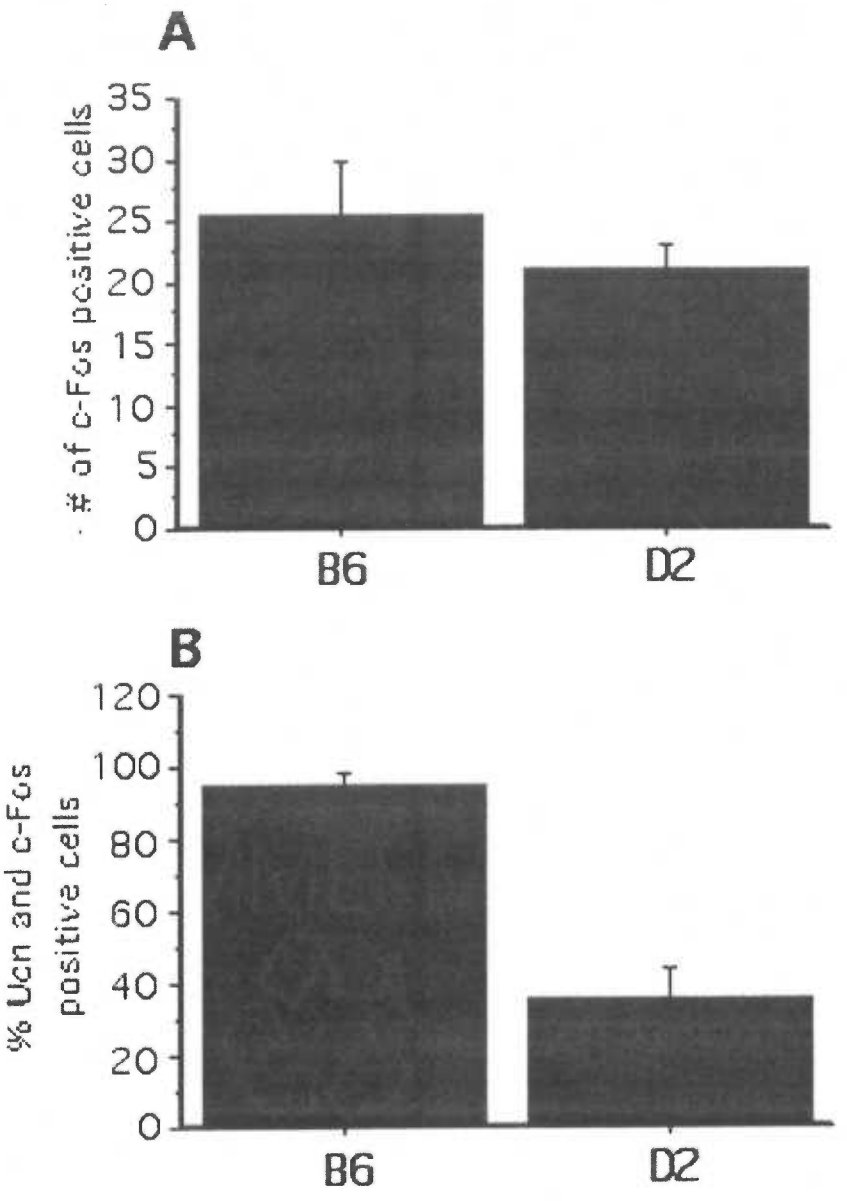

A
# of c-Fos positive cells
35
30
25
20
15
10
5
0
B6
D2
B
% Ucn and c-Fos positive cells
120
100
80
60
40
20
0
B6
D2

Figure 18. c-Fos expression patterns following saline, acute ethanol and repeated ethanol. Both acute and repeated ethanol treatments led to significant elevations of EW c-Fos expression ($p < .0001$). Also of importance, was the significant difference between acute and repeated ethanol ($p < .05$). No statistical strain differences were noted.

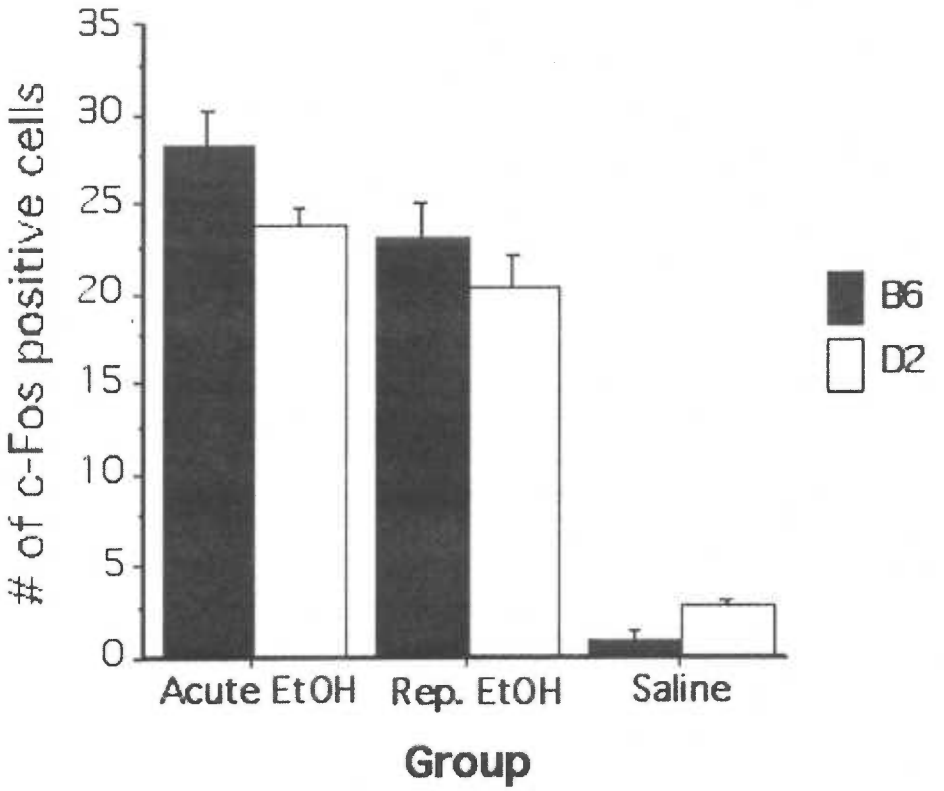

# of c-Fos positive cells
35
30
25
20
15
10
5
0
Acute EtOH
Rep. EtOH
Saline
Group
B6
D2

Figure 19. Repeated and acute ethanol treatment produced no significant alterations in the expression of Ucn (top panel) or the numbers of Ucn-expressing cells (bottom panel) using immunohistochemistry. As revealed previously, the D2 strain had significantly lower levels of Ucn compared with the B6 strain ($p < 0.001$), however, there were no interactive effects between strain and treatment group.

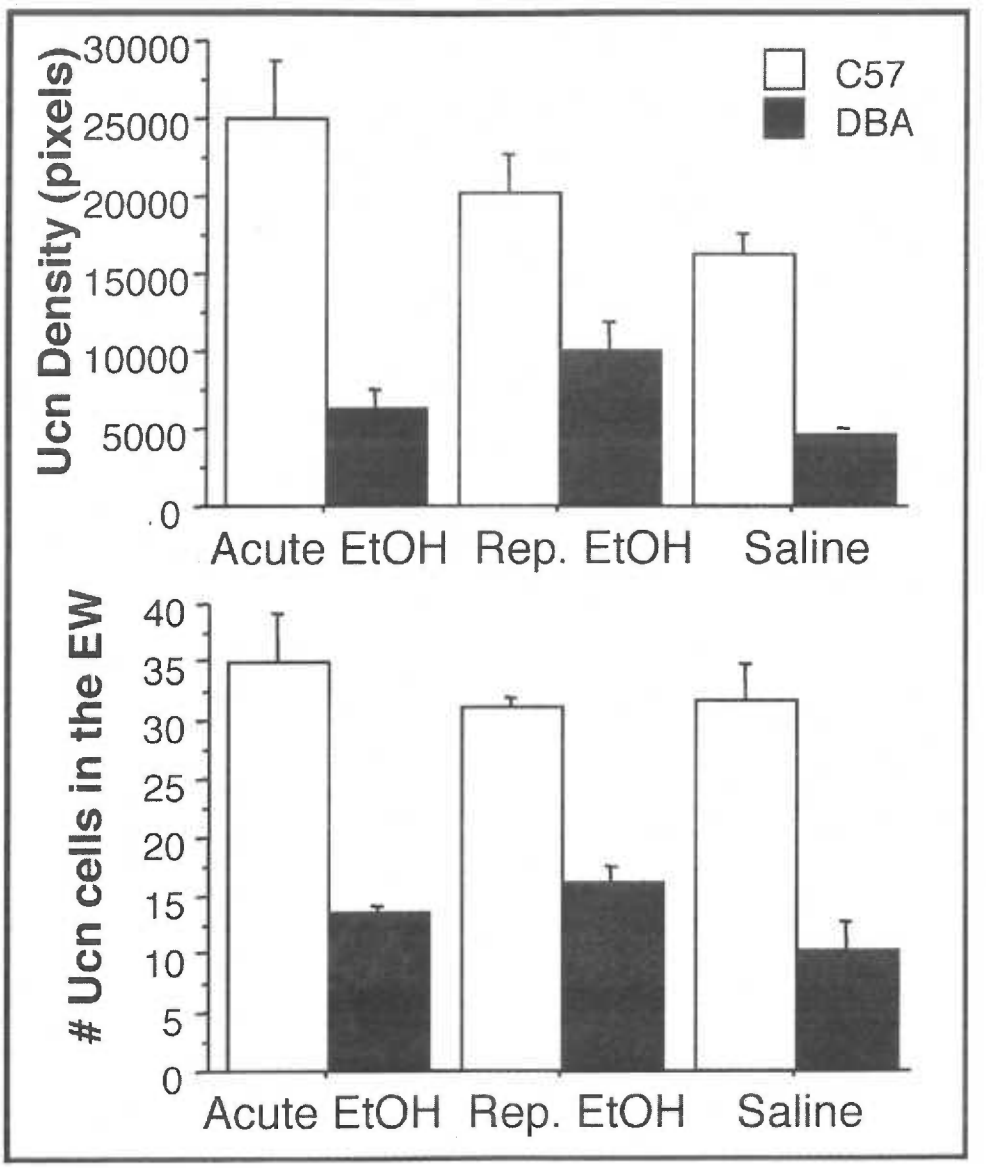

Ucn Density (pixels)
30000
25000
20000
15000
10000
5000
0
C57
DBA
Acute EtOH
Rep. EtOH
Saline
Ucn cells in the EW
40
35
30
25
20
15
10
5
0
Acute EtOH
Rep. EtOH
Saline

Figure 20. Frequency distributions of several phenotypic measures performed in the B6D2 F2 population of mice including Ucn measures (A and B), ethanol-induced hypothermia (C), locomotor activation/depression (D), baseline body temperatures (E), duration of loss of righting reflex (F), central/total activity on Day 1 (G) and latency to LORR (H). Preliminary screening revealed normality of the distributions and were therefore used in subsequent correlation analyses.

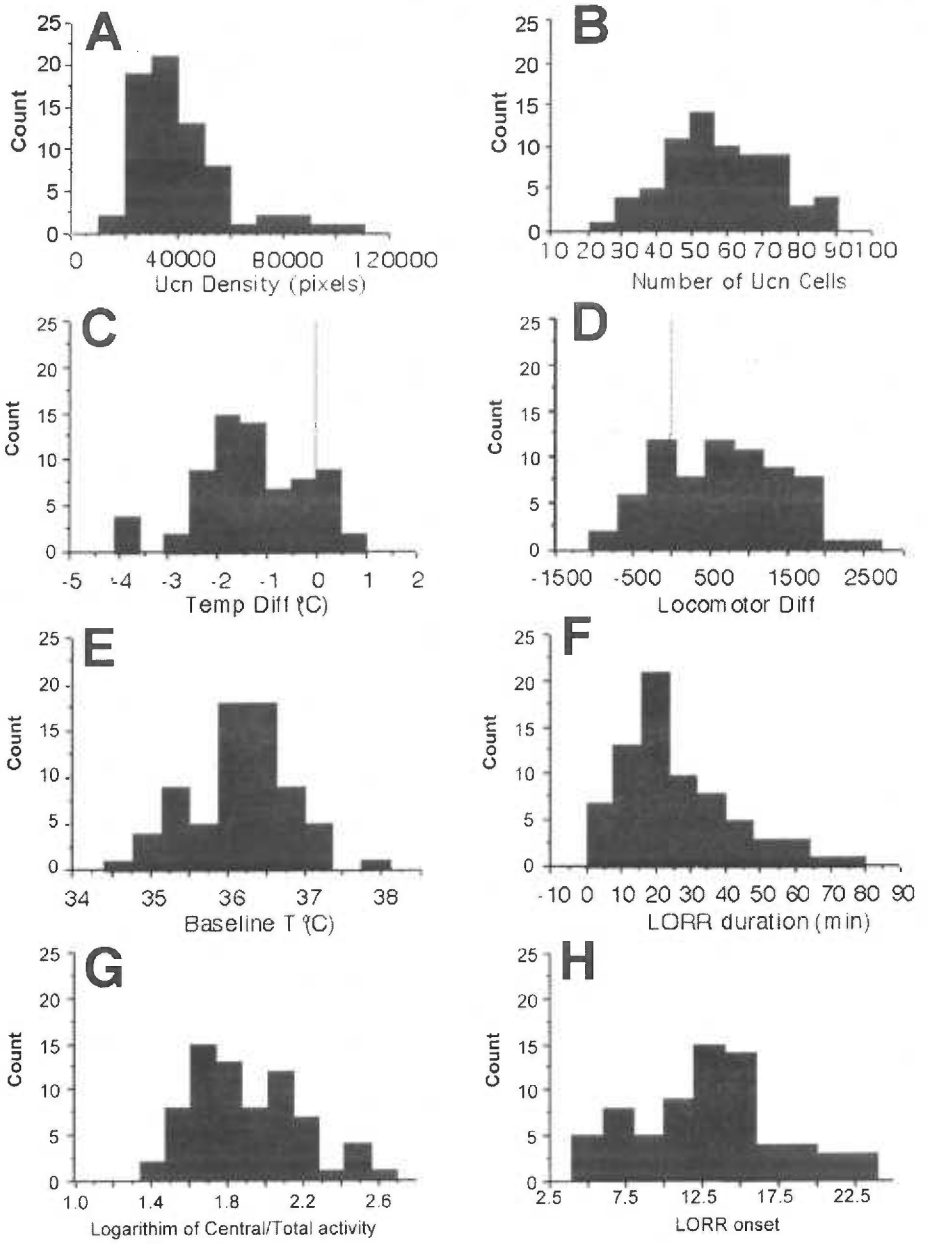

A
Count
Ucn Density (pixels)
B
Number of Ucn Cells
C
Temp Diff (°C)
D
Locomotor Diff
E
Baseline T (°C)
F
LORR duration (min)
G
Logarithim of Central/Total activity
H
LORR onset

Figure 21. Frequency distributions of B6D2 F2 intercross mice during the continuous 2-bottle choice procedure (2 Left panels) and the 30-minute limited access ethanol/sucrose procedure (Right panel). The left panel corresponds to total consumption of the ethanol solution at 3% (top), 6% (middle) and 10% (bottom) concentrations. The middle panel corresponds to preference ratio (ethanol consumed/total fluid consumed) at the 3% (top), 6% (middle) and 10% (bottom) concentrations. Note the skewed distributions in both consumption and preference in the 24 h 2-bottle procedure. The tendencies to not drink in this procedure may have limited the ability to detect significant relationships with Ucn expression. The right panel corresponds to the consumption of a 3% ethanol/10% sucrose (top), 6% ethanol/10% sucrose (middle), and 10% ethanol/10% sucrose (bottom).

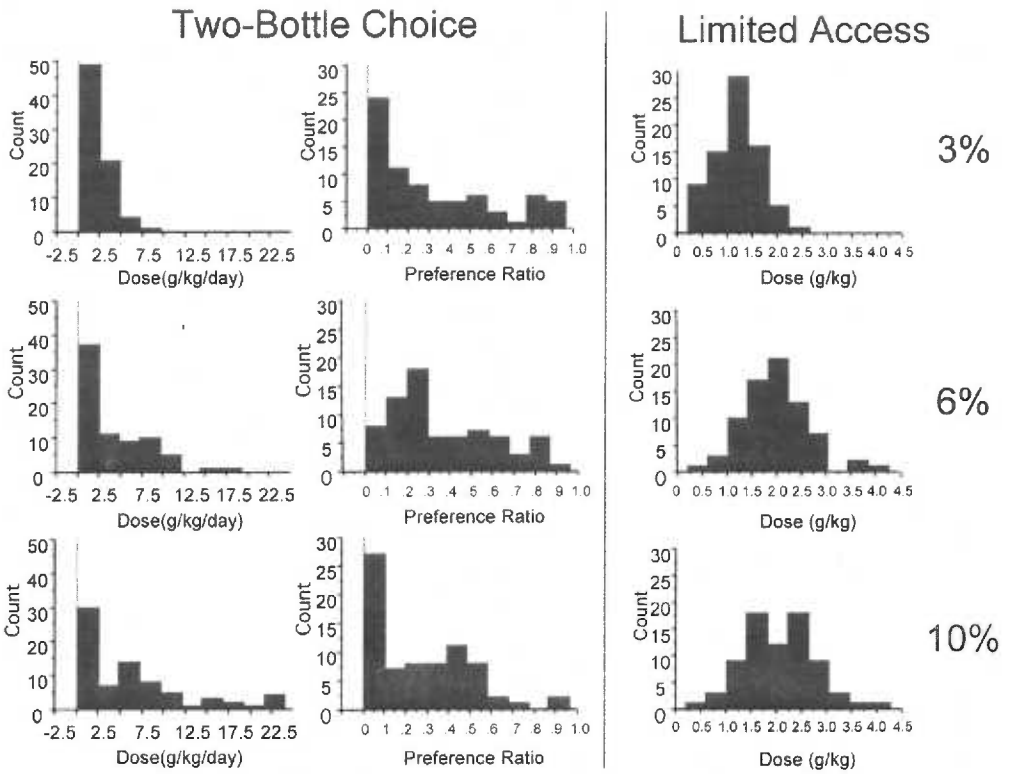
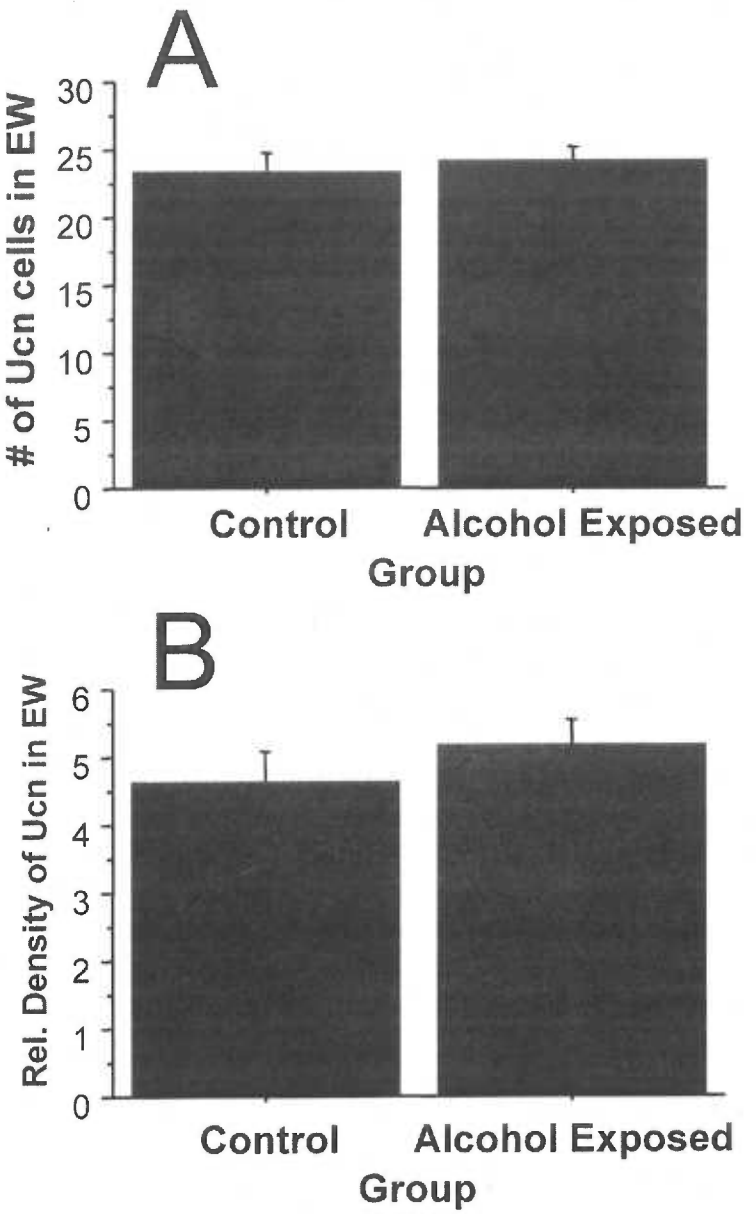

Two-Bottle Choice
Limited Access
Count
Dose(g/kg/day)
Preference Ratio
Dose (g/kg)
3%
6%
10%

Figure 22. Consumption of ethanol in the B6D2 F2 mice had no effect on the number of Ucn-positive cells (A) or expression of Ucn in EW (B).


A
# of Ucn cells in EW
30
25
20
15
10
5
0
Control
Alcohol Exposed
Group
B
Rel. Density of Ucn in EW
6
5
4
3
2
1
0
Control
Alcohol Exposed
Group

CHAPTER 3: Assessment of Edinger-Westphal nucleus lesions on ethanol-related behavioral measures

The neurobiological study of alcoholism has primarily focused on neural systems associated with ethanol's rewarding components such as the mesocorticolimbic dopamine system and the extended amygdala. However, it is likely that ethanol also targets neural systems outside these conventional systems. Actions within these systems may represent modes through which ethanol produces behavioral effects that contribute to drug consumption such as initial drug sensitivity or the development of tolerance and sensitization to drug effects. While an overlap in these neural systems may exist, it is likely that this overlap is limited and additional neural systems are involved.

The EW represents a novel neural target given its unique sensitivity to ethanol. Chang and colleagues (1995) first observed that an acute injection of ethanol resulted in elevated c-Fos expression in the EW. It was also observed that while the c-Fos response of most ethanol-sensitive brain structures desensitized to repeated ethanol injections, c-Fos expression in the EW persisted. In another report, elevated c-Fos expression was observed exclusively in the EW of rats self-administering ethanol-containing beer during daily 30-min sessions over a 21-day period (Topple et al., 1998). Similar findings have been repeatedly shown using a variety of voluntary ethanol consumption models (Bachtell et al., 1999; Ryabinin et al., 2001; Weitemier et al., 2001; Ryabinin et al., 2003). This evidence strongly suggests that the EW is an important, novel neural target for ethanol.

The EW is the primary source of the neuropeptide Ucn in the brain. Ucn is structurally and functionally similar to CRF and it binds to CRF receptors with equal or greater affinity than CRF itself (Vaughan et al., 1995; Reyes et al., 2001). Recent data from our lab demonstrates that ethanol-induced c-Fos expression occurs within Ucn cells of the EW (Bachtell et al., 2002; Ryabinin et al., 2003). In addition, Ucn expression in the EW was also observed to correlate with several ethanol-mediated responses (Bachtell et al., 2002; Bachtell et al., 2003b). A positive relationship exists between EW Ucn expression and ethanol-induced hypothermia. This was observed both in a heterogeneous population of B6D2 F2 mice and the HOT/COLD selected lines of mice, which were selectively bred for their resistance/susceptibility, respectively, to ethanol-induced hypothermia.

Support from another population of B6D2 F2 mice suggests that EW Ucn is also related to ethanol consumption and preference (Bachtell et al., 2002; Bachtell et al., 2003b). A positive correlation between ethanol consumption and the number of EW Ucn cells was observed in this population of mice. This relationship was supported by analyzing EW Ucn in the high alcohol preferring and low alcohol preferring selected lines of mice, where a similar positive relationship exists. Taken together, these results suggest that Ucn in the EW may be a region important for detecting ethanol intoxication states, which subsequently regulates ethanol intake. These relationships would be aided by further confirmation with direct manipulation of the EW Ucn system. The present studies utilized electrolytic lesions localized to the EW to establish a confirmation

of the relationship of EW Ucn with ethanol-induced hypothermia and ethanol consumption.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) were placed four or five per cage for one week. At this time, animals underwent surgery as detailed below. All animals were 7-8 weeks of age at the beginning of the surgical procedure. C57BL/6J mice were selected for these analyses because of the well-characterized behavioral responses (e.g. ethanol-induced hypothermia and ethanol drinking) and thorough neurochemical characterization performed in Chapter 2. Animals were maintained on a 12 h light-dark cycle with lights on beginning at 6 a.m. Water and food were available ad libitum throughout the experiment in the home cage. All animal procedures were in accordance with National Institutes of Health guidelines.

Surgical Procedures

Mice were initially anesthetized with 4 ml/kg body weight of a cocktail containing ketamine (30 mg/ml), xylazine (3.0 mg/ml), and acepromazine (0.6 mg/ml) for the surgical procedure. Heart (30-50 beats/5 sec) and breathing rates (12-18 breaths/5 sec) were monitored throughout the session to assess appropriate anesthesia level. Mice are known to show extreme hypothermia under anesthesia. Therefore, rectal temperatures were monitored throughout surgery

and a heating pad was used to maintain the animal's normal temperature (approx. 37^{O} C). While anesthetized, mice were positioned in a stereotaxic apparatus (Model EW40M, Cartesian Research, Inc., Sandy, OR). This apparatus allows for angular insertion of electrodes. Insulated (except for 500μm tip) stainless steel electrodes (SNE-300, Rhodes Medical Instruments, Inc, Woodland Hills, CA) were stereotaxically guided into the EW nucleus (A/P: 3.2 mm, M/L: 0.0 mm, D/V: 3.75 mm from Bregma) at a twenty-degree angle through a small hole drilled in the skull (Franklin and Paxinos, 1997). The angled placement was necessary to avoid damaging the midsaggital sinus. The electrode was connected to the positive terminal of a lesion making current device (Model 3500, Ugo Basile, Comerio, Italy). The negative terminal was used to ground the animal via a connection to the animal's ear. Preliminary experiments have revealed that 5 s of 400 μA current is sufficient to produce a lesion approximately 200 μm in diameter. Following application of electrical current, electrodes were removed, the skin sutured and animals were removed from the apparatus. The electrode was lowered into the EW of sham-operated animals however no current was applied. During the immediate post-operative period, animals were kept warm with a heating pad and monitored for unobstructed respiration and appropriate body temperature until the effects of general anesthesia had dissipated (generally 3 hr). Food and fluid intake were monitored daily during the subsequent two days of recovery. Each subject was also monitored for normal signs of alertness and motor activity during the subsequent two days of the post-operative recovery period.

Ethanol Sensitivity Measurements

Mice from both sham and lesion groups were tested for various ethanol-related phenotypes including hypothermia, locomotor activation, anxiolysis, and sedation. For the sensitivity experiments, two cohorts of 30 B6 mice were used. Animals from both cohorts were tested for the hypothermic effects of ethanol. To evaluate these effects, animals were first assessed for baseline rectal temperatures, which was immediately followed by an injection of 3.0 g/kg ethanol (i.p.). Rectal temperatures were subsequently measured at 15 and 30 minutes post-ethanol injection.

One week following the hypothermia assessments, pupil measurements were made in all mice. These assessments were performed in a dark room. A fiber optic light, which was positioned 50 mm from the animals nose provided indirect lighting. In preliminary assessments, it was observed that this indirect source of lighting provided optimal visualization of the pupil. An infrared camera, capable of video recording in low light environment, was positioned 50 mm from the animal's left eye. While the animal was gently restrained by the scruff at the nape of the neck, the pupillary response to the light was recorded for 2-3 sec. Pupil diameters were later measured by an observer blind to the animal's group assignment using the ImageJ software. Total area in pixels was used as the dependent variable.

One week after pupil assessments, mice from cohort 1 were then tested for ethanol-induced locomotion and anxiolysis using a two-day procedure.

Animals were first habituated to a saline injection and the activity chambers during the first day. On the next day, all animals were injected with 1.8 g/kg ethanol (i.p.) dissolved in saline (20% v/v). On both days, animals were placed into dimly lit activity chambers enclosed in sound attenuating boxes two minutes following the injection. Activity was assessed by an automated photobeam system that included a 10 X 12 array of photocells, situated 1 cm off the floor, surrounding a 21 X 25 X 18 cm Plexiglas arena. Activity was assessed in five-minute intervals for a total of 15 min. The distance traveled during each interval was used as a measure of activity. For a more detailed analysis, the arena was separated into 60 equal zones, 48 of which were located next to the arena wall and defined as the peripheral region. Thus, activity was divided into both peripheral and central activity. Central activity includes consecutive beam breaks across both horizontal dimensions in one of the12 central zones. Based on this division of activity, a ratio of central to total activity was created and used to assess anxiety-like behavior on both testing days.

Two weeks following pupil assessments (intervening week included morphine hypothermia testing), animals from cohort 2 were tested for the loss of righting reflex using a 4.0 g/kg dose of ethanol. Animals were first injected and the time until the onset of loss of the righting reflex was measured. The time to recover the righting reflex (two rightings within 30 sec) was then recorded. Upon recovery of righting reflex, mice were sacrificed by cervical dislocation and trunk blood was collected for blood ethanol concentration (BEC) measurements. Brains were removed for verification of lesion placement using the thionin

staining protocol below.

Several dependent variables resulted from these behavioral assessments. Responses following the 3.0 g/kg dose of ethanol include: baseline rectal T (°C) and ethanol-induced hypothermia (°C) at the 15 and 30 minute time points. The 15 and 30 minute time points following the 3.0 g/kg dose was based on the well-characterized hypothermic response in C57BL/6J mice. This method is different to that used in the F2 population (Chapter 2), wherein hypothermic responses were measured following variable time points post-ethanol injection, and is believed to allow more accurate assessments of hypothermia. Locomotor activity testing was analyzed using a difference score calculated by subtracting Day 1 activity (saline vehicle) from Day 2 activity (1.8 g/kg ethanol). Additionally, locomotor activity testing allowed us to assess the degree of anxiolysis produced by ethanol by calculating a ratio of central activity to total activity, which was a cursory measure of anxiety-related behavior. However, this measure does not provide the best measure of anxiety because the test environment was not designed to be maximally anxiogenic. The following three measures were evaluated following the 4.0 g/kg dose of ethanol: (1) time until LORR onset, (2) duration of LORR and (3) BEC from trunk blood at recovery. This procedure also differs from that used in Chapter 2 where a 3.6 g/kg dose was used. Preliminary findings showed that the 4.0 g/kg dose produced a more reliable onset and duration of LORR.

Morphine Hypothermia Test

Mice from cohort 2 were assessed for morphine-induced hypothermia during the week intervening pupil assessment and LORR testing. Since morphine is capable of significantly increasing c-Fos expression in the EW (see Chapter 1) and induces hypothermia, this assessment provided a control for the specificity for EW lesions to affect temperature regulation. As in previous hypothermia experiments, animals were first assessed for baseline rectal temperatures, which was immediately followed by an injection of 30 mg/kg morphine (i.p.). This dose was previously shown to be effective in producing maximal hypothermic responses in C57BL/6J mice (Belknap et al., 1998). Rectal temperatures were subsequently measured at 30 minutes post-morphine injection.

Ethanol Drinking Procedures

Ethanol-naive B6 mice were used for assessment of the effects of EW lesions on ethanol drinking. Following surgical procedures and post-operative recovery (5-8 days), the experiment was initiated by individually housing the animals in metal hanging racks. The animals were allowed 5 days to habituate to the new housing conditions. During this time, animals were given continuous access to one bottle containing tap water. On the first through the fourth day of the experiment, animals were given continuous access (24-h) to two 50 ml cylinders (one containing tap water and one containing 3% ethanol in tap water). On the fifth through eighth and ninth through twelfth day of the experiment, the ethanol concentration was 6% and 10%, respectively. On days 13 and 14, the ethanol

was increased to 20%. Body weights and fluid consumption (g) from both cylinders were recorded daily at 9 AM (two hours after light onset). Bottle positions were alternated daily to avoid development of a position preference. Both preference measures (g ethanol consumed/ g total fluid consumed) and consumption measures (g/kg/day) were calculated and used as dependent variables.

Sucrose and Quinine Consumption

In an effort to rule out differences in taste sensitivity that would affect drinking an ethanol-containing solution, consumption tests of sucrose and quinine were performed (Thiele et al., 1998). Immediately following the 2-bottle choice ethanol drinking procedure, consumption and preference for sucrose and quinine solutions were compared was analyzed in both the EW-lesioned and sham-operated group. On days 15 and 16, animals were presented with one bottle of 1.7% sucrose and one bottle of tap water. On days 17 and 18, the sucrose concentration was increased to 4.3% sucrose. On day 19 and 20, the animals were presented with one bottle of 0.03 mM quinine and one bottle of tap water. On days 21 and 22, the quinine concentration was increased to 0.1 mM quinine. Body weights and fluid consumption (g) from both cylinders were recorded daily at 9 AM (two hours after light onset). Bottle positions were alternated daily to avoid development of a position preference. Preference measures for sucrose and quinine were calculated at each concentration and consumption measures (ml/kg) were calculated and used as dependent variables.

Thionin Histology

Dissected brains were postfixed overnight in 2% paraformaldehyde in PBS, cryoprotected with 30% sucrose in PBS and sectioned coronally on a cryostat. Thionin staining was performed on 40 μm coronal brain sections mounted on gelatin-coated glass slides.

Blood Ethanol Measurement

Trunk blood was collected in microcentrifuge tubes upon sacrifice and placed on ice. All samples were immediately centrifuged at 14,000 rpm for 20 minutes. Serum was collected and stored overnight at –20^{O}C. Blood samples (3 μl) from individual animals were used to assess NAD-ADH reactivity with the spectrophotometric NAD-ADH Detection System (Sigma Diagnostics, St. Louis, MO, USA). A set of 10 standards ranging from 10-3000 mg/dl was run periodically to assess the accuracy of the NAD-ADH Detection system.

RESULTS

Histology

Coronal brain sections encompassing the EW were examined under a microscope for accuracy of the lesion and the degree of tissue damage. Accuracy of the lesions were judged based on the extent of midline destruction of the large cell bodies known to be contained within the EW (see Chapter 2). For the most part, tissue damage was limited to a 200 μm diameter region, which

was sufficient to destroy a large portion of the EW with little damage to the surrounding areas (Figure 23). Accurate lesions were observed in approximately one-third of the animals that underwent surgery for EW lesions (32 out of 90). Animals with accurate lesions had no overt behavioral side effects that impeded the ability to record any of the measures outlined below. Some animals (n=19) recovered from surgery with a significant list and possessed varying degrees of circling locomotor behavior. This behavior ceased within a few days following the surgery. Of the animals that developed this behavior post-operatively, most (n=17) possessed lesions missing the EW laterally. Some animals (n=10) with electrical current delivery did not fully recover from surgery and died post-operatively. Preliminary analyses revealed that animals with misplaced lesions performed similar to the sham control animals, however because of unknown effects of the electric current application and disparate locations of the missed lesions, these animals were eliminated from the final analyses.

Effect of Edinger-Westphal nucleus lesions on ethanol sensitivity measures

Animals with EW lesions were compared to sham-operated controls on a number of ethanol sensitivity measures including ethanol-induced hypothermia, sedation, and locomotor activation. These three sensitivity measures were selected based on previous experiments showing that a positive relationship between EW Ucn exists for ethanol-induced hypothermia, but not for ethanol-induced sedation or activation. In support of this relationship, a comparison of sham-operated control animals with EW-lesioned animals demonstrated

disruption of thermoregulatory responses in the EW-lesioned animals. This effect was apparent in both the basal state and the ethanol-induced hypothermic state (Figure 24). Although not statistically significant, animals with EW lesions tended to have lower baseline body temperatures ($F(1,34) = 3.73$, $p = 0.062$). Ethanol-induced hypothermia was significantly blunted at both 15-minute and 30-minute post injection in EW-lesioned animals compared with sham-operated animals ($F(1,34) = 9.64$, $p < 0.004$). This effect was not a result of differences in the metabolism of ethanol as BEC measurements were equivalent between the two groups ($F(1,33) < 1$, NS, Figure 24).

Further tests of ethanol sensitivity, revealed specificity for thermoregulatory responses over other sensitivity measures. This is demonstrated in the tests of ethanol-induced sedation, which was indexed by the duration of loss of righting reflex (Figure 25). No significant differences between EW-lesioned and sham-operated control animals were observed ($F(1,16) < 1$, NS). Furthermore, locomotor activity in response to a low dose of ethanol (1.8 g/kg) was also comparable between EW-lesioned and sham-operated control mice ($F(1,30) < 1$, NS, Figure 25). It should be noted that mice displayed no locomotor activity increases in response to ethanol. This is characteristic of the C57BL/6J mouse strain and may prohibit the detection of differences in this response. Assessment of basal anxiety using the ratio of central activity to total activity on day 1 of locomotor activity testing revealed no significant difference between the sham-operated (0.14 ± 0.021) and EW-lesioned (0.14 ± 0.037) groups ($F(1,15) < 1$, NS). Likewise, the groups did not differ on the Central/Total

ratio following ethanol administration nor did ethanol appear to have an effect on the Central/Total ratio (EW-lesioned: 0.15 ± 0.031 and sham-operated: 0.15 ± 0.032, $F(1,15) < 1$, NS).

Effect of Edinger-Westphal nucleus lesions on morphine-induced hypothermia

If the EW is necessary for thermoregulation, it would be expected that lesions of the EW would result in a disruption of all forms of thermoregulatory challenges. Therefore, we sought to test the specificity of an EW lesion to disrupt ethanol-induced hypothermia by testing EW-lesioned animals for morphine-induced hypothermia. Morphine was selected for two reasons. First, the hypothermic response to morphine is often more dramatic than ethanol. Second, morphine was observed to induce c-Fos expression, albeit to a lesser extent, at both high and low doses (see Chapter 1). Evaluation of morphine-induced hypothermia revealed that EW-lesioned mice had normal hypothermic responses at 30-minutes post injection when compared to sham-operated control mice, suggesting that the EW is not sufficient to disrupt morphine-induced hypothermia ($F(1,16) < 1$, NS, Figure 26).

Effect of Edinger-Westphal nucleus lesions on pupillary responses

The EW has been implicated in oculomotor functions where disinhibition of the nucleus produces a mydriatic response to light (Westphal, 1887; Warwick, 1954; Roste and Dietrichs, 1988; Burde and Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). It is therefore of interest to test the influence of EW lesions

on pupillary responses. In comparison with sham-operated control mice, there was no significant difference in the basal pupil diameter of the EW-lesioned mice ($F(1,32) = 1.617$, $p = 0.21$). It is believed that the EW is under tonic inhibition, which when removed, results in pupillary dilation. Removal of this tonic inhibition through an EW lesion would thus be expected to produce a persistently large pupil. This was not observed and in fact, a trend toward a smaller pupil was observed in the EW-lesioned animals (336.28 ± 21.58) vs. the sham-operated (378.56 ± 25.59). Visualization of the pupillary constriction response was made in response to a brief flash of light. Although quantification of this response was not performed, there were no detectable differences between the pupil constriction response of sham-operated and EW-lesioned mice.

Effect of Edinger-Westphal nucleus lesions on ethanol consumption

Comparison of EW-lesioned and Sham-operated animals on the two-bottle choice ethanol consumption test revealed that EW-lesioned mice consumed significantly less ethanol at the 3, 6, and 10% concentrations of ethanol (Figure 27A). A mixed design analysis of variance revealed that there was an overall effect of lesion ($F(1,60) = 18.66$, $p < 0.005$). The daily dose of ethanol significantly increased as the concentration of ethanol was increased ($F(3,60) = 92.93$, $p < 0.001$). In addition, significant interactive effects between Group and concentration were detected and are shown in Figure 26 ($F(3,60) = 4.67$, $p < 0.006$).

Likewise, analysis of the preference ratios at 3%, 6%, and 10% ethanol concentrations also demonstrated that EW-lesioned mice had significantly diminished preference for ethanol compared with sham-operated animals (Figure 27B). A mixed design analysis of variance for the preference ratios detected significant group differences ($F(1,60) = 12.78$, $p < 0.0002$). The preference ratios across the ethanol concentrations significantly increased as the concentration of ethanol was increased ($F(3,60) = 6.80$, $p < 0.0005$). In addition, significant interactive effects between Group and concentration were detected and are shown in Figure 27 ($F(3,60) = 7.34$, $p < 0.0005$).

The differences observed between the EW-lesion and sham-operated groups are likely not due to altered taste sensitivity because animals from both groups strongly preferred both the 1.7% and 4.3% sucrose solution (Figure 28A). Animals from both groups preferred the 4.3% sucrose significantly more than the 1.7% sucrose ($F(1,20) = 108.46$, $p < 0.0001$). There were no significant interactive effects between the groups and preference across the two sucrose concentrations ($F(1,20) = 1.22$, $p = 0.28$). Despite using low concentrations of sucrose, animals consumed nearly all fluid during the sucrose days from the sucrose-containing bottle, suggesting that animals were displaying equal taste sensitivity at these concentrations. However, it is possible that no group differences were revealed because of a ceiling effect. Perhaps at lower sucrose concentrations group differences would be more apparent because animals would be less willing to consume from the sucrose-containing bottle. Consumption of the sucrose solution was significantly higher in the sham-

operated animals compared with the EW-lesioned animals ($F(1,20) = 5.94$, $p < 0.03$, Figure 28B). Animals from both groups consumed significantly more of the 4.3% sucrose compared to the 1.7% sucrose ($F(1,20) = 43.15$, $p < 0.0001$). There were no significant interactive effects of group and sucrose consumption ($F(1,20) = 3.47$, $p = 0.08$).

In addition, animals from both groups avoided the fluid bottle containing 0.03mM and 0.1 mM quinine (Figure 28C). There were no group differences in either preference ratio or consumption of the quinine at either concentration ($F(1,20) < 1$, NS and $F(1,20) < 1$, NS, respectively). Animals of both groups avoided the 0.1 quinine significantly more and consumed significantly less of the 0.1 mM quinine compared with 0.03 mM quinine ($F(1,20) = 25.75$, $p < 0.0001$ and $F(1,20) = 19.58$, $p < 0.0005$, respectively). There were no significant interactive effects between group and quinine concentration for quinine avoidance or quinine consumption ($F(1,20) = 2.25$, $p = 0.14$ and $F(1,20) = 2.44$, $p = 0.13$, respectively).

Differences between the EW-lesioned animals and sham-operated animals were observed in total fluid consumption and water consumption throughout the 2-bottle procedure (Figure 29). No overall group differences were detected for total fluid consumption ($F(1,133) < 1$, NS). However, there was a significant change in total fluid consumption during the 2-bottle procedure across days ($F(7,133) = 49.30$, $p < 0.0001$). There were significant interactive effects between group and total fluid consumption across days (Figure 29A, $F(1,133) = 7.17$, $p < 0.0001$). In addition, analyses performed on water consumption during

the 2-bottle choice test revealed a significant effect of group $F(1,140) = 10.02$, $p < 0.005$), Days ($F(7,140) = 60.37$, $p < 0.0001$), and Group X Days interaction ($F(7,140) = 3.73$, $p < 0.001$). In general, EW-lesioned animals consumed more water when ethanol was presented in the alternative bottle (Figure 29B).

DISCUSSION

This series of experiments assessed the effects of electrolytic lesions of the EW on several ethanol-mediated responses in male C57BL/6J mice. It was observed that EW lesions selectively disrupted ethanol-induced hypothermia, while other measures of ethanol sensitivity such as locomotor activity and ethanol-induced sedation were unchanged compared with sham-operated control animals. In addition, mice with EW lesions displayed decreased preference for ethanol-containing solutions and decreased ethanol consumption using the 2-bottle choice test.

We failed to observe a difference in ethanol consumption and preference between EW-lesioned and sham-operated animals at the 20% ethanol concentration. The lack of differences in ethanol preference are likely due to the substantial decrease in preference observed in the sham-operated animals, which displayed strong preferences for the 6% and 10% ethanol concentrations. The consumption measure used in the analysis corresponds to the dose consumed per day, which is obscured by the increasing concentrations. While the volume of ethanol consumed slightly increased for the sham-operated animals, the EW-lesioned animals displayed no increase and the resulting

increase in dose of ethanol consumed was simply a result of the increasing concentration and not consumption alone.

The EW has attracted recent interest given its sensitivity to both voluntary and involuntary ethanol administration using c-Fos as a marker for neuronal activity (Chang et al., 1995; Topple et al., 1998; Bachtell et al., 1999; Ryabinin et al., 2001). In addition, the EW is the primary source of the neuropeptide Ucn in the brain (Vaughan et al., 1995). Both involuntary and voluntary ethanol-induced c-Fos expression in the EW has been shown to occur within Ucn-containing neurons (Bachtell et al., 2002; Ryabinin et al., 2003). Using several genetic models, a characterization of the EW Ucn system and its relationship to ethanol-mediated responses was performed (Bachtell et al., 2002; Bachtell et al., 2003b). These experiments demonstrated that Ucn in the EW was positively correlated with a hypothermic response to ethanol as well as ethanol consumption. These results concur with the altered behaviors observed following EW lesions. Thus, by disrupting the EW Ucn system with electrolytic lesions, ethanol-induced hypothermia and ethanol consumption were dramatically decreased.

While the electrolytic lesion strategy used in these experiments significantly disrupted the Ucn system within the EW, it is likely that many inputs and outputs were disrupted as well. Thus, these lesions are not specific to the Ucn neurons in the EW. An alternative strategy in determining the role of Ucn would be gene deletion strategies. The development of Ucn-deficient mice has been used to identify the functions of Ucn. The results, however, are not consistent. Thus, two separate groups have developed Ucn-deficient mice with

conflicting outcomes (Vetter et al., 2002; Wang et al., 2002). One study reported that Ucn-deficient mice display increased anxiety-like behavior and hearing impairments. The other study reported normal anxiety responses and normal hearing, but impaired startle response in Ucn-deficient mice. While these strategies are advantageous in their specificity for deleting Ucn, the abundance of Ucn in peripheral tissues obscures the significance of Ucn in the brain, where it is very discretely localized.

Based on the findings in EW-lesioned animals, it could be hypothesized that Ucn-deficient mice may have altered baseline thermoregulatory systems. It has been shown that members of the CRF/Ucn family such as CRF, sauvagine, and urotensin, produce thermogenic actions when administered centrally (Le Feuvre et al., 1989). Thus, central and peripheral administration of CRF and sauvagine induce a dose-dependent hypothermia in rats. Ucn is known to produce potent and long-lasting hypotension and vasodilation (Vaughan et al., 1995; Lubomirov et al., 2001). These alterations may contribute to alterations in thermoregulatory processes. The poorly understood role of the CRF/Ucn family of peptides in thermoregulation demands further investigation.

The finding that EW lesions decreased preferences for ethanol solutions and ethanol consumption suggests that the EW regulates ethanol consumption patterns. While this is a robust effect, it is likely a secondary effect of the diminished ethanol-induced hypothermia. It is typically believed that decreased ethanol sensitivity results in increased ethanol consumption. For example, Cunningham and Niehus (Cunningham and Niehus, 1989) demonstrated that

ethanol consumption is inversely related to ethanol-induced hypothermia. Thus, higher ethanol consumption was observed in rats subsequently exposed to elevated ambient temperatures, a manipulation that eliminates ethanol-induced hypothermia. Based on these findings, it would be expected that, if animals have reduced ethanol-induced hypothermia, they would consume more ethanol. This was not observed in the EW-lesioned mice, where decreased hypothermic responses relate to decreased ethanol consumption. In addition, as already noted, B6 and D2 mice do not show consistent differences in ethanol-induced hypothermia, yet the differ reliably and dramatically in ethanol consumption.

One possible explanation for our temperature results is that the EW is responsible for maintaining basal temperatures and elimination of the EW would result in a disruption of this maintenance process. A trend for this effect was identified in the present experiments where lesioned mice tended to have lower baseline body temperatures. This finding supports the significant positive correlation between Ucn in the EW and baseline body temperature, which was identified in B6D2 F2 mice (Bachtell et al., 2002). Lowered baseline body temperature would essentially render the mice more similar to an intoxicated state, at least from a thermoregulatory standpoint, which may result in a decreased desire to consume ethanol. Therefore, the avoidance of ethanol-containing solutions would result not from the decreased sensitivity to ethanol, but from the altered baseline state of the animal.

Regardless of mechanisms, these findings contradict the traditional functions assigned to the EW. For many years, the EW was considered a primary

component of the neurocircuitry of the oculomotor response. We observed that EW lesions had no effect on the pupil diameter and the pupillary response to light. Other recent evidence also suggests that the function of the EW may be more diverse. For example, the presence of Ucn in the EW suggests that it may mediate anxiety behaviors. In fact, EW Ucn was shown to upregulate in response to a 3-hr restraint stress (Roste and Dietrichs, 1988; Spence and Saint-Cyr, 1988a; Bittencourt and Sawchenko, 2000). Interestingly, this type of stress has been associated with hypothermic responses (Herbert and Howes, 1993; Johnson et al., 2000). Based on this, it could be hypothesized that Ucn in the EW is important in hypothermic responses to other stimuli as well. Based on our findings, it appears that, depending on the stimulus, hypothermic responses may be generated through an EW-dependent or EW-independent mechanism. This notion is based on the observed hypothermic effects of morphine, which were nearly identical in EW-lesioned and sham-operated animals. The failure to detect differences between the groups could have resulted from the exceedingly high morphine dose or from the diminished differences in baseline temperature prior to morphine administration.

Our findings suggest that ethanol-induced hypothermia occurs through an EW-dependent pathway. Other studies using a variety of stimuli support also the EW's role in temperature regulation. Thus, transneuronal labeling was observed in EW neurons following injection of a pseudorabies virus injected into the wall of the ventral tail artery of rats, which is a primary mediator of increased sympathetic blood flow for heat loss (Smith et al., 1998). The EW has also been

shown to play a role in mediating the choroidal vasculature that is responsible for temperature modulation in the eye (Parver, 1991). Finally, animals acutely exposed to both warm and cold ambient temperatures show elevated c-Fos expression in the EW compared with control animals (Bachtell et al., 2003a). Therefore, the EW, as previously suggested, may represent a novel midbrain component of a thermoregulation detection system (Nagashima et al., 2000).

SUMMARY AND CONCLUSIONS

The experiments presented here represent a logical continuation of previous findings demonstrating that the EW Ucn system is highly responsive to ethanol and may regulate ethanol-induced hypothermic responses and ethanol consumption. The results reported use an electrolytic strategy to manipulate the EW Ucn system and support previous findings. Thereby, animals with EW-lesions demonstrate decreased ethanol-induced hypothermia and ethanol consumption. These findings contradict the traditional functions attributed to the EW and represent a novel role for the EW to regulate temperature and consumption.

Figure 23. Thionin staining was used for histological verification of placement and damage assessment caused by the EW-lesion surgery. An example of the tissue damage caused by the surgery in (A) sham-operated and (C) EW-lesioned animals. To assess the degree of damage to the urocortin cells in the EW, immunohistochemistry was performed in (B) sham-operated and (D) EW-lesioned animals. Note the relatively localized disruption of the EW nucleus and Urocortin cells with little damage to peripheral regions.

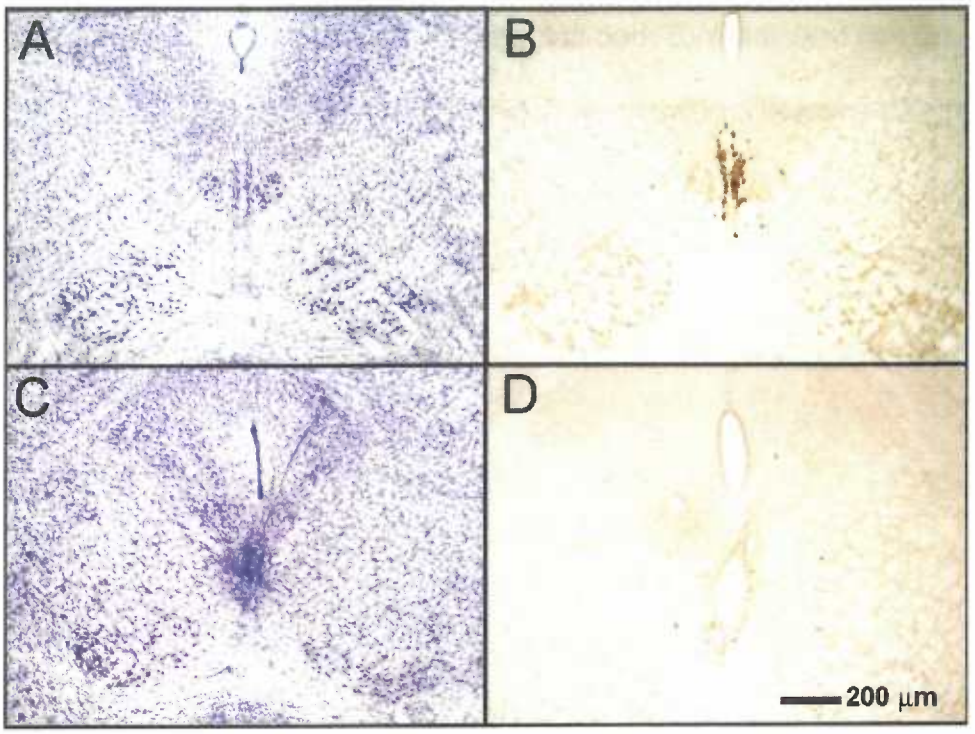
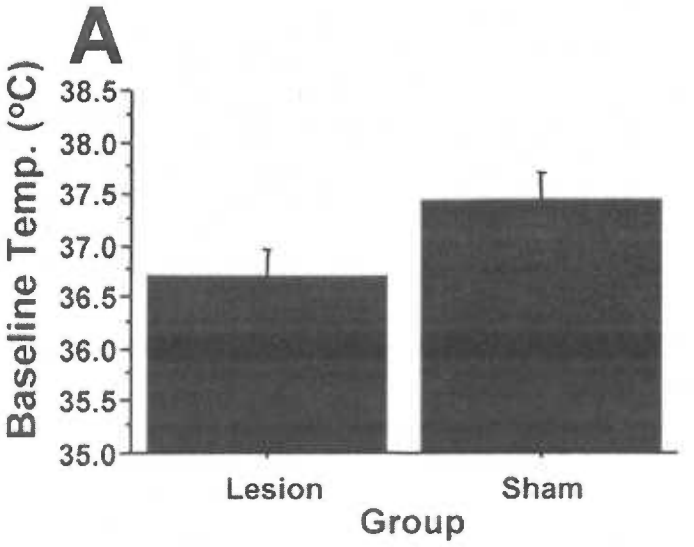
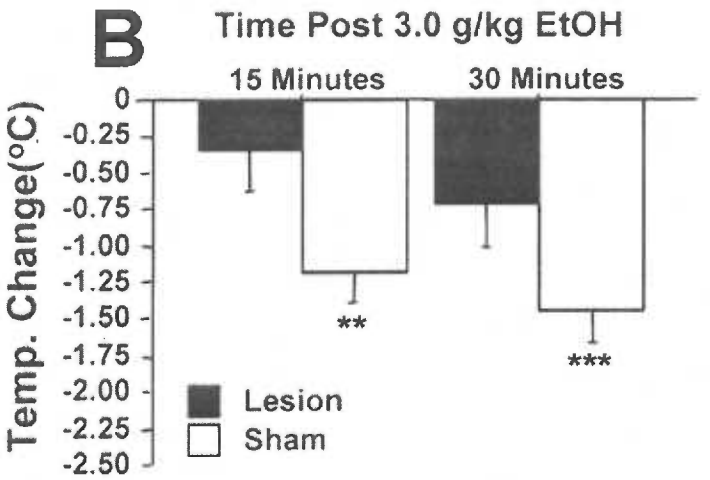

A
B
C
D
200 μm

Figure 24. EW-lesions caused disruptions in thermoregulatory processes compared with sham-operated animals. (A) Slight disruptions in basal thermoregulation were apparent in the EW-Lesioned mice (n=20) compared with sham-operated mice (n=16). (B) At both 15- and 30-min after an injection of 3.0 g/kg (i.p.) ethanol, EW-lesioned animals (n=20) displayed a significantly blunted hypothermic response compared with sham-operated animals (n=16). ** $p < 0.01$, *** $p < 0.005$ (C) Blood ethanol concentrations were measured 60 minutes following an i.p. injection of 4 g/kg ethanol in both EW-lesioned (n=19) and sham-operated (n=16) animals. No differences were observed between the two groups.


A
Baseline Temp. (°C)
38.5
38.0
37.5
37.0
36.5
36.0
35.5
35.0
Lesion
Sham
Group


B
Time Post 3.0 g/kg EtOH
15 Minutes
30 Minutes
Temp. Change(°C)
0
-0.25
-0.50
-0.75
-1.00
-1.25
-1.50
-1.75
-2.00
-2.25
-2.50
**
***
Lesion
Sham

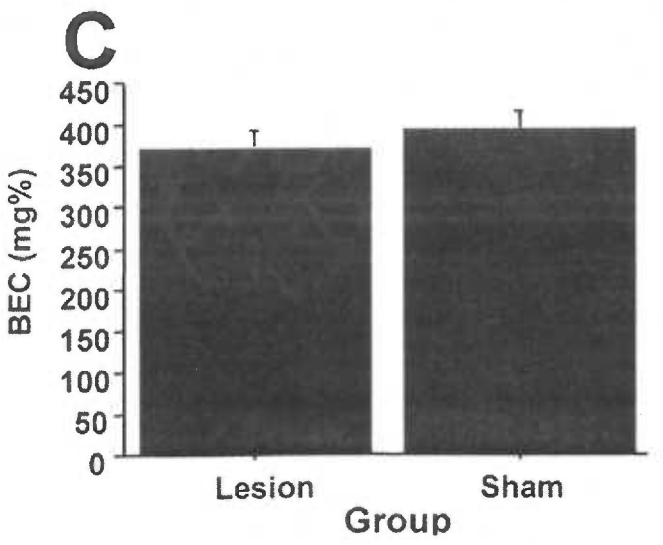
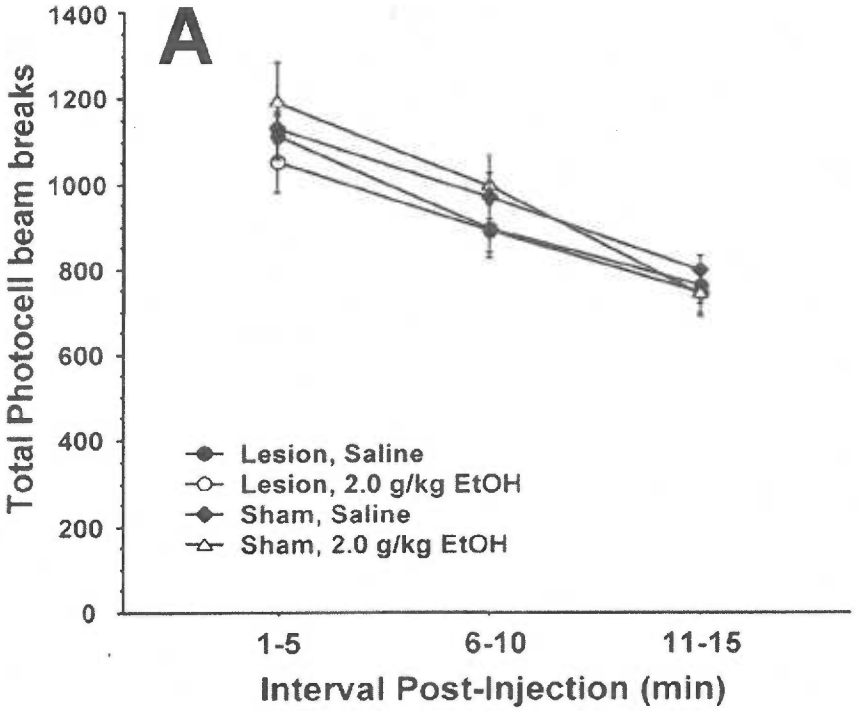

C
BEC (mg%)
450
400
350
300
250
200
150
100
50
0
Lesion
Sham
Group

Figure 25. Ethanol sensitivity, as measured by locomotor activity and ethanol-induced sedation, were not altered by EW lesions. (A) Locomotor responses were recorded 15-min following both a saline injection and a 1.8 g/kg dose (i.p.) of ethanol on successive days. Both EW-lesioned (n=10) and sham-operated (n=8) animals performed similarly and no significant differences were observed. (B) The duration of the loss of righting reflex was measured following a 4 g/kg injection (i.p.) of ethanol. EW-lesioned animals (n=10) displayed nearly identical responses to sham-operated animals (n=8).


A
Total Photocell beam breaks
1400
1200
1000
800
600
400
200
0
Lesion, Saline
Lesion, 2.0 g/kg EtOH
Sham, Saline
Sham, 2.0 g/kg EtOH
1-5
6-10
11-15
Interval Post-Injection (min)

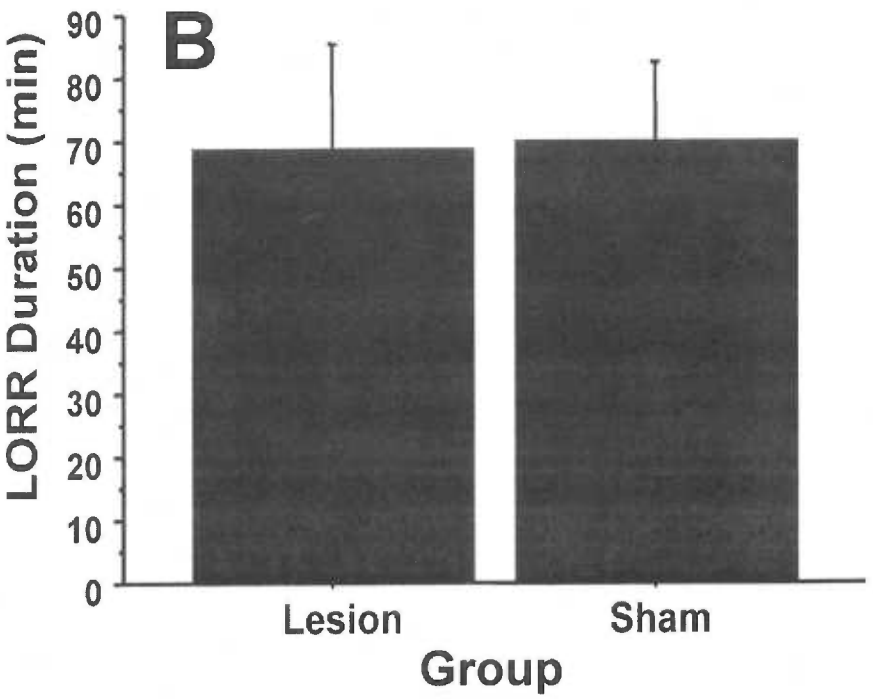

B
LORR Duration (min)
90
80
70
60
50
40
30
20
10
0
Lesion
Sham
Group

Figure 26. Lesions of the EW had no effects on thermoregulatory responses following an acute injection of morphine. (A) Baseline body temperatures were not significantly different, but EW-Lesioned mice (n=20) tended to have lower baseline temperatures compared with sham-operated mice (n=16). (B) Hypothermic responses to 30 mg/kg (i.p.) morphine were measured 30 minutes following injection in both EW-lesioned (n=10) and sham-operated (n=8) animals. No statistically significant effects were observed between the groups.

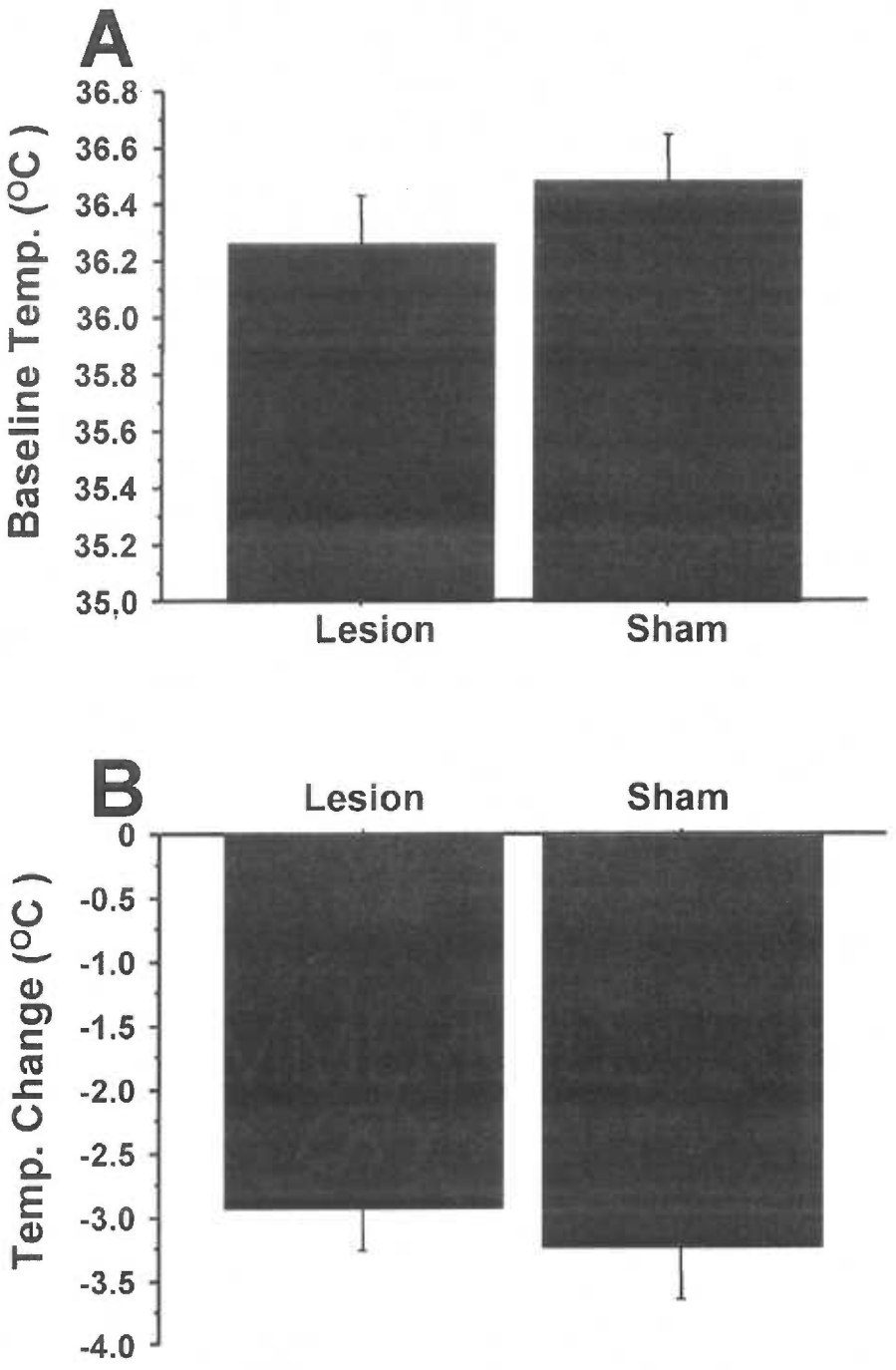

A
Baseline Temp. (°C)
36.8
36.6
36.4
36.2
36.0
35.8
35.6
35.4
35.2
35,0
Lesion
Sham
B
Lesion
Sham
Temp. Change (°C)
0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0

Figure 27. Lesions of the EW diminished ethanol consumption. (A) EW-lesioned animals (n=12) consumed less daily ethanol across the 3%, 6% and 10% ethanol concentrations compared with sham-operated animals (n=10) (** significant post-hoc comparison, $p < 0.0001$). Consumption of 20% ethanol was not significantly different. (B) EW-lesioned animals demonstrate a decreased preference for ethanol compared with sham-operated animals (* significant post-hoc comparison, $p < 0.005$). EW-lesioned and sham-operated animals preferred ethanol similarly at the 20% concentration.

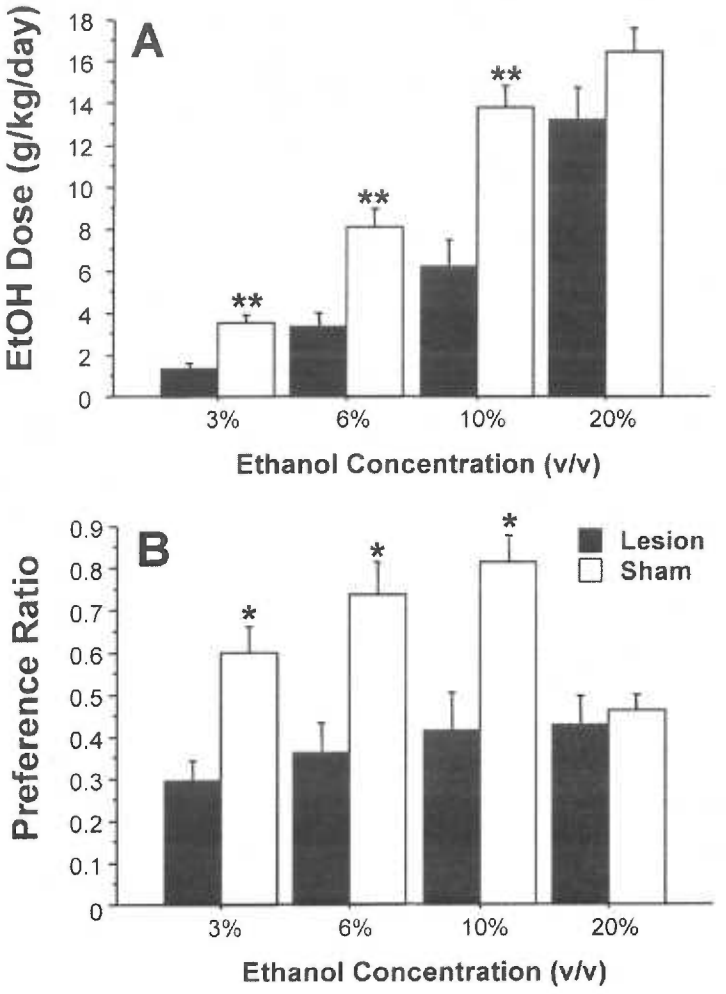

A
EtOH Dose (g/kg/day)
18
16
14
12
10
8
6
4
2
0
**
**
**
3%
6%
10%
20%
Ethanol Concentration (v/v)
B
Preference Ratio
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
Lesion
Sham
3%
6%
10%
20%
Ethanol Concentration (v/v)

Figure 28. EW-lesioned (n=12) and sham-operated (n=10) animals performed similarly on the taste sensitivity tests. (A) Both EW-lesioned and sham-operated animals strongly preferred sucrose at the 1.7% and 4.3% concentrations. (B) Consumption of the sucrose solution was significantly different between EW-lesioned and sham-operated animals. Sham-operated animals consumed more sucrose than EW-lesioned animals. (C) Both EW-lesioned and sham-operated animals avoided quinine at the 0.03 and 0.1 mM concentrations. (D) Consumption of quinine at both concentrations was equivalent between the two groups.

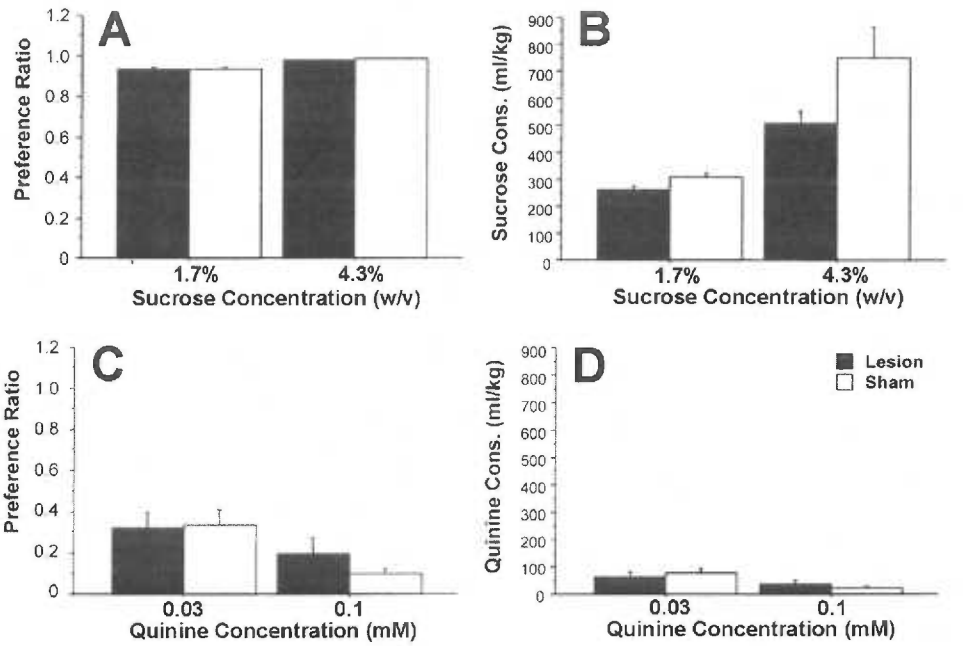
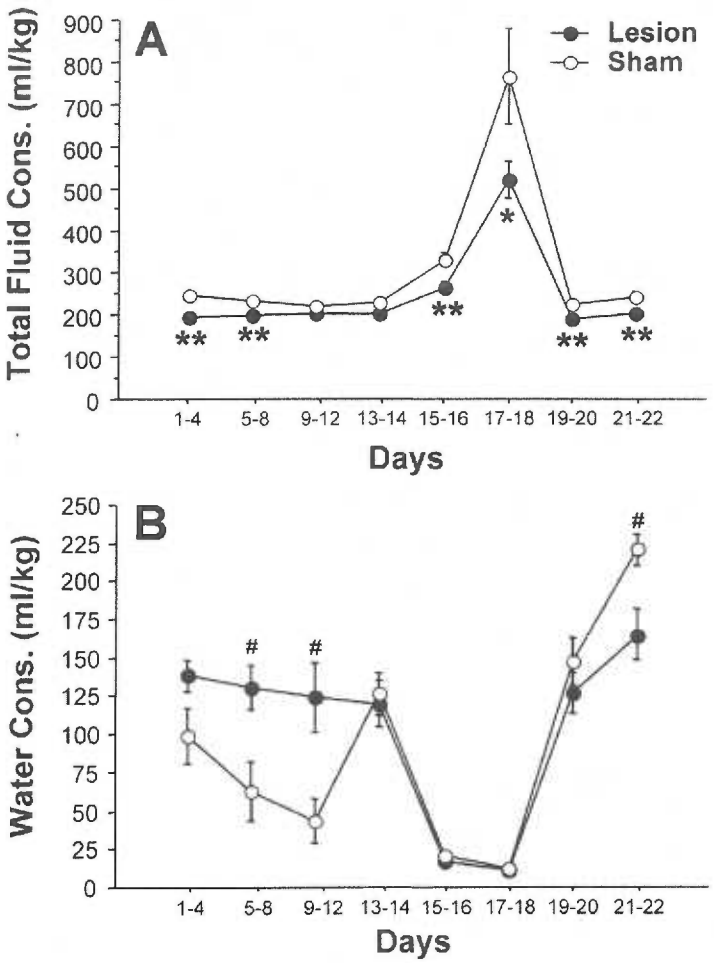

A
Preference Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0
1.7%
4.3%
Sucrose Concentration (w/v)
B
Sucrose Cons. (ml/kg)
900
800
700
600
500
400
300
200
100
0
1.7%
4.3%
Sucrose Concentration (w/v)
C
Preference Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0
0.03
0.1
Quinine Concentration (mM)
D
Lesion
Sham
Quinine Cons. (ml/kg)
900
800
700
600
500
400
300
200
100
0
0.03
0.1
Quinine Concentration (mM)

Figure 29. Total fluid consumption and water consumption during the 2-bottle procedure. Animals always had access to one bottle containing tap water. The other bottle was changed as follows: Days 1-4 3% ethanol, Days 5-8 6% ethanol, Days 9-12 10% ethanol, Days 13-14 20% ethanol, Days 15-16 1.7% sucrose, Days 17-18 4.3% sucrose, Days 19-20 0.03 mM quinine, Days 21-22 0.1 mM quinine. Bottle positions were alternated daily to avoid the development of a side preference. (A) An analysis of total fluid consumption throughout the procedure demonstrated that EW-lesioned animals (n=12) had reduced fluid consumption during some portions of the procedure compared with sham-operated animals (n=10) (* significant post-hoc comparison, $p < 0.05$, ** significant post-hoc comparison, $p < 0.005$). (B) Analysis of water consumption during the procedure revealed significant group differences that depended on the fluid alternative. When 6% ethanol and 10% ethanol were the alternative to water, EW-lesioned animals consumed more water than the sham-operated animals ($^{\#}$ significant post-hoc comparison, $p < 0.01$). When 0.1 mM quinine was the alternative to water, sham-operated animals consumed more water than EW-lesioned animals ($^{\#}$ significant post-hoc comparison, $p < 0.01$).


A
Lesion
Sham
Total Fluid Cons. (ml/kg)
900
800
700
600
500
400
300
200
100
0
**
**
**
*
**
**
1-4
5-8
9-12
13-14
15-16
17-18
19-20
21-22
Days
B
Water Cons. (ml/kg)
250
225
200
175
150
125
100
75
50
25
0
#
#
#
1-4
5-8
9-12
13-14
15-16
17-18
19-20
21-22
Days

GENERAL DISCUSSION

The primary objective of this dissertation was to characterize the EW and its responsiveness to ethanol. This was achieved by identifying the pharmacological and intracellular mechanisms that contribute to ethanol-induced c-Fos expression in the EW. The cellular composition of the EW was identified with an emphasis on the cell types in the EW that are targeted by ethanol. Finally, two approaches were used to ascertain the functional significance of ethanol's actions on the EW. The first approach utilized a heterogeneous genetic model to develop relationships between Ucn expression in the EW and ethanol-mediated behaviors. The second approach identified the impact of electrolytic lesions of the EW on ethanol-mediated behaviors. The results of these combined approaches provided great insight into the role of the EW during ethanol exposure.

Ethanol's effects on the Edinger-Westphal nucleus

The focus on the EW in this dissertation was derived from a series of ITF brain mapping studies aimed at identifying ethanol-sensitive neural structures. Chang and colleagues (1995) were the first to observe elevations in c-Fos expression following an acute ethanol injection. In this report, c-Fos expression was also characterized following a repeated ethanol injection regimen. While the c-Fos response of most ethanol-sensitive brain structures desensitized to repeated ethanol injections, the expression of c-Fos in the EW persisted. This

finding suggested that ethanol's effects in the EW differ from that in other ethanol-sensitive sites.

The unique sensitivity of the EW to ethanol was again demonstrated by Topple and colleagues (1998), who observed an increase in c-Fos expression in the EW of rats self-administering ethanol-containing beer during daily 30-min sessions over a 21-day period. Not only did this provide support for the persistence of ethanol-induced c-Fos expression in the EW, it demonstrated that the EW was responsive to ethanol that was voluntarily administered. Importantly, other structures, such as the central nucleus of the amygdala, the bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus, and paraventricular nucleus of the thalamus that are quite sensitive to acute administration of involuntary ethanol administration, were not responsive to voluntary administration. Similar findings have been repeatedly shown using a variety of ethanol consumption models (Bachtell et al., 1999; Ryabinin et al., 2001; Weitemier et al., 2001; Ryabinin et al., 2003). Taken together, the induction of c-Fos in the EW following both voluntary and involuntary administration of ethanol, and its sensitivity to repeated ethanol treatments indicates that this is an important neural target for ethanol.

Given that the EW is a sensitive site during ethanol intoxication, the question remained about the cell type within the EW that expresses c-Fos. Previous reports observed that benzodiazepine treatment was not only capable of inducing c-Fos in the EW, but that long-term benzodiazepine treatment upregulated Ucn mRNA in the EW (Cutrera et al., 1993; Skelton et al., 2000).

These data suggest that ethanol, which acts as a positive modulator of the $GABA_A$ receptor much like benzodiazepines, would target Ucn cells in the EW as well. Indeed, using double immunohistochemistry, we observed that ethanol-induced c-Fos expression colocalized with Ucn in the EW. This finding has been replicated in mice voluntarily consuming large quantities of ethanol (Ryabinin et al., 2003). Since the EW is the predominant site of Ucn expression in the brain (Vaughan et al., 1995; Kozicz et al., 1998; Yamamoto et al., 1998; Bittencourt et al., 1999), ethanol-induced expression of c-Fos in Ucn-positive cells of the EW highly implicates an interaction between ethanol and the CRF/Ucn system.

The Corticotropin-releasing factor/urocortin system and ethanol

The role of the CRF/Ucn system in drug and alcohol abuse has become an area of interest primarily because of the HPA axis' responsiveness to drugs of abuse (Sarnyai et al., 2001). Acute ethanol administration, for example, increases HPA activity as measured by elevations in plasma adrenocorticotropin hormone and corticosterone levels (Rivier et al., 1984). In addition, acute ethanol administration elevates c-Fos expression and CRH-R1 levels in the paraventricular nucleus of the hypothalamus (Rivier and Lee, 1996; Lee and Rivier, 1997). While these changes represent physiological perturbations that may translate into behavioral changes contributing to an addicted state, the story is complicated by the ability of the CRF system to habituate and adapt to repeated ethanol exposure (Spencer and McEwen, 1990; Lee et al., 2001; Rivier and Lee, 2001).

The data presented here suggest that Ucn in the EW is another component of the CRF system that may contribute to alcoholism. Thereby, not only are Ucn neurons in the EW sensitive to acute ethanol administration, they are sensitive to repeated ethanol administration. This finding supports data demonstrating a slower habituation of ethanol-induced c-Fos in the EW (Chang et al., 1995; Topple et al., 1998). In addition, a recent report demonstrated that voluntary consumption of ethanol during 30-min daily sessions over a 14-day period results in robust elevations in c-Fos expression in EW Ucn neurons (Ryabinin et al., 2003).

Further support that Ucn may contribute to alcoholism stems from the characterization of Ucn in the EW of B6D2 F2 mice. In this population of mice, a positive relationship existed between Ucn expression in the EW and ethanol consumption. This relationship likely stems from basal Ucn differences in the EW between the ethanol-preferring B6, and ethanol-avoiding, D2, mouse strains where B6 mice possess higher Ucn expression in the EW compared with D2 mice. In addition to these findings, mounting evidence from other genetic models of ethanol consumption suggests an important relationship between Ucn expression in the EW and ethanol intake. For example, mice selectively bred for high ethanol consumption (i.e. HAP mice) in a two-bottle choice test possess higher levels of Ucn in the EW compared with mice simultaneously bred for low ethanol consumption (i.e. LAP mice) (Grahame et al., 1999; Whatley et al., 1999; Bachtell et al., 2003b). In addition, a B6 congenic mouse strain carrying a D2 chromosomal segment including *Alcp1*, which is a D2 allele relating to reduced

ethanol consumption in a primed free-choice paradigm, demonstrates a similar relationship (Whatley et al., 1999). Thereby, analysis of Ucn expression in these mice showed that ethanol-naive male B6.D2 *Alcp1* mice, possessing ethanol-avoidance drinking patterns, had lower levels of EW Ucn compared with male B6.B6 mice, which strongly prefer ethanol-containing solutions (Bachtell et al., 2003b). Taken together, this data not only supports a role for Ucn's involvement in ethanol consumption, but also suggests that Ucn expression in the EW may infer genetic predispositions dictating a willingness to consume ethanol.

The notion of genetic relationships between the CRF/Ucn system and alcoholism is not a novel concept. Roberts and colleagues (1992) demonstrated that D2 mice show significantly higher elevations in both the release of adrenocorticotropin hormone and CRF upon acute ethanol administration compared with B6 mice. In addition, George and colleagues (1990) demonstrated that Wistar rats showing high ethanol preferences possess elevated CRF-like immunoreactivity in the hypothalamus. Comparisons in CRF levels and electroencephalographic activity between alcohol preferring and non-preferring selected lines of rats show that preferring rats have depressed CRF levels in the hypothalamus, amygdala, and cortex while also having enhanced electroencephalographic responses to exogenous CRF (Ehlers et al., 1992).

Further behavioral characterization of the EW revealed that the relationship between EW Ucn and ethanol consumption interrelates with EW Ucn expression and temperature regulation. Correlations in the B6D2 F2 mice suggest that another consequence of differences in EW Ucn expression is an

altered ethanol-induced hypothermia. The use of the HOT/COLD selected lines of mice, which were selectively bred for their susceptibility or resistance to ethanol-induced hypothermia, provide support not only for the EW's role in thermoregulation, but also support the EW's role in ethanol consumption (Crabbe et al., 1987; Cunningham et al., 1991; O'Connor et al., 1993). Thereby, mice from replicate 1 of the HOT line (HOT-1) show substantially lower numbers of Ucn cells in the EW compared with mice from replicate 1 of the COLD line (COLD-1) (Bachtell et al., 2002). Interestingly, Cunningham and colleagues (1991) showed that COLD-1 mice willingly ingest ethanol solutions at high concentrations compared with HOT-1 mice. Such differences in the hedonic effects of ethanol were not observed in the replicate 2 HOT/COLD mice, where there were also no Ucn differences. Given this evidence, it is likely that genetic differences in the CRF/Ucn system may predispose animals to high and low ethanol sensitivity and/or high and low alcohol consumption.

The functional consequences of ethanol-induced c-Fos expression in urocortin neurons of the Edinger-Westphal nucleus

The experiments outlined in this dissertation used two strategies to identify the functional consequences of ethanol's action on Ucn neurons in the EW. First, a genetic strategy was used where relationships were made between Ucn in the EW and several ethanol-mediated responses. These experiments were followed by electrolytic lesions of the EW and measurements of ethanol-mediated responses. Both strategies yielded results demonstrating that EW Ucn neurons

are involved in ethanol-induced hypothermic responses as well as ethanol consumption.

Based on the results of the experiments presented here, the EW Ucn system appears to play a primary role in thermoregulatory responses. This coincides with previous findings suggesting that the EW is involved with thermoregulation (Parver, 1991; Smith et al., 1998). In addition, animals acutely exposed to both warm and cold ambient temperatures show elevated c-Fos expression in the EW compared with control animals (Bachtell et al., 2003a). Because EW neurons robustly express c-Fos following many routes of administration, it can be hypothesized that the EW regulates a behavioral response that is common across all routes of administration. One such response is the hypothermic response to ethanol.

Ethanol-induced sedation, as measured by the loss of righting reflex, typically occurs at high doses of ethanol (e.g. > 3.5 g/kg) and mice do not voluntarily consume amounts sufficient to evoke a sedative response. Likewise, ethanol-induced locomotor activation is generally observed at low doses (1.5 g/kg-2.5 g/kg). While this is comparable with the ethanol doses typically consumed voluntarily by mice, the dose-responsiveness of the locomotor stimulation does not agree with the findings demonstrating the dose-responsiveness of c-Fos expression in the EW. Elevations in EW c-Fos can be detected at very low ethanol doses (0.6 g/kg) and continue to rise until approximately 2.4 g/kg. At this dose, ethanol-induced c-Fos expression plateaus, reaching levels comparable with those seen at 3.6 g/kg and 4.8 g/kg.

Interestingly, the dose-responsiveness of EW c-Fos expression is comparable to that observed for ethanol-induced hypothermia, thus supporting a role for EW involvement in ethanol-induced hypothermia.

The EW also appears to play a role in the consumption of ethanol. While the mechanisms of this effect are still unclear, animals with lower EW Ucn cells tend to prefer ethanol to a lesser extent and consume less ethanol than animals with higher numbers of EW Ucn cells (Bachtell et al., 2003b). It is likely that this effect occurs as a secondary effect to the thermoregulation differences, which may lead to a different subjective effect of ethanol intoxication. Alternatively, the EW may be a component of an alternative reward pathway. Given its connectivity with the ventral tegmental area, it is possible that enhanced dopaminergic output, caused by ethanol's actions in the ventral tegmental area (Gessa et al., 1985; Brodie et al., 1990; Brodie et al., 1999), stimulates Ucn neurons in the EW. These neurons are known to project to the lateral septum where it may influence ethanol consumption. Confirmation of these hypotheses would be aided by further experimentation.

Limitations in the interpretations of the functional aspects of the Edinger-Westphal nucleus

While two strategies were used to ascertain the functional significance of elevated c-Fos expression in the EW following ethanol exposure, caution should be made in the interpretations of the given findings. The use of a heterogeneous population of mice to establish relationships between Ucn in the EW and

behavioral responses is faulty in that no cause and effect relationship can be determined. In addition, the correlations obtained in the analyses represent phenotypic correlations, which can be influenced by both genetic and environmental factors. Regardless of genetic or environmental factors that contributed to the observed relationships, this model simply provided a cursory examination of potential responses that may be influenced by an increased or decreased level of Ucn in the EW. In addition, the desire to gather data on as many behavior responses while limiting repeated testing could have detrimentally affected the accuracy of some of the measurements. For example, hypothermic responses to ethanol were measured at varying intervals post-ethanol injections, which likely led to increased variability and decreased reliability in the measurements. In addition, anxiety was measured using the generic thigmotaxic behavior, recorded during locomotor activity tests in an environment that was not constructed to directly assess anxiety-like behaviors. This is a less than ideal model and should be followed up using more direct measures of anxiety such as the elevated plus maze, light-dark box, or open field testing. This is especially necessary given the potential relationship of Ucn to anxiety-related behaviors (Vetter et al., 2002; Wang et al., 2002).

The other model that was used to validate some of the relationships identified was an electrolytic lesion strategy. While this strategy was fruitful in substantiating the relationships, some concerns with the electrolytic lesion strategy may obscure the interpretation of results. First, not only were cell bodies of the EW ablated, all fibers of passage were likely disrupted. The vast

interconnections of the EW make this an extremely important consideration. For example, we demonstrated that tyrosine hydroxylase-containing fibers pass through and terminate in the EW. Disruption of these fibers would not only influence the input to the EW, but may also affect a neurocircuit that passes through the EW. In addition, ablation of the cell bodies in the EW, Ucn-containing or otherwise, also disrupts the functioning of nuclei receiving input from the now-destroyed neurons. In the case of Ucn, a large number of Ucn fibers project to the spinal cord and the lateral septum (Bittencourt et al., 1999). Elimination of these fibers would likely lead to altered functioning in these regions as well. Thus, while it is likely that the cell bodies in the EW are part of the circuitry involved in the behavioral changes observed, it is unclear how much is contributed by the EW-proper and how much is contributed by effects in projection regions.

A second major issue with lesion studies includes the inferences that are made about the function of the ablated nucleus in an otherwise intact animal. Removal of a nucleus reveals information about an animal without the nucleus, but reveals no reliable information about what the activity of the nucleus actually is. Some of the baseline differences in the EW-lesioned animals compared to the sham-operated animals highlight the concern for this issue. For example, EW-lesioned animals tended to have a decreased basal body temperature. This suggests that the EW may be important for regulating normal homeostatic processes. In addition, animals generally consumed less fluid in the drinking studies, again suggesting that the EW is involved in some homeostatic process

that regulates fluid balance. However, the interpretability of both of these observations is obscured by the fact that EW-lesioned animals are fundamentally different animals and reveal no information about how the EW may contribute to these homeostatic processes in an animal with a fully functioning EW. The altered homeostatic systems may result from a compensatory response occurring in other regions. In addition, a partial recovery of function may occur following the lesion, which may indicate a compensatory response or an incomplete neurocircuit. Overall, the strategies used in these experiments provided a first look at the functional consequences of the EW upon ethanol exposure and further understanding would be gained by using techniques minimizing these concerns.

The Edinger-Westphal nucleus: A functional and morphological anomaly

The findings of this dissertation suggest that Ucn in the EW does not contribute to the function traditionally assigned to the EW such as oculomotor adaptation (Roste and Dietrichs, 1988; Burde and Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). While other groups have supported a role apart from this traditional view of EW (Innis and Aghajanian, 1986; Lanteri-Minet et al., 1993 Weninger, 1999 #2432; Weninger et al., 2000), it is important to consider the questions recently raised concerning what constitutes the EW (Erichsen and May, 2002). The EW is frequently described as a component of the oculomotor complex containing preganglionic neurons projecting to the ciliary ganglion. However, it has been suggested that this may not be the case for all species and

there may be an important species-specific subregion exclusively projecting to the ciliary ganglion.

For example, Erichsen & May (Erichsen and May, 2002) reported that only 5% of the cat preganglionic motoneurons are found in the EW proper. In another report, Kourouyan and Horton (Kourouyan and Horton, 1997) demonstrate that, in the primate, only the lateral visceral cell column is involved with the oculomotor response, while the medial visceral cell column is not. Therefore, it appears that the areas involved in the oculomotor response may only include a portion of what is commonly referred to as the EW. In addition, there has also been some debate about whether the EW is homologous in primate and non-primate species (Erichsen and May, 2002). Morphologically, the EW is primarily composed of peptidergic neurons with central projections related to visceral autonomic responses (Burde et al., 1982; Maciewicz et al., 1983; Maciewicz et al., 1984; Innis and Aghajanian, 1986; Roste and Dietrichs, 1988; Vaughan et al., 1995; Bittencourt et al., 1999). In contrast, the portions of the EW involved in the oculomotor response contain medium-sized preganglionic motoneurons and smaller interneurons, which are generally located laterally within the supraoculomotor area and in the anterior median nucleus (Sekiya et al., 1984; Burde, 1988; May et al., 1992). The observations in this dissertation support the conclusion that Ucn in the EW of rodents may function differently than previously thought and is involved in regulating sympathetic autonomic functions beyond the oculomotor response.

Mechanisms of ethanol's effects in the Edinger-Westphal nucleus

The EW is a compact midbrain nucleus that is most often recognized as a brain structure involved in relaying information through cholinergic neurons to the ciliary ganglion (Burde and Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). However, EW projections to areas such as the lateral septum, spinal cord, facial nucleus, inferior olive, parabrachial nucleus, periaqueductal gray, reticular formation, trigeminal nucleus, cerebellum, and vestibular nuclei implicate its position in more complex neural circuits (Roste and Dietrichs, 1988; Spence and Saint-Cyr, 1988b; Klooster et al., 1993; Bittencourt et al., 1999). Adding to this complexity are the various sources of input. The olivary pretectal nucleus sends projections to the EW, which is thought to play a role in the oculomotor response (Klooster et al., 1995; Klooster et al., 2000). On the other hand, input from several hypothalamic nuclei (Saper et al., 1976; Koss, 1986; Zheng et al., 1995) and the locus coeruleus implicate other neuroanatomical inputs potentially contributing to other behavioral or physiological responses (Loewy et al., 1973; Breen et al., 1983).

Given this complexity, it is important to characterize the mechanisms through which ethanol acts in the EW. Two mechanisms were addressed in this dissertation. First, a detailed analysis of several neurotransmitter systems was performed in order to ascertain the pharmacological mechanisms. Second, the intracellular mechanisms were identified by generating time courses of signaling proteins using immunohistochemistry. Congruent with the degree of neural

connectivity with this nucleus, the results suggest that complex neurotransmitter interactions occur during ethanol intoxication to influence activity in EW neurons. $GABA_A$ receptor modulation is necessary and sufficient for increased expression of c-Fos in EW neurons suggesting that ethanol is acting directly upon $GABA_A$ receptors. In agreement with our findings are reports showing elevated c-Fos expression and increased urocortin mRNA in EW neurons upon treatment with benzodiazepines (Cutrera et al., 1993; Skelton et al., 2000). Ethanol actions at $GABA_A$ receptors do not, however appear to be the only transmitter system involved.

Complete blockade of ethanol-induced c-Fos expression was observed by pretreatment with $\alpha_{2A/D}$-adrenoceptor and dopaminergic receptor antagonists, suggesting the necessity of these systems for ethanol-induced c-Fos in the EW. The noradrenergic and dopaminergic systems, however, are not sufficient to produce c-Fos expression in the EW. These findings support previous data showing no c-Fos expression in the EW following cocaine administration, which potently enhances extracellular levels of dopamine (Ryabinin et al., 2000). Taken together, the data in this series of experiments suggests that ethanol is acting directly at $GABA_A$ receptors, and this activity is modified by actions at $\alpha_{2A/D}$ and dopaminergic receptors. Support for this claim stems from data in which CDP-induced c-Fos expression (via $GABA_A$ receptors) is blocked by the non-selective α_2-adrenoceptor antagonist, yohimbine.

It appears that there are several neurotransmitter systems operating to influence the EW upon ethanol intoxication. Furthermore, these data implicate

the involvement of other brain nuclei upstream from the EW (Figure 30). Although thorough tracing experiments have not been performed to map the nuclei projecting to the EW upon ethanol exposure, data suggest that dopaminergic/noradrenergic neurons expressing tyrosine hydroxylase may play an important role in EW neuronal activity. These neurons likely represent noradrenergic projection neurons of the ventral noradrenergic bundle perhaps stemming from the locus coeruleus (Breen et al., 1983).

Support for this notion comes from a report emphasizing that EW neurons are under the influence of two primary sources of input (Szabadi and Bradshaw, 1996). The first source is an ascending tonic inhibitory input originating in the locus coeruleus. Physiological and pharmacological evidence supports this idea showing that this pathway releases noradrenaline onto inhibitory postsynaptic α_2-adrenoceptors to influence autonomic functions (Koss, 1986). Supporting the notion that the locus coeruleus projections to the EW can be influenced by ethanol is evidence demonstrating that ethanol decreases noradrenergic activity in the locus coeruleus (Pohorecky and Brick, 1977). This decrease is reversed by antagonists acting at α_2-adrenoceptors (Verbanck et al., 1991). In any case, the influence of locus coeruleus input onto EW neurons demands further study given the pharmacological evidence presented here.

The opioid system was shown to influence c-Fos expression in the EW, but pretreatment with naltrexone was ineffective in modulating ethanol-induced c-Fos expression. This is thought to result from morphine interacting with μ-opioid receptors on GABA interneurons and noradrenergic/neuropeptide Y neurons in

the locus coeruleus (Figure 30). Morphine's action at these receptors would result in disinhibition of the EW from its noradrenergic tone. Because the actions of ethanol and morphine are independent in the locus coeruleus, naltrexone has no effect on EW disinhibition caused by ethanol, but blocks morphine from disinhibiting the noradrenergic tone to the EW from the locus coeruleus.

The second source of input to the EW involves an indirect pathway that is mediated through a non-noradrenergic mechanism. Given the evidence presented here, it is tempting to assign this non-noradrenergic input to dopamine neurons originating in the VTA. This may occur via ethanol's actions in the VTA to increase dopaminergic activity (Bunney et al., 2000). Contradictory to this hypothesis is data showing that agonists for dopamine do not produce c-Fos induction in EW, which is supported by data demonstrating no c-Fos induction in EW neurons following cocaine administration (Ryabinin et al., 2000). Therefore, it is extremely likely that another source of input is modulating EW activity. Physiological as well as morphological data suggest that EW neurons are influenced by additional inhibitory input from the hypothalamus (Saper et al., 1976; Koss, 1986; Zheng et al., 1995). Although the transmitter systems targeted here do not address this notion, the existence of Neuropeptide Y Y5 receptors in the EW give credence to this hypothesis (Grove et al., 2000). Other evidence suggests that Substance P projections from the olivary pretectal nucleus mediate EW activity through neurokinin1 receptors (Klooster et al., 1995; Klooster et al., 2000). The studies here primarily targeted the classic neurotransmitter systems,

but it is apparent that the Substance P and neuropeptide Y, neuropeptide systems may also be involved in ethanol-induced c-Fos expression.

The characterization of the pharmacological mechanisms suggests that not only are there complex interactions between these neurotransmitters systems, but that the intracellular mechanisms may be unique. This is highlighted by the fact that all of the receptors suspected to modulate ethanol-induced c-Fos expression in EW neurons (including Y5 and neurokinin 1 receptors) act to downregulate adenylate cyclase activity. The observation that c-Fos, but not Egr1 is induced following ethanol administration is also suggestive of a unique signaling pathway (Bachtell et al., 1999; Bachtell and Ryabinin, 2001).
The experiments presented highlight this unique sequence of events in that no elevations in phospho-CREB were detected at any of the time points analyzed. Congruent with the pharmacological mechanisms, this indicates that adenylate cyclase activation of protein kinase A, and subsequent phosphorylation of CREB is not involved with ethanol-induced c-Fos expression in EW neurons. It was also observed that Elk1 phosphorylation was not upregulated at any of the time points suggesting that its activity at the serum response element is not responsible for ethanol-induced c-Fos expression.

Elevations in the serine-727 phosphorylated Stat3 concurrent with elevations of phospho-ERK 1/2 suggest that these signaling proteins are involved in ethanol-mediated events in the EW. Pharmacological blockade of ethanol-induced c-Fos in the EW through manipulation of the upstream kinase of ERK 1/2, MEK 1/2, presents evidence that signaling proteins in this pathway are

necessary for ethanol-induced c-Fos. The elevation of serine-727 phosphorylated Stat3 suggests that interactions at the c-Sis inducible element on the c-fos promotor may lead to ethanol-induced c-Fos expression. Functional activity of Stat3, however, generally requires dual phosphorylation at tyrosine-705 and serine-727 sites (Levy and Lee, 2002). The present experiments observed elevations only in the serine-727 phosphorylated form, which challenges the idea of Stat3 promoting c-fos expression. Our findings, however, are similar to a report showing that, in tissue culture, MEK 1/2 phosphorylation of ERK 1/2 occurs simultaneously with serine-727 phosphorylation, but not tyrosine-705 phosphorylation, of Stat3 (Ceresa et al., 1997). It is possible that interactions are occurring between these signaling molecules, however further investigation of the signaling pathways would be aided by studies aimed at determining whether a direct relationship between MEK 1/2-ERK1/2 occurs with Stat3.

Relationships between the mechanisms of ethanol actions in the Edinger-Westphal nucleus and the functional consequences

The experiments outlined in this dissertation have established that Ucn in the EW is important in mediating the hypothermic response to ethanol as well as ethanol consumption. In addition, it was determined that several pharmacological mechanisms are involved in ethanol's actions on the EW. A further understanding of the EW would be aided by an investigation of the relationships between the identified pharmacological systems impacting EW activity and the ethanol-mediated behaviors operating through the EW.

If the EW is associated with hypothermic responses to ethanol, then it could be expected that other compounds producing hypothermic responses would also result in enhanced c-Fos expression in the EW. This hypothesis is true for the positive modulators of the $GABA_A$ receptor, including allopregnanolone, chlordiazepoxide, and pentobarbital, which all produce significant increases in EW c-Fos expression, while also producing hypothermic responses (Greeley and Cappell, 1985; Melchior and Allen, 1992; Palmer et al., 2002). This is also true for morphine, which acts through μ-opioid receptors. Morphine administration results in both enhanced EW c-Fos expression and dramatic hypothermic responses (Belknap et al., 1998). The data gathered in the EW lesioned animals, however, contradict the notion that morphine-induced hypothermia occurs through an EW-dependent mechanism because morphine-induced hypothermia was unaffected by EW lesions. The noradrenaline and dopamine systems, however, appear to contradict this hypothesis. In the case of the noradrenaline system, the α-agonist clonidine produces hypothermic responses (McLennan, 1981; Livingston et al., 1984), however, no increases in EW c-Fos expression were observed after any doses sufficient for hypothermic responses. Likewise, apomorphine, an agonist of the dopamine system is also not sufficient to induce c-Fos expression in the EW, but does produce hypothermia at similar doses (Barnett et al., 1972; Sanchez, 1989). Taken together, these studies suggest that the GABA system may operate to influence hypothermia through an EW-dependent system, while noradrenaline and dopamine influence hypothermia through an EW-independent system.

An analysis of the receptor systems necessary for ethanol-induced c-Fos complicates the relationship between ethanol-induced EW activity and ethanol-induced hypothermia. It could be hypothesized that the receptor systems that block or reduce ethanol-induced c-Fos expression would also block or reduce ethanol-induced hypothermic responses. This hypothesis, however, is only partially supported. Using pharmacological antagonists of the noradrenaline and dopamine systems it was established that signaling through these systems was necessary for ethanol-induced c-Fos expression. The same compounds capable of blocking ethanol-induced c-Fos expression in the EW are also capable of reducing or blocking ethanol-induced hypothermia. For example, RX821001 significantly reduced ethanol-induced hypothermia (Durcan et al., 1991). Likewise, acute treatment of haloperidol is also capable of blocking ethanol-induced hypothermia (Yamawaki et al., 1984). Analysis of the GABA system challenges the existence of a relationship between EW activity and ethanol-induced hypothermia. Thereby, the $GABA_A$ antagonists, PTZ and bicuculline, which were quite potent in blocking ethanol-induced c-Fos in the EW, actually potentiate ethanol-induced hypothermia (Beleslin et al., 1997).

Taken together, the relationship between the pharmacological systems involved in EW c-Fos expression and those involved in hypothermic responses appears to involve two interacting components. The first is the noradrenaline/dopamine component where these systems are necessary for ethanol-induced EW activity and for ethanol-induced hypothermia, but not sufficient for EW activity alone. This component is, however, sufficient for

producing hypothermia as evidenced by hypothermic responses produced by agonist administration. The other component is the GABA component, which is necessary and sufficient for EW activity, while being sufficient but not necessary for hypothermic responses. It is believed that the noradrenaline/dopamine component provides tonic inhibitory influence on the EW, which is modulated by GABA. However, the obvious lack of consistencies underscores the need for additional study using systematic approaches aimed at the identification of the pharmacological influences on EW activity in the production of hypothermic responses.

Less complicated are the pharmacological relationships with ethanol consumption behaviors. It was observed that EW-lesioned animals preferred ethanol-containing solutions less, which was evident in the reduced daily ethanol dose consumed. Therefore, it would be expected that pharmacological manipulations reducing activity in the EW would result in decreased ethanol consumption. This hypothesis is true for all compounds that were observed to significantly block ethanol-induced c-Fos expression. Thus, pretreatment of chronic PTZ, yohimbine, RX 821001, and haloperidol resulted in decreased ethanol consumption (Fuchs et al., 1984; Reid et al., 1994; Buczek et al., 1998). Further support was observed with other antagonists of these systems, which produce similar effects on ethanol consumption. For example, Ro 15-4513, which is an inverse agonist at the benzodiazepine site, significantly reduces ethanol consumption (June et al., 1994). The serotonergic/dopaminergic antagonist, risperidone, also diminished ethanol consumption, an effect observed to be quite

similar to that produced by haloperidol (Panocka et al., 1993). These results support the EW's involvement in ethanol consumption behaviors. Even though these relationships appear to be clearer, further study using appropriate mechanistic tools would greatly aid the knowledge of the mechanisms associated with the EW in ethanol consumption.

SUMMARY AND CONCLUSIONS

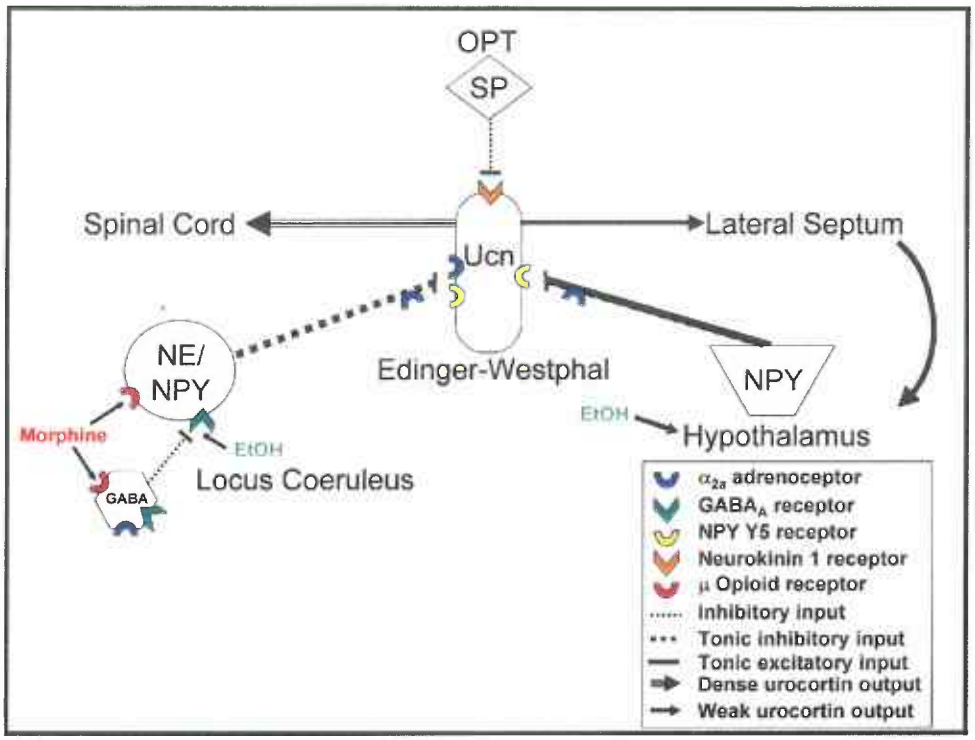
The actions of ethanol in the brain are wide spread and a primary focus has been on the involvement of the mesocorticolimbic dopamine system and extended amygdala on reward. However, it is likely that structures lying outside of these systems are important for producing behavioral effects that indirectly contribute to the development of alcoholism. Using c-Fos immunohistochemistry as a mapping tool, several studies have shown that the EW is a novel site that is highly sensitive to ethanol. Characterization of ethanol's effects on the EW, implicate the involvement of the Ucn system as identified through double-labeling immunohistochemistry. The involvement of the Ucn system for behavioral responses to ethanol was also evaluated where it was discovered that thermoregulatory responses to ethanol and ethanol consumption are mediated through actions in the EW. This provides the first demonstration implicating the EW Ucn system's involvement in behavioral responses to ethanol.

The mechanisms associated with ethanol-induced c-Fos expression were also characterized. These mechanisms include a complex set of transmitter systems including GABA, noradrenaline and dopamine. The notion that

neuropeptide systems such as Neuropeptide Y and Substance P are also mediating EW activity suggests that the identified transmitters are not the only pharmacological mechanisms involved in ethanol's actions on the EW. A characterization of the intracellular mechanisms of ethanol-induced c-Fos expression suggests that c-Fos expression is preceded by signal transduction pathway including MEK/ERK/Stat3. While the experiments contained within this dissertation begin to explore the involvement of the EW Ucn system in alcoholism, the continued study of the EW and its major projection regions will certainly help to clarify its involvement.

Figure 30. Ethanol (EtOH) causes elevations in c-Fos expression in urocortin (Ucn) neurons in the EW. Urocortin neurons in the EW are known to project primarily to the spinal cord with fewer projections to the lateral septum (Bittencourt et al., 1999). It is thought that EW neurons are under the influence of two primary sources of input (Szabadi and Bradshaw, 1996). The first source is an ascending tonic inhibitory input originating in the locus coeruleus, which is thought to contain noradrenergic/neuropeptide Y. The second source of input to the EW involves an indirect pathway from the hypothalamus that is tonically active and non-noradrenergic. Other evidence suggests that Substance P (SP) projections from the olivary pretectal nucleus (OPT) mediate EW activity through neurokinin1 receptors (Klooster et al., 1995; Klooster et al., 2000). As depicted in this hypothesized neurocircuit, ethanol's ability to induce c-Fos in the EW is thought to occur through an interaction of events occurring in these input pathways. There is considerable evidence to suggest that alcohol acts on the locus coeruleus neurons to decrease noradrenergic activity through $GABA_A$ receptor activation (Pohorecky and Brick, 1977; Verbanck et al., 1991). This effectively inhibits the tonic noradrenergic tone on EW neurons (i.e. disinihibits the EW). Ethanol is also known to act in the hypothalamus (Rivier et al., 1984; Rivier et al., 1990; Rivier and Lee, 1996; Lee and Rivier, 1997). It is hypothesized that tonically active neurons projecting to the EW from the hypothalamus become hyperactive during ethanol administration, while remaining inactive during morphine, chlordiazepoxide or allopregnanolone. This explains the inability of morphine, chlordiazepoxide and allopregnanolone to elevate c-Fos expression to

the levels achieved following ethanol administration. The hyperactivity of the hypothalamic pathway coupled with the decreased noradrenergic inhibition from the LC ultimately leads to robust c-Fos expression following ethanol administration. While it is unclear which hypothalamic nuclei project to the EW and what the nature of these projections is, it appears that neuropeptide Y (NPY) is a likely candidate. Not only does the EW contain a high density of NPY Y5 receptors (Grove et al., 2000), the NPY system has been implicated in ethanol sensitivity and ethanol consumption (Thiele et al., 1998). In addition, strong projections from the lateral septum to the hypothalamus suggest a possible feedback loop that may influence both fluid consumption and ethanol sensitivity (Raisman, 1966; Swanson and Cowan, 1979; Sawchenko and Swanson, 1983).


OPT
SP
Spinal Cord
Lateral Septum
Ucn
NE/
NPY
Edinger-Westphal
NPY
EtOH
Hypothalamus
Morphine
EtOH
Locus Coeruleus
GABA
α_{2a} adrenoceptor
$GABA_A$ receptor
NPY Y5 receptor
Neurokinin 1 receptor
μ Opioid receptor
Inhibitory input
Tonic inhibitory input
Tonic excitatory input
Dense urocortin output
Weak urocortin output

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