2013

CHARACTERIZING CONSUMPTION, DEPENDENCE, AND THE ROLE OF GLUCOCORTICOIDS IN AN ANIMAL MODEL OF VOLUNTARY ETHANOL CONSUMPTION

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Characterizing Consumption, Dependence, and the role of Glucocorticoids in an Animal
Model of Voluntary Ethanol Consumption

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Science
at the University of Kentucky

By

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Lexington, Kentucky

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2013

ABSTRACT OF DISSERTATION

Characterizing Consumption, Dependence,
and the role of Glucocorticoids in an
Animal Model of Voluntary Ethanol Consumption

Alcohol abuse disorders (AUD) represent a serious worldwide health problem with far reaching social, financial, and interpersonal implications. One of the most devastating facets of these disorders is the propensity to relapse following periods of abstinence. Ethanol withdrawal (EWD) is believed to promote relapse by increasing anxiety and craving, and may contribute to the development of cognitive decline associated with long-term dependence. Clinical data suggest that stress also plays a main role in both the development of AUD as well as relapse to drinking. As a physiological stressor, EtOH elevates levels of stress hormones (cortisol in humans, corticosterone (CORT) in the rat). Both CORT and EtOH have been shown to alter the composition, function, and activity of the N-methyl-D-aspartate (NMDA) receptor, and in particular, the NR2B subunit of this receptor. These alterations have been suggested to mediate EWD, which may negatively impact abstinence rates. This synergistic interaction between EtOH and CORT may present a therapeutic target for the treatment of EWD. In fact, data suggest that blocking the glucocorticoid receptor, which is a main target for CORT, with RU-486 could promote abstinence, as treatment with the drug has been shown to reduce consumption and the development dependence, as well as the severity of EWD and the cognitive deficits following EWD. However, these latter effects have not been validated in models of voluntary EtOH consumption. As there is considerable evidence that active versus passive intake can significantly impact neuroadaptations to ethanol this is an important consideration. These studies sought to characterize consumption and evaluate the development of dependence in a chronic voluntary model of intermittent access (IA) to EtOH. CORT plasma levels and protein expression of the glucocorticoid and NR2B receptors were measured during and/or following exposure. Finally, to assess the role of CORT in EtOH consumption and the development of dependence, the glucocorticoid receptor antagonist ORG-34517 was administered during access to EtOH. IA access to 20% EtOH produced varying levels of consumption (2.0-6.7g/kg/24hr exposure) and blood EtOH levels (6.3-116.9 mg/dl), but did not significantly affect food consumption or weight gain. Baseline

CORT levels were found to be predictive of subsequent EtOH consumption and levels of consumption were sufficient to elevate CORT levels following one hour of EtOH exposure. Further, IA to EtOH was sufficient to produce dependence, as measured by elevations in the acoustic startle reflex following 26 hours and five days of withdrawal. No alteration in protein expression was observed regarding either the NR2B or glucocorticoid receptors and exposure to ORG-34517 had no effect on consumption or withdrawal.

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Characterizing Consumption, Dependence, and the role of Glucocorticoids
in an Animal Model of Voluntary Ethanol Consumption

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ACKNOWLEDGEMENTS

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INTRODUCTION

Issues with Alcohol Use

Alcohol use is prevalent in America, with 60-70% of the population reportedly consuming at least one alcoholic beverage during his or her lifetime (NIH, 2010). It is present when we celebrate with our family and friends, or when we struggle through our most difficult times. A question that has plagued the public and scientists alike is why some individuals can use alcohol socially while others develop an addiction to the drug, which can take a lifetime to break. Indeed, in the United States an estimated 30-40% of people who drink will develop an alcohol use or abuse disorder, with an estimated 76 million people diagnosed with an alcohol use disorder (AUD) worldwide (WHO, 2004). The effects on society are many and include poor health and increased mortality, increased violence and crime, marginalization of those affected, and loss of productivity. In fact, the cost of excessive alcohol use in the United States yielded a median of $2.9 billion dollars in 2006, which results in an average cost of $1.91 for each drink, per state (Sacks et al, 2013). Treating AUDs is difficult, indicative of the high rates of relapse associated with the disorder. Reports from alcohol dependent individuals indicate that stress is a main factor in relapse to drinking (Pohorecky, 1991; Weiss & Porrino, 2002), a finding that can be replicated in preclinical research (recently reviewed by Martin-Fardon & Weiss, 2013). The association between stress and alcohol is complex, but provides researches with a possible therapeutic target in the treatment against alcohol use and abuse disorders.

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Alcohol and the Hypothalamic-Pituitary-Adrenal Axis

The stress response is a biological adaptation that is elicited when there is a transition in the physiological or behavioral state. Such changes include the transition from sleep to wakefulness or from restfulness to escape. In the latter transition the stress response plays a crucial role in the overall health and survival of the organism. The hypothalamic-pituitary-adrenal (HPA)-axis is an integral component of the stress response, activation of which culminates in the release of glucocorticoids (cortisol in human; corticosterone (CORT) in the rat or mouse). Glucocorticoids can release and redirect stored energy in times of crisis but can also initiate long lasting adaptations in several different organs, including the brain. Activation of the HPA-axis begins with the periventricular nucleus (PVN) of the hypothalamus, which receives input from many different regions of the central nervous system (CNS). When one of these regions detects a challenging or aversive stressor, it initiates the release of corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) from the PVN into the portal blood circulation. When released onto the anterior pituitary gland, these neurohormones act in concert to stimulate the release of adrenocorticotropin hormone (ACTH). ACTH then travels to the adrenal gland via systemic blood circulation to initiate the synthesis and release of glucocorticoids. The secretion of glucocorticoids provide a mechanism of both positive (amygdala) and negative (hippocampus and medial prefrontal cortex) feedback for the system (reviewed by Herman et al, 2003; Herman, 2012). Glucocorticoids can exert both genomic and nongenomic changes via the

adrenocorticosteroid receptors, the mineralocortocoid (MR) and glucocorticoid receptors (GR).

Alcohol has been characterized as a promiscuous drug, as it affects an array of biological processes through a myriad of different mechanisms. One such process, which has received particular attention, is the influence alcohol exerts upon the HPA-axis. Though a host of factors can influence this interaction, including dose, duration of exposure, route and type of administration (active vs. passive), and prior drug exposure, there are common mechanisms by which ethanol (EtOH) activates the stress response. As explained above, CRF synthesis and release from the PVN represents the initial step in HPA-axis activation. EtOH administered directly into the PVN can increase CRF release and upregulate CRF receptor (CRFR) expression (Rivest & Rivier, 1994; Lee et al., 2005). Using a hypothalamic primary cell line, Li et al. (2005) found that exposure to EtOH increased CRF gene transcription and mRNA levels, resulting in elevated CRF secretion, effects which were blocked with the addition of either a cAMP antagonist or PKA inhibitor. Together, these data suggest that EtOH can act directly to stimulate the stress response. However, EtOH can affect the HPA-axis through less direct mechanisms as well, including activation of the catecholaminergic system (reviewed by Allen et al., 2011). As the main noradrenergic region of the brain, the locus coeruleus (LC) plays a central role in this interaction (Reyes et al, 2006). Lesioning this region resulted in the elimination of ACTH release following EtOH injection, while lesions to the minor medullary cell groups that project onto the PVN resulted in a decrease in CRF immunoreactivity (Allen et al, 2011). Together, these studies demonstrate but a portion of the ways in

which EtOH exposure can produce elevations in HPA-axis activity and are suggestive of how complex the problem of treating AUDs can become.

Alcohol Use and Stress in the Human Population

Clinical research conducted over the past 20 years has demonstrated a complex association between stress and drug abuse, including the development AUDs. Anecdotally, it is almost a tradition to relax with a glass of wine or other alcoholic drink following a particularly stressful day (e.g. writing one's dissertation). More insidiously, a recovering alcoholic may be driven to relapse following a difficult day or major life stressor. In fact, major stressors such as losing a job, moving, or experiencing a catastrophic event are associated with lifetime alcohol consumption and risk of developing an AUD (Veenstra et al, 2006). However, the question as to why these external risk factors induce some individuals to alcohol dependence, while others remain unaffected, can be answered in part by genetics.

Identifying genes that may contribute to the development of alcoholism has been difficult, as the disease is complex and likely results from the interaction of many genes. However, while there are numerous candidate genes that researches are investigating, there remains only two that have been definitively linked to the disease (reviewed by Zimmerman, 2004). These genes code for enzymes that regulate alcohol metabolism, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), and are unequally distributed among individuals with and without AUDs. Genetic polymorphisms that confer high levels of alcohol metabolism are less likely to occur in alcohol dependent populations, whereas the converse is

true of polymorphisms resulting in low metabolic activity (reviewed in Zimmermann et al., 2004). It is understandable how a biological process such as alcohol clearance can influence an individual's propensity for alcoholism, but how an individual reacts to stress also appears to be a contributing factor, and which is in turn mediated by one's genetic profile. Twins studies have been used to determine that the stress response is a heritable characteristic, with rates for HPA-axis reactivity as high as 97% (Federenko et al., 2004), while heredity for basal cortisol levels are around 62% (Bartels et al, 2003). As having an alcoholic in the family acts as a predictor for developing the disease, studies compared the stress response between individuals who are family history negative (FHN) to those who are family history positive (FHP) for alcoholism. Data reveal that FHP subjects displayed a significantly altered stress response under clinical conditions with regards to ACTH and cortisol secretion, including a hypersensitive response to psychosocial stressors (reviewed by Uhart & Wand, 2008; and also Zimmerman et al, 2007). Interestingly, polymorphisms in the GR have been shown to confer elevated levels of basal cortisol secretion (Rosmond et al, 2002), greater glucocorticoid sensitivity (Lambert et al., 1998), increased salivary cortisol response following psychosocial stress (Wust et al., 2004), and to be associated with age of onset of alcohol abuse in adolescents (Desrivieres eta al., 2010). These data suggest that cortisol levels and GC receptor state can confer sensitivity for developing an AUD and that these factors are influenced by genetics. Further investigations into this system may result in identifying cellular and molecular targets, which could lead to pharmacotherapies for the treatment of AUDs and the relapse to drinking.

The Study of Ethanol Exposure and Stress in Animal Studies

Preclinical investigations into the interaction between stress and EtOH consumption have yielded equivocal results, which appear to be dependent upon the type of stress (chronic or acute/sub-chronic) or EtOH (chronic or intermittent) exposure used. A recent meta-analysis on this issue was conducted by Becker et al. (2013) and concluded that, in general, EtOH consumption was either decreased or remained unchanged following exposure to acute or sub-chronic stress procedures (e.g., foot shock, restraint, isolated housing), while chronic stress (e.g., maternal separation, shift in circadian cycle, chronic variable stress) elevated consumption with the most robust elevations occurring in adulthood following stress exposure during adolescence. Interestingly, the group determined that the stress of EtOH exposure, in and of itself, is sufficient to reliably elevate EtOH consumption when the drug is presented in a chronic yet intermittent fashion. This final observation is particularly reflective of the escalation into alcohol dependence seen in the human population, which is characterized by bouts of intoxication, punctuated by periods of abstinence, following which, consumption often escalates.

Models of Ethanol Exposure in Rodents

Preclinical studies of EtOH exposure are presented with a particular challenge, as rodents, which are most often used, do not readily self-administer the drug in levels sufficient to produce intoxication and dependence. It is common to use inbred or selectively bred rodents (e.g., C57/BL6 mouse, P rat, HAD rat) that

have been developed to express key traits of alcoholism, such as high EtOH preference and consumption, as intoxication and dependence can be readily induced. Significant contributions to our understanding of the mechanisms behind alcohol abuse have been gained by using such genetically homogeneous strains. However, these animal models can't fully represent the disease of alcoholism, as it is influenced by multiple genetic and environmental factors, as well as by gene x environment and gene x gene interactions (Becker, 2012; Crabe, 2002; Enoch & Goldman, 2001; Johnson et al., 1998).

Researchers have developed several investigative techniques to obtain intoxication in the outbred rodent. These include passive models of exposure such as intragastric intubation, intraperitoneal injection, and exposure via EtOH vapor chambers. However, passive versus active drug administration can alter release and turnover of neurotransmitters, including those important to the development of alcohol use disorders (as reviewed by Jacobs et al., 2003). For example, release and turnover of dopamine and glutamate are differentially affected in the nucleus accumbens depending on a passive (yolked control) versus active (operant self-administration) delivery of cocaine (Dworkin, 1993; Hemby et al, 1997). Delivery dependent changes have been found with other drugs of abuse including amphetamine (Di Ciano, et al. 1998) and morphine (Smith et al, 2003). Most recently, voluntary but not passive (gavage) administration of EtOH produced alterations in anxiety following 14 days of exposure (Mitchell et al., 2012). Together, these findings suggest that the effects of drugs of abuse, including EtOH, may not be derived simply by their pharmacological actions but instead manifest

due to an interaction between drug and cognitive processes (Jacobs et al., 2003). As the development of pharmacological targets is driven by neuroadaptations resulting from drug administration, it is crucial that preclinical models properly reflect these neuroadaptive states.

Alcohol, Stress, and the NMDA Receptor

Excitatory tone in the CNS is primarily regulated via the gamma-amino butyric acid (GABA) and glutamate neurotransmitter systems, with GABA providing inhibitory control and glutamate providing excitatory control. Acutely, EtOH targets these systems, producing an overall inhibitory effect (Popp et al., 1999). As a consequence of prolonged EtOH exposure and the accompanying neural inhibition, compensatory neuroadaptions alter the CNS to regain and maintain a homeostatic level of activity (reviewed by Kumari and Ticku, 2000). It is suspected that these neuroadaptations are associated with the development of dependence, as well as symptoms of ethanol withdrawal (EWD), including elevated anxiety and seizure activity (reviewed by Becker, 2010). These neuroadaptations include changes in the composition, function, and activity of the NMDA receptor (elegantly reviewed in Prendergast & Mulholland, 2012). Of particular interest have been the EtOH-mediated alterations in the stoichiometry of the NMDA receptor, as subunit composition has direct implications regarding channel kinetics (Erreger et al., 2005; Vicini et al., 1998), synaptic localization (Kohr, 2006), and protein binding partners (Hardinham et al., 2002; Vanhoutte & Bading, 2003; Ivonov et al., 2006). Using recombinant NMDA receptors, Erreger and others (2005) have shown that

NR1/NR2A heteromers had faster gating time than did NR1/NR2B. However, NR1/NR2B heteomers had longer channel activation duration and therefore conferred greater total conductance of calcium. Additionally, the NR2B subunit can alter channel-ligand binding and confer increased excitotoxicity (Butler et al., 2010). Chronic EtOH exposure has been associated with increased mRNA (Anji & Kumari, 2006) and protein expression (Carpenter-Hyland et al, 2006; Pian et al., 2010) of the NR2B subunit; however others have found no such changes (Chandler et al., 2007; Rudolph et al., 1997). Interestingly, exposure to CORT has also been associated with elevations of NR2B protein (Weiland et al, 1997). Several groups have found that trafficking of the NR1/NR2 to the synaptic membrane is mediated by EtOH (Carpenter-Hyland et al., 2004; Hendrickson et al., 2003; Qiang et al., 2007). Others have found increased neurotransmission following EtOH-dependent phosphorylation of the NR2B by the tyrosine kinsase Fyn (Miyakawa et al., 1997; Yaka et al., 2003; Yaka et al., 2003). Additionally, the NR2B subunit includes a positive allosteric modulatory site for polyamines (Mony et al, 2009; reviewed by Prendergast & Mulholland, 2012). Exposure to CORT (Cousins et al., 1984) and EtOH (Williams 1994), including EWD (Williams, 1994), elevates synthesis of polyamines by upregulating the ornithine decarboxylase (ODC) enzyme within the brain (Cousin et al., 1982). Recently, it was reported that long-term co-exposure to EtOH and CORT sensitized hippocampal tissue to insult following the withdrawal of EtOH, the effects of which were mediated by the NR2B receptor (Butler et al, in press). Together, these data provide overwhelming evidence that stress and EtOH have

several different routes by which they can independently and jointly alter NMDAr-dependent neurotransmission.

Alcohol and the Glucocorticoid Receptor

The MR and GR are the primary targets for corticosteroid hormones in both humans and rodents. The receptor names are derived from the primary processes they influence in the periphery, namely mineral balance and gluconeogenesis. Both receptor types have been found in brain tissue. MR distribution is restricted, but includes high expression within the hippocampal formation. GRs are ubiquitously expressed on both neurons and glia but are more concentrated in certain brain regions, including the hippocampus and PVN. CORT has a much higher affinity for the MR, with full occupation occurring at low physiological levels (~10ng/ml=29nM); whereas the GR is only partially occupied at such levels and becomes fully occupied during times of stress, when a CORT surge occurs (~400ng/ml=1.12μM) (deKloet et al, 1993). For this reason, GRs have been of specific interest with regards to EtOH related changes in the CNS. GRs are found within the cytoplasm of cells, where they are inactively bound to protein chaperones. Once occupied by CORT, the chaperone dissociates, and the receptor complex translocates to the nucleus, where either transactivation or transrepression may occur. Transactivation is initiated when the receptor complex merges with DNA to form a homodimer at the glucocorticoid response element (GRE), most often resulting in gene transcription (reviewed in Jenkins et al., 2001). Alternately, the receptor complex can interact with other transcription factors,

thereby suppressing or facilitating their effects, a phenomena referred to as crosstalk (reviewed in Kassel & Herrlich, 2007). Through transactivation and crosstalk, and coupled with the fact that there are many known isoforms of the receptor, GRs are able to produce wide influence on protein expression, cellular activity, and behavioral outcomes. While this is infinitely adaptive to the organism, this level of complexity presents a challenge for researchers when attempting to investigate the already complex stress response.

GR activation has important consequences on EtOH consumption, which has well been characterized in animal models. For example, elevated levels of CORT can enhance EtOH consumption (Fahlke et al., 1994a, b; Falke & Hansen, 1995; Hansen et al., 1995), whereas adrenalectomy (ADX) (de Witte et al, 1996; Hansen et al., 1995, Fahlke et al, 1994) or antagonizing the GR are effective in reducing voluntary consumption (Koeig & Olive, 2004). Additionally, antagonism of the GR has been shown to reduce behavioral signs of (Sharrett-Field et al., 2013), and cognitive deficits (Jaquot et al., 2008) resulting from EWD. Further, ADX prior to EtOH exposure has been shown to decrease subsequent EtOH intake (Fahlke & Eriksson) and EtOH preference (Lambin & de Witte, 1996) in selectively bred rat lines, while administration of CORT restored consumption in both experiments. It is clear that, at least in animal models of EtOH exposure, GR activation is able to mediate consumption.

While GRs can affect EtOH intake, EtOH intake can also modulate GR expression. For example, a decline in GR mRNA in the hippocampus (Eskay et al., 1995), cortex (Roy et al., 2002; Vendruscolo et al., 2012), amygdala (Vendruscolo et

al., 2012), and PVN (Roy et al., 2002) has been shown following EtOH exposure in rodents. Such declines have been shown to persist up to 24 hours following the withdrawal of EtOH, while elevations in GR mRNA were found during protracted abstinence (Vendruscolo et al., 2012). However, data are mixed regarding receptor alterations both during and following EWD (see Rachamin et al., 1989; Spencer & McEwen, 1990). As previously discussed, the interaction between EtOH and GRs have behavior consequences, as manipulating levels of CORT or GR activation can alter consumption, the escalation of dependence, and the severity of EWD.

Acoustic Startle Reflex

The startle reflex can be elicited by a sufficiently sudden and intense stimulus and likely manifests to defend an organism from predation or a blow, and to prepare for fight or flight. The reflex begins with eye-lid closure, which is followed by the contraction of facial, neck, and skeletal muscles (Davis, 1979). This contraction of musculature throughout the body is accompanied by the cessation or freezing of ongoing behaviors and the acceleration of heart rate (Davis, 1979). The startle reflex can be experimentally initiated by the presentation of an acoustic, tactile, or visual stimulus in most mammals, including humans and rodents (reviewed by Koch, 1998). Importantly, the acoustic startle reflex (ASR) found in rodents can generalize to humans, a fact that suggests similar neural mechanisms mediate the response in both species (Geyer & Braff, 1987; Braff et al., 2001). An additional benefit of employing ASR is that acoustic, tactile or visual stimuli can be combined to either attenuate or enhance responding. For example, prior to the presentation of a

startling tone, the delivery of an acute aversive stimulus, such as a foot shock, will elevate the ASR, while the presentation of a non-startling tone (prepulse) will attenuate the response (Davis 1979; Geyer & Dulawa, 2003; Koch 1998). Additionally, ASR can be manipulated through conditioning with either aversive or appetitive conditioned stimulus/unconditioned stimulus pairing (fear-potentiated). ASR can be altered in magnitude by several stimuli, including stimulus intensity (Pilz et al., 1987), interstimulus interval (Davis, 1979), genetics (Pilz et al., 2002), diurnal rhythm (Chabot & Taylor, 1992), sensory environment (ie, background noise) (Geyer & Dulawa, 2003) illumination (Walker & Davis 1997), and drug manipulation (last reviewed by Davis, 1979). In general, anxiogenic drugs (e.g. yohimbine) elevate ASR in humans (Morgan III et al., 1993) and rats (Powell et al., 2005), while anxiolytics, including benzodiazpaines, attenuate responding in humans (Rodríguez-Fornells et al., 1999) and also rodents (Commissaris et al., 2003).

Indicative of most reflex responses, the fundamental neural circuitry believed to conduct ASR is rather simple. Briefly, the auditory signal is conducted by a small group of cells within the auditory nerve, the cochlear root neurons, which terminate directly onto the nucleus reticularis pontis caudalis. Projections from this structure terminate onto the motor neurons of the face (eye blink and facial contraction) and spinal cord (whole body musculature contraction) (Davis et al., 1982; Lee, et al., 1996). Glutamate is the main excitatory neurotransmitter mediating this circuit, including activity at the glutamatergic AMPA and NMDA receptors (Ebert & Koch, 1992; Krase et al., 1993), which is opposed by GABA

inhibition (Birnbaum et al., 1997). With one distinction regarding a bi-phasic response to the eye blink reflex that is seen in humans, a similar pattern of responding and latency have been observed across human and rat species (Meincke et al., 2002). While the basic ASR is mediated by three synapses, the neural inputs that are able to modulate the response are complex, involving several brain structures. The neural pathway believed to mediate the sensory-gating measure of prepulse inhibition (PPI) involves a cortico-striatio-pallido-pontine (CSPP) circuit that can be regulated by the limbic system (reviewed in Braff et al, 2001 (human); Swerdlow et al., 2001 (animal)). Regions of the limbic system that are important in mediating PPI include the hippocampus, prefrontal cortex (PFC) and medial prefrontal cortex (mPFC), and the BLA (Swerdlow, 2001).

Acoustic Startle Reflex in Humans

Alterations of ASR and PPI have been used to study numerous conditions occurring in the human population. Alterations in PPI have been associated with schizophrenia (Braff et al., 2001; Weike et al., 2000), obsessive-compulsive disorder (Swerdlow et al., 1993), Tourette syndrome (Swerdlow et al., 2001), attention deficit disorder (Ornitz et al., 1992), and post-traumatic stress disorder (PTSD)(Grillon et al., 1996; reviewed by Braff et al., 2001). Further, increases in the acoustic startle reflex have been found in individuals who suffer from anxiety (Grillon et al., 1997), depression (Grillon et al., 1997;Mneime et al., 2008), childhood abuse, or experience substance abuse (Grillon et al., 2008). In healthy subjects, alcohol suppresses the expression of ASR while PPI is either unaffected or

moderated by baseline responding (Grillon et al., 1994; Hutchison et al., 1997). In a small study of alcohol dependent individuals experiencing EWD (n=8), a decrease in PPI was found, with individuals previously experiencing delirium tremens (n=3) showing the greatest deficits (Keedwell et al., 2001). In a group of recently detoxified heavy drinkers (meeting DSM-III-R requirements), ASR was found to be elevated as compared to control subjects (Krystal et al., 1997). Further, a positive relationship was shown between the amplitude of the response and number of detoxification episodes (Krystal et al., 1997). A recent study suggests these effects are biphasic, as decreased ASR and PPI were found after one month of abstinence in a similar population (Marin et al., 2012). Evidence suggests that ASR and PPI are potential vulnerability biomarkers for AUD as children with a family history positive (FHP) for alcoholism show elevated baseline ASR (Zimmermann et al., 2004) and impaired PPI (Grillon et al., 1997). Additionally, in young adult males who are FHP, baseline PPI was reduced and exposure to alcohol did not dampen ASR to the same levels as those seen in control subjects (Grillon et al., 2000).

Acoustic Startle Reflex in Animal Models

Preclinical investigations using ASR and PPI to study the effects of alcohol have found no alterations in adulthood in either measure as a result of prenatal (Poter & Bernston 1987) or early postnatal (Woolfey et al., 2003) administration of EtOH. However, when exposure occurred during adolescence, adult animals did show evidence of decreased inhibition in the presence of the prepulse (Slawecki & Ehlers, 2005). Data regarding EtOH-induced changes in PPI in outbred animals are

mixed, with Jones et al (1999), finding a disruption in PPI, while other groups found no significant differences (Sandback et al., 1999; Chester & Barrenha, 2007). During EWD, elevated ASR has been found in the outbred rodent following chronic forced exposure to EtOH (Pohorecky & Roberts, 1992; Rassnick et al., 1992; Vandergriff et al., 2000). Data collected in animals selectively bred for EtOH preference show reductions in EWD in animals with high preference for EtOH, as compared to low-preferring animals (Jones et al., 2000; Chester et al., 2003, 2004, 2005; Chester & Barrenha 2007). In fact, it has been demonstrated that EWD-associated changes in startle response and habituation to startle is mediated in part by factors such as EtOH preference and sensitivity to EWD, which is linked to preference (Gilliam & Collins 1986; Jones et al, 2000; Dess et all, 2005; Ponomarev & Crabbe, 1999). Further, under EtOH naïve conditions, high preferring animals (P-rat and Wistar High Preferring) display an elevated ASR prior to EtOH exposure, as compared to non-preferring animals of the same strain (Jones et al, 2000; Acewicz et al., 2012). However these results, both regarding ASR activity during EWD and EtOH-naïve conditions, are in direct contrast with the human literature, discussed above. Differences regarding baseline ASR may be evident due to previous EtOH exposure, as it is difficult to control for lifetime alcohol experience in humans. As for the disparity in EWD-associated changes in ASR, it should be noted that the studies cited above used forced methods of EtOH exposure. Therefore, low-consuming animals, which tend to have high sensitivity to the effects of EWD, were forced to consume EtOH and served as the comparison groups for these studies. Additionally, experience with alcohol can also influence EWD-associated elevations in ASR in the

human population. Despite this conflicting data, it is evident that ASR could be a valuable biomarker for the development of alcohol dependence.

Experimental Rationale

Alcohol abuse disorders represent a serious worldwide health problem with far-reaching social, financial, and interpersonal implications. One of the most devastating facets of these disorders is the propensity to relapse following periods of abstinence. EWD has been linked to neuroadaptations that promote relapse by increasing anxiety and craving. Further, EWD may contribute to the development of cognitive decline associated with long-term dependence. EtOH exposure changes the composition, function, and activity of the N-methyl-D-aspartate receptor (NMDAr), and in particular, the NR2B subunit of this receptor. Stress similarly affects activity of this receptor system. CORT, the primary stress hormone found in non-human mammals, has also been shown to alter the expression and/or function of the NMDAr. As EtOH is a physiological stressor, it is reported to increase levels of plasma CORT. This synergistic interaction between EtOH and CORT as it affects the NMDAr and GR could present a therapeutic target in the treatment of EWD. In fact, data suggest that the GR antagonist mifepristone could promote abstinence, as treatment with the drug has been shown to reduce consumption (Koenig & Olive, 2004), the development and maintenance of EtOH dependence (Vendruscolo et al, 2012), as well as and decreasing the severity of EWD (Sharrett-Field et al, 2013) and cognitive deficits following EWD (Jaquot et al, 2008). However, these effects have not been validated in preclinical models of voluntary EtOH consumption. As there is

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considerable evidence that active versus passive drug intake can significantly impact neuroadaptations to EtOH, this is an important consideration (Jacobs et al, 2003; Dworking et al, 1995).

Study one of the following studies characterized consumption patterns resulting from a voluntary model of EtOH exposure and also the ability of this model to produce dependence, as defined by the manifestation of EtOH withdrawal and as measured by the acoustic startle reflex (ASR). It was hypothesized that voluntary intermittent access to 20% EtOH solution for 7 weeks would produce elevations in ASR and reductions in the habituation and inhibition of ASR during EWD. To determine the influence of CORT on consumption and dependence, plasma CORT levels were also assessed. Higher levels of baseline CORT were predicted to be associated with elevated consumption and resulting blood EtOH content. CORT levels assessed during acute and protracted EWD were expected to be elevated; levels were also predicted to associate with levels of EtOH consumption. Following EtOH exposure, protein levels of the NMDA NR2B subunit and GR were assessed in the pre-frontal cortex (PFC) and hippocampus. Elevated levels of both GR and NR2B in EtOH exposed animals were hypothesized at this time point.

Study two was conducted to determine the effects administration of the selective GR antagonist, 11,21-Bisphenyl-19-norpregnane (ORG-34517) would have on voluntary consumption levels and subsequent EWD. EtOH exposure methods as well as CORT and ASR assessment followed those described above. However, during weeks 7 and 8, a subset of animals received ORG-34517 or placebo control (Nutella®) prior to EtOH access. It was hypothesized that ORG-34517 would

decrease voluntary consumption of 20% EtOH. Attenuation of EtOH-dependent changes in ASR during EWD were also anticipated. However, elevations in CORT were hypothesized in the ORG-34517 treated animals, as blocking the GR also blocks the natural negative feedback mechanism for the stress response. Assessment of protein levels were similar to those conducted in Study 1, however, the medial region of the pre-frontal cortex (mPFC) was isolated in addition to the hippocampal region. GR and NR2B protein expression was assessed, as well the phosphorylation state of the NR2B receptor, which plays an important role in channel kinetics of NR2B-containing receptors. EtOH-associated elevations in GR, NR2B, and pNR2B were expected.

Chapter Two

INTERMITTENT ACCESS TO 20% ETHANOL PRODUCES MEASURABLE

WITHDRAWAL AND ALTERATIONS IN PLASMA CORTICOSTERONE LEVELS

(Study 1; Sharrett-Field, et al.)

Introduction

An estimated 60-70% of the American population will consume alcohol during his or her lifetime, and 30-40% of these individuals will develop an alcohol use or dependence disorder (NIH, 2010; WHO, 2004). Clinical studies suggest that people often drink following a stressful day or event in an effort to "relax". In stark contradiction, alcohol is a pharmacological stressor, activating the hypothalamic-pituitary adrenal axis and resulting in the synthesis and release of stress hormones (cortisol in the human; corticosterone (CORT) in the rodent). Understanding the interaction between stress and alcohol is imperative, as not only does stress encourage social drinkers to imbibe, but the occurrence of a stressful event is the most commonly reported trigger for relapse to drinking in abstinent alcoholics (Pohorecky, 1991; Weiss & Porrino, 2002). In fact, prior exposure to a stressor can enhance self-administration of several drugs of abuse, including EtOH, in both humans (reviewed by Enoch, 2011) and animals (reviewed by Becker, 2010).

It is well accepted that both alcohol and CORT are reinforcing agents, as both activate the mesolimbic dopamine system (Piazza et al, 1998; Gessa et al, 1985; Mereu et al, 1984). Elevated levels of CORT can enhance EtOH consumption in

animals (Fahlke et al., 1994a, b; Falke & Hansen, 1995; Hansen et al., 1995), whereas adrenalectomy (de Witte et al, 1996; Hansen et al., 1995, Fahlke et al, 1994) or antagonizing the glucocorticoid receptor (GR) (a primary receptor for CORT) are effective in reducing voluntary consumption (Koeig & Olive, 2004). Additionally, antagonism of the GR has been shown to reduce behavioral signs of (Sharrett-Field et al., 2013) and cognitive deficits resulting from EWD (Jaquot et al., 2008). Developing treatments for use during EWD is crucial, as abstinence rates are likely affected by neurochemical alterations that occur during withdrawal. Such alterations include changes in protein expression and phosphorylation state of the NR2B subunit of the NMDA receptor. In fact, over-activation of this subunit is thought to contribute to symptoms associated with EWD. EtOH exposure has been found to elevate levels of NR2B mRNA and/or NR2B subunit protein, although these findings are not consistent (discussed in Prendergast & Mulholland, 2012). Recently, increases in tyrosine phosphorylation of the subunit were found following IA EtOH exposure in the dorsal striatum (Wang et al, 2011), and models of forced EtOH exposure have revealed elevations in the hippocampus (Wu et al, 2010). Additionally, there is a link between stress and NR2B activation, as elevations in CORT lead to the synthesis and release of polyamines, which allosterically increase activity at the NR2B subunit. Together, these studies provide evidence that the GR presents a likely target for both reducing EtOH consumption and promoting abstinence by decreasing the severity and consequences of EWD.

Most preclinical investigations into the effects of AUDs use involuntary methods of EtOH delivery, as rodents do not readily self-administer levels of the

drug sufficient to produce intoxication and dependence. However, there is accumulating evidence that EtOH promotes behavioral and neurochemical changes that are dependent upon voluntary EtOH consumption (Mitchell et al., 2012; Moolten & Kornetsky, 1990; Pautassi, Truxell et al., 2008; Weise-Kelly & Siegel, 2001). Data also suggest that individual differences in HPA-axis reactivity and GR polymorphisms can, at least in part, mediate drinking behavior and the development of dependence (Desrivieres et al, 2010). Therefore, using voluntary models of EtOH exposure, which allows for individual differences in consumption patterns to arise, can provide insights concerning factors that contribute to AUDs.

The following investigation uses a voluntary intermittent access (IA) model of EtOH exposure to investigate the interaction between CORT and consumption. We sought to characterize the pattern of drinking that occurs in this model and to determine if dependence is produced following 7 weeks of exposure, as measured by alteration in the ASR during periods of acute (T1) and prolonged withdrawal from EtOH (T2). The expression and state of the NMDA NR2B receptor were assessed, as EtOH consumption, dependence, and withdrawal have been closely linked to this receptor protein. It was hypothesized that expression and tyrosine phosphorylation of the NR2B subunit would be elevated in EtOH exposed animals. To further explore the interaction between EtOH and CORT, the expression of GRs were also evaluated; expression was expected to increase during protracted EWD (T20). Immunoblotting for both proteins was conducted within the hippocampus and prefrontal cortex at the conclusion of the studies.

Materials and Methods

Subjects

Male, Long-Evans rats (60 days old; Harlan Laboratories, Indianapolis, IN) were single housed and exposed to 12 hour reversed dark/light cycle (lights off at 0900hrs). During a one week acclimation period, animals were handled for 20min/day for 3 days. Animal and food weights were taken weekly between 0800 and 0900hrs on Monday. Animals had *ad libitum* access to food and water throughout the experimental procedure.

Intermittent Access to Ethanol

Animals were given 24hrs of concurrent access to 1 standard bottle containing a 20% (v/v) EtOH/water solution and one standard bottle containing tap water. After 24hrs, EtOH bottles were removed and all animals received 2 bottles containing tap water (for Experimental Timeline, see Table 2.1). Placement of EtOH and water bottles were alternated to avoid side preference. This pattern of access (24hrs with EtOH followed by 24hrs without EtOH) continued for 45 days (21 days of EtOH exposure on M-W-F). Two empty cages containing no animal received identical treatment regarding bottle changes and served to account for bottle leakage (leak controls). A separate group of animals (water controls) received identical treatment except neither bottle contained EtOH.

Table 2.1 Experimental Timeline

	M	*T*	*W*	*TH*	*F*	*SA*	*SU*
WK1	*T0* *Baseline ASR*		*Blood Draw* *EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	
WK2	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	
WK3	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	
WK4	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	
WK5	*EtOH/Water*	*Water/Water*	*Blood Draw* *EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	
WK6	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	
WK7	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	*EtOH/Water*	*Water/Water*	
WK8	*EtOH/Water*	*Water/Water*	*T1 ASR* *Water/Water*	*Water/Water*	*Water/Water*	*Water/Water*	
WK9	*T2 ASR* *Sacrifice-* *Trunk Blood*						

Measures of Ethanol Consumption

EtOH consumption was measured in two manners. First, bottles given to animals (water and EtOH) were weighed prior to presentation and again following 24hrs of access. This value, less the weight change detected in the leak control bottles, was expressed as mL/kg of fluid intake. Twenty percent of this value was multiplied by the specific gravity of 200proof EtOH ($0.794g/cm^3$) to determine the EtOH dose (g/kg) each animal consumed. Additionally, a subset of animals (n=12) were video recorded for 24hrs during week 7 of the study. Videos were rated for drinking behavior, which was easily identifiable. Drinking criteria included contact between the animals' mouth and the bottle spout and both front feet leaving the cage floor. This measure yielded a pattern defining the times of day in which drinking occurred, number of drinking episodes per day, and time spent drinking per episode.

Blood Ethanol and Corticosterone Concentration

Blood was collected (approximately 140 μl/animal/time point) by placing a lateral nick in the tip of the tail prior to EtOH exposure and again 1hr following the introduction of EtOH during week 5. Six days following the removal of EtOH, animals were euthanized via rapid decapitation and trunk blood was collected. Upon collection, blood samples were placed on ice and centrifuged (5 min at 15,000 g/min); blood plasma was collected and immediately stored at -80°C.

Blood EtOH levels (BEL) were determined using the Analox AM1 instrument (Analox Instruments, Lunenburg, MA), which measures blood EtOH concentrations

indirectly through measurement of molecular oxygen levels. In the presence of molecular oxygen, EtOH is oxidized by the enzyme alcohol oxidase to form acetaldehyde and hydrogen peroxide. Under the conditions of the assay, oxygen consumption is directly proportional to EtOH concentration in the plasma sample.

Blood CORT levels (BCLs) were determined using a competitive EIA Corticosterone kit (IDS Limited, Fountain Hills, AZ). Briefly, 100 μl of each diluted sample (1:20), calibrator, and control and 100μl of enzyme (corticosterone labeled with horseradish peroxidase) were added to an antibody coated plate (polyclonal rabbit anti-corticosterone) in duplicate and incubated at 4°C for 24 hrs. Following incubation, the plate was thrice washed and 200 μl of substrate (tetramethylbenzidine and hydrogen peroxide) was added. After 30 min, stop solution (0.5M hydrochloric acid) was added and the microplate was read using a Beckman Coulter DTX 880 Mulitmodal Detector (Lagerhausstrasse, Austria) with Beckman Coulter Multimode Detection Software (v.20.0.12). Mean absorbance values for samples, controls, and calibrators were measured at 450nm producing a mean value; this value was used to calculate percent binding (B/B0%=[(mean absorbance)/(mean absorbance for "0" calibrator)]×100). Mean concentration of CORT for each sample (ng/ml) was determined based upon the calibration curve defined by known samples that were run with the experimental samples.

Behavioral Measures

Acoustic Startle Reactivity was measured in a commercially available startle system (S-R Lab, San Diego, CA, USA). Chambers (35x33x38.5 cm, length x width x

height) contained an overhead light (3 lux) and a Plexiglas holding tube (8.9cm in diameter x 20.3cm in length), in which animals were placed for testing. The holding tube was designed to reduce but not eliminate voluntary movement of the animal, thereby reducing stress. A piezoelectric accelerometer within each tube transduced large movement by the animal and was attached to a PC, on which responses were recorded using S-R Lab software (v5.0). A speaker located in the ceiling of the chamber delivered acoustic startle stimuli as well as 75dB white noise background. At the beginning of each trial (defined below), data were recorded over a 100ms window and were sampled at 1ms intervals. Data were recorded in two different chambers. Both input (dB) and output (amplitude of response) were calibrated prior to each testing session using San Diego Instruments calibration devices.

Data consisted of two measures, startle reactivity (first startle response (FSR)) and startle plasticity (habituation of the startle response and prepulse inhibition). ASR data was collected prior to EtOH exposure (T0) and again 26hrs (T1) and 6 days (T2) after EtOH exposed animals received the final EtOH exposure. These time points were chosen to reflect both acute and protracted EWD in the EtOH exposed animals. During IA access, EtOH exposed animals become accustomed to experiencing 24hrs between EtOH exposures. Therefore, at 26hrs (T1), it was reasoned that animals would have expected, but not received EtOH, a situation that would elevate anxiety in dependent animals.

Prior to all ASR testing a 5 min acclimation period consisting of background noise (75dB white noise) was presented and remained on throughout testing. For measures of FSR and habituation, animals were presented with 6 120dB stimuli

(Sandbak et al, 2000). The FSR is equivalent to the amplitude of the startle response to the initial presentation of a 120dB stimulus tone. Habituation score was calculated by subtracting the average amplitude of the last three startle responses from the average of the initial three responses. Prepulse inhibition (PPI) was assessed over 8 blocks consisting of a random presentation of the following 5 trials:

(i)   Pulse stimulus (110 dB for 40ms)
(ii) Prepulse A(4dB for 20ms) + pulse stimulus
(iii) Prepulse B(8dB for 20ms) + pulse stimulus
(iv) Prepulse C(16dB for 20ms) + pulse stimulus
(v) No stimulus (background noise was applied)

PPI was defined as a decrease in the amplitude of the startle response in the presence of the prepulse stimuli and expressed as: PPI = (100-(mean startle reflex with the prepulse/mean startle reflex without prepulse)) x 100 (Geyer, 2001). Interval between the presentation of the prepulse and pulse was 100ms, intertrial intervals were varied randomly in the range of 10 to 30s. Total time in startle chamber for each animal was approximately 25 minutes.

Protein Expression

Following rapid decapitation, whole brains were removed from a subset of animals. Olfactory bulbs were discarded and prefrontal cortex was isolated from each hemisphere; following sagittal section, hippocampi were bi-laterally removed. Tissue from each brain region was placed on dry ice immediately following removal and stored at -80°C. Frozen tissue was placed in lysis buffer ((Radio-Immunoprecipation Assay (RIPA) Buffer (1:100; Thermo Scientific, Rockford IL), 99.8M^{-6} PMSF/EtOH in PBS (1:100), HALT Protease & Phosphotase Inhibitor (1:10;

Thermo Scientific, Rockford IL)). Tissue was coarsely ground using a pestle and centrifuged at 4°C for 20min at 12000rpm. Subsequently, supernatant was collected and assayed using the Pierce® BCS Protein Assay Kit (Fisher) to determine the protein concentration of each sample.

Immunoblotting was conducted in the Mini-Protean® System using TGX Gradient Precast Gels (4-15%) (Bio-Rad, USA). Prepared samples (10uL containing 50ug of protein) or Bio-Rad Precision Plus Protein™ standards (7uL) were vortexed and loaded into lanes. Samples were electrophoresed in 1x Tris/glycine/sodium dodecyl sulfate buffer (Bio-Rad, Hercules, CA, USA) at room temperature for approximately 2.5 hrs at 80-90V. Protein was transferred onto a membrane (nitrocellulose paper) in buffer (.25M^{-3} Tris Base, .19M^{-3} Glycine, 10%Methanol) for 1hr at 100V, carriage was chilled by the addition of an ice pack. Membrane was removed and rinsed twice in 1xTBS and allowed to rock at room temperature for 15min. Membrane was removed and rinsed twice in 1xTBS and agitated in blocking agent (5% non-fat dried milk in TBS) for 1hr at room temperature. Membrane was rinsed in 1xTBS with the addition of 10% Tween20 (TTBS) then agitated in TTBS for 15min. Following a final rinse, membrane was placed in primary antibody solution (5% non-fat dried milk or bovine serum albumin in TTBS with anti-NR2B (1:1000; Millipore), -GR (1:5000; Santa Cruz), or -pNR2B (1:500; Cell Signaling) and agitated overnight at 4°C. On day 2, membranes were rinsed and agitated in TTBS in 5min intervals for a total of 20min. Following placement in light-proof boxes, membranes were agitated in fluorescent secondary anti-body solution (5% milk in TTBS with IRDye800 (Rockland Immunochemicals, Gilbertsville, PA, USA) for 1hr at room

temperature. Following three 5min rinse/agitation cycles in TTBS, membranes were imaged on Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and quantified using Image J software (NIH).

Statistical Analysis

All data were analyzed using either mixed or repeated measure ANOVA. When appropriate, treatment was used as the between subjects and time (week or timepoint) as within subjects factors. Correlations were determined using Pearsons's *r*. When appropriate, post hoc testing utilized Bonferroni t-test. Significance was determined at $p < 0.05$.

Results

Body Weight and Food Consumption

Body and food weights were taken weekly on Monday prior to the introduction of EtOH and continued through Monday of week 7. Weights did not differ significantly between EtOH exposed (n=24) and water control (n=8) group prior to experimentation. Analysis of weekly weight gain during the study revealed no interaction or main effect of treatment (final weights (g): EtOH= 425 ±35.6, Ctrl = 415 ±30.1), but a main effect of time ($F(6,180) = 52.13$ (6, 180) $p< 0.001$) occurred. Similarly, analysis of food consumption revealed no significant difference in weekly food intake between the groups (total food intake (g): EtOH = 1119 ±67.1, Ctrl = 1150 ±83.4), but a main effect of time was observed ($F(6,180)=6.28$ $p < 0.001$), with a significant elevation in food consumption occurring during week 7 (fig. 2.1).

Figure 2.1.
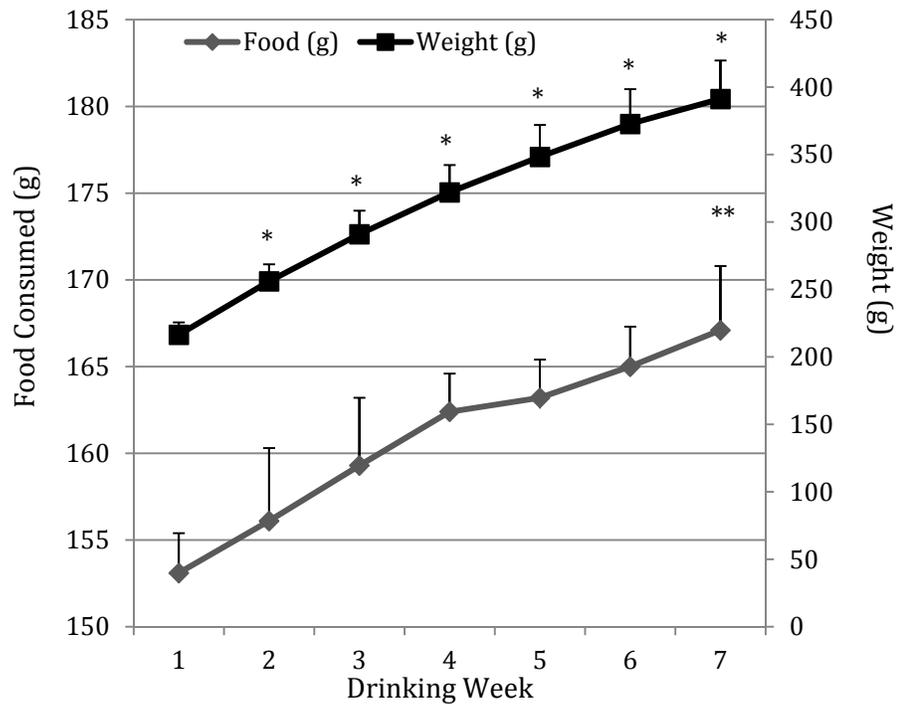
Fig 2.1. Weekly food consumption and weight gain during weeks 1-7. No differences were found between EtOH (n=24) and control animals (n=8) for either variable. Gradual increases in food consumption were accompanied by gradual but significant increases in weight. Average food consumption (g)= 160.9 ±2.97; average weight gain (g) = 231.1 ±11.95. (*= as compared to week 1, $p < 0.05$; **=as compared to weeks 1-4, $p < 0.05$.)

Ethanol Consumption and Blood Ethanol Concentration

Total fluid consumption (calculated as: total volume of EtOH (ml/kg) plus total volume of water (ml/kg)) for EtOH exposed animals (n=24) was compared to total consumption of water treated controls in a two-way mixed measure ANOVA (treatment x time (week)) revealed a significant interaction (F(6,180) = 4.475, p < 0.001). Water control animals consumed significantly more fluid than did EtOH treated animals during weeks 1 and 2, after which total fluid consumptions between the groups did not differ significantly (fig 2.2).

Figure 2.2.
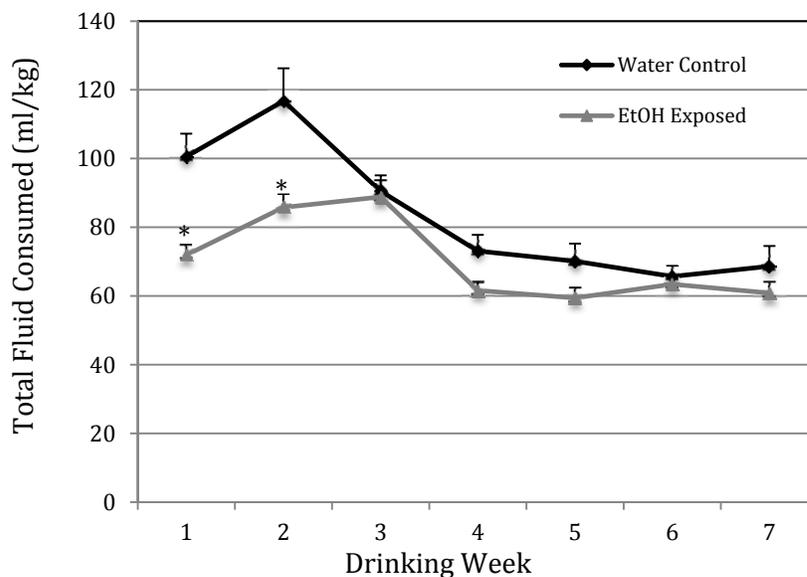
Figure 2.2. Total fluid consumed by water control (n=8) and EtOH exposed (n=24) animals. During weeks 1 and 2, water control animals consumed significantly more fluid than did EtOH exposed animals, no significant difference was found during later weeks. (* = as compared to water controls, p < 0.001)

To analyze the consumption pattern within the EtOH treated animals, a two-way repeated measure ANOVA (fluid type (EtOH or water) x time (week)) was conducted. Analysis revealed a significant interaction between the type of fluid consumed and time (F(6, 138)= 8.63, p < 0.001). Water consumption in these animals declined significantly between week 3 and 5, stabilizing thereafter (fig 2.3a). EtOH consumption elevated during the first three weeks of exposure, followed by a decline during week four (the 8th EtOH exposure) where it remained stable until week seven (fig 2.3a). Additionally, EtOH consumption was significantly lower than that of water during all weeks, except in weeks 5 and 7, when water consumption decreased. Figure 2.4a depicts the concentration of EtOH (g/kg) animals consumed, on average, during a 24hr period of exposure (average dose (g/kg/24hr) 3.79, ± 0.43). Blood EtOH concentrations resulting from these doses ranged from 6.3 - 116.9 mg/dl, and the average BEC obtained was 42.0, ±7.6 mg/dl (fig 2.4b).

Figure 2.3a.
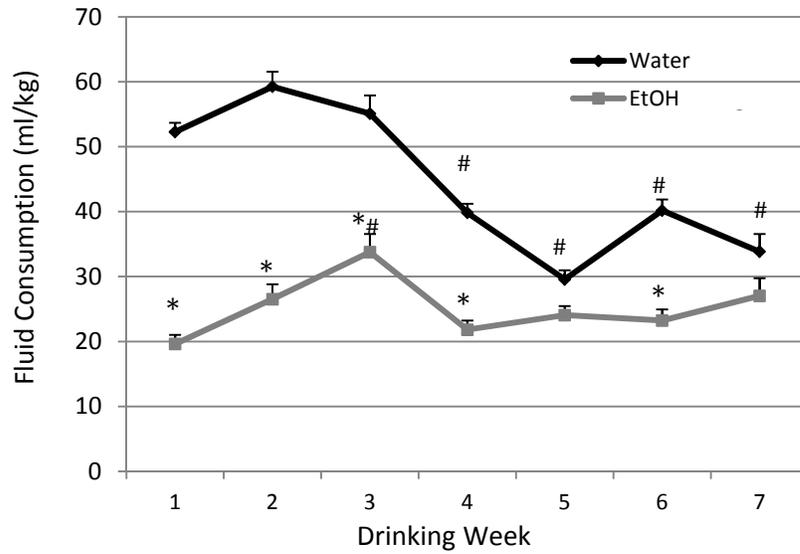
Fig 2.3a. Fluid consumption in animals given intermittent access to 20% EtOH. Animals consumed more water than EtOH during all but weeks 5 and 7. Average consumption (ml/kg): water = 44.3, $\pm$1.90; EtOH = 25.2, $\pm$2.94. (*=as compared to water control animals, $p < 0.05$; #=as compared to week 1, $p < 0.05$)

Figure 2.3b.
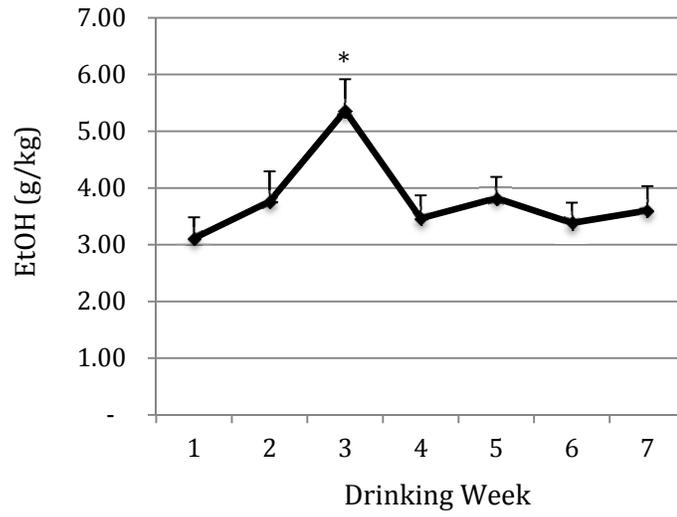
Fig 2.3b. Average weekly dose of EtOH was stable between weeks 1and 2. Consumption increased during week 3, and remained steady during weeks 4-7. Average weekly dose of EtOH (g/kg) = 3.79 ±0.43. (p <0.05; *=as compared to week 1, p < 0.05)

Distribution of EtOH Consumption
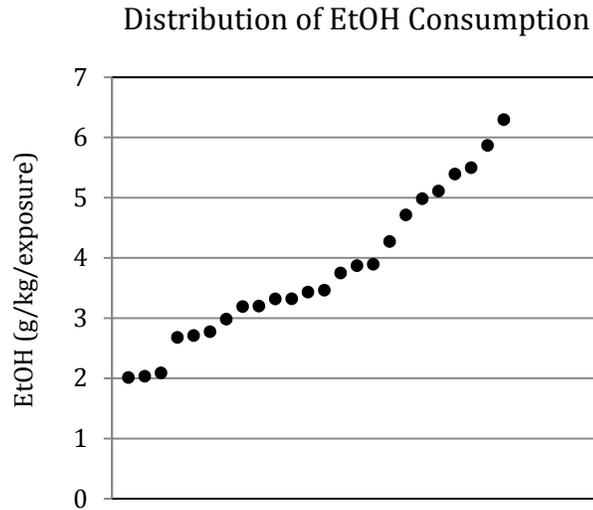
Fig 2.4a. Distribution of the average EtOH dose (g/kg/exposure) (n=24) given intermittent access to 20% EtOH. IA model produced a wide array of EtOH consumption, ranging from 2.0- 6.7g/kg/exposure, with the average animal self-administering 3.79 ±0.28 g/kg/exposure.

Figure 2.4b.

Distribution of BEC
Following 1Hr of EtOH Access
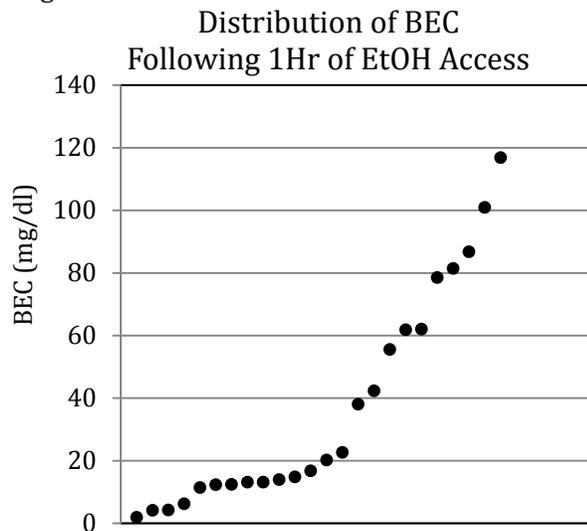
Fig 2.4b. Distribution of the average blood EtOH concentration (mg/dl) in animals (n=24) given intermittent access to 20% EtOH. IA model produced a wide array of BECs following 1Hr of EtOH access, ranging from 6.3-116.9 mg/dl and averaged 42.0, ±7.1 mg/dl (b).

Analysis of video data revealed that animals engaged in EtOH drinking on average 7.6 times per day, spending an average of ~56sec of drinking per visit. The majority of drinking (82.6%) occurred during the dark cycle (0900-2000hrs) (drinking time (min): total=83.8; dark cycle= 69.3; light cycle =14.6), with 23.3% of all drinking taking place within the first 4hrs of exposure. The 30min following 1130hr was the most active time for EtOH consumption (8 of 12 animals consumed EtOH at this time point for a total of 10.1min). Overall, drinking behavior did not remain consistent during the dark cycle but rather periods of elevated drinking were followed by periods of reduced drinking (fig 2.5).

Figure 2.5.
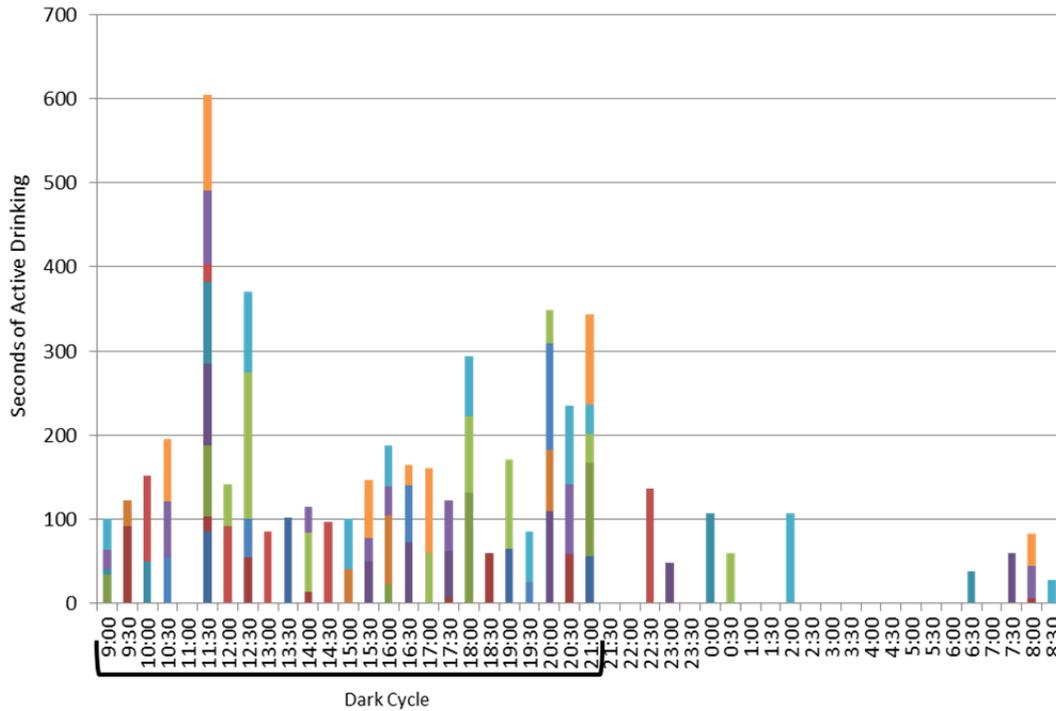
Fig 2.5. Drinking behavior recorded during a 24hr period of exposure to 20% EtOH, data for each animal (n=12) is represented by a different color, the length of the bar is indicative of the total time spent drinking at each visit to the EtOH bottle. Animals spent a greater amount of time drinking during the dark cycle (82.6% of total time), with 23.3% of all drinking taking place during the initial 4hrs of exposure (09:00-12:00). A spike in consumption occurred just prior to and after lights were turned on at 21:00hrs. 72 visits to EtOH bottles were recorded during the dark cycle, with an average of 57.8 sec spent drinking per visit. This is compared to 18 visits (48.5 sec/visit), which occurred during the light cycle. Animals engaged in a total of 83.8 min of drinking, 90 individual visits were recorded during the 24hr period.

Corticosterone Analysis

Data from one EtOH animal was excluded from this analysis, as the quantity of plasma remaining after the determination of BEC was insufficient to conduct the CORT assay (EtOH n=23, control n=8). Further, CORT data collected at the final timepoint (T2) represents a subset of animals. Due to procedural issues, one data point (control animal) from this subset was excluded from analysis (EtOH n= 12; control n= 3). Due to the reduced number of control subjects represented at the final timepoint, statistical analysis was not performed. Average and SEM were calculated for each group at each timepoint (T0, following 1 hr of EtOH exposure, and T2), these values are represented in fig 2.6a. Plasma CORT concentration in both the water control animals and EtOH exposed animals were elevated at T2, just prior to sacrifice.

Fig. 2.6a.
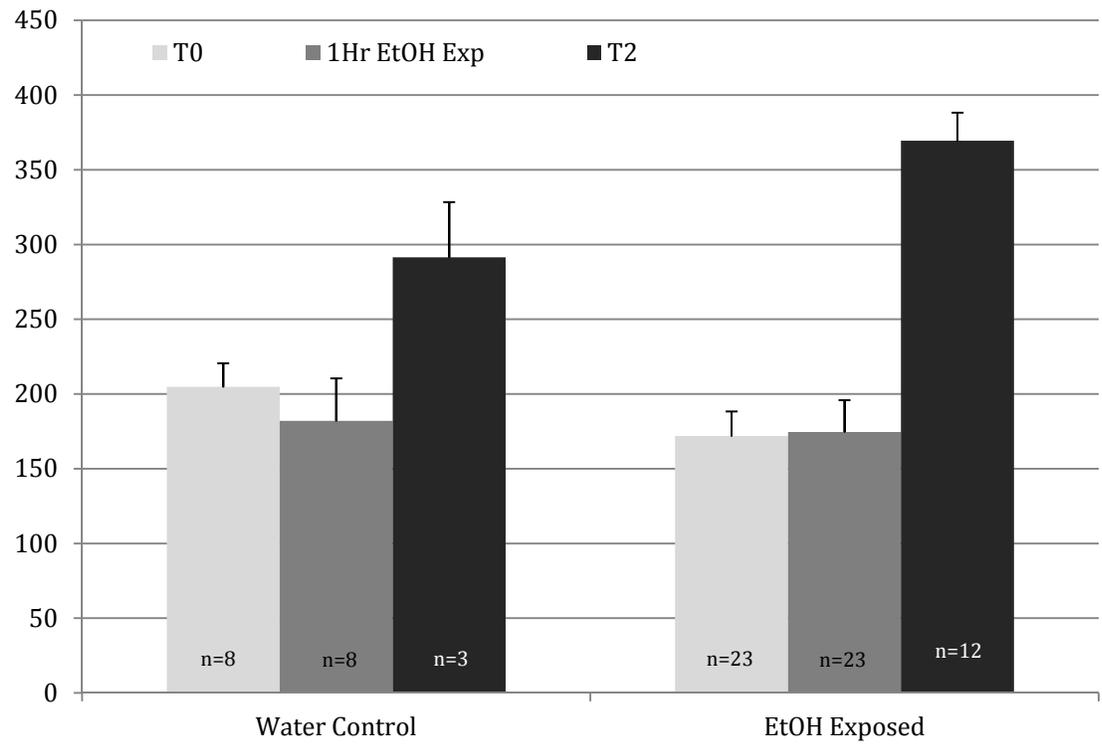
Figure 2.6a. Plasma CORT levels recorded at baseline (T0), 1hr following exposure to 20% EtOH, and prior to sacrifice (T2). Plasma CORT levels are elevated in EtOH exposed animals at the final time point. Data were not subjected to statistical analysis.

Further analysis of CORT levels within EtOH treated animals yielded several significant correlations relative to EtOH consumption. First, EtOH consumption (g/kg/24hr) was positively correlated with baseline (T0) CORT values (r(21)=0.543, p=0.0074), suggesting that CORT levels are predictive of subsequent drinking behavior (fig 2.6b). A positive correlation was also found between CORT levels following 1hr of EtOH exposure and blood EtOH level assessed at the same time point (r(21)=0.418, p=.0419) (fig 2.6c). Finally, CORT levels taken following 1hr of EtOH exposure were positively correlated with samples taken following 6 days of EWD (T2), immediately prior to sacrifice (r(21)=0.646, p=0.0234) (fig 2.6d).

Figure 2.6b.
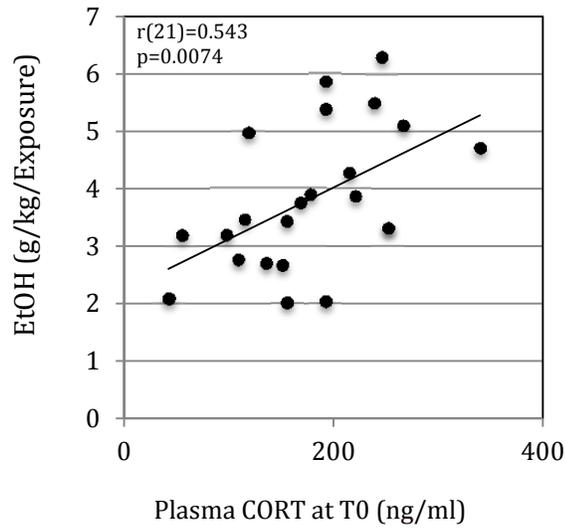
Figure 2.6b. Plasma CORT concentration prior to (T0) and following 1Hr of EtOH exposure. Animals with higher levels of plasma CORT prior to EtOH exposure consumed significantly higher levels of 20% EtOH.

Figure 2.6c
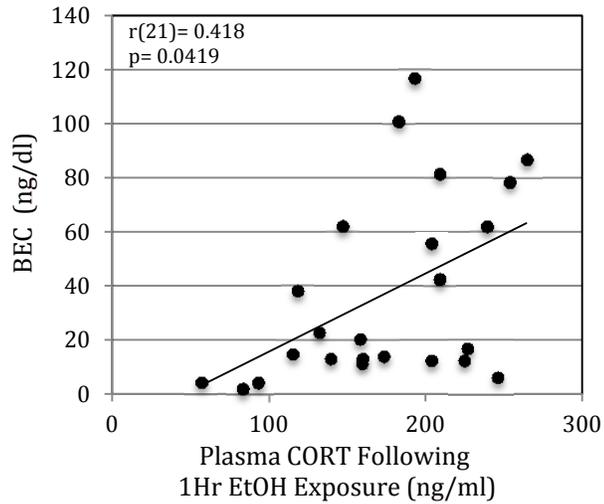
Figure 2.6c. Plasma CORT concentration following 1Hr of EtOH exposure is associated with BEC at the same time point.

Figure2.6d.
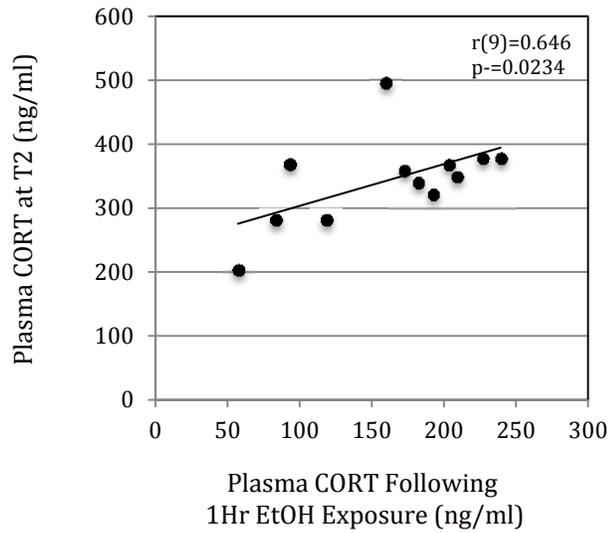
Figure 2.6d. Plasma CORT levels following 1Hr of EtOH exposure and following 6 days of EWD (T2). A significant correlation between CORT levels assessed following 6 days of EWD and levels measured following 1hr of EtOH exposure were found.

Acoustic Startle Reflex

Data from four animals were excluded from startle analysis. Startle reactivity for these animals was assessed in a first generation ASR chamber. Upon analysis it was observed that animals evaluated in these chambers had greater within session response variability and that, on average, responses were greater than animals assessed in the newer model chambers. Personal communication with San Diego Instruments verified that chambers of this age were susceptible to producing unreliable results and therefore, data collected in these chambers were excluded from analysis. Additionally, the data detailed below include values of three water treated control animals that were collected in a separate pilot study. These animals were exposed to the same experimental conditions described above. EtOH treated animals from this pilot study were excluded from analysis, as methods concerning EtOH exposure were not deemed consistent with those detailed herein (for ASR data: EtOH n=21; Ctrl n=10).

FSR (fig 2.7): A significant interaction was found between treatment and day (F(2,58)= 3.49, p = 0.037). Water treated control animals demonstrated no elevation in FSR at either T1 or T2, as compared to baseline value (T0). However, a similar comparison to baseline (T0) value revealed that EtOH treated animals displayed an elevated startle response following 26hrs (T1) and 6 days of EWD (T2).

Figure 2.7.
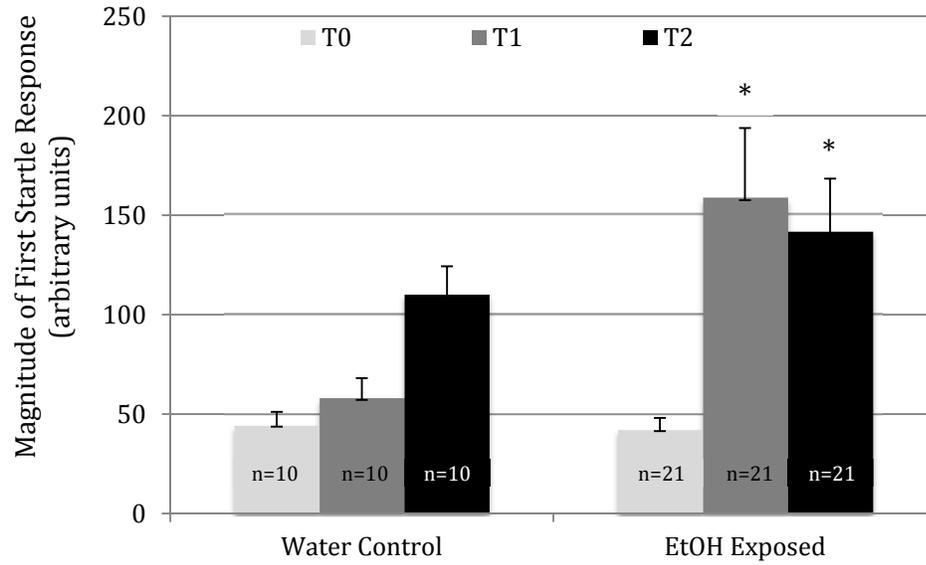
Figure 2.7. Magnitude of the startle reflex to the first presentation of a 120dB pulse as measured at baseline (T0), 26hrs (T1) and 6days (T2) following EtOH withdrawal. No differences were detected among the water control animals. Animals exposed to 20% EtOH had significantly higher response during both acute (T1) and protracted (T0) withdrawal. (*= compared to T0, p < 0.05)

Habituation (fig 2.8): Data regarding the ability of animals to habituate to repeated presentations of a startling pulse (120dB) revealed no effect of treatment, but a main effect of timepoint ($F(2,58) = 6.79$, p=0.002) was significant. Post hoc testing revealed a significant difference between habituation at baseline as compared to values measured following 26hrs (T1) or 6 days (T2) of EWD. A virtual lack of habituation was observed during baseline evaluation, whereas habituation was observed at later time points (M (arbitrary units): baseline (T0) = -0.8, ±9.13; 26hrs EWD = 33.9, ±9.13; 6 days = 28.3, ±9.13).

Figure 2.8.
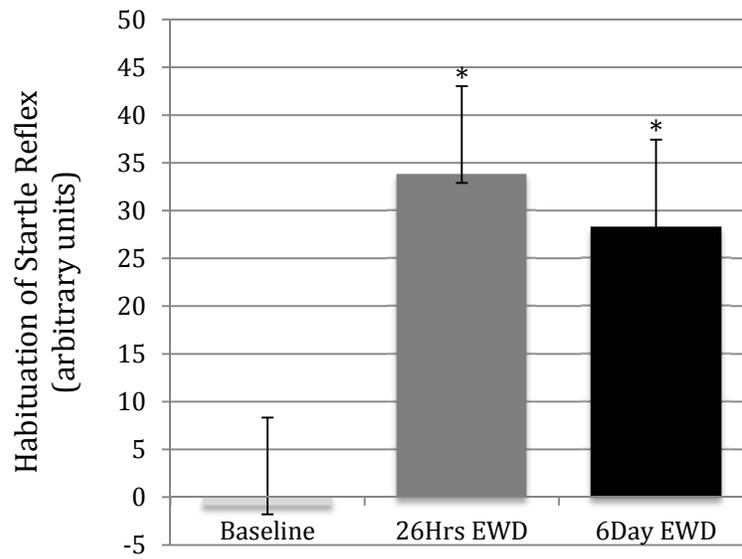
Figure 2.8. Habituation to the repeated presentation of a 120dB pulse (n=31, all time points). No differences were found regarding treatment. Habituation was evident at the latter two time points measured, although animals showed virtually no habituation at baseline (T0). (*= compared to T0, $p < 0.05$)

PPI (fig 2.9a & b):   PPI data was initially analyzed for treatment effects within each level of the prepulse (treatment (2) x level (3)). As no effects of treatment were found, data were collapsed across the variable and a repeated two-way ANOVA (timepoint (3) x level (3)) was conducted.

A main effect of timepoint ($F_{(2, 60)}$ = 33.00, p < 0.001) and level ($F_{(2, 60)}$ = 14.522, p < 0.001) was found. Inhibition of startle in the presence of the prepulse was lowest at T0 and improved at both subsequent timepoints (baeline vs 26hrs EWD, p < 0.001; BL vs 6 day EWD, p < 0.001) (fig 9a). Regarding the levels of prepulse, the highest decibel prepulse (87dB) produced the largest inhibition (79dB vs 89dB; 83dB vs 89dB) with no significant difference between the 79 and 82dB prepulse (9b).

Figure 2.9a.
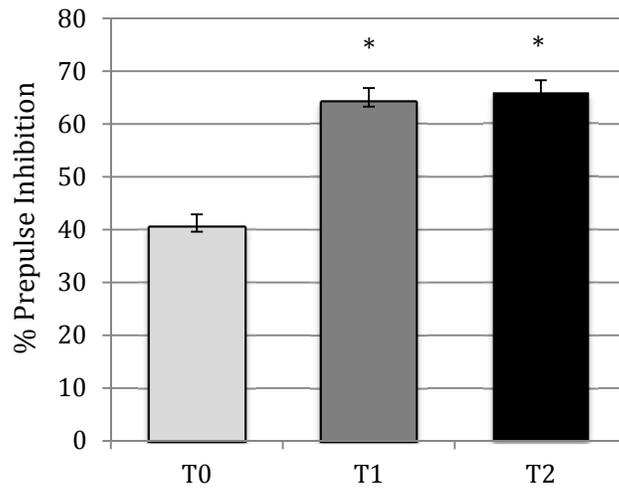
Fig 2.9a. Percent of Prepulse inhibition (PPI) is the ability of animals (n=31) to inhibit the startle response to a pulse (12dB) when the pulse is preceded by a non-startling level of prepulse. No differences were found regarding treatment, data were collapse to analyze for the effect of timepoint and level of prepulse (79dB, 82dB, 87dB). At T0a 40% reduction in the startle response was observed, whereas reductions of ~65% were seen at later timepoints (* = as compared to T0, p< 0.05)

Figure 2.9b.
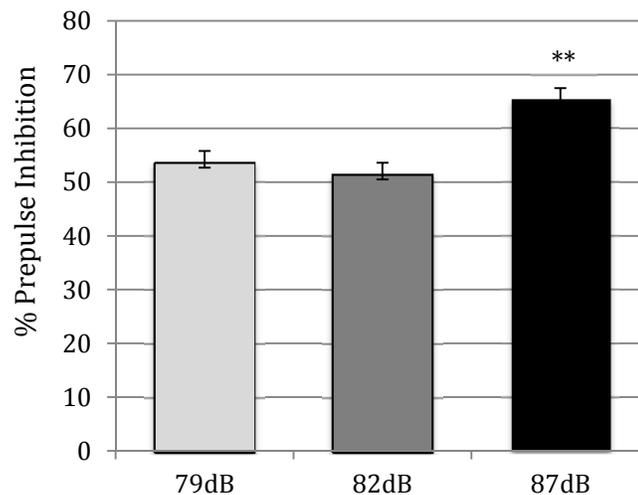
Fig 2.9b. Inhibition of the prepulse was most robust at the highest level (87dB) of prepulse. No effect of EtOH treatment was observed. (**= as compared to 79 and 82dB level prepulse, p < 0.05).

Protein Analysis

Due to procedural issues, tissue from one EtOH treated animal was excluded from analysis (EtOH n=11; Ctrl n=4). A one-way ANOVA was used to analyze treatment effects in protein receptor expression of NR2B subunits and GR, as well as changes in phosphorylation state of the NR2B subunit. Immunoblotting experiments using the pNR2B antibody failed to produce measurable results. Communication with Cell Signaling determined that the antibody was ineffective at targeting the protein. Replacement antibody was subsequently delivered and verified to be functional, however, time restraints did not allow for re-analysis of protein. No effect was found regarding EtOH induced changes in NR2B or GR. However, it should be noted that effects regarding changes in GR expression did approach significance (F=(1,14)=4.015, p = 0.065) (mean arbitrary units) (EtOH= 4260.8 ±481.1 vs Ctrl = 2680.1 ±473.6). Power analysis revealed the performed test (0.354) was below the desired power of 0.800 due to the low sample size (EtOH n= 12; Ctrl n=4).

Discussion

Characterization of Drinking in the Intermittent Access Model of Ethanol Exposure

The current study sought to characterize voluntary drinking produced by intermittent access to 20% EtOH, and the results yielded several interesting findings. Figure 1 demonstrates that no differences in weight gain or food intake were observed between EtOH exposed and water treated animals during the 7 weeks of experimentation. In models of forced EtOH exposure (e.g., single-bottle choice, intra-gastric gavage, vapor chamber exposure) weight loss is typical and can be problematic when interpreting data, as body weight loss can be an indicator of adverse health. As normal weight gain and appetite were observed in the present study, this extraneous variable does not need consideration when using this exposure paradigm.

A difference was observed when comparing total fluid intake between EtOH exposed and water control animals, with water control animals consuming more fluids during the first two weeks of the study (fig 2.2). However, no differences were observed following week 3, as total fluid consumption in both groups declined, remaining steady following week 4. A broad distribution of EtOH consumption levels was observed, ranging from 2.0-6.3 g/kg/exposure, with an average consumption of 3.79 ±0.43 (fig 2. 4a). In humans, this equates to approximately 3-10 drinks in a male and 4-11 drinks in a female (average weight of the American adult (kg): male 88, female= 75.4 (Fryar et al, 2012). These levels of consumption reflect moderate (3 drinks/day) to problem drinking (> 3 drinks/day) in the human population (NIAAA) and suggest the data collected in these experiments can be

generalized to individuals in these groups. Similarly, BEC data reported here are also widely distributed, ranging from 6.3 – 116.9mg/dl (fig 2.4b). However, it should be noted that blood samples for BEC analysis were collected following 1hr of EtOH exposure, at 1000hrs, while the most robust period of drinking didn't occur until the 30 minutes between 1130 and 1200hrs (fig 5). Therefore, while data reveal the wide range of BECs produced by the model, the data are less likely to reflect maximum levels that resulted.

Corticosterone

These studies sought to investigate the relationship between CORT and EtOH consumption and revealed several associations between the variables. Perhaps the most revealing finding was that basal levels of CORT were predictive of subsequent EtOH consumption. Similarly, Prasad & Prasad (1995) compared the drinking and HPA-axis profiles of selectively bred alcohol preferring (P) and non-preferring (NP) rats to that of the Holtzman Sprague-Dawley rats, which are selectively bred for their CORT profile. Specifically, the profile of the high-low (H-L) animal is one of high basal secretion and low stimulation upon fasting (24hrs), whereas the low-high (L-H) animal's profile is reversed with low basal levels and a high fasting response. The researchers found both drinking and CORT profiles between the animals to be similar, such that the P and H-L rats both display elevated levels of consumption and baseline CORT levels. Similarly, the alcohol preferring AA rat displays elevated baseline CORT levels as compared to its non-drinking counterpart, the ANA rat. Interestingly, this situation is reversed if the animals are group housed (Apter &

Eriksson, 2005). The current findings are consistent with these data and suggest that elevated levels of CORT tend to be associated with increased EtOH consumption. Together, they suggest that basal CORT levels may provide a valuable biomarker for EtOH preference that and that this trait may be genetically conferred.

In humans, elevated baseline plasma cortisol is often found in alcohol dependent but abstinent individuals, while both blunted and elevated HPA-axis reactivity to stressors has been observed. However, lowered basal levels of cortisol and ACTH have been recorded in offspring of alcoholics. These somewhat conflicted studies demonstrate that measuring one hormone level, whether basal or under stress, only provide a glimpse of the total HPA-axis profile. A host of factors including receptor sensitivity and the influence of other hormones (i.e., CRH and ACTH) need to be considered when contemplating the system. In addition, the importance of individual differences in HPA-axis sensitivity and reactivity is becoming increasingly evident. For example, Kerns et al. (2005) recently identified an array of genes within the limbic system that were responsive to the acute administration of EtOH (e.g., single i.p. dose of 0.5-2.0 g/kg). Among these were glucocorticoid-responsive genes that are differentially expressed in mouse strains (DBA2/J and C57BL/6) based upon EtOH preference and sensitivity (i.e., locomotor activation and sensitization) (Costin et al., 2013). Exploring how these genes confer different properties regarding acute EtOH exposure could provide insight into why some individuals escalate from casual drinking to alcoholism and perhaps yield a molecular target for its treatment. Providing a different perspective on the role of glucocorticoids as they pertain to addiction, a recent review by Bartlett and

colleagues (Srinivasan et al., 2013) explored the possible protective effects of the hormone. These authors point out that the ability to persevere under stress (i.e. resilience), whether conferred via genetics or the environment (i.e., learned), predicts resilience to the development of EtOH dependence. They also suggest cellular mechanisms by which glucocorticoids may mediate this association. In total, this research underscores the intricate influence that glucocorticoids can have on the development of AUDs.

In the present study, the influence of CORT was evident not only on EtOH consumption, but also on EWD, as hormone levels were found to be at T2 in EtOH exposed animals but not in water treated controls (fig. 2.6a). Finding elevated CORT in EtOH exposed animals during withdrawal is consistent with a breadth of data regarding HPA-axis dysregulation that occurs in the human population during EWD. Elevations in cortisol have been reported during the early withdrawal phase (Esel et al., 2001), which is followed by a decline in hormone levels as abstinence progresses (Adinoff et al., 1998; von Bardeleben & Holsbeer., 1989). In some alcohol-dependent individuals, dysregulation of HPA-axis activity is extreme, mirroring that seen in patients with Cushing's Syndrome (Besemer et al., 2011). Supporting data have been gathered regarding the stress response using long-term models of EtOH exposure in rodents. During the initial phase of EWD and following chronic EtOH treatment, elevations in plasma CORT (Alele & Devaud, 2007; Rasmussen et al., 2000) and reductions in GR mRNA (Vendruscolo et al., 2012) were observed, while increases in GR mRNA were observed during protracted EWD (Vendruscolo et al., 2012). These elevations can have deleterious results, as demonstrated by *in vivo* studies in which

exposure to CORT was shown to promote neuronal injury within the hippocampus following EtOH exposure (Mulholland et al., 2005), the effects of which were glutamate receptor-dependent (Butler et al., in press; Mulholland et al., 2006). Additionally, neurochemical adaptations that occur during the withdrawal phase appear to be integral to the progression of dependence. This is demonstrated as escalation of drinking and worsening of withdrawal symptoms that have been seen in animals exposed intermittently to EtOH (allowing for EWD periods), but not in those exposed to the continuous treatment (O'Dell, 2004). Further, there is evidence that these alterations are GR-dependent, as blocking the receptor is efficacious at reducing the escalation to drinking and the manifestation of withdrawal behavior (Cippitelli, et al, 2012; Sharrett-Field et al., 2013; Simms et al, 2012; Vendruscolo et al, 2012).

At issue with the interpretation that EtOH exposure was found to mediate elevations in CORT during withdrawal (fig 2.6d), is the lack of association between hormone elevations and EtOH consumption (presented in fig 2.10). One possibility for this occurrence is that EtOH consumption of any level sufficient in elevating CORT during protracted withdrawal. However, the results may also arise from an extraneous variable. Just prior to sacrifice, animals were placed in a restraining device (DecapiCone, Braintree Scientific, USA) to aid in decapitation. This restraint could have provided a stressor, resulting in a rise in CORT levels. In support of this, hormone levels measured following 1hr of EtOH exposure were significantly associated with those measured at T2, prior to decapitation (fig. 2.6d). This association may have occurred because animals were stressed under both

conditions, at 1hr from the presentation of EtOH, and at the final time point, due to restraint stress. Additionally, CORT elevation was evident in water control animals prior to decapitation, although the effect was not significant (fig 2.6a). Therefore, it is possible that rise in CORT levels observed in Figure 2.6a. is mediated by stress, independent of EtOH exposure.

Figure 2.10.
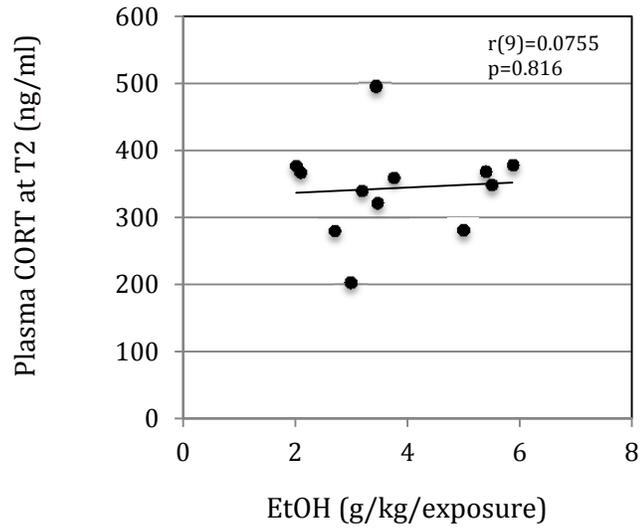
Figure 2.10. Plasma CORT levels following 6 days of EWD (T2) was not associated with EtOH exposure.

Finally, it is interesting to note that, although EtOH is known to activate the HPA-axis, thereby increasing CORT levels, no elevation is evident in animals following 1hr of access to 20% EtOH (fig 2.6a). This is likely because only modest EtOH consumption occurred within the first hour of access (fig 2.5). As mentioned previously, a more accurate representation of BEC could be assessed at the 1200 time point, when drinking is more robust (fig 5). Additionally, although drinking levels following 1hr of access were insufficient to detect an overall change from baseline CORT values (fig 2.6a), a significant correlation was found between CORT and BEC (fig 2.6c). This demonstrates that, in animals consuming higher levels of EtOH during the first hour, consumption was sufficient to produce elevations in CORT.

In sum, the CORT data suggested that basal CORT levels could provide a valuable biomarker for EtOH preference. Further, outbred animals consume sufficient levels of EtOH during intermittent exposure to elevate CORT levels, though further investigations would be needed to determine if elevations in CORT occur during protracted EWD.

Acoustic Startle Reflex

Testing conducted using ASR was sensitive to the effects of EWD in these studies, as EtOH exposed animals showed significantly elevated responding to the first presentation of a 120dB level pulse during both acute and protracted EWD (fig 2.7). Importantly, EWD is a defining characteristic of dependence (American Psychiatric Association, 1994). The manifestation of EWD in rodents can present as

local or whole-body tremor, rigidity (tail and body), enhanced seizure susceptibility (handling- and auditory-induced) or less overtly in anxiety-like behaviors (i.e., decreased exploratory behavior) (Helig et al., 2010). Similarly, humans can experience seizures and increased anxiety during EWD. Alterations in ASR have been used to assess anxiety disorders in humans (Grillon et al., 1997), as well as depression (Grillon et al., 1997; Mneime et al., 2008), childhood abuse, post-traumatic stress disorder (PTSD) (Grillon et al., 1996; reviewed by Braff et al., 2001), or substance abuse (Grillon et al., 2008), which all have in common an interaction with the stress response. In fact, injections of corticotropin-releasing hormone (CRH) are able to enhance the startle response, an affect that is mediated by the efferent projections of the ventral hippocampus onto the bed nucleus of the stria terminalis (BNST), as lesions to either resulted in decreased CRH enhancement of ASR (Lee & Tsai, 1989; Lee & Davis, 1997). Additionally, administration of the centrally acting hypertension drug moxondine was able to reduce EWD-enhanced ASR (Vandergriff et al., 2000). This drug is an agonist at the imidazoline(I)(1) receptor, which are centrally distributed and are expressed in the medulla, ventral tegmental area, and peripherally in the adrenal medulla. Interestingly, intensity of ASR has been associated with the number of withdrawal episodes an individual has experienced, in which a threshold of 2 or more detoxifications has been suggested (Krystal, 1997). These findings suggest that ASR is sensitive to the kindling effects associated with EWD, indicative of a hypersensitivity within the CNS (Ballenger & Post, 1978; recently reviewed by Breese et al, 2011). Because the IA model of EtOH

exposure allows for repeated periods of withdrawal from EtOH, it is possible that elevations in ASR are due to the effects of kindling.

Although no treatment effect was found regarding measures of startle plasticity (HAB and PPI), the data did yield important information that may impact the overall interpretation of this study. In analyzing both HAB and PPI data, a main effect of day was observed, such that baseline values were significantly different than those of either later time points (figs 2.8 & 2.9a). In fact, very little habitation or inhibition was observed during baseline measurements. This pattern of reduced responding is known to occur in adolescent animals. Regarding PPI, younger animals are believed to lack the ability to filter out irrelevant stimuli, thereby reducing effectiveness of the prepulse to predict the subsequent pulse (Ellwanger et al., 2003; van den Buuse, 2003; and reviewed by Geyer et al., 2001). It has been suggested that this lack of inhibition results from an underdeveloped prefrontal cortex (Ellwanger et al., 2003; van den Buuse, 2003). In the current study, presentation of the prepulse reduced baseline responding by ~40%, whereas reductions exceeding 60% were observed at later time points (fig 2.9a). Similar results, published by Brunell & Spear (2006), showed significantly less PPI at baseline in adolescent Sprague-Dawley rats (post natal day (PND) 30-31) (~30%) than in adults (PND 69-71) (~60%). The acceptable age range for reaching adulthood in rats has been debated in the literature, but based upon factors such as growth spurt, loss of NMDA in the PFC, and exiting of the nest in the wild, post-natal day (PND) 28-43 appears to be a conservative range for the end of adolescence (reviewed by Spear, 2000). However, signaling changes associated with maturation

can occur as late as PND55 (250-275g) (Spear, 2000). Animals used in the present studies were PND60, with an average weight of 220g, placing them on the edge of these parameters. If, in fact, these animals were still undergoing development, the effects demonstrated here may be exclusive to adolescent EtOH exposure.

Suggestive of this interpretation, abstinent alcohol-dependent males who began drinking during adolescence (mean age of onset =18) had elevated ASR (eye-blink) as compared to abstinent individual who began drinking later in life (mean age of onset=30) (Schellekens et al, 2012). While these results may indicate possible genetic differences between these groups similar to those seen in relatives of alcoholics, it is important not to exclude the effect of EtOH exposure during the final stages of development. In fact, early onset drinking is a strong predictor of later alcohol abuse and dependence, with probabilities increasing significantly if drinking begins prior to age 19 (DeWit et all, 2000), which has been defined as the end of adolescence by the World Health Organization. Preclinical research supports this, as adolescent but not adult rodents, experience enhanced voluntary drinking following the presentation of minor environmental stressors (e.g., isolated housing) (Doremus et al, 2005). Indeed, there is accumulating evidence that adolescents experience fewer effects that are associated with the regulation of drinking (e.g., sedation, anxiety upon withdrawal), while experiencing more of the neurodegenerative effects that can promote addiction (reviewed by Nixon & McClain, 2010). A recent study into mechanisms that may contribute to early onset drinking identified several GR polymorphisms to be associated with alcohol use and abuse in a group of 14 year old adolescents (Desrivieres et al, 2011).

Collectively, the ASR data collected here suggest that consumption resulting from IA EtOH exposure is sufficient to produce dependence. However, it remains to be seen if these effects are dependent upon the age of the animal during initial exposure.

Protein Analysis

Literature regarding upregulation of NR2B receptor subunits following EtOH exposure within the hippocampus and PFC are not without precedent, although studies have produced mixed results. For example, chronic EtOH exposure was found to elevate levels of NR2B in the forebrain (Narita et al, 2000) and cerebral cortex (Henniger et al, 2003; Kumari & Ticku, 1998) (but see also Narita et al., 2000) and hippocampus (Butler et al, in press). These elevations have been implicated in CNS hyperexcitiblity that results upon EWD (reviewed by Kumari & Ticku, 2000). Lack of evidence in the present study should not be interpreted as conclusive, as there is evidence of region-specificity regarding EtOH-induced alterations of the NR2B and the current studies investigated alteration in the entire are of the PFC and hippocampus.

As previously discussed, EtOH is able to affect the expression of GRs in several different brain regions during EtOH intake and withdrawal. (Eskay et al., 1995; Roy et al., 2002; Vendruscolo et al., 2012; Vendruscolo et al., 2012; Roy et al., 2002). In the present study, no significant alterations in GR were found following 6 days of EWD. However, a trend toward upregulation was. This finding is consistent with results of a recent publication in which EtOH exposure via vapor chamber

resulted in decreases in GR mRNA (hippocampus, PFC, nucleus accumbens, BNST) 24hrs following the withdrawal of EtOH and elevations in GR mRNA (nucleus accumbens, BNST, amygdala) 3 weeks later evident (Vendruscolo et al, 2013). An upregulation of GR during protracted withdrawal may have implications regarding stress-induced relapse, as abstinent individuals may experience increased sensitivity HPA-axis activation. In fact, preclinical studies have shown an escalation in drinking following protracted withdrawal (O'Dell et al, 2004), which is escalated by stress (reviewed by Breese et al, 2010), but which is also blocked by GR antagonists (Simms et al, 2012). Together, these data suggest the GR is as a possible therapeutic target in the treatment of alcohol dependence and relapse.

Chapter Three

EFFECTS OF THE ADMINISTRATION OF THE SELECTIVE GLUCOCORTICOID

RECEPTOR ANTAGONIST ORG-34517 ON VOLUNTARY ETHANOL CONSUMPTION

AND SUBSEQUENT ETOH WITHDRAWAL

(Study 2; Sharrett-Field, et al.)

Introduction

Alcoholism is a devastating disorder, by some accounts affecting up to 15% of the American population. Individuals often repeatedly shift from periods of dependence, withdrawal, abstinence, relapse, and back to dependence. In fact, relapse rates range from 20-80%, underscoring the cyclic nature of the disease. Repeated alcohol withdrawal is problematic in that subsequent withdrawal episodes have been associated with a worsening of symptoms (withdrawal sensitization), which can culminate in seizure activity and persistent neuroadaptations. Currently, individuals presenting with alcohol withdrawal are treated with barbiturates and other CNS depressants or anti-consultants. While these drugs are successful in reducing seizure activity and increasing short-term abstinence, they have not been shown to reduce withdrawal sensitization or promote long-term abstinence. Medications such as disulfiram, acamprosate, and, naltrexone have demonstrated some, yet limited, success in achieving long term abstinence. However, they have not shown efficacy in reducing withdrawal sensitization. Importantly, withdrawal sensitization has been associated with changes in the rewarding characteristics of alcohol, increasing both negative (i.e., anxiety) and positive (i.e., increased consumption following relapse) reinforcement;

these factors are believed to lead to increased rates of relapse. Therefore, developing treatments that are efficacious at reducing consumption and withdrawal could offer additive protection against relapse to drinking.

Stress is a known contributor to the development and escalation of alcoholism and also plays a main role in relapse drinking. Major life stressors (ie., loss of a job, moving, chronic health related issues) are associated with lifetime alcohol consumption and risk of developing an AUD (Veenstra et al, 2006). However, the question as to why these external risk factors induce some individuals to abuse alcohol, while others seek more adaptive means of coping, remains an unanswered question. Accumulating evidence suggests that how an individual reacts to stress can be a contributing factor to the development of an alcohol abuse disorder (AUD), which in turn is mediated by one's genetic profile. Twin studies suggest that the basal state and reactivity of the body's main stress system, the HPA-axis, is a heritable trait (Bartels et al, 2003). Additionally, children of individuals who are alcohol dependent have both an altered stress response and a propensity for developing alcoholism, suggesting a genetic link for both traits (Uhart & Wand, 2008; Zimmerman et al, 2007). Preclinical data support the link between stress and the development of AUD, as altering levels of the primary stress hormone corticosterone (CORT) in rodents can elevate or decrease EtOH consumption (Fahlke et al., 1994a, b; Falke & Hansen, 1995; Hansen et al., 1995; de Witte et al, 1996; Hansen et al., 1995, Fahlke et al, 1994). Further targeting the glucocorticoid receptor (GR), a primary receptor of CORT, with the antagonist mifepristone can reduce voluntary consumption (Koenig & Olive, 2004; Vendruscolo et al, 2012),

stress-induced EtOH seeking (Simms et al., 2011), behavioral signs of ethanol withdrawal (EWD) (Sharrett-Field et al., 2013) and cognitive deficits resulting from EWD (Jaquot et al., 2008). While the traditional antagonist mifepristone is effective in treating these correlates of alcohol abuse in preclinical models, implications for its clinical use are less optimistic, as it is a potent progesterone receptor (PR) antagonist. Activity at the PR is needed to sustain pregnancy; therefore prescription of mifepristone would likely be limited to the male population. A derivative of mifepristone, 11, 21 Bisphenyl-19norpreganane (ORG-34517) displays a high affinity for GR binding with negligible binding to the PR (affinity ration for GR/PR of 488) (Gebhard et al, 1997), suggesting it is a suitable replacement for mifepristone.

The following study was conducted to determine the effects administration of the selective GR antagonist ORG-34517 would have on voluntary consumption levels and subsequent EWD. An intermittent access model of EtOH exposure was used and drug was delivered via inclusion in the consumer grade product, Nutella®. It was hypothesized that ORG-34517 would decrease voluntary consumption of 20% EtOH. Attenuation of EtOH-dependent changes in ASR during EWD was also anticipated. However, elevations in CORT were anticipated in the ORG-34517 treated animals, as blocking the GR also blocks the natural feedback mechanism for the stress response. Assessment of protein levels was conducted in the hippocampus and the medial pre-frontal region of the frontal cortex (mPFC) to assess the state of GR and NR2B protein expression, as well as the phosphorylation state of the NR2B receptor. EtOH-associated elevations in GR and pNR2B were expected.

Materials and Methods

The following section closely follows methods detailed in Chapter 2. For the reader's
convenience, changes and additions have been underlined.

Subjects

Male, Long-Evans rats (60 days old; Harlan Laboratories, Indianapolis, IN)
were single housed and exposed to reversed dark/light cycle (lights off at 0900hrs).
During a one week acclimation period, animals were handled for 20min/day for 3
days. Animal and food weights were taken weekly between 0800 and 0900hrs on
Monday. Animals had *ad libitum* access to food and water throughout the
experimental procedure.

Intermittent Access to Ethanol

Animals were given 24hrs of concurrent access to 1 standard bottle
containing a 20% (v/v) EtOH/water solution and one standard bottle containing tap
water. After 24hrs, EtOH bottles were removed and all animals received 2 bottles
containing tap water (for Experimental Timeline, see Table 3.1). Placement of EtOH
and water bottles were alternated to avoid side preference. This pattern of access
(24hrs with EtOH followed by 24hrs without EtOH) continued for 55 days (27 days
of EtOH exposure on M-W-F). Two empty cages containing no animals received
identical treatment regarding bottle changes and served to account for bottle

leakage (leak controls). A separate group of animals (water controls) received identical treatment except neither bottle contained EtOH.

Table 3.1 Experimental Timeline

	M	T	W	TH	F	Sa	Su
WK1	T0 Baseline ASR		Blood Draw EtOH/Water	Water/Water	EtOH/Water	Water/Water	
WK2	EtOH/Water	Water/Water	EtOH/Water	Water/Water	EtOH/Water	Water/Water	
WK3	EtOH/Water	Water/Water	EtOH/Water	Water/Water	EtOH/Water	Water/Water	
WK4	EtOH/Water	Water/Water	EtOH/Water	Water/Water	EtOH/Water	Water/Water	
WK5	EtOH/Water	Water/Water	Blood Draw EtOH/Water	Water/Water	EtOH/Water	Water/Water	
WK6	EtOH/Water	Water/Water	EtOH/Water	Water/Water	EtOH/Water	Water/Water	
WK7	ORG or Nutella EtOH/Water	Water/Water	ORG or Nutella EtOH/Water	Water/Water	ORG or Nutella EtOH/Water	Water/Water	
WK8	ORG or Nutella EtOH/Water	Water/Water	ORG or Nutella EtOH/Water	Water/Water	ORG or Nutella EtOH/Water	Water/Water	
WK9	ORG or Nutella EtOH/Water	Water/Water	ORG or Nutella EtOH/Water	Water/Water	ORG or Nutella EtOH/Water	Water/Water	
WK 10	ORG or Nutella EtOH/Water	Water/Water	Water/Water T1 ASR	Water/Water	Water/Water	Water/Water	
WK 11	T2 ASR Sacrifice- Trunk Blood						

EtOH consumption was measured in two manners. First, bottles given to animals (water and EtOH) were weighed prior to presentation and again following 24hrs of access. This value, less the weight change detected in the leak control bottles, was expressed as ml/kg of fluid intake. Twenty percent of this value was multiplied by the specific gravity of 200 proof EtOH (0.794cm) to determine the EtOH dose (g/kg) each animal received. Additionally, blood EtOH concentration was measured following 1Hr of access, as described below. No video recordings were conducted in these experiments.

Drug Treatment

These studies used the selective GR antagonist ORG-34517 to investigate the effect of GR antagonism on EtOH consumption and EWD. ORG-34517 is chemically similar to RU-486, which has been shown to reduce voluntary EtOH consumption, EtOH reinstatement, EWD and cognitive deficits resulting from EtOH exposure and subsequent withdrawal, but does have some beneficial properties over RU-486. For example, while RU-486 shows high affinity for the GR, it does show activity at the progesterone receptor (PR) as well. Blocking this receptor produces spontaneous termination of pregnancy, making administration of the drug unsuitable for women who are or may become pregnant, thus limiting the drug's clinical use. ORG-34517 is selective for the GR and shows little to no affinity for the PR. Additionally, the mechanism of action for each drug differs slightly. The GR is a cytosolic receptor, which is inactively bound by chaperone proteins within the cytosol. Binding of CORT

to the receptor/chaperone complex initiates a conformation change and the chaperone dissociates. The CORT/GR complex is then able to translocate to the nucleus where the complex can begin the process of protein synthesis. Both RU-486 and ORG-34517 compete with CORT for the GR binding in the cytosol. However, co-exposure of RU-486 and CORT results in no reduction in translocation of the GR to the nucleus whereas co-exposure with ORG-34517 and CORT results in a 75% reduction in translocation (Peeters et al., 2008). Due to the chemical and mechanistic similarities between RU-486 and ORG-34517, it was hypothesized that the latter would be effective at reducing consumption in the present model. Due to its selectivity for the GR, ORG-34517 would be available to a larger clinical population, as compared to RU-486.

Following week 5, EtOH exposed animals were matched for consumption levels (g/kg) and distributed across drug treatment, such that both high and low consumers were equally represented, and water control animals received random assignment (average consumption (g/kg): ORG-34517=4.1, ±1.3, placebo=3.4, ±3.4. This time point was selected based upon previous data, which suggest that voluntary consumption has stabilized at this point. The selective GR antagonist ORG-34517 or placebo (compliments of Pop Test, Cliffside Park, NJ) was delivered M-W-F, 1hr prior to the presentation of EtOH bottles. Capsules containing drug (150mg) or placebo were opened and added to Nutella® (150mg/2g) and mixture was placed in a Ziploc® bag. Through a cut in the corner of the bag, 0.53mg/kg was measured out, resulting in a 40mg/kg dose at each time point. Nutella® mixture was delivered by placing the entire dose on the inside wall of each animal's cage (drug delivery

visually detailed in figures 3.1a, b, c). At each presentation, most animals readily (within 5min) consumed the entire dose of mixture, and all animals consumed the entire amount of mixture in the 30min prior to the delivery of EtOH. This method of drug delivery was selected, as it made use of the capsules as they had been formulated (i.e., for oral ingestion) and had the added benefit of minimizing the stress incurred by the experimental animals.

Figure 3.1a.

Figure 3.1b.
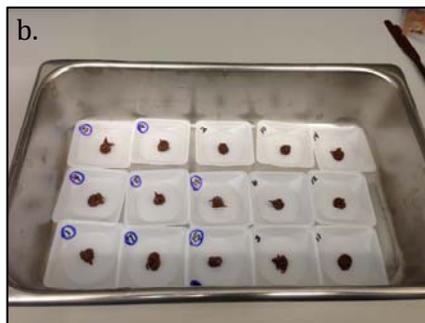
Figure 3.1c.

Figure 3.1a-c. Delivery of ORG-34517 or placebo in vehicle (Nutella®). Capsules containing drug (150mg) or placebo were opened and added to Nutella® (150mg/2g), mixed and placed in a Ziploc® bag (a). Through a cut in the corner of the bag, 0.53mg/kg was measured out, resulting in a 40mg/kg dose at each time point (b.). Nutella® mixture was delivered by placing the entire dose on the inside wall of each animal's cage (c.).

Blood Ethanol and Corticosterone Concentration

Tail blood was collected (approximately 140 µl/animal/time point) prior to EtOH exposure and again 1hr following the introduction of EtOH during week 6. Six days following the removal of EtOH, animals were euthanized via rapid decapitation and trunk blood was collected. Upon collection, blood samples were placed on ice and centrifuged (5 min at 15,000 g/min); blood plasma was collected and immediately stored at -80°C.

Blood EtOH levels (BEL) were determined using the Analox AM1 instrument (Analox Instruments, Lunenburg, MA), which measures blood EtOH concentrations indirectly through measurement of molecular oxygen levels. In the presence of molecular oxygen, EtOH is oxidized by the enzyme alcohol oxidase to form acetaldehyde and hydrogen peroxide. Under the conditions of the assay, oxygen consumption is directly proportional to EtOH concentration in the plasma sample.

Blood CORT levels (BCLs) were determined using a competitive EIA Corticosterone kit (IDS Limited, Fountain Hills, AZ). Briefly, 100 µl of each diluted sample (1:20), calibrator, and control and 100µl of enzyme (corticosterone labeled with horseradish peroxidase) were added to an antibody coated plate (polyclonal rabbit anti-corticosterone) in duplicate and incubated at 4°C for 24 hrs. Following incubation, the plate was thrice washed and 200 µl of substrate (tetramethylbenzidine and hydrogen peroxide) was added. After 30 min, stop solution (0.5M hydrochloric acid) was added and the microplate was read using a Beckman Coulter DTX 880 Mulitmodal Detector (Lagerhausstrasse, Austria) with

Beckman Coulter Multimode Detection Software (v.20.0.12). Mean absorbance values for samples, controls, and calibrators were measured at 450nm producing a mean value; this value was used to calculate percent binding (B/B0%=[(mean absorbance)/(mean absorbance for "0" calibrator)]×100). Mean concentration of CORT for each sample (ng/ml) was determined based upon the calibration curve defined by known samples that were run with the experimental samples.

Behavioral Measures

Acoustic Startle Reactivity was measured in a commercially available startle system (S-R Lab, San Diego, CA, USA). Chambers (35x33x38.5 cm, length x width x height) contained an overhead light (3 lux) and a Plexiglas holding tube (8.9cm in diameter x 20.3cm in length), in which animals were placed for testing. The holding tube was designed to reduce but not eliminate voluntary movement of the animal, thereby reducing stress. A piezoelectric accelerometer within each tube transduced large movement by the animal and was attached to a PC, on which responses were recorded using S-R Lab software (v5.0). A speaker located in the ceiling of the chamber delivered acoustic startle stimuli as well as 75dB white noise background. At the beginning of each trial (defined below), data were recorded over a 100ms window and were sampled at 1ms intervals. Data were recorded in two different chambers. Both input (dB) and output (amplitude of response) were calibrated prior to each testing session using San Diego Instruments calibration devices.

Data consisted of two measures, startle reactivity (first startle response (FSR)) and startle plasticity (habituation of the startle response and prepulse

inhibition). ASR data was collected prior to EtOH exposure (T0) and again 26hrs (T1) and 6 days (T2) after EtOH exposed animals received the final EtOH exposure. These time points were chosen to reflect both acute and protracted EWD in the EtOH exposed animals. During IA access, EtOH exposed animals become accustomed to experiencing 24hrs between EtOH exposures. Therefore, at 26hrs (T1), it was reasoned that animals would have expected, but not received EtOH, a situation that would elevate anxiety in dependent animals.

Prior to all ASR testing a 5 min acclimation period consisting of background noise (75dB white noise) was presented and remained on throughout testing. For measures of FSR and habituation, animals were presented with 6 120dB stimuli (Sandbak et al, 2000). The FSR is equivalent to the amplitude of the startle response to the initial presentation of a 120dB stimulus tone. Habituation score was calculated by subtracting the average amplitude of the last three startle responses from the average of the initial three responses. Prepulse inhibition (PPI) was assessed over 8 blocks consisting of a random presentation of the following 5 trials:

(i) Pulse stimulus (110 dB for 40ms)
(ii) Prepulse A(4dB for 20ms) + pulse stimulus
(iii) Prepulse B(8dB for 20ms) + pulse stimulus
(iv) Prepulse C(16dB for 20ms) + pulse stimulus
(v) No stimulus (background noise was applied)

PPI was defined as a decrease in the amplitude of the startle response in the presence of the prepulse stimuli and expressed as: PPI = (100-(mean startle reflex with the prepulse/mean startle reflex without prepulse)) x 100 (Geyer, 2001). Interval between the presentation of the prepulse and pulse was 100ms, intertrial

intervals were varied randomly in the range of 10 to 30s. Total time in startle chamber for each animal was approximately 25 minutes.

Protein Expression

Following rapid decapitation, whole brains were removed. Olfactory bulbs were discarded and medial prefrontal cortex (mPFC) was isolated from each hemisphere; following sagittal section, hippocampi were bi-laterally removed. Tissue from each brain region was placed on dry ice immediately following removal and stored at -80°C. Frozen tissue was placed in lysis buffer (Radio-Immunoprecipation Assay (RIPA) Buffer (1:100) (Thermo Scientific, Rockford IL), 99.8M^{-6} PMSF/EtOH (Sigma-Aldrich, St. Louis MO) in PBS (1:100), HALT Protease & Phosphotase Inhibitor (1:10) (Thermo Scientific, Rockford IL)), coarsely ground using a pestle, and centrifuged at 4°C for 20min at 12000rpm. Supernantant was collected and assayed using the Pierce® BCS Protein Assay Kit (Fisher) to determine the protein concentration of each sample.

Immunoblotting was conducted in the Mini-Protean® System using TGX Gradient Precast Gels (4-15%) (Bio-Rad, USA). Prepared samples (10uL containing 50ug of protein) or Bio-Rad Precision Plus Protein™ standards (7uL) were vortexed and loaded into lanes. Samples were electrophoresed in 1x Tris/glycine/sodium dodecyl sulfate buffer (Bio-Rad, Hercules, CA, USA) at room temperature for approximately 2.5 hrs at 80-90V. Protein was transferred on the membrane (nitrocellulose paper) in buffer (.25M^{-3} Tris Base, .19M^{-3} Glycine, 10%Methanol) for 1hr at 100V, carriage was place on a stir plate and contained an ice pack. Membrane

was then rinsed twice in 1xTBS and allowed to rock at room temperature for 15min. Membrane was rinsed twice in 1xTBS and agitated in blocking agent (5% non-fat dried milk in TBS) for 1hr at room temperature. Membrane was rinsed in 1xTBS with 10% Tween20 (TTBS) then agitated in TTBS for 15min. Following a final rinse, membrane was placed in primary antibody solution (5% milk in TTBS with anti-NR2B 1:1000 (Millipore), -GR 1:5000 (Santa Cruz), or -pNR2B (Cell Signaling)) and agitated overnight at 4°C. On day 2, membranes were rinsed and agitated in TTBS in 5min intervals for a total of 20min. They were placed in light-proof boxes and then agitated in fluorescent secondary anti-body solution (5% milk in TTBS with IRDye800 (Rockland Immunochemicals, Gilbertsville, PA, USA) for 1hr at room temperature. Following a final rinse in TTBS, membranes were imaged on Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and quantified using Image J software (NIH).

Statistical Analysis

Data were subjected to analysis of variance for the effects of drug treatment (ORG-34517 or placebo control Nutella®) occurring within each fluid treatment (EtOH or water). No main effect or interaction was found regarding treatment with ORG-34517 or placebo on any measure assessed. Therefore, all data were collapsed across the variable and analyzed for the effects of fluid treatment. When appropriate, treatment was used as the between subjects and time (week or timepoint) as the within subjects factors. Correlations were determined using

Pearsons's *r*. When appropriate, post hoc testing utilized Bonferroni t-test. Significance was determined at $p < 0.05$.

Results

Body Weight and Food Consumption

Body and food weights were taken weekly on Monday prior to the introduction of EtOH and continued through Monday of week 10. Data were subjected to analysis of variance and, while no differences were found between EtOH exposed and water treated control animals, an interaction was observed ($F_{(8,112)}$ = 2.093, p= 0.042). Post hoc analysis determined that water treated controls consumed more food during week 6, as compared to values observed in weeks 1 and 3. A reduction in food consumption was found in EtOH exposed animals during week 3, as compared to week 1 (fig 3.2a) (Food Intake (g): EtOH =165.1, ±3.46, Ctrl =179.1, ±5.99).

Figure 3.2a.
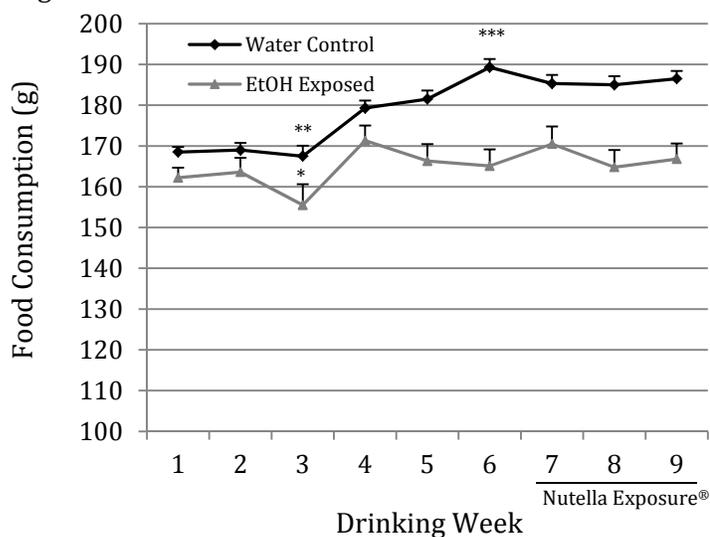
Fig 3.2a. No effects of ORG-34517 or placebo were observed in either EtOH or control animals regarding food consumption. Weekly food consumption did not differ significantly between water control (n=4) and EtOH exposed (n=12) animals at any week. Within EtOH exposed animals, consumption varied only between weeks 3 and 4. In water treated control animals, week 6 consumption levels differed from those observed during week 3 and week 3 levels were elevated from week 1. Average food consumption (g) = 172.0 ±4.65 (*=as compared to week 4; **=as compared to week 1; ***=as compared to week 3, p<0.005)

Weights did not differ significantly between treatment groups prior to experimentation. No significant effects of treatment were found during any of the 9 weeks. A main effect of time was observed ($F(8,112) = 502.8$, $p<0.001$), such that animals gained a significant amount of weight during each week of the experiment (fig 3.2b) (Final weights (g): EtOH= 451.9, ±10.2, Ctrl = 475.5, ±23.7).

Figure 3.2b.
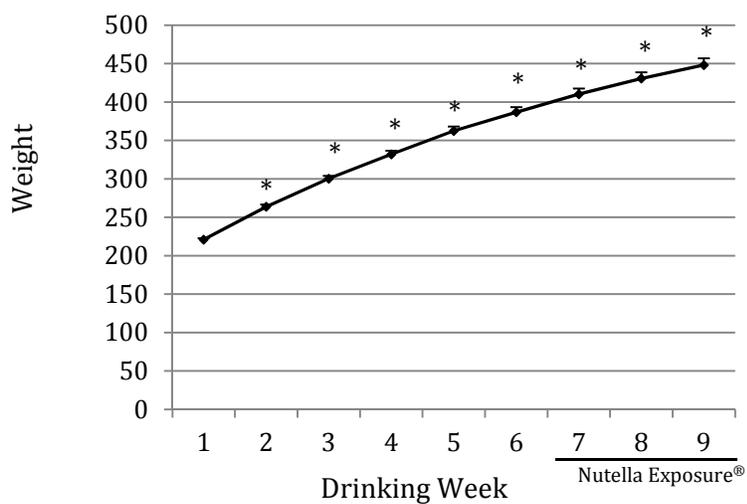
Fig 3.2b. No effects of ORG-34517 or placebo were observed in either EtOH (n=12) or control animals (n=4). No treatment dependent changes in weight gain were found; animals gained a gradual but significant amount of weight over the course of the study. Average weight gain (g)= 239.2 ±9.36. (*= as compared to week 1; $=as compared to week 4; **=as compared to week 6; p<0.005 for all).

Ethanol Consumption and Blood Ethanol Concentration

Total fluid consumption was calculated for EtOH exposed animals by adding total volume of EtOH (ml/kg) to the total volume of water (ml/kg) consumed. This value was compared to total volume of water (ml/kg) consumed by control animals. A significant interaction was found between treatment and drinking week (F(8,112) = 31.2, p<0.01), although no difference in total fluid consumption was found between animals exposed to EtOH and those receiving only water at any week. However, post hoc testing reveal that water treated control animals consumed significantly less fluid in the final 4 weeks (weeks 6-9) as compared to the first 5 weeks, while this decline occurred later in EtOH exposed animals, during weeks 7-9 (fig 3.3).

Figure 3.3.
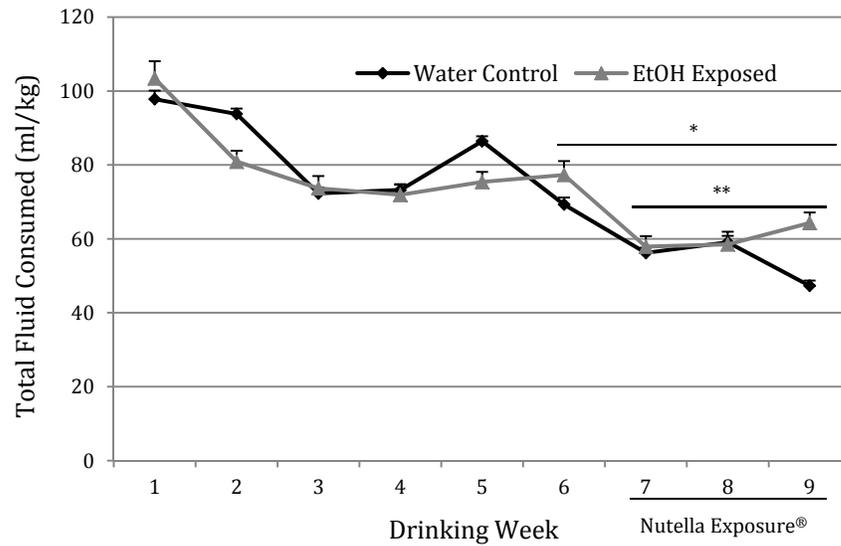
Figure 3.3. Total fluid consumed by water control (n=4) and EtOH exposed (n=12) animals. No significant effect of ORG-34517 or placebo was found, no significant differences were found between water control animals and EtOH exposed animals. Fluid consumption in control animals decreased during week 6, remaining stable thereafter; in EtOH animals, this decrease was evident beginning in week 7, suggesting a possible, though not significant, influence of Nutella® (* = as compared to weeks 1-5, p<.05; **=as compared to weeks 1-6 water controls, p < .05).

Analysis of the consumption pattern within EtOH exposed animals revealed a significant interaction between the type of fluid consumed (EtOH or Water) and time (week) ($F(8, 88)= 13.2$, $p<0.001$). Animals consumed more water than EtOH during all but weeks 3, 5 and 7-9 (fig 3.3a). EtOH consumption remained steady, with a decrease in consumption observed only during week 7 (3.4a). Figure 3.4b depicts the average dose of EtOH (g/kg) animals consumed each week (average weekly dose (g/kg): 3.17, ±0.544). Distribution of the average BEC (mg/dl) is detailed in figure 3.4c and depicts a wide array of BECs, ranging from 4.2-101.0, with the average animal obtaining a BEC of 32.3 ±9.5 (fig 3.4d).

Figure 3.4a.
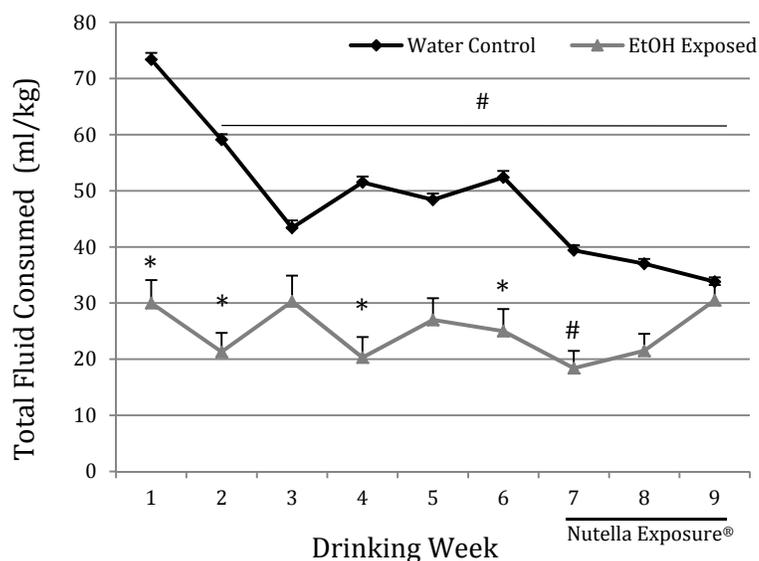
Fig 3.4a. No effects of ORG-34517 or placebo were observed in either EtOH or control animals regarding food consumption. Weekly food consumption did not differ significantly between water control (n=4) and EtOH exposed (n=12) animals at any week. Within EtOH exposed animals, consumption varied only between weeks 3 and 4. In water treated control animals, week 6 consumption levels differed from those observed during week 3 and week 3 levels were elevated from week 1. Average food consumption (g) = 172.0 ±4.65 (*=as compared to week 4; **=as compared to week 1; ***=as compared to week 3, p<0.005)

Figure 3.4b.
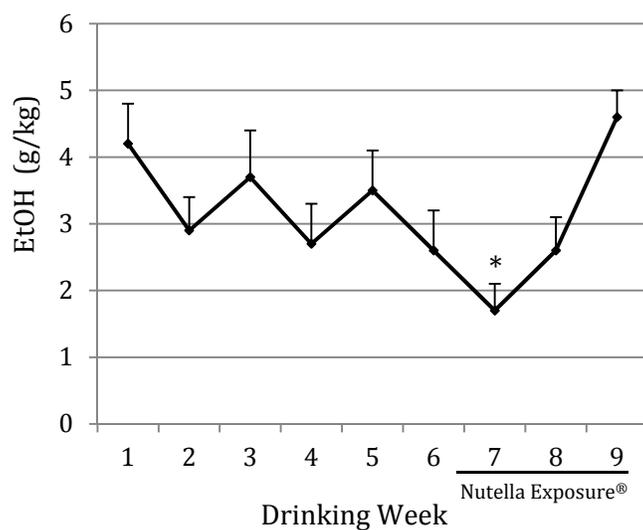
Fig 3.4b. Average weekly dose of EtOH (g/kg) remained steady throughout the experiments (n=12), with the exception of a decrease in consumption occurring during week 7. Average dose (g/kg) = 3.17 ±0.54. (*=as compared to week 1, p < 0.05)

Figure 3.4c.

Distribution of EtOH Consumption
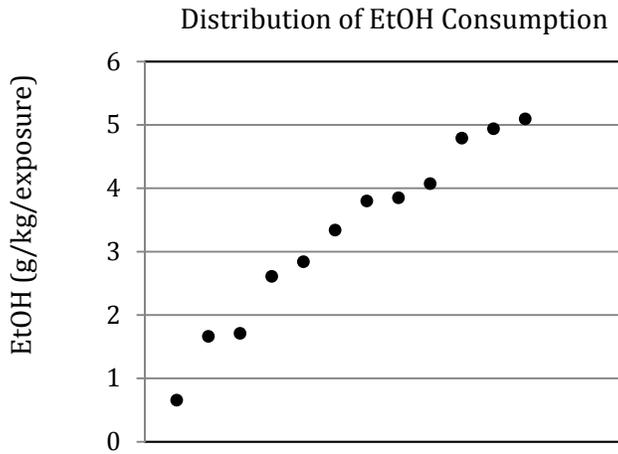
Fig 3.4c. Distribution of the average EtOH dose (g/kg/exposure) (n=12) given intermittent access to 20% EtOH (n=12). IA model produced a wide array of EtOH consumption, ranging from 0.6-5.1 g/kg/exposure, with the average animals self-administering 3.28, ±0.54 g/kg/exposure.

Figure 3.4d.

Distribution of BEC
Following 1 Hr of EtOH Access
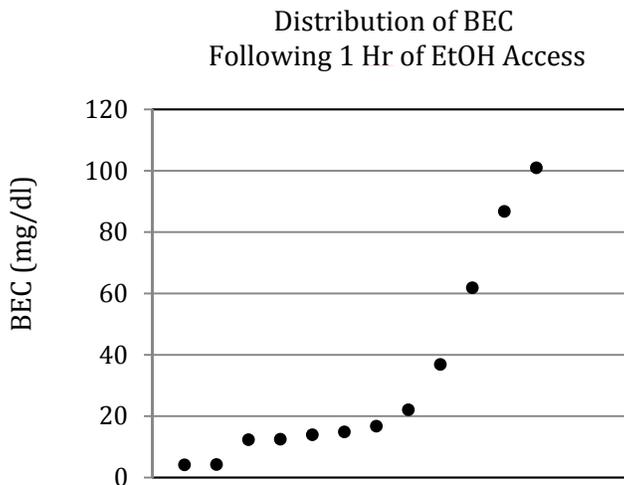
Fig 3.4d. Distribution of the average BEC (mg/dl) (n=12) given intermittent access to 20% EtOH. IA model produced a wide array of BECs, ranging from 4.2-101.0, with of 32.3 ±9.5.

Corticosterone Analysis

Analysis of plasma CORT levels revealed no effect of EtOH treatment, but a main effect of day (F(2, 28)=19.57, p<0.001). CORT plasma levels assessed at T2 (6 days following the withdrawal of EtOH) were significantly elevated in all animals, as compared to those measured at either baseline or following 1Hr of EtOH exposure (fig 3.5a). However, data did trend toward patterns of elevations previously demonstrated by these investigators (table 3.2). Specifically, CORT elevations were greater in EtOH exposed as compared to water treated control animals following 1hr of access to EtOH as well as during protracted EWD, though levels did not reach significance.

Figure 3.5a.
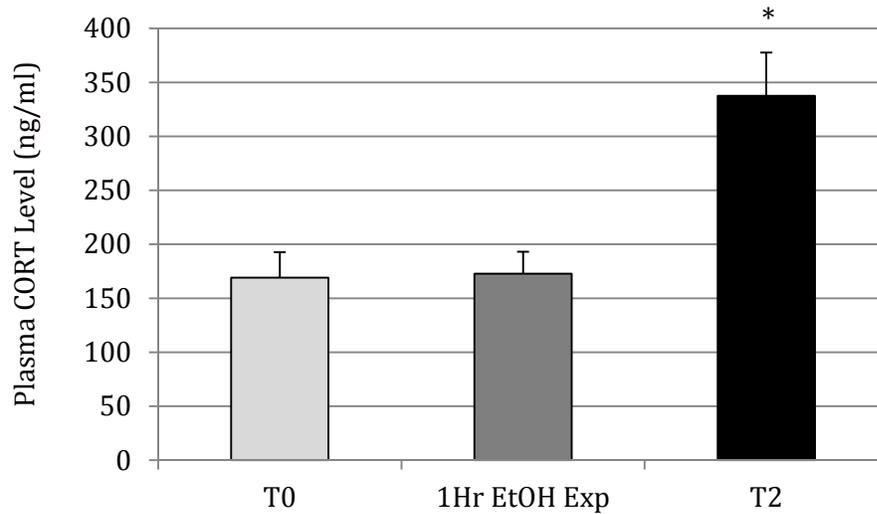
Figure 3.5a. Plasma CORT levels recorded at baseline, 1hr following exposure to 20% EtOH, and T2 (time of sacrifice). No effect of EtOH treatment was found. Plasma CORT levels are significantly elevated in all animals at the final time point, no significant difference was found among EtOH exposed (n=12) and water control animals (n=4). (*= compared to T0 and following 1Hr of EtOH exposure, $p < 0.05$)

Table 3.2
Plasma Corticosterone Levels (ng/ml)

	T0		1Hr EtOH Exposure		T2	
	M	SEM	M	SEM	M	SEM
Water Control (n=4)	164.8	±47.8	130.0	±34.0	290.9	±60.9
EtOH Exposed (n=12)	173.4	±23.3	215.0	±18.6	384.1	±40.5

Correlational analysis regarding EtOH exposed animals revealed significant findings. As previously demonstrated, CORT levels assessed prior to EtOH exposure were positively correlated (r= 0.651, p=0.0219) with subsequent EtOH intake (g/kg) (fig 3.5b). However, contrary to previous findings, neither BEC (3.5c), nor CORT levels obtained during protracted EWD (T2) (3.5d) correlated with those assessed after 1Hr of EtOH exposure.

Figure 3.5b.
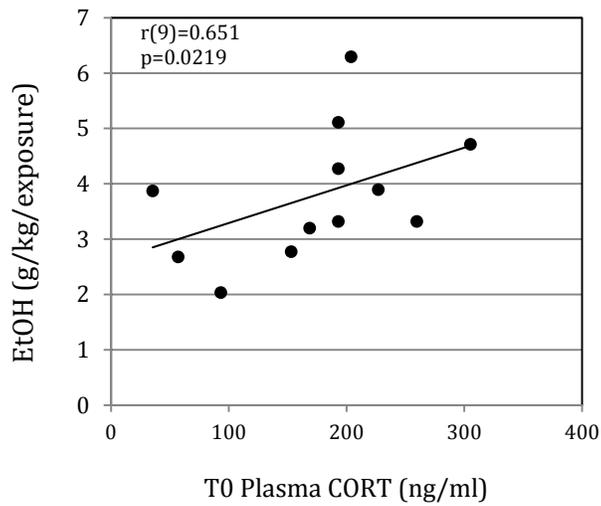
Figure 3.5b. Plasma CORT concentration prior to and following 1Hr of EtOH exposure. Animals with higher levels of plasma CORT prior to EtOH exposure consumed significantly higher levels of 20% EtOH.

Figure 3.5c.
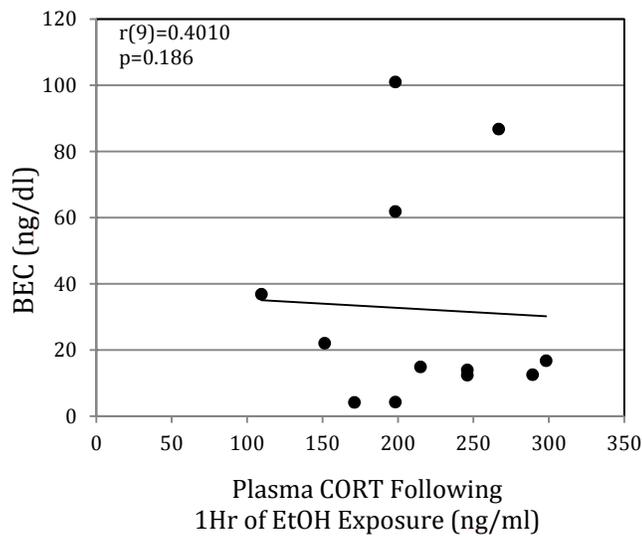
Figure 3.5c. Plasma CORT concentration following 1Hr of exposure to EtOH is not associated with BEC at the same time point.

Figure 3.5d.
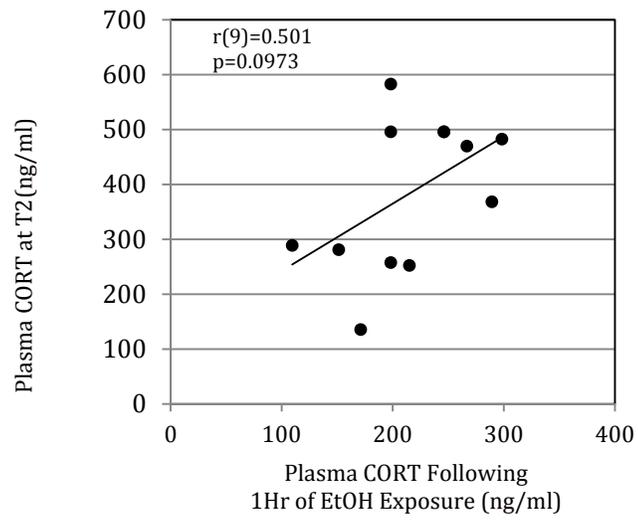
Figure 3.5d. Plasma CORT levels measured at baseline do not significantly correlate with those following 6days of EWD (T2).

Acoustic Startle Reflex

FSR (fig 3.6): Data analysis revealed no effects of treatment, but a main effect of time ($F_{(2,28)}$= 4.5, p = 0.019). Post hoc testing revealed that all animals displayed an elevated FSR at T2, when EtOH treated animals had experienced 26hrs of EWD. Interestingly, and consistent with previous data, EtOH exposed animals (n=12) responded more robustly during this time point than did control animals (n=4), though these differences did not reach significance (table 3.3).

Figure 3.6.
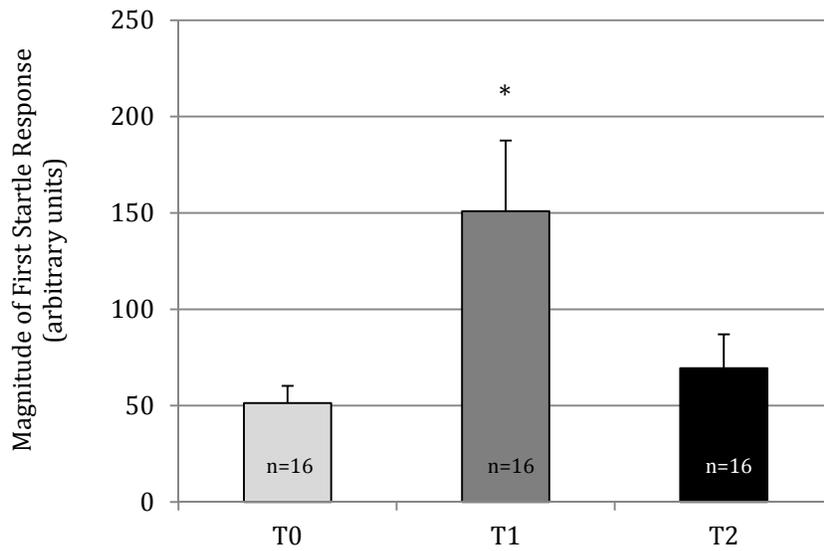
Figure 3.6. Magnitude of the startle response to the first presentation of a 120dB pulse as measured at baseline(T0) and 26hrs (T1)and 5days (T2) following EtOH withdrawal. No significant differences were found regarding EtOH treatment, however, all animals demonstrated elevations in response during acute EWD.. (*= compared to T0, p < 0.05)

Table 3.3
Magnitude of Startle Reflex (arbitrary units)

	T0		T1		T2	
	M	SEM	M	SEM	M	SEM
Water Control (n=4)	57.3	±16.9	112.5	±23.5	72.8	±35.2
EtOH Exposed (n=12)	45.4	±16.9	189.3	±40.6	66.1	±17.5

Habituation: Analysis of habituation data revealed no significant effects regarding either treatment or day.

PPI (fig 3.7): Data were initially analyzed within each level of prepulse for the effects of treatment and time. As no main effect or interaction was found regarding treatment, data were collapsed across the variable and analyzed in a two-way repeated measure ANOVA (level x time (day)). A significant interaction was detected ($F(4,60) = 4.383$, $p = 0.004$. Post hoc testing revealed that both the 79 and 87dB level prepulse was sufficient to significantly reduce inhibition, as compared to baseline values (fig 3.7). Reductions of 38 and 46% were observed, respectively.

Figure 3.7.
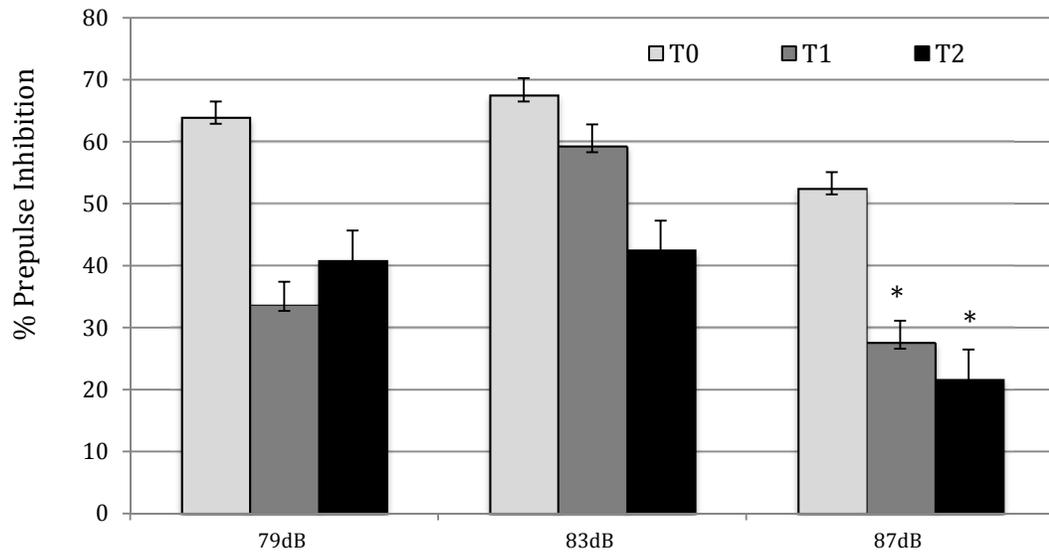
Fig 3.7. Percent of prepulse inhibition (PPI) is the ability of animals (n=16) to inhibit the startle response to a pulse (12dB) when the pulse is preceded by a non-startling level of prepulse. The 79 and 87dB prepulse levels were able to inhibit startle response at T2, as compared to baseline (* = as compared to T0, $p < 0.05$).

Protein Analysis

A one-way ANOVA was used to analyze treatment effects in protein receptor expression of NR2B subunits and GR. Initial immunoblotting experiments using the pNR2B antibody failed to produce measurable results. Communication with Cell Signaling determined that the antibody was ineffective at targeting the protein. Replacement antibody was subsequently delivered and verified to be functional and re-analysis of tissue revealed detectable levels of protein. However, due to a high background signal data were not able to be analyzed. Subsequent experiments will work toward optimizing antibody concentrations. No effect was found regarding EtOH induced changes in NR2B or GR.

Discussion

The most surprising finding in the present study was that ORG-34517 had no significant effect upon EtOH consumption or EWD. The structure of ORG-34517 is similar to that of RU-486 and was therefore expected to have the same properties with regards to EtOH consumption. Further, the efficacy of ORG-34517 was comparable to that of an equal dose of RU-486 at blocking learning and memory, as administration of either drug was as effective at reducing immobility in an inescapable forced swim test (Bilang-Bleuel et al, 2005). As previously explained, the drugs differ largely based upon their pharmacological actions at the PR, with RU-486 but not ORG-34517, acting to antagonize this receptor. In this study 100mg/kg of either drug was administered subcutaneously (s.c.), 15min prior to testing procedures. As route of administration can have a large impact on the bioavailability of a drug, it is possible that both the dose and timing of drug delivery

could be adjusted to accommodate the protocol used in these studies. First, the dosage may need to be elevated, as p.o. administration allows for first-pass metabolism, likely reducing the drug-plasma concentrations (doses as high as 50mg/kg i.p. have been reported (Jaquot et al., 2008). Additionally, as compared to s.c. administration, p.o. delivery results in slower absorption, therefore 30mins may be insufficient time for the drug to reach maximal concentrations in the blood stream. Subsequent studies should include an analysis of the pattern of consumption elicited by the animals, as it is possible that administration of ORG-34517 may shift this pattern. While these studies suggest there are advantages to using an outbred rodent to study EtOH consumption, a selectively bred line may be better suited for ORG-34517dosing studies. Using such a line would greatly reduce EtOH exposure times prior to administration of ORG-34517, as these animals tend to acquire and stabilize elevated levels of consumption quickly. Because the P rat shows similar CORT and EtOH preference as individuals who have a genetic predisposition to developing AUDs, they present as a likely candidate for such studies.

The present study replicated much of the data discussed in the previous chapter; data from both experiments are presented in tables 3.4-3.7.

Table 3.4

Average Food Consumption and Weight Gain (all animals)

		Food (g)		Weight (g)	
		M	SEM	M	SEM
Study 1	(n=32)	160.9	±2.97	231.1	±11.95
Study 2	(n=16)	172.0	±4.65	239.2	± 9.36

Table 3.5

Average Fluid/Ethanol Consumption and BEC (EtOH animals only)

		Water (ml/kg)		EtOH (ml/kg)		EtOH (g/kg)		BEC (mg/dl)	
		M	SEM	M	SEM	M	SEM	M	SEM
Study 1	(n=24)	44.3	±1.90	25.2	±2.94	3.79	±2.94	42.0	±7.10
Study 2	(n=12)	48.7	±1.04	24.9	±3.59	3.28	±0.54	32.3	±9.50

Table 3.6
Corticosterone (ng/ml)

	T0		T1		T2	
	M	SEM	M	SEM	M	SEM
Study 1						
Water Control (n= 8)*	204.8	±20.8	182.0	±20.5	291.4	±38.9
EtOH Treated (n=23)*	171.9	±12.4	174.6	±12.0	369.5	±19.1
Study 2						
Water Control (n= 4)	164.9	±47.8	130.0	±34.0	290.9	±60.9
EtOH Treated (n=12)	173.4	±23.3	215.0	±18.6	384.1	±40.5

(*At T2: Water control n=3; EtOH treated n=12)

Table 3.7
First Startle Reflex (arbitrary units)

	T0		T1		T2	
	M	SEM	M	SEM	M	SEM
Study 1						
Water Control (n=10)	44.7	±3.67	58.1	± 9.93	110.3	±14.02
EtOH Treated (n=21)	42.4	±5.67	158.5	±35.34	141.4	±26.94
Study 2						
Water Control (n= 4)	57.3	±16.9	112.5	±23.5	72.8	±35.2
EtOH Treated (n=12)	45.4	±16.9	189.3	±40.6	66.1	±17.5

General Discussion

This dissertation used a voluntary model of EtOH consumption to examine the relationship between CORT levels and EtOH consumption. Study 1 revealed a positive correlation between baseline CORT and subsequent levels of EtOH consumption. The study also characterized the pattern of consumption associated with IA access to 20% EtOH in the Long-Evans rat and suggests that this pattern is sufficient to produce physical dependence as evidenced by alterations in ASR during EWD. Finally, ASR data suggest the animals used in these studies may not have reached full maturity prior to the first EtOH exposure. Study 2 tested the hypothesis that blocking the GR with the selective antagonist ORG-34517 would result in a reduction in both EtOH consumption and subsequent EWD as measured by ASR. Contrary to the hypothesis, ORG-34517 produced no effects on EtOH consumption or EWD.

Corticosterone

The interaction between CORT and EtOH consumption has been well studied. As a positive reinforcer, the presentation or elevation of CORT can have a positive influence on behavior. Therefore, it is not surprising that exogenous administration of CORT or stimulating the HPA-axis through the presentation of a stressor can elevate EtOH consumption (Becker et al, 2013; Falke et al, 1994a,b; Falke & Hansen, 1995; Hansen et al, 1995). However, fewer studies have investigated how basal levels of CORT could influence consumption. The finding that basal CORT levels are

predictive of subsequent EtOH consumption in the outbred Long-Evans rat is consistent with pre-clinical investigations using selectively bred rat lines. For example, elevations in both basal CORT levels and EtOH consumption are evident in the EtOH preferring P rat as compared to the non-EtOH preferring NP rat (Prasad & Prasad, 1995). While the data presented here are correlational and do not allow us to infer causation, a possible reason that animals with elevated levels of CORT demonstrate increased consumption may be due to variations in the GR protein. Recent investigations have indicated that polymorphisms in the GR are associated with elevated cortisol secretion following the presentation of psychosocial stress (i.e., public speaking) (Wust et al, 2004). Genetic variations in GR are also associated with elevations in basal cortisol levels at bedtime (Rosmond et al, 2000). Additionally, Disrivieres and colleagues (2011) investigated the role of the NR3C1 GR gene in a large population of 14 year olds (n=4534). This group found that variation in this gene was associated with initiation of drinking, as female subjects with the minor allele reported using alcohol repeatedly as compare to non-alcohol using subjects. Together these data suggest a role for the GR in mediating HPA-axis activity and sensitivity, and that this may influence alcohol use. While Subsequent investigations are necessary to further understand the correlation between CORT and EtOH consumption, the current findings may be helpful in the clinical setting, as elevated levels of baseline CORT could aid in identifying individuals who may be at risk for developing AUD.

Intermittent Access Model of EtOH Exposure as a Model for Dependence

To fully investigate the complex problem of alcohol abuse, it is necessary to have numerous methods by which use and dependence can be explored. Such models should include the use of genetically manipulated and selectively bred animals, models of forced EtOH administration, and models of voluntary consumption, in which individual variations can be expressed. In utilizing a voluntary model of EtOH consumption, individual differences in CORT levels, which were predictive of EtOH intake, were detected. This finding would have been masked in a model of forced EtOH exposure. As individual differences are known to influence alcohol consumption and dependence in the human population, having preclinical models in which they can be explored is important to better understand AUD.

Data presented here suggest the IA model could be used to explore the development of dependence in the outbred laboratory rat, despite levels of consumption that may be characterized as modest. Specifically, in study 1 EtOH exposed animals demonstrated elevated FSR during both acute and protracted EWD, as compared to animals receiving only water. While not significant, data collected in study 2 were similar in that FSR was elevated in EtOH exposed animals during the acute phase of EWD. Following IA to 20% EtOH alterations in NMDA-induced currents and changes in phosphorylation of the NR2B subunit have been found in regions of the striatum (Wang et al, 2010). This is important as alterations in these receptor proteins have been implicated in mediating signs of EWD, including anxiety. In fact, short-term exposure to moderate concentrations of EtOH

has been shown to produce measurable changes in anxiety-related EWD behavior (Bonassoli et al, 2011; Cabral et al, 2006; Kayir et al, 2010). Data reported here support these findings and suggest that IA to EtOH may produce measurable signs of EWD, which is indicative of physical dependence. However, a recent study employing the IA model found no elevations in anxiety, as measured by performance on the elevated plus maze (EPM). In this study by Cippitelli et al (2012) EPM performance was assessed following 21 exposures to EtOH (equivalent to that in study 1) and 30hr following the removal of EtOH (FSR assessment began at 26hr in study 1). In comparing the Cippitelli research and the current study, two procedural differences were noted. The current studies used Long-Evans rats that were PND60, while the Cippitelli study used Wistar rats at PND75-79. A direct comparison of consumption levels between the Cippitelli and current study is not possible, as the former does not mention controlling for leakage when calculating the amount of EtOH consumed. However, both studies assessed BEC following 1hr of EtOH exposure. A comparison of these values show BEC levels in study 1 are almost twice those seen the Cippitelli study. In fact, IA exposure in the Cippitelli study resulted in one animal having BEC higher than 40mg/dl (n=8; ~12%), whereas 8 of 24 animals obtained this level in study 2 (~33%). From this comparison it cannot be concluded that that the animals in the Cippitelli study never reached higher BEC levels during EtOH exposure, but it does provided evidence that the pattern of drinking differs between the two studies. This is surprising, as Simms et al (2008) compared consumption and BEC between the two strains using IA access to EtOH and reported no significant differences in either measure. Further, Simms and

colleagues found that, following 30min of EtOH access, 25% of the Long-Evans and 70% of the Wistar rats had BEC above 40mg/dl. In the current study, there is evidence that animals drink rapidly during initial exposure to EtOH and subsequently obtain elevated BEC levels. Evidence of this occurrence is lacking in the Cippitelli study and may account of the lack of EWD anxiety.

ASR data suggest that the animals used in these studies may not have fully matured prior to the introduction of EtOH. Specifically, ASR data collected at baseline revealed very little habituation or inhibition in the presence of the prepulse, a state that is indicative of adolescence (Brunell & Spear, 2006). Research suggests that adolescents experience fewer effects that are associated with the negative regulation of EtOH such as sedation and withdrawal, but are especially sensitive to the neurodegenerative effects that are associated with the development of dependence. Preclinical data support this, as adolescent rats consume more EtOH than adult animals (Brunell and Spear, 2005; Doremus et al., 2005) and animals display greater EWD when exposure begins in adolescence as compare to adulthood (Wills et al, 2009). Regarding the previously discussed Cippitelli study, it is possible that age is the underlying reasons these animals display a different pattern of consumption as compared to both the present study and that conducted by Simms and colleagues. Specifically, in the latter 2 studies, animals were PND60 when EtOH exposure began as compared to the Cippitelli study, in which animals were PND75-79. As more labs are using the IA model, it will be important to determine if age is a crucial factor regarding EtOH consumption and the development of dependence.

Glucocorticoid Receptor Antagonists

It has long been accepted that the environment, genes, and their interactions play defining roles in the addiction process. Indicative of the complex behavioral profile for alcoholism, it is likely that an array of gene combinations are able to confer susceptibility for the disorder, including those involved in the synthesis, secretion, and expression of glucocorticoids and their receptor proteins. Stress can contribute to the escalation of alcoholism and also promote relapse. Preclinical data support the importance of CORT in mediating EtOH consumption and its consequences. For example, Koenig and Olive (2004) induced EtOH consumption in the outbred Long-Evans rat using sucrose fading and water deprivation. The researchers then allowed the animals one hour of access to both water and 10% EtOH. Administration of RU-486 (40mg/kg, i.p.) delivered immediately prior to introduction of EtOH reduced consumption by 40%, while water consumption was unaffected. Recently, Vendruscolo and colleagues (2012) showed that administration of RU-486 (sub-cutaneous pellet) during exposure to EtOH via vapor chamber (21days) prevented escalated responding on a progressive ratio task during acute EWD (24hr). Similar results were reported by Simms et al (2011), as administration of RU-486 (30mg/kg, i.p.) attenuated reinstatement of EtOH responding following the delivery of the pharmacological stressor yohimbine (2mg/kg, i.p.) in the Long-Evans rat. Research regarding the efficacy of RU-486 in treating EWD includes studies using both short- and long-term EtOH exposure. In a short-term model of binge-like EtOH exposure, where EtOH is delivered intra-gastrically (i.g.) three times daily for 4 days, RU-486 (40mg/kg, s.c.) significantly

decreases the severity of behavioral abnormalities (e.g. tremor and "wet dog shakes") 10-12hr following the final dose of EtOH (Sharrett-Field et al, 2013). Following 8 months of exposure to 24% v/v EtOH as the sole source of fluid, Jacquot and colleagues (2008) found that a single dose of RU-486 (50mg/kg, i.p.) reduced handling-induced hyperexcitability in the C57/BL10 strain mice. Further, following assessment of withdrawal symptoms, animals received initial exposure to an object recognition task. Upon retest, those animals *not* receiving RU-486 spent and equal amount of time exploring the novel and familiar objects, which is indicative of memory deficits. This effect was attenuated in animals treated with RU-486. As RU-486 and ORG-34517 have similar chemical structures and both are antagonists of the GR, it was hypothesized that administration of ORG-34517 would both reduce EtOH consumption and attenuate signs of EWD. However, analysis of the data revealed that drug administration had no effect on consumption or EWD. This failure likely arose due to improper scheduling and drug dosage. However, the differences regarding activity at the PR cannot be overlooked. Specifically, RU-486 is a GR/PR antagonist whereas ORG-34517 is a selective GR antagonist, with minimal PR binding even at high doses. Therefore, the possibility that the effects of RU-486 are PR mediated should be considered. In fact, several studies have shown that administration the progesterone metabolite allopregnanalone (ALLO), which is an agonist of the GABA-A receptor, can produce tolerance to the anxiolytic effects of EtOH (Sharma et al, 2007), increase responding for EtOH (Janak et al, 1998; Nie & Janak, 2003), and reduce EWD-induced anxiety (Sharma et al., 2007). It is unclear how blocking the PR with administration of RU-486 affects levels of progesterone in

the brain. However, it is conceivable that RU-486 could decrease EtOH consumption by decreasing levels of progesterone and subsequently, levels of ALLO. Evaluating levels of these neurosteroids in the brain following administration of RU-486 would be helpful in determining their possible role in the attenuation of EtOH consumption.

In sum, these findings add to the growing body of literature that advocates for the use of voluntary intermittent access to EtOH. This model is advantageous in that it reflects real world factors such as voluntary consumption in a genetically heterogeneous population, and allows for individual differences in EtOH usage to manifest. This was demonstrated in the current studies, as individual basal levels of CORT were found to be correlated with subsequent levels of EtOH consumption, a fact that would have gone unobserved in a model of forced exposure. Characteristics regarding the pattern of EtOH consumption produced by IA are demonstrated here and it is suggested that this pattern is capable of producing physical dependence, as measured by ASR. However, ASR data suggest that the age of the animals used in these and several other studies may influence findings regarding consumption patterns, which may subsequently influence the manifestation of EWD. Finally, these studies revealed that baseline CORT levels are predictive of subsequent EtOH consumption, providing further evidence that the hormone and its receptors may be a pharmacological target for the treatment of alcohol abuse disorders.

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Vitae
Lynda Sharrett-Field

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EDUCATION

Degree Awarded 2010	Master of Science in Experimental Psychology, Behavioral Neuroscience and Psychopharmacology
Department of Psychology
University of Kentucky, Lexington, KY
Mentor: Mark A. Prendergast, Ph. D.

Thesis: *Somatic injury precedes distal atrophy following excitotoxic hippocampal insult.*

Degree Awarded 2008	Bachelor of Science in Psychology
Department of Psychology
University of Southern Indiana, Evansville, IN

RESEARCH EXPERIENCE

University of Kentucky	Graduate Research Assistant	2008-Present
University of Southern Indiana Department of Psychology	Laboratory Assistant	2007

TEACHING EXPERIENCE

<u>University of Kentucky</u>

Psychology 312-Brain and Behavior	Fall 2013
Psychology 459-Drugs, Brain, and Behavior	Fall 2013

Psychology 459-Drugs and Behavior.
Co-Instructor with Dr. Mark Prendergast	Fall 2012

Psychology 496: *The Nervous System, Psychotherapeutics.*
Guest lectures	Spring 2012

Psychology 456: *Amino Acids.*
Guest lecture	
	Fall 2011

<u>Eastern Kentucky University</u>

Psychology 311-Brain and Behavior	Spring 2013

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University of Southern Indiana
Psychology 101*: An introduction to memory.*   Spring 2007
Teaching assistant lecture
Psychology 101: *An introduction to learning.*   Fall 2007
Teaching assistant lecture
Supplemental Instructor for Dept. of Academic Skills   Fall/Spring 2006

PUBLICATIONS

1. Butler TR, Berry JN, **Sharrett-Field LJ** & Prendergast MA (2013). Long-term ethanol and corticosterone co-exposre sensitize the hippocampus to insult droning ethanol withdrawal in an NMDA GLUN2B subunit-dependent manner. *Alcoholism: Clinical and Experimental Research.*

2. **Sharrett-Field LJ**, Butler TR, Reynolds AR, Berry JN, Prendergast, MA (2013) Sex differences in neuroadaptation to alcohol and withdrawal toxicity. *Pflügers Archiv European Journal of Physiology.*

3. **Sharrett-Field LJ**, Butler TR, Berry JN, Prendergast, MA (2013) Mifepristone Pretreatment Reduces Ethanol Withdrawal Severity In Vivo. *Alcohol Clin Exp Res.* 2013 Aug;37(8):1417-23

4. Berry, JN, **Sharrett-Field LJ**, Butler TR, Prendergast, MA (2012). Temporal dependence of cysteine protease activation in hippocampus following excitoxic insult. *Neuroscience*; 20;165(2): 525-34.

5. Butler TR, Self RL, Smith KJ, **Sharrett-Field LJ**, Berry JN, Littleton JM, Pauly JR, Mulholland PJ, Prendergast, MA. (2010). Selective vulnerability of hippocampal cornu ammonis 1 pyramidal cells to excitotoxic insult is associated with the expression of polyamine-sensitive N-methyl-D-asparate-type glutamate receptors. *Neuroscience*; 165(2):525-34.

6. Butler TR, Smith KJ, Berry JN, **Sharrett-Field LJ**, Prendergast, MA. (2009). Sex differences in caffeine neurotoxicity are exacerbated by prior EtOH exposure and withdrawal. *Alcohol Alcoholism*, 44(6):567-74.

PROFESSIONAL PRESENTATIONS

Sharrett-Field LJ, Butler TR, Berry JN, Reynolds, AR, Prendergast MA. Short term ethanol exposure induces calpain dependent α-spectrin proteolysis and neurodegeneration in the hippocampus. Poster presentation, Research Society on Alcoholism. SanFrancisco, CA (June 2012).

Sharrett-Field LJ, Butler TR, Berry JN, Prendergast MA. Glucocorticoid antagonist diminishes alcohol withdrawal severity in rats following EtOH exposure using a novel binge paradigm. Poster presentation, Research Society on Alcoholism. Atlanta, GA (2011).

Sharrett-Field LJ, Berry JN**,** Butler TR, Prendergast MA. Somatic injury precedes distal atrophy following excitotoxic hippocampal insult. Poster presentation, Bluegrass Neuroscience. Lexington, KY (2010). *Award Winner.*

Palladino, J., Bloom, C. & **Sharrett-Field, L.** SDIQ-R; Reevaluating popular misconceptions concerning sleep and dreams. Talk presented at the annual meeting of Mid-America Undergraduate Psychology Research Conference at Thomas Moore College (2008).

Bloom, C., Harden, M., Venard, J., Slevin, R., Fritz, C.,& **Sharrett-Field, L.** Obsessive-compulsive symptoms and superstitious conditioning in a non-clinical sample. Poster presentation, American Psychological Society. Washington, D.C. (2007).

SERVICE

Brain Awareness Week Community Outreach Program	2009-2012
Bluegrass Chapter of the Society for Neuroscience	
Community Outreach	2009-2010

AWARDS

Research Society for Alcoholism	Student Merit Travel Award	2011-13
Bluegrass Chapter of the SfN	Outstanding Poster Award	2010
UK Graduate School	Academic Non-Service Fellowship	2008-2009
University of Southern Indiana	Research Potential Award	2009
University of Southern Indiana	Outstanding Psychology Student	2009
University of Southern Indiana	Internship Travel Grant	2008
Palladino/Assante Memorial	Scholarship Award	2007

MEMBERSHIPS

Bluegrass Chapter of the Society for Neuroscience	2009-Current
Research Society on Alcoholism	2009-Current
Society for Neuroscience	2009-2010
Association for Psychological Science	2007-Current
Psi Chi National Honor Society	2006-Current