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Entitled
ALTERATIONS IN THE SEEKING AND SELF-ADMINISTRATION OF ETHANOL AND ANXIETY-LIKE BEHAVIOR FOLLOWING EXPOSURE TO YOHIMBINE IN RATS SELECTIVELY BRED FOR HIGH ALCOHOL INTAKE

For the degree of Doctor of Philosophy

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ALTERATIONS IN THE SEEKING AND SELF-ADMINISTRATION OF ETHANOL AND ANXIETY-LIKE BEHAVIOR FOLLOWING EXPOSURE TO YOHIMBINE IN RATS SELECTIVELY BRED FOR HIGH ALCOHOL INTAKE

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Megan Lee Bertholomey

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

May 2011

Purdue University

Indianapolis, Indiana

ACKNOWLEDGMENTS

I would like to thank my family, friends, and students and faculty in the Psychobiology of Addictions program for their tremendous support and relentless belief in my ability to succeed. Special thanks go to my dissertation committee members, including my two wonderful mentors, for guiding me through this process. Additional thanks go to my partners-in-crime who were always ready to promote or prevent productivity depending upon which was in greater need. You all know who you are.

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ABSTRACT

Bertholomey, Megan Lee. Ph.D., Purdue University, May 2011. Alterations in the Seeking and Self-Administration of Ethanol and Anxiety-Like Behavior Following Exposure to Yohimbine in Rats Selectively Bred for High Alcohol Intake. Major Professor: Nicholas Grahame

Stress has been shown to contribute to alcohol drinking; however, inconsistencies in both the clinical and pre-clinical literature speak to the need for better paradigms to study this interaction. The present experiments compared animal models of the propensity to consume ethanol, the selectively bred alcohol-preferring (P) and high-alcohol-drinking (HAD) rat lines, in their response to yohimbine on ethanol seeking and self-administration and anxiety-like behavior. The P and HAD lines consume similar amounts of ethanol, yet differ in apparent motivation to drink ethanol, in anxiety-like behavior, and response to stress in alcohol drinking. Therefore, it was of interest to determine whether stress may differentially affect ethanol-motivated behaviors between the P and HAD lines. Acute administration of yohimbine, an α-2 adrenoreceptor antagonist that increases anxiety and activate stress systems, increased operant ethanol self-administration and reinstatement of ethanol seeking in P rats, and free-choice ethanol drinking in both P and HAD rats. However, acute yohimbine administration decreased ethanol drinking when given limited access in the home cage, an effect that was diminished by extending the pre-treatment interval or increasing the number of ethanol

exposure sessions. Yohimbine did not alter appetitive responding during a non-reinforced trial, nor did yohimbine alter the acquisition of free-choice ethanol drinking. Exposure to alcohol deprivation resulted in modest increases in ethanol intake, but yohimbine did not potentiate this effect. While acute yohimbine administration increased anxiety-like behavior, prior experience with repeated yohimbine exposures or with repeated deprivation periods did not. P rats were shown to be more active and less anxious and to display greater responding during a non-reinforced trial than HAD rats. Taken together, the results of these experiments demonstrate that the timing of yohimbine exposure relative to ethanol access is a critical component to determining its effects on ethanol seeking and self-administration and anxiety-like behavior. Further investigation into the parameters under which stress alters the motivation to seek and consume ethanol between these selectively bred lines is warranted, and future work that incorporates therapeutic agents aimed at reducing stress reactivity and alcohol drinking could elucidate effective strategies in the treatment of alcoholism.

INTRODUCTION

Stress is a component of everyday life that can impact a number of different behaviors. Anecdotally, it is not uncommon for drinkers to express a desire to consume alcohol to "take the edge off" or relax after experiencing periods of stress. Further, the prevalence of "happy hours" at bars and restaurants speaks to the idea that work-related stress may contribute to alcohol drinking. While the use of alcohol to relieve stress is accepted as a general concept, the extent to which stress contributes to problem drinking has been the focus of numerous empirical studies and several reviews (Breese et al. 2005a; Martin-Fardon et al. 2010; Mason et al. 2009; Piazza and Le Moal 1998; Piazza and Le Moal 1996; Pohorecky 1990; 1991; Silberman et al. 2009; Sinha 2001; Weiss 2005). However, the field of research concerned with the interaction between stress, anxiety, and alcohol is fraught with inconsistencies. While it is generally accepted that stress and anxiety contribute to alcohol drinking, and that alcohol drinking can contribute to stress and anxiety, the type and timing of stress exposure, the stage of alcohol drinking, family history/genetics, age, and gender are but a few of the factors that can add to the variance seen in the stress-alcohol relationship. In order to disentangle the effects of some of these factors – in particular, genetics and the timing of stress exposure relative to alcohol drinking – have on alcohol drinking, the research presented here was undertaken

to determine the effects stress on ethanol seeking and self-administration and anxiety-like behavior in genetic animal models of the propensity to drink alcohol.

Definition of stress and its effects on the nervous system

Although stress is a widely used term, its origin can be traced to Hans Selye who coined the term "stress" in the 1940s to refer to the nonspecific biological reaction to noxious agents (Selye 1984). However, despite its eventual acceptance in scientific nomenclature, there are various connotations of the word. Generally, stress can refer to either the physiological and psychological response to a stressor, or to the stressor itself. A stressor is characterized by a stimulus that causes distress, anxiety, and uneasiness in an organism (Sayette 1999) and is associated with activation of the HPA axis. All organisms experience some degree of stress, which can elicit either beneficial or detrimental effects. For example, the classic Yerkes-Dodson law demonstrates that moderate levels of arousal enhance performance compared to low or high levels (Yerkes and Dodson 1908). Some sensation-seeking individuals also actively engage in stressful activities and find mild stress to be reinforcing (Zuckerman et al. 1984). Rats have been shown to develop a place preference for a location associated with intensive handling (Bozarth 1987), and mice will engage in intracranial self-stimulation into "aversive" brain areas such as the mesencephalic central gray (Cazala et al. 1985). Importantly, stress has also been implicated in the development of substance abuse as drugs of abuse stimulate overlapping brain reward and stress pathways (Piazza and Le Moal 1998).

Activation of central stress pathways in the limbic system leads to the release of monoamine and opioid neurotransmitters that have direct and indirect effects on the

hypothalamic-pituitary-adrenal (HPA) axis (Brady and Sonne 1999). HPA axis activation is integral to the physiological response to stress; indeed, the stress response is often quantified by glucocorticoid levels. The hypothalamus produces corticotropin-releasing hormone/factor (CRH/F), which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland, which in turn activates the release of glucocorticoid hormones from the adrenal glands. The principal glucocorticoid in humans is cortisol, and in rodents is corticosterone. Drugs of abuse also have effects on these hormones; for example, cocaine, morphine, and alcohol all elevate ACTH following acute administration (Ellis 1966; Guaza et al. 1983; Nikodijevic and Maickel 1967; Rivier et al. 1984; Rivier and Vale 1987), which, in turn, stimulates corticosteroid release – yet humans and animals will voluntarily consume these drugs. Interestingly, rats will self-administer corticosterone at doses equivalent to those present following restraint stress (Piazza et al. 1993), and will also self-administer analogues of ACTH (Jouhaneau 1979). These findings provide support for the interaction between stress and reward, but also demonstrate that this relationship is quite complex.

Impact of stress and anxiety in the etiology of alcoholism

The notion that in times of stress, individuals will self-administer alcohol and other drugs to minimize the stress response by altering its affective and physiological concomitants makes intuitive sense. Building upon this generally accepted relationship, several theories have been put forth to understand why stress tends to increase alcohol self-administration. One of the first and most widely recognized of these is the tension-reduction hypothesis (TRH) that posits that alcohol drinking reduces stress, and that in

times of stress individuals will have increased motivation to drink alcohol (Conger 1956). Levenson and colleagues (1980) built upon the TRH by proposing that alcohol consumption can reduce the level of an organism's response to stress in a phenomenon called the stress-response dampening effect (SRD). Later, Sayette (1993) modified the conceptualization of the SRD and put forth the appraisal-disruption model based on findings that the response dampening effect is more likely if alcohol consumption precedes the stressor. Taking a more pathological approach, Khantzian (1985) set forth the self-medication hypothesis, suggesting that those who abuse drugs do so to alleviate the negative affect associated with comorbidity between substance use and other psychiatric disorders, such as anxiety and major depressive disorder. Moving a bit further into the course of drug and alcohol use, Volpicelli's (1987) 'relief' hypothesis, Koob and colleagues' (Koob 2006; 2008; Koob and Le Moal 1997; 2001; Piazza and Le Moal 1996) allostasis model, and Marlatt and Gordon's (1985) relapse-prevention hypothesis all implicate the risk of resumption of problem drinking following stress exposure due to a specific sensitivity to stress that develops during the transition to dependence. While each of these theories have their merits, they also have received criticism and, of course, none alone are able to fully explain how stress modulates alcohol drinking or how alcohol modulates stress responses. Importantly, these models presuppose the use of alcohol as a negative reinforcer, which has two implications: one, other motivational mechanisms by which stress may affect ethanol drinking (e.g., positive reinforcement) are not explored; and two, that alcohol is effective in reducing anxiety.

Some evidence for the anxiolytic effects of ethanol can be found by examining the relationship between alcohol use and anxiety disorders. Significant comorbidity exists

between these classes of disease as evidenced by epidemiological data showing that individuals with alcohol use disorders are 2-3 times more likely to have an anxiety disorders and vice versa compared to the general population (Burns and Teesson 2002; Grant et al. 2007; Kessler et al. 1997; Kushner et al. 2000; Regier et al. 1990; Schuckit et al. 1997; Swendsen et al. 1998). Further, alcoholics with comorbid anxiety disorders drink more ethanol and are more disabled by their drinking than those without comorbidity (Burns et al. 2005). Taking this relationship into consideration, it could be that anxiety leads to alcohol dependence through a self-medication mechanism, that alcohol dependence generates an anxious state, or that both disorders arise from a common underlying vulnerability. Evidence exists in support of these theories (Cosci et al. 2007; Finney and Moos 1991; Grant et al. 2004; King et al. 2003; Kushner et al. 2000; Kushner et al. 1990; Leeies et al. 2010; Roelofs 1985; Schuckit et al. 1990), and importantly, each of these studies support a relationship between anxiety and alcohol drinking. Anxiety is also associated with an increased risk of relapse in detoxified alcoholics (Bradizza et al. 2006; Driessen et al. 2001; Finney and Moos 1991; Kushner et al. 2005; Voltaire-Carlsson et al. 1996; Willinger et al. 2002), indicating that anxiety reduction should be used as a therapeutic target to reduce drinking. In experimental settings, alcohol has been shown to decrease self-reported anxiety in anxious subjects (Chutuape and de Wit 1995; Kushner et al. 1996; Zack et al. 2007). However, alcohol does not consistently reduce anxiety or minimize the effects of stress nor does stress always increase the seeking or self-administration of ethanol (de Wit et al. 2003; Nesic and Duka 2006; Soderpalm and de Wit 2002).

While anxiety has been shown to have a positive relationship with alcohol drinking, it is important to determine to what extent stress is associated with both anxiety and the propensity to consume alcohol. Neurobiological systems regulating stress responses, implicating norepinephrine, CRF, and glucocorticoids in brain structures in the extended amygdala, are also involved in the pathology of anxiety and alcohol use disorders (Koob 2009; Martin et al. 2009; Mathew et al. 2008; McEwen 2007). Considering this overlap in brain stress and anxiety systems, it is not surprising that "clinically anxious patients appear overly sensitive to stressful contexts" (Grillon 2002, p. 964). Evidence of this connection between anxiety and stress is also demonstrated by an increase in salivary cortisol in individuals with anxiety disorders (Vreeburg et al. 2010). However, while numerous studies have been conducted to determine both basal and stress-related corticosterone and CRF levels in individuals diagnosed with anxiety disorders, there is considerable variability in these measures of stress reactivity across the different types of anxiety disorders (e.g., social anxiety disorder, panic disorder, generalized anxiety disorder, and post-traumatic stress disorder; Martin et al. 2009; Mathew et al. 2008). Presumably, this heterogeneity of symptomatology in subtypes of anxiety disorders in clinical populations is not as extensive in rodent models of anxiety-like behavior, which may make interpretations of the stress-anxiety relationship more consistent in animal models. Several inbred strains and selectively bred lines of rodents have been characterized as displaying anxiety-like behavior (Clement et al. 2002). The inbred Lewis (Berton et al. 1997; Windle et al. 1998a), Wistar-Kyoto (Pardon et al. 2003), and Roman (Gentsch et al. 1982; Steimer and Driscoll 2003) strains of rats and the C57/BL6 (van Bogaert et al. 2006) strain of mice all show anxiety-like behavior and

enhanced stress reactivity. The high anxiety behavior (HAB) rat line, selectively bred for anxiety-like behavior in the elevated plus maze, also show elevated levels of stress-related hormones and peptides following stress exposure compared to their low anxiety behavior (LAB) counterparts (Landgraf et al. 1999; Liebsch et al. 1998). It is reasonable to assume, therefore, that enhanced stress reactivity is evident in organisms displaying greater anxiety. Interestingly, each of these inbred strains display a positive relationship between anxiety-like behavior and alcohol drinking (Adams et al. 2002; Li and Lumeng 1984; McClearn and Rodgers 1961; Pare et al. 1999; Satinder 1975; Suzuki et al. 1988; Yaroslavsky and Tejani-Butt 2010), although selectively-bred HAB rats show a negative relationship between anxiety-like behavior and alcohol drinking (Henniger et al. 2002). Nonetheless, there is evidence to suggest that rodents possessing basal anxiety-like behavior are more responsive to stress and in many cases also display elevated alcohol drinking.

Testing the effects of stress on alcohol drinking in alcoholics seeking treatment in an experimental setting raises ethical issues, and as such, studies directly assessing stress-related drinking in alcohol dependent subjects are few. However, chronic alcohol drinking is associated with alterations in the HPA axis, stress-related craving, and stress-related relapse. For example, acute and chronic alcohol intoxication (Adinoff et al. 2003; Mendelson and Stein 1966) as well as alcohol withdrawal (Adinoff et al. 1991; Keedwell et al. 2001; Mendelson et al. 1971) produces robust increases in cortisol. While alcohol dependent individuals show higher levels of ACTH and cortisol, they produce blunted glucocorticoid responses to stress (Bernardy et al. 1996; Errico et al. 1993; Lovallo 2006; Lovallo et al. 2000; Margraf et al. 1967). This alteration in the stress system is associated

with an increased risk of relapse (Junghanns et al. 2003; Junghanns et al. 2005; Walter et al. 2006). Further, abstinent alcohol dependent subjects report greater craving following stress in experimental settings (Cooney et al. 1997; Fox et al. 2007; Miller et al. 1974; Sinha et al. 2009) and stress exposure is significantly associated with relapse (Cooney et al., 1997; Brady et al., 2006; Brown et al., 1990). Taken together, these findings suggest that individuals who have underlying anxiety or alcohol use disorders are more sensitive to the effects of stress on drinking than non-anxious individuals and social drinkers.

Animal models of the interaction between anxiety, stress, and alcohol

The relationship between anxiety, stress, and alcohol has been explored extensively in animals. Despite the inconsistencies in the human literature concerning the effects of alcohol on anxiety, alcohol has been shown to reduce anxiety-like behavior in several animal studies (Blanchard et al. 1993; Durcan and Lister 1988; File et al. 1976; Stewart et al. 1993). Rats demonstrating higher basal levels of anxiety-like behavior have been shown to drink significantly more ethanol than their low-anxiety-expressing counterparts. Further, intraperitoneal (i.p.) injections of ethanol that produced similar blood alcohol concentrations (BACs) as those seen in the drinking component of the study elicited reduced anxiety-like behavior in the elevated plus maze, indicating that the anxious rats were likely drinking alcohol for its anxiolytic properties (Spanagel et al. 1995). Withdrawal from ethanol has also been shown to increase anxiety-like behavior in animals (Baldwin et al. 1991; Doremus et al. 2003; File et al. 1989; File et al. 1991; File et al. 1992; Knapp et al. 2004; Moy et al. 1997; Overstreet et al. 2004; Rassnick et al. 1993; Valdez et al. 2002; Valdez et al. 2004). Thus, the inverse relationship between

alcohol and anxiety evidenced in humans is also present in animal models, and withdrawal-related anxiety may promote further alcohol use.

Given the importance of underlying risk factors discussed above in the impact stress has on ethanol drinking, animal models of the stress-alcohol interaction that take these factors into consideration should provide the most fruitful data. While some studies have shown stress to increase ethanol drinking in continuously drinking, non-dependent outbred rodents (Anisman and Waller 1974; Bond 1978; Caplan and Puglisi 1986; Casey 1960; Croft et al. 2005; Mills and Bean 1978; Mills et al. 1977; Nash and Maickel 1985; 1988; Powell et al. 1966; Rockman et al. 1987; Roman et al. 2005; Von Wright et al. 1971) others have failed to find this effect (Champagne and Kirouac 1987; Kinney and Schmidt 1979; Mills and Bean 1978; Sprague and Maickel 1994; Weisinger et al. 1989). When given limited, binge-like (>80 mg% in <2 hours; NIAAA 2004) ethanol access, ethanol intake was increased following restraint stress (Chester et al. 2006; Lynch et al. 1999), and yohimbine (Le et al. 2009; Le et al. 1998), but not following social defeat stress (van Erp and Miczek 2001), indicating that type of stress used is important in determining its effect on ethanol drinking. In studies using male rodents selectively bred for alcohol preference and inbred strains that show high ethanol intake, stress tends to increase ethanol consumption (Breese et al. 2004; Chester et al. 2008; Chester et al. 2004b; Chester et al. 2006; Croft et al. 2005; Matthews et al. 2008; Overstreet et al. 2007; Roman et al. 2003). However, high alcohol intake is not necessarily related to increased post-stress drinking as other studies have shown that stress tends to reduce ethanol consumption in outbred rats expressing a preference for alcohol (Bond 1978; Rockman et al. 1986; Rockman et al. 1987; Volpicelli et al. 1990). On the other hand, rats that have

been exposed to intermittent ethanol vapor exposure (Sommer et al. 2008) or have undergone cycles of deprivation from voluntary alcohol drinking (Breese et al. 2004; Funk et al. 2004; Overstreet et al. 2007; Vengeliene et al. 2003) typically display stress-related increases in ethanol intake, although this effect is not always found (Bertholomey et al. 2011; Dayas et al. 2004). It is possible, therefore, that sensitivity to stress-related increased in ethanol intake is greater in rodents that have a genetic susceptibility to consume alcohol or those that have undergone repeated cycles of ethanol exposure and abstinence.

In addition to stimulating self-administration of ethanol, stress is also able to elicit reinstatement of ethanol seeking. Following extinction of operant responding for ethanol, exposure to footshock or yohimbine stress reliably increases responding on the lever previously associated with ethanol availability despite not receiving ethanol access (Cippitelli et al. 2010; Le et al. 2009; Le et al. 2005; Le et al. 2000; Le et al. 1999; Le et al. 1998; Marinelli et al. 2007; Richards et al. 2009; Richards et al. 2008; Simms et al. 2010). However, stress type may also be important, as social defeat suppresses ethanol consumption (van Erp and Miczek 2001), and fails to elicit reinstatement of ethanol seeking (Funk et al. 2005). Interestingly, cues associated with social defeat (Funk et al. 2005) and footshock (Liu and Weiss 2003) have been shown to reinstate ethanol seeking. To the extent that reinstatement procedures model craving and risk of relapse (Koob 2000; Le and Shaham 2002; Li 2000; Mason et al. 2009; Rodd et al. 2004b; Shaham et al. 2003; Weiss 2005), these findings in animals are consistent with the notion that stress increases craving, alcohol drinking, and relapse in humans.

Changes in ethanol reinforcement and vulnerability to stress as a function of ethanol drinking history

Each stage in the progression of drinking could potentially represent a sensitive period during which external factors such as stress may influence further drinking. As reviewed above, stress has been shown to promote the initiation and maintenance of alcohol drinking and to contribute to relapse. Theories of addiction have posited that both positive and negative reinforcement can contribute to the escalation of drug and alcohol use (Koob and Le Moal 1997; 2001; 2008; Robinson and Berridge 1993; Verheul et al. 1999). While both reward and relief may account for stress-related craving (Grusser et al. 2007; Heinz et al. 2003), several lines of research converge on the notion that negative reinforcement is highly implicated in mediating stress-related alcohol drinking due to its tension- and anxiety-reducing effects (Brown et al. 1990; Brown et al. 1995; Cooper et al. 1992; Kushner et al. 1994). Since withdrawal from ethanol has been shown to promote anxiety in both humans (Hershon 1973; Roelofs 1985) and in animal models (Baldwin et al. 1991; Becker 2000; Doremus et al. 2003; File et al. 1989; File et al. 1991; File et al. 1992; Holter et al. 1998; Knapp et al. 2004; Moy et al. 1997; Rassnick et al. 1993), it is possible that negative reinforcement mediates stress-related increases in ethanol seeking and self-administration following periods of abstinence.

Changes in ethanol reinforcement as a function of ethanol exposure can be assessed in several ways. First, as problem drinking is typically first identified by binge drinking (Bonomo et al. 2004; Chassin et al. 2002; Hill et al. 2000; Pitkanen et al. 2005), animal models that result in binge-like ethanol self-administration are useful. Since the definition of a binge is reaching 80 mg% within two hours (NIAAA 2004) free-choice

home cage and operant self-administration procedures in which animals consume similar amounts of ethanol can be used to assess modulations in ethanol reinforcement. One particularly useful method for assessing binge-like operant self-administration is the sipper tube model (Samson et al. 2000; Samson et al. 1999; Samson et al. 1998) that procedurally separates ethanol seeking from ethanol consumption by imposing a response requirement that the animal must emit before gaining 20-minute access to ethanol. Using this procedure, the operant behavior is not impinged upon by the pharmacological effects of the reinforcer. Further, if a non-reinforced extinction trial is imposed, it is possible to assess appetitive aspects of ethanol reinforcement without ethanol "on board" (Samson and Chappell 2001; Samson et al. 2001). In a similar fashion, reinstatement procedures that use priming doses of ethanol, cues previously associated with ethanol, or stress to stimulate previously extinguished responding for ethanol can be used to assess ethanol reinforcement (Shaham et al. 2003). Transitioning from binge-like ethanol exposure, chronic self-administration and relapse paradigms are also useful in modeling the cyclic pattern of drinking often seen in human alcoholics. When ethanol is returned following a period of abstinence, a transient increase in ethanol consumption is often seen (Sinclair and Senter 1967). This pattern of drinking is known as the alcohol deprivation effect (ADE) and has been seen in several species (Burish et al. 1981; McKinzie et al. 1998b; Salimov and Salimova 1993). The ADE is enhanced following repeated deprivations in terms of quantity (Holter et al. 2000; Rodd-Henricks et al. 2000a; Spanagel and Holter 1999) and duration (Rodd et al. 2003; Rodd-Henricks et al. 2000b) of drinking. Importantly, appetitive responding in the sipper tube model, reinstatement of ethanol seeking, and the alcohol deprivation effect have all been described as models of craving

(Epstein et al. 2006; Heyser et al. 1997; Koob 2000; Le and Shaham 2002; Li 2000; Mason et al. 2009; Rodd et al. 2004b; Samson et al. 2001; Shaham et al. 2003; Weiss 2005). As such, application of stressors during these specific time points in the course of ethanol drinking history could elucidate the impact stress has on the escalation of ethanol use and also ascertain differences in the reinforcing properties of ethanol as a function of stress.

Yohimbine as a stressor

It is clear that stress type plays a critical role in its impact on altering behavior. The principal types of stressors used in preclinical research are physiological in nature, such as shock, restraint, social defeat, and maternal separation. Each of these stressors has been shown to alter anxiety-like behavior (Breese et al. 2005b; Huot et al. 2001; Kalinichev et al. 2002; Kinsey et al. 2007; Pare and Glavin 1986; Rodgers and Cole 1993; van Dijken et al. 1992) and sympathetic nervous system activation (Handa et al. 1994; Kant et al. 1983; Keim and Sigg 1976; Martinez et al. 1998; Pitman et al. 1988; Sgoifo et al. 1996). However, use of these types of stressors can be problematic in several ways. First, there is a great degree of variability between subjects in the experience of a stressor; individual differences in any number of factors, such as size, sensitivity in sensory systems, affective traits, could potentially mediate the stress response. Second, there is limited face validity in these types of stressors. For example, the types of stressors that contribute to escalated alcohol use in humans tend to be social, occupational, and legal in nature rather than physical, and often are related to alcohol use itself (Brown et al. 1990; Dawson et al. 2005). Stress models that involve social interaction (e.g., social defeat) and

those that implicate the negative consequences of alcohol use (e.g., punishment tests) should therefore provide greater insight into the effects of stress on alcohol intake. However, social defeat stress tends to decrease ethanol drinking (Funk et al. 2005; van Erp and Miczek 2001), and conflict tests have not examined punished drinking with ethanol as the reinforcer. Further, these stressors still require some level of physical threat.

Because of the potential drawbacks to using physical stressors, there has been a renewed interest in using pharmacological stressors. A benefit to the use of a pharmacological stressor is that the dose can be systematically varied and is able to target more "downstream" processes in HPA axis activation, circumventing some of the variability that may be seen in a physical stressor and potentially minimizing the impact of individual differences. In addition, pharmacological stressors can be assessed for predictive/face validity. One such example is yohimbine, an α-2 adrenoreceptor antagonist that increases norepinephrine release in several brain areas including the amygdala, a brain site associated with fear and anxiety (Tanaka et al. 2000). Yohimbine elevates glucocorticoid levels in both rats (Marinelli et al. 2007; Suemaru et al. 1989) and humans (Gurguis et al. 1997; Mattila et al. 1988; Vythilingam et al. 2000), which is an identifying feature of the stress response. In addition, yohimbine elicits self-reports of anxiety in humans (Charney et al. 1989; McDougle et al. 1995) and anxiety-like behavior in rats (Guy and Gardner 1985; Johnston and File 1989; Pellow et al. 1985a), rendering this stressor more translatable between rats and humans and high in predictive validity. Given the anxiogenic and HPA-activating profile of yohimbine, researchers began characterizing it as a pharmacological stressor (Bremner et al. 1996a; b) and using it to elicit reinstatement to drug seeking (Lee et al. 2004; Shepard et al. 2004).

In addition to its effects on anxiety, yohimbine has also been shown to interact with alcohol. For example, yohimbine blocks ethanol-induced inhibition of locus coeruleus neurons in vitro at physiologically relevant ethanol concentrations (100 nM), suggesting an interaction between these two chemicals at the molecular level (Verbanck et al. 1991). Further evidence for this interaction at the molar level is shown in a study by McDougle et al. (1995), which found that ethanol and yohimbine had additive effects on subjective reports of anxiety, intoxication, and cortisol levels in humans. However, while yohimbine was found to increase anxiety and cortisol levels in recently detoxified alcoholics, yohimbine failed to substitute for ethanol using subjective ratings, indicating that the discriminative stimulus effects of yohimbine are different from those of alcohol (Krystal et al. 1994). Yohimbine has also been shown to reinstate alcohol seeking in rats (Cippitelli et al. 2010; Le et al. 2009; Le et al. 2005; Marinelli et al. 2007; Richards et al. 2009; Richards et al. 2008; Simms et al. 2010) and induces the expression of c-fos and CRF mRNA in limbic brain areas associated with reward to a similar degree to footshock stress (Funk et al. 2006). While the effects of yohimbine on reinstatement of ethanol seeking have been replicated in several studies, the effects of yohimbine on alcohol drinking are scant. Yohimbine significantly increased operant self-administration of alcohol, and these effects were not due to general increases in locomotor activity as responding on the inactive lever was unchanged (Le et al. 2009; Le et al. 2005). Yohimbine was able to block clonidine-induced reductions in ethanol intake in alko-alcohol (AA) rats when given 12-hour limited access to 10% ethanol (Opitz 1990). However, to our knowledge, no studies directly assessing the effects of yohimbine alone on voluntary, free-choice ethanol intake have been undertaken. Since yohimbine is

capable of producing stress and anxiety responses in both rats and humans, and given that yohimbine interacts with systems regulating alcohol's effects, yohimbine may prove to be an effective tool in determining the effects of stress on appetitive and consummatory aspects of ethanol self-administration.

Lines of rats selectively bred for high ethanol intake

Selective breeding for divergent voluntary ethanol intake has proven to be a valuable tool in understanding genetic contributions to behavior associated with alcohol consumption. One widely used animal model of the propensity to drink alcohol is the alcohol-preferring P rat, selected from a heterogeneous stock of Wistar rats for consumption of over 5.0 g/kg/day of 10% v/v ethanol when given continuous, free-choice access (Lumeng et al. 1977). In addition to expressing high, pharmacologically relevant levels of daily ethanol intake (Bell et al. 2006; Murphy et al. 1986), P rats also prefer ethanol to water and palatable sweet solutions (Lankford et al. 1991), demonstrate a willingness to work for ethanol (Czachowski and Samson 2002; Murphy et al. 1989), develop tolerance (Gatto et al. 1987a; b) and dependence under free-choice drinking conditions (Kampov-Polevoy et al. 2000; Waller et al. 1982), will self-administer ethanol via non-oral routes, e.g., into the ventral tegmental area of the brain and intragastrically (Gatto et al. 1994; Waller et al. 1984), and find alcohol to be less aversive than the non-preferring NP line in conditioned place preference test (Stewart et al. 1996) and conditioned taste aversion tests (Froehlich et al. 1988). Taken together, these behaviors indicate that the P rat satisfies the proposed criteria of an animal model of alcoholism (Cicero 1979; Lester and Freed 1973).

The replicate high-alcohol-drinking HAD-1 and HAD-2 lines of rats were selected using the same criteria as the P rats but were generated from a foundation stock of N/Nih rats, which themselves were developed by crossing eight inbred rat strains (Li et al. 1993; Spuhler and Deitrich 1984). While less characterized than P rats, HAD rats show many of the same phenotypes shared by the other alcohol-preferring line (for a review, see Murphy et al. 2002). However, the HAD replicate lines differ in some important aspects from P rats. First, while P rats prefer ethanol to other palatable solutions (Lankford et al. 1991), this effect is not present in HAD rats (Lankford and Myers 1994). In addition to their elastic ethanol preference (as evidenced by the ability of alternative palatable reinforcers to diminish ethanol drinking), HAD rats also appear to have lower motivation to self-administer alcohol. For example, while P rats robustly express the alcohol deprivation effect (ADE), a proposed model of craving (Heyser et al. 1997; Sinclair and Li 1989; Spanagel and Zieglgansberger 1997) after a single deprivation period (McKinzie et al. 1998b; Sinclair and Li 1989), HAD rats fail to show an ADE unless multiple deprivation periods are imposed (Oster et al. 2006; Rodd-Henricks et al. 2000a). In addition, using the sipper tube model, P rats demonstrated greater appetitive responding for both ethanol and sucrose in both progressive ratio and extinction sessions, while HAD rats showed comparatively lower levels of responding similar to those seen in unselected Long-Evans rats (Czachowski and Samson 2002). Reduced motivated responding by HAD rats compared to P rats was previously found in a study by Files et al. (1998) wherein HAD-1 and -2 rats failed to adjust responding for requirements exceeding an FR8, while P rats maintained responding up to an FR32. Taken together, it appears as though P rats show greater motivation to seek and consume

ethanol than HAD rats. Thus, it is of interest to determine whether exposure to stress might differentially alter these behaviors in these two selectively bred lines.

Important to the present investigation is the notion that selective breeding for a specific phenotype such as high alcohol drinking will fix trait-relevant alleles. As such, if anxiety and stress reactivity are associated with alcohol drinking, selection for high ethanol intake may also select for high anxiety-like behavior. Consistent with this, P rats show a greater acoustic startle response (Chester et al. 2003; 2004a; McKinzie et al. 2000), enhanced footshock-induced suppression of operant responding, greater latency in a passive avoidance test (Stewart et al. 1993), and decreased time spent in the open arms of the elevated plus maze (Pandey et al. 2005; Stewart et al. 1993), all of which point to an anxious phenotype. However, increased anxiety-like behavior in P rats compared to NP or outbred rats was not found in other studies (Overstreet et al. 1997; Roman et al. 2011; Viglinskaya et al. 1995). To the extent that anxiety-like behavior may be related to stress reactivity, P rats show more acute elevated post-restraint stress-induced alcohol drinking (Chester et al. 2004b) than NP rats. While P rats show greater anxiety-like behavior than NP rats, the same pattern in not seen in HAD and LAD rats. For example, HAD-1 rats showed an attenuated acoustic startle response compared to low-alcohol-drinking LAD-1 rats (Chester et al. 2003; 2004a), while HAD- and LAD-2 rats did not differ in acoustic startle response (Chester et al. 2003). In addition, HAD and LAD rats do not differ in anxiety-like behavior when tested in the elevated plus maze (Badia-Elder et al. 2003; Hwang et al. 2004). Finally, HAD rats have been shown to exhibit post-stress increases in ethanol drinking after footshock stress (Vengeliene et al. 2003), but not restraint stress (Chester et al. 2004b). Adding support for these behavioral differences

between the selected lines, analysis of amygdalar CRF content showed that while P rats have greater levels of CRF mRNA/immunoreactivity compared to NP rats, this difference was not evident when comparing HAD and LAD rats (Hwang et al. 2004). Taken together, P and NP rats show greater differences in behavior than HAD and LAD rats, and P rats appear to be more anxious and sensitive to stress than HAD rats. However, few studies have directly assessed behavioral differences between P and HAD rats (Overstreet et al. 1997; Roman et al. 2011). Thus, it is of interest to compare P and HAD rats within the same paradigm to determine if anxiety-like phenotypes are associated with alcohol consumption in these lines.

Study aims and predicted results

The present study sought to determine the role of stress in altering ethanol seeking and self-administration in rats selectively bred for high alcohol drinking. Specifically, it was of interest to determine (1) whether yohimbine would alter the acquisition and maintenance of drinking in a continuous access paradigm and deprivation-related drinking in an intermittent access paradigm; (2) whether yohimbine and/or ethanol deprivation would alter the motivation to seek and consume ethanol in a binge-drinking procedure; (3) whether yohimbine, binge-like ethanol drinking, and/or ethanol deprivation would alter anxiety-like behavior; and (4) whether P and HAD rats differ in yohimbine-related alterations in ethanol seeking and self-administration and anxiety-like behavior. In order to achieve this goal, the following five experiments were undertaken. In Experiment 1, the effects of three doses of yohimbine on binge-like operant self-administration were examined, as well as the effects of an established dose of yohimbine

on the reinstatement of ethanol seeking in alcohol-preferring rats. Experiment 2 explored the effects of yohimbine at different pretreatment intervals on limited access ethanol and water drinking, and the effects of yohimbine and ethanol (separately or in combination) on anxiety-like behavior in P and HAD rats. Experiment 3 sought to determine the effects of yohimbine treatment before and during the first two weeks of ethanol drinking on the acquisition of ethanol intake, while Experiment 4 sought to determine the effects of yohimbine on the maintenance of chronic ethanol drinking and on deprivation-related drinking, as well as changes in anxiety-like behavior as a function of ethanol drinking and stress history in P and HAD rats. Finally, Experiment 5 assessed yohimbine- and alcohol deprivation-related changes in ethanol seeking and operant self-administration in P and HAD rats. In these experiments it was predicted that (1) yohimbine stress would enhance the acquisition and maintenance of drinking in a continuous access paradigm and potentiate deprivation-related drinking in an intermittent access paradigm; (2) yohimbine and ethanol deprivation would increase the motivation to seek and consume ethanol in a binge-drinking procedure, with the possibility of an interaction between yohimbine and ethanol deprivation; (3) yohimbine and ethanol deprivation would increase anxiety-like behavior, while binge-like ethanol drinking would decrease anxiety-like behavior and counteract the effects of yohimbine; and (4) P rats would show greater anxiety-like behavior, a more robust ADE, and greater yohimbine-related increases in ethanol self-administration.

EXPERIMENTS 1-5: METHODS, RESULTS, DISCUSSION

General Methods

Subjects

Male P (S68-69), HAD-1 (S56), and HAD-2 (S54) rats aged approximately 6 weeks upon arrival were used in the following experiments. Rats were singly housed in plastic shoebox cages in a temperature- and humidity-controlled vivarium maintained on either a reversed light/dark cycle (lights off at 10:00 am, Experiments 2-4) or a regular light/dark cycle (lights on at 7:00 am, Experiments 1 and 5). Water and food were available ad lib throughout the experiment except where noted.

Drugs

Yohimbine HCl (Sigma) was prepared with sterile water at doses of 0.625, 1.25, or 2.5 mg/kg body weight in a 1.0 mg/ml injection volume and was administered i.p. Ethanol solutions were prepared using 95% ethanol diluted with water to a 10% v/v concentration.

Elevated Plus Maze

The elevated plus maze (EPM) consisted of two open arms (50 x10 cm) and two closed arms (50 x 10 x 50 cm) situated at right angles to each other elevated 90 cm off of the ground. EPM testing was conducted in a separate room under low illumination and behavior was recorded using a video camera. During this 5-minute session, number of entries into and amount of time spent in the center, closed arms, and open arms, as well as number of fecal boli were measured.

Operant Self-Administration

Operant sessions were conducted in Med-Associates chambers (St. Albans, VT, USA; 30 x 30 x 24.5 cm) equipped with a houselight, retractable levers, and a retractable sipper tube. The sipper tube was a graduated cylinder tube fitted with a rubber stopper and a stainless steel spout with double ball bearings to prevent leakage. The levers were located on the wall opposite to the sipper tube. Operant chambers were housed in sound-attenuated cubicles equipped with exhaust fans to mask external noise. Electrical inputs and outputs of each chamber were controlled using Med-Associates software (Med-Associates). During the initial operant training sessions rats were water restricted in their home cages and were shaped in 1-hour operant sessions on an FR1 schedule for 60-second access to a sipper tube containing a 10% sucrose solution. Following this, the session length was reduced to 30 minutes and the sipper access time was reduced to 30, then 15 seconds. Prior to each session, the house light was off and the levers and sipper tube were retracted in the operant chamber until the session began. To acclimate rats to the ethanol solution, a modified sucrose-fading procedure was used (Samson 1986). Rats

received 1-2 sessions on an FR1 schedule at each of the following concentrations: 10% sucrose/2% ethanol; 10% sucrose/5% ethanol; 10% sucrose/10% ethanol; 5% sucrose/10% ethanol; 2% sucrose/10% ethanol; and finally 10% ethanol. The response requirement increased on successive sessions from an FR1 to an FR4. Next, a response requirement (RR) was implemented in which a number of responses were required to gain access to the sipper tube for 20 minutes. This requirement increased from an RR4 to an RR10. The entire training procedure took 3-4 weeks to complete.

Statistical Analyses

In Experiment 1, oneway ANOVAs with yohimbine dose (0, 0.625, 1.25, or 2.5 mg/kg) as the between-subjects factor were performed for ethanol intake as well as active and inactive lever presses. Paired-samples t-tests compared lever pressing during extinction and reinstatement, and independent-samples t-tests compared lever pressing between rats injected with yohimbine versus vehicle. In Experiment 2, separate mixed factorial ANOVAs were performed for water and for ethanol intake during baseline or during the injection period, with line (P vs. HAD-1) and yohimbine dose (vehicle vs. 1.25 mg/kg yohimbine) as between-subjects factors, and day as the within-subjects factor. Behaviors in the EPM (open/closed/center/total arm entries; open/closed/center time) were analyzed using factorial MANOVAs with the same factors listed for the drinking data above. In Experiment 3, mixed factorial ANOVAs with line (P vs. HAD-2) and treatment condition (pretreatment, acquisition, control) as between-subjects factors and day as the within-subjects factor were performed for fluid intake. In Experiment 4, mixed factorial ANOVAs were performed for water and for ethanol intake, with line (P vs.

HAD-2), yohimbine dose (vehicle vs. 1.25 mg/kg yohimbine), and ethanol access pattern (continuous vs. intermittent) as between-subjects factors, and averaged weekly intake for each cycle or each day as the within-subjects factor. Behaviors in the EPM (open/closed/center/total arm entries; open/closed/center time) were analyzed using factorial MANOVAs with the same between-subjects factors as above. Ethanol intake on the yohimbine challenge day was subjected to a factorial ANOVA, with line (P vs. HAD-2) and treatment condition (control, pretreatment, acquisition, continuous plus yohimbine, deprivation plus yohimbine, and deprivation plus vehicle) as between-subjects factors. In Experiment 5, mixed factorial ANOVAs were performed for ethanol intake, licks, and lever presses during extinction, with line (P vs. HAD-2), yohimbine dose (vehicle vs. 1.25 mg/kg yohimbine), and ethanol access pattern (continuous vs. intermittent) as between-subjects factors, and deprivation period as the within-subjects factor. Since rats were tested in several identically-treated cohorts, cohort was added as a covariate in all analyses. Fisher's LSD pairwise comparisons were made where appropriate. The significance level was set at $p < 0.05$ in all experiments.

Experiment 1: Effects of yohimbine on ethanol self-administration and reinstatement of ethanol seeking

Methods

A total of 24 male P rats were used in the present experiment. Rats were given a week to acclimate to the vivarium during which time they were weighed, handled, and habituated to transport. Operant sessions were run five days a week during the light cycle.

After one week of RR training, a second, inactive lever was introduced upon which responses were recorded but elicited no programmed consequences. Rats were then given a one-week baseline drinking period before beginning injections. All rats were injected with either vehicle or one of three doses of yohimbine (0.625, 1.25, or 2.5 mg/kg) 15 minutes prior to being placed in operant chambers on test days. Each dose was given a week apart in a balanced design. Samples for blood ethanol concentration analysis were taken following the final treatment session. Rats were then given two weeks of non-injection reinforced sessions before extinction sessions began. During extinction sessions, rats were able to emit operant responses but did not receive access to ethanol. The extinction criterion was set at <10 active lever presses for at least two consecutive sessions, which was met by all rats by 14 extinction sessions. On the 15th session (reinstatement), rats were injected with 1.25 mg/kg of yohimbine prior to a non-reinforced session.

Results

Dose-Response

Three rats did not reach the response requirement after injection of vehicle (n = 1) or 2.5 mg/kg yohimbine (n = 2) and therefore were excluded from the analysis. The final sample size was n = 21. Repeated-measures ANOVAs with yohimbine dose (vehicle, 0.625, 1.25, or 2.5 mg/kg yohimbine) as the between-subjects factor revealed a significant main effect of dose for both ethanol intake [$F(3,60) = 7.63$, $p < 0.001$] and licks [$F(3,60) = 7.75$, $p < 0.001$] on injection days (Figure 1a). Pairwise comparisons

revealed that rats exhibited higher ethanol intake after being injected with the 0.625 (M = 0.81 g/kg) and the 1.25 (M = 0.90 g/kg) mg/kg doses than after the 2.5 mg/kg dose (M = 0.59 g/kg) or vehicle (M = 0.57 g/kg). Likewise, licks were greater after the 0.625 (M = 766) and the 1.25 (M = 738) mg/kg doses than after the 2.5 mg/kg dose (M = 497) or vehicle (M = 482). Inactive lever presses and latency to press on the active lever did not differ between groups. Correlational analysis revealed that there was a significant correlation between ethanol intake and blood alcohol level (BAC), $r = 0.617$, $p = 0.002$ (Figure 22a).

Reinstatement

One rat in the yohimbine group failed to extinguish lever-pressing behavior and was not tested in reinstatement. In addition, one rat injected with vehicle was determined to be an outlier based on box plots and was excluded from the analysis. Final sample sizes were $n = 11$ in both the yohimbine and vehicle groups. An independent-samples t-test revealed that rats injected with yohimbine exhibited significantly higher responding during reinstatement (M = 16) than those injected with vehicle (M = 5.5; $t(21) = 2.48$, $p = 0.022$) (Figure 1b). Paired-samples t-tests showed that yohimbine-injected rats pressed more during reinstatement than during the final day of extinction [$t(11) = 3.27$, $p = 0.007$], while this difference was not seen in vehicle-injected rats.

Discussion

In Experiment 1, P rats exhibited higher ethanol intake and number of licks following injection of the low (0.625 mg/kg) and middle (1.25 mg/kg) doses of

yohimbine compared to vehicle and the high (2.5 mg/kg) dose. However, yohimbine did not alter inactive lever pressing or the latency to respond on the active lever. The middle (1.25 mg/kg) dose of yohimbine elicited reinstatement to ethanol seeking as evidenced by significantly higher active lever presses following yohimbine injection compared to vehicle and to extinction responding. These findings are consistent with previous research demonstrating the efficacy of yohimbine in reinstating ethanol seeking (Le et al. 2009; Le et al. 2005; Marinelli et al. 2007) and indicate the P rats show stress-related increases in binge-like ethanol drinking and motivation to seek ethanol.

Experiment 2: Effects of yohimbine on limited access ethanol drinking and on anxiety-like behavior

Methods

Phase I: 15-minute pretreatment with yohimbine

A total of 48 male P and 47 male HAD-1 rats weighing 294.9±4.74 g (P rats) and 186.4±2.39 g (HAD-1 rats) were used in the present experiment. After acclimatization to the vivarium, rats were assigned to the following four groups balanced for body weight: ethanol plus yohimbine; ethanol plus vehicle; water plus yohimbine; water plus vehicle. Rats received access to their assigned fluid two hours into the dark cycle. Drinking sessions occurred every other day for a total of 10 exposure days over the course of three weeks. The regular water bottle was removed from the home cage and replaced with a bottle containing either water or a 10% v/v ethanol solution. During the first four sessions

(baseline), no injections were given. Days 1, 3, and 4 of baseline were 20-minute access sessions, while day 2 was a 2-hour session utilized as a probe to determine the extent to which intake might be increased if a longer session was used. Rats were exposed to sham injections on the final day of baseline. During the final six sessions, rats were given an injection of either 1.25 mg/kg yohimbine or vehicle 15 minutes prior to limited access. On the last day of injection/limited access, all rats were tested in the elevated plus maze immediately after the drinking session to assess anxiety-like behavior. One additional injection/limited access session was given for the purposes of collecting tail blood samples for blood alcohol level analysis.

Phase IIa: 8- and 24-hour pretreatment with yohimbine

In order to test time-specific effects of yohimbine treatment on ethanol drinking, the same rats from Phase I were exposed to a similar procedure during Phase II with the exception that the yohimbine pretreatment interval was lengthened. To avoid potential carry-over effects, rats began Phase II after a 10-day washout period during which time no injections or exposure to ethanol were given. Group assignments were as follows:

Phase I		Phase II
Ethanol + Yohimbine	→	Ethanol + Vehicle (8-hour pretreatment)
Ethanol + Vehicle	→	Ethanol + Yohimbine (8-hour pretreatment)
Water + Yohimbine	→	Ethanol + Vehicle (24-hour pretreatment)
Water + Vehicle	→	Ethanol + Yohimbine (24-hour pretreatment)

The 8-hour pretreatment interval was chosen as this is the half-life of yohimbine (Hubbard et al. 1988), and at which time the effects of yohimbine should be diminished compared to the more acute 15-minute pretreatment interval. The 24-hour pretreatment interval was chosen to represent an "unpaired" group. As such, rats in the 24-hour pretreatment groups were those assigned to the water access group in Phase I and were therefore ethanol-naïve prior to yohimbine exposure. Fluid access was given every other day for a total of 14 exposure days, including four baseline drinking days during which no injections were given, six drinking days preceded by an injection at the appropriate time for each group, and four post-injection sessions.

Phase IIb: 8-hour pretreatment with yohimbine in stress-naïve HAD-1 rats

To reduce the potential confound of prior exposure with yohimbine, a separate group of 23 stress-naïve HAD-1 rats were tested using the same protocol described above. It should be noted, however, these rats were not experimentally-naïve; HAD-1 rats initially trained for Experiment 1 were used in this experiment. These rats were unable to be trained to lever press for a 10% sucrose reinforcer, thus showing either an inability to learn or lack of motivation to perform the operant response. Therefore, these rats were tested using a limited access home cage drinking paradigm. Rats were handled for one week prior to limited access sessions. Drinking and injection procedures were similar to those in Phase IIa with the exception that all rats received ethanol and injections occurred 8 hours prior to ethanol access.

Results

Phase I: Drinking

Mixed factorial ANOVAs with line (P, HAD-1), and yohimbine dose (yohimbine, vehicle) as between-subjects factors, cohort (1, 2) was a covariate, and day as the within-subjects factor were analyzed separately for water and ethanol drinking during the injection period. Final samples sizes were as follows: P/EtOH/YOH = 12; P/EtOH/VEH = 11; P/H2O/YOH = 12; P/H2O/VEH = 12; HAD-1/EtOH/YOH = 12; HAD-1/EtOH/VEH = 10; HAD-1/H2O/YOH = 12; HAD-1/H2O/VEH = 11. For the water group, a significant main effect of line [$F(1,39) = 11.55$, $p < 0.001$] and an interaction between day and dose [$F(5,195) = 11.56$, $p < 0.001$] were found (Figure 2a). HAD-1 rats drank more water (M = 16.95 g/kg) than P rats (M = 12.09 g/kg) during the injection period. Yohimbine stimulated water drinking following the first and second injection, but this effect dissipated with subsequent injections.

For the ethanol group, significant main effects of line [$F(1,43) = 4.49$, $p = 0.004$] and dose [$F(1,43) = 35.89$, $p < 0.001$] and interactions between day and dose [$F(5,215) = 10.74$, $p < 0.001$] and line [$F(5,215) = 2.79$, $p = 0.018$] were found (Figure 2b). Overall, HAD-1 rats drank more ethanol (M = 0.98 mg/kg) than P rats (M = 0.81 g/kg), and rats injected with vehicle drank more (M = 1.15 g/kg) than those injected with yohimbine (M = 0.64 g/kg). In P rats, ethanol drinking did not differ systematically as a function of day, while HAD-1 rats showed an inverted-U shaped pattern of drinking, with significant decreases in ethanol intake on injection days 2, 3, and 5 compared to the first injection day. In rats injected with yohimbine, ethanol intake was reduced on injection days 2-6

compared to the first injection day, while ethanol drinking in the vehicle group increased as a function of time. Correlational analysis revealed that there was a significant correlation between ethanol intake and blood alcohol level, $r = 0.631$, $p = 0.001$ (Figure 22b).

Phase I: Elevated Plus Maze

A multivariate factorial ANOVA with number of center, open arm, closed, and total arm entries, and duration of time spent in the center, open, closed arms, and the percent of time spent in the closed and open arms as the dependent variables, and line (P, HAD-1), fluid (water, ethanol), and yohimbine dose (yohimbine, vehicle) as independent variables and cohort (1, 2) as a covariate was performed to analyze behavior in the EPM. A significant main effect of line was found for number of entries into the center [$F(1,76) = 7.74$, $p = 0.007$], and the open arms [$F(1,76) = 20.7$, $p < 0.001$], total arm entries [$F(1,76) = 9.64$, $p = 0.003$] (Figure 3), time spent in the open [$F(1,76) = 13.28$, $p < 0.001$] and closed arms [$F(1,76) = 14.15$, $p < 0.001$] as well as percent time spent in the open [$F(1,76) = 13.28$, $p < 0.001$] and closed arms [$F(1,76) = 14.15$, $p = 0.007$] (Figure 4). P rats had greater center, open and total arm entries and spent more time on the open arms than HAD-1 rats, while HAD-1 rats spent more time in the closed arms than P rats. A significant main effect of dose was found for total [$F(1,76) = 7.33$, $p = 0.008$] and open arm entries [$F(1,76) = 12.18$, $p = 0.001$] as well as time spend in the closed arms [$F(1,76) = 4.8$, $p = 0.032$]. Rats injected with yohimbine entered the center and the open arms less than those injected with vehicle, but spent more time in the closed arms. A significant main effect of cohort was also found for total [$F(1,76) = 40.38$, $p < 0.001$] and open arm

entries [$F(1,76) = 8.44$, $p = 0.005$], as well as time spent in the closed [$F(1,76) = 6.16$, $p = 0.015$] and open arms [$F(1,76) = 9.12$, $p = 0.003$]. Cohort 1 entered and spent more time in the open arms than cohort 2, while cohort 2 had more total arm entries and spent more time in the closed arms than cohort 1.

Since cohort clearly had a strong effect on EPM behavior, separate factorial ANOVAs were performed for cohort 1 and cohort 2. In cohort 1, main effects of line on open [$F(1,76) = 6.54$, $p = 0.015$] and total arm entries [$F(1,76) = 5.19$, $p = 0.028$] were found, but no other effects were evident. However, all of the main effects of dose on behavior shown in the initial analysis were present when analyzing cohort 1 alone. In contrast, main effects of line were seen for the behaviors shown in the initial analysis, but none of the effects of dose were evident, when analyzing cohort 2 alone.

Because ethanol intake was decreased as a function of yohimbine, it was of interest to determine whether the ability of ethanol to significantly alter EPM behavior compared to water was hampered by low intake following yohimbine injection. A correlational analysis was performed for ethanol intake (g/kg) on the day of EPM testing and number of entries made into the open arms of the EPM in rats assigned to the ethanol access group. A significant correlation between ethanol intake and open arm entries was found, $r = 0.033$, $p = 0.025$, indicating that greater amounts of ethanol drinking was associated with reduced anxiety-like behavior (Figure 5).

Phase IIa: 8- and 24-hour pretreatment with yohimbine

Mixed factorial ANOVAs with line (P, HAD-1), yohimbine dose (yohimbine, vehicle) as between-subjects factors, day as the within-subjects factor, and cohort (1, 2)

as a covariate were analyzed separately for the 8-hour pretreatment group and the 24-hour pretreatment group during both the Phase IIa baseline and injection periods. Final sample sizes were as follows: 8-hour: P/YOH = 11; P/VEH = 12; HAD-1/YOH = 11; HAD-1/VEH = 10; 24-hour: P/YOH = 12; P/VEH = 11; HAD-1/YOH = 10; HAD-1/VEH = 10. In the 8-hour pretreatment group, main effects of day [$F(2,78) = 15.27$, $p < 0.001$], dose [$F(1,39) = 11.16$, $p = 0.002$], and cohort [$F(1,39) = 6.57$, $p = 0.014$] were found for ethanol drinking during the Phase IIa baseline, as was a significant day by cohort interaction [$F(2,78) = 4.88$, $p = 0.010$; Figure 6a]. In general, rats showed day-dependent increases in ethanol drinking across days. Rats that had experienced yohimbine during Phase I demonstrated decreased ethanol intake (M = 0.85 g/kg) during Phase IIa baseline compared to those that had previously experienced vehicle (M = 1.230 g/kg). Rats in cohort 1 drank more ethanol (M = 1.16 g/kg) and accelerated their ethanol intake faster than those in cohort 2 (M = 0.91 g/kg). During the injection period, a significant main effect of dose was found [$F(1,39) = 7.41$, $p = 0.010$], as were significant interactions between line and dose [$F(1,39) = 5.49$, $p = 0.024$] and day [$F(5,195) = 2.51$, $p = 0.031$]. Rats injected with yohimbine drank more ethanol (M = 1.82 g/kg) than those injected with vehicle (M = 1.44 g/kg). However, given that differences in yohimbine treatment during Phase I affected drinking during the Phase IIa baseline period, this finding is likely due to carry-over effects of prior experience with yohimbine. Follow-up oneway ANOVAS revealed that while HAD-1 rats showed differences in ethanol drinking as a function of yohimbine [$F(1,22) = 9.14$, $p = 0.006$], this was not true for P rats. Both lines of rats increased their drinking as a function of day, and although this increase was evident in different patterns, these differences were not systematic.

In the 24-hour pretreatment group, significant main effects of day [$F(2,80) = 7.29$, $p = 0.001$] and dose [$F(1,40) = 5.63$, $p = 0.023$] were found during the Phase IIa baseline, as was a significant day by dose by line interaction [$F(2,80) = 5.47$, $p = 0.006$; Figure 6b]. Follow-up 2-way ANOVAs separated by yohimbine dose revealed that rats that had previously been treated with yohimbine during Phase I showed significant main effects of day [$F(2,42) = 14.53$, $p < 0.001$] and interactions between day and line [$F(2,42) = 14.52$, $p < 0.001$], while rats that had previously been treated with vehicle showed only a main effect of day [$F(2,40) = 17.78$, $p < 0.001$] and exhibited increased ethanol drinking on days 2 and 3 compared to day 1. Subsequent oneway ANOVAs showed that while P rats previously treated with yohimbine increased their ethanol intake as a function of day, this was not true for HAD-1 rats. Taken together, it appears as though carry-over effects of yohimbine exposure in Phase I also affected the acquisition of ethanol drinking in HAD-1 rats in the unpaired group. During the injection period, only a main effect of day was found [$F(2,40) = 3.40$, $p = 0.006$]. Pairwise comparisons did not reveal a systematic change in ethanol drinking between each injection day, although drinking increased across the injection period.

Phase IIb: 8-hour pretreatment with yohimbine in stress-naïve HAD-1 rats

A repeated-measures ANOVA with yohimbine dose (yohimbine, $n = 12$; vehicle group, $n = 11$) as the between-subjects factor and day as the within-subjects factor revealed significant main effects of day [$F(5,105) = 2.74$, $p = 0.023$], dose [$F(1,21) = 6$, $p = 0.024$], and a day by dose interaction [$F(5,105) = 2.31$, $p = 0.049$], during injections (Figure 7). Rats drank significantly more ethanol following yohimbine injection [M =

1.58 g/kg] than vehicle (M = 1.25 g/kg), and pairwise comparisons showed that this effect was apparent on injection day 3 [$t(21) = -2.31$, $p = 0.031$] and 4 [$t(21) = -4.63$, $p < 0.001$]. No differences were seen between groups during baseline or following the post-injection baseline period.

Discussion

In Experiment 2, acute (15-minute) administration of yohimbine prior to 20-minute fluid access elicited transient increases in water drinking, an effect that was more pronounced in HAD-1 rats. In contrast, this pre-treatment interval elicited robust reductions in ethanol intake. Extending the pre-treatment interval to the half-life of yohimbine (8 hours; Hubbard et al. 1988) yielded significant increase in ethanol intake compared to vehicle, although this finding is confounded by carryover effects of previous stress exposure, especially in HAD-1 rats. Specifically, HAD-1 rats treated with yohimbine and those treated with vehicle demonstrated continued differences in ethanol intake even after a 10-day washout period and a four-day ethanol re-acquisition period (Phase IIa baseline). In contrast, P rats treated with yohimbine and with vehicle did not differ in ethanol intake during this phase of Experiment 2. While this may suggest that acute yohimbine treatment may have promoted an aversion to ethanol that did not extinguish in HAD-1 rats, the finding that prior exposure with yohimbine also retarded acquisition of ethanol drinking in the 24-hour pretreatment group provides evidence against a yohimbine-related aversion to ethanol. As in the 8-hour pretreatment group, P rats in the 24-hour pretreatment group did not show the persistent differences between yohimbine and vehicle evident in HAD-1 rats. When a group of stress-naïve HAD-1 rats

were injected with yohimbine 8 hours prior to ethanol access, a transient but significant increase in drinking was evident. Thus, it appears that yohimbine-related changes in ethanol drinking are time- and experience-dependent in HAD-1 rats, while P rats are less sensitive to this effect.

While yohimbine significantly increased anxiety-like behavior in the EPM, as evidenced by rats spending less time in the open arms and more time in the closed arms, locomotor activity was not affected as evidenced by a lack of an effect of yohimbine on total arm entries. Self-administered ethanol failed to alter anxiety-like behavior compared to self-administered water, and ethanol and did not block the effects of yohimbine. However, taking the reduced ethanol drinking as a function of yohimbine into consideration, rats in the ethanol access group demonstrated a positive correlation between ethanol intake and number of open arm entries, which suggests that ethanol was exerting an anxiolytic effect. Interestingly, P rats were less anxious and more active than HAD-1 rats, indicated by greater total and open arm entries and time spent in the open arms. Thus, it is possible that the sensitivity to the time-dependent effects of yohimbine on limited access ethanol drinking in HAD-1 rats might be due to their increased basal anxiety-like behavior. Finally, significant changes in EPM behavior were seen as a function of cohort. As such, environmental factors that were not controlled by the experimenter, such as season and changes in animal care staff, may have contributed to the effects seen in this portion of the experiment. The sensitivity to changes in behavior in the EPM as a function of environment, despite attempts to control as many extraneous variables as possible, has previously been shown (Crabbe et al. 1999; Wahlsten et al. 2003).

Experiment 3: Effects of yohimbine administration before and during ethanol acquisition on subsequent ethanol intake

Methods

A total of 37 male P and 32 male HAD-2 rats weighing 276.5±8.72 g (P rats) and 214.7±3.28 g (HAD-2 rats) were used in the present experiment. After acclimatization to the vivarium, rats were divided into the following three groups balanced for body weight: yohimbine pretreatment (PRE); yohimbine during acquisition (ACQ); vehicle control (CONTROL). Each group of injections was administered every other day (three times/week) for two weeks. Rats in the pretreatment group were injected with 1.25 mg/kg yohimbine for the two weeks preceding ethanol access while rats in the acquisition group were injected with yohimbine during the first two weeks of ethanol access. Rats in all three groups received vehicle injections on days that the test rats received yohimbine such that all rats received the same number of injections. Ethanol and water were provided continuously on the home cage, and the position of the bottles was alternated every day to avoid confounds associated with side preference. Injections and fluid intake measures occurred just prior to the start of the dark cycle. Ethanol access continued for a period of 12 weeks, after which time all rats received a challenge injection of yohimbine. Post-injection intakes were then taken for an additional week.

Results

Final sample sizes were as follows: P/CONT = 11; P/ PRE = 13; P/ACQ = 12; HAD-2/CONT = 10; HAD-2/PRETX = 11; HAD-2/ACQ = 11. A mixed factorial ANOVA with line (P, HAD-2) and treatment group (CONTROL, PRE, ACQ) as between-subjects factors, day as the within-subjects factor, and cohort (1, 2) as a covariate revealed a significant main effect of day [$F(85,4845) = 38.88$, $p < 0.001$] for ethanol intake, and pairwise comparisons indicated that intake increased over the course of the experiment (Figure 8). No other main effects or interactions were evident in this analysis. Analyzing the data averaged across weekly blocks of drinking also failed to identify any main effects, although there was a trend for HAD-2 rats in the acquisition group to show slower escalation of ethanol drinking compared to HAD-2 rats in the control group. A multivariate ANOVA using the same between-subjects factors described above failed to find any significant main effects of these factors on ethanol drinking on any of the injection days. The effects of the challenge dose of yohimbine on ethanol drinking in these rats will be discussed in the results section for Experiment 4.

Similar analyses were performed for water intake. A mixed factorial ANOVA with line (P, HAD-2) and treatment group (CONTROL, PRE, ACQ) as between-subjects factors, day as the within-subjects factor, and cohort (1, 2) as a covariate revealed a significant main effect of day [$F(85,4845) = 38.88$, $p < 0.001$] for water intake, and pairwise comparisons indicated that intake increased over the course of the experiment (Figure 9). However, consistent with the findings from the ethanol intake data, no effects were seen on water drinking on injection days. Analyzing the data averaged across weekly blocks of drinking revealed significant main effects of block [$F(12,732) = 10$, $p <$

0.001], line [$F(1,61) = 44.24$, $p < 0.001$] and a significant block by line interaction [$F(12,732) = 2.99$, $p < 0.001$]. In general, water intake decreased as a function of block, and HAD-2 rats drank more water (M = 56.78 ml/kg) than P rats (M = 29.9 ml/kg). Follow-up oneway ANOVAs run separately for P and HAD-2 rats revealed that both lines exhibited significant differences in drinking across the blocks, and although the pattern of drinking varied between the lines these differences were not systematic.

Discussion

Exposure to yohimbine prior to ethanol access or during the initial two weeks of ethanol drinking failed to significantly alter drinking acquisition, although there was a tendency for both groups of yohimbine-treated HAD-1 rats to demonstrate diminished ethanol intake compared to those treated with vehicle. This finding is consistent with other studies that failed to find stress exposure to alter the acquisition of ethanol drinking in adult animals (Chester et al. 2006; Ng Cheong Ton et al. 1983). Line differences in water intake were present, but not systematic, in this experiment.

Experiment 4: Effects of yohimbine on drinking in rats given continuous or intermittent access to ethanol

Methods

A total of 46 male P and 42 male HAD-2 rats weighing 276.5±8.72 g (P rats) and 214.7±3.28 g (HAD-2 rats) were used in the present experiment. Rats from the control group in Experiment 3 were also used as the control group in Experiment 4. After

acclimatization to the vivarium, rats were divided into the following four groups balanced for body weight: intermittent ethanol access plus yohimbine (INT/YOH); intermittent ethanol access plus vehicle (INT/VEH); continuous ethanol access plus yohimbine (CONT/YOH); and continuous ethanol access plus vehicle (e.g., the control group from Experiment 3). All rats were given continuous access to 10% v/v ethanol and water on their home cage for a period of six weeks prior to yohimbine (1.25 mg/kg) and vehicle injections. Ethanol and water were provided continuously on the home cage, and the position of the bottles was alternated every day to avoid confounds associated with side preference. Injections and fluid intake measures occurred just prior to the start of the dark cycle. Rats in the intermittent ethanol access groups were deprived of ethanol at weekly intervals with a week of ethanol access in between deprivation periods. These cycles of intermittent ethanol access were repeated three times. Injections of vehicle or yohimbine were given every other day during the deprivation period (or at a parallel time in the continuous ethanol access group) for a total of three injections per week. Twenty-four hours prior to the third and final cycle of resumed ethanol access, and 48 hours following the third injection, all rats were tested in the EPM. After a week of resumed drinking, all rats were given a challenge injection of yohimbine. Post-injection drinking measures were taken for an additional week.

Results

Drinking

Final sample sizes were as follows: P/CONT = 11; P/DEP/VEH = 11; P/DEP/YOH = 12; P/CONT/YOH = 12; HAD-2/CONT = 10; HAD-2/DEP/VEH = 11; HAD-2/DEP/YOH = 11; HAD-2/CONT/YOH = 10. A mixed factorial ANOVA with line (P, HAD-2), ethanol access pattern (continuous, intermittent), and yohimbine dose (yohimbine, vehicle) as between-subjects factors, average ethanol intake for each cycle (baseline, reinstatement 1, reinstatement 2, reinstatement 3) as the within-subjects factor, and cohort (1, 2) as a covariate revealed significant main effects of cycle [$F(3,231) = 2.83$, $p = 0.039$], cohort [$F(1,77) = 8.25$, $p = 0.005$], and dose [$F(1,77) = 4.21$, $p = 0.043$] as well as a cycle by access pattern by dose interaction [$F(3,231) = 2.63$, $p = 0.002$] on ethanol intake (Figure 10). In general, rats injected with yohimbine drank significantly more ethanol (M = 7.12 g/kg) than those injected with vehicle (M = 6.59 g/kg), and rats in cohort 1 drank more ethanol (M = 7.26 g/kg) than rats in cohort 2 (M = 6.52 g/kg). Ethanol drinking was higher during each reinstatement period compared to baseline and highest during the final reinstatement cycle compared to all other periods. Follow-up 2-way ANOVAs run separately for yohimbine and vehicle groups revealed that rats injected with yohimbine showed a significant main effect of cycle [$F(3,123) = 15.41$, $p < 0.001$] with a pattern of drinking consistent with that described above. In the vehicle group, however, a main effect of cycle [$F(3,123) = 8.58$, $p < 0.001$] and cycle by access pattern interaction [$F(3,123) = 3.81$, $p = 0.012$] were found for ethanol drinking. Subsequent oneway ANOVAs revealed that rats given continuous and deprived access

both showed main effects of cycle on drinking, but in the continuous access group, drinking was elevated only during the final drinking period, whereas rats in the deprived group showed elevations in drinking during each period compared to the baseline drinking period.

Separate factorial ANOVAs with line (P, HAD-2) and dose (yohimbine, vehicle) as between-subjects factors, day as the within-subjects factor, and cohort as a covariate during each of the three cycles were performed for rats given continuous ethanol access to determine the effects of yohimbine on the maintenance of ethanol drinking. Since between-groups differences were evident on the day before the first injection, drinking scores were converted to percent of baseline (the final week of drinking before the injections began). During cycle 1, main effects of day [$F(5,190) = 3.09$, $p = 0.010$] and cohort [$F(1,38) = 5.32$, $p = 0.027$] were found, as well as a day by cohort interaction [$F(5,190) = 2.99$, $p = 0.013$; Figure 11). Follow-up oneway ANOVAs run separately for each cohort revealed no main effect of day in cohort 1, and a significant main effect of day [$F(5,105) = 4.17$, $p = 0.002$] in cohort 2, but differences across day were not systematic. During cycle 2, main effects of day [$F(5,190) = 4.25$, $p = 0.001$] and a significant day by cohort interaction [$F(5,190) = 3.43$, $p = 0.005$] as well as a day by line by group interaction [$F(5,190) = 2.71$, $p = 0.022$] were found. Separate 2-way ANOVAs run separately for P and HAD-2 rats revealed that both groups showed main effects of day [$F(5,190) = 2.99$, $p = 0.013$ and $F(5,190) = 2.99$, $p = 0.013$, respectively], but no main effects of or interactions with group were found. Again, changes in drinking as a function of day were not systematic. During cycle 3, a significant day by group interaction was found [$F(5,190) = 3.28$, $p = 0.007$]. Separate oneway ANOVAs revealed

that both the vehicle and yohimbine groups showed main effects of day [$F(5,100) = 3.32$, $p = 0.008$ and $F(5,105) = 4.08$, $p = 0.002$, respectively], but once again, changes in drinking as a function of day were not systematic.

When ethanol intake on the yohimbine challenge injection day from rats in Experiment 3 and Experiment 4 was analyzed using a factorial ANOVA with group (CONTROL, PRE, ACQ, INT/VEH, INT/YOH, CONT/YOH) and line (P, HAD-2) as between-subjects factors and cohort (1, 2) as a covariate, a main effect of cohort was found [$F(23,111) = 19.04$, $p < 0.001$; Figure 12]. Overall, rats in cohort 1 drank more ethanol (M = 7.03 g/kg) than those in cohort 2 (M = 5.54 g/kg). When analyzed separately by cohort, no main effects or interactions emerged.

Similar analyses were performed for water drinking. A mixed factorial ANOVA with line (P, HAD-2), ethanol access pattern (continuous, intermittent), and yohimbine dose (yohimbine, vehicle) as between-subjects factors and average ethanol intake for each cycle (baseline, reinstatement 1, reinstatement 2, reinstatement 3) as the within-subjects factor revealed a significant main effect of cycle [$F(3,231) = 11.16$, $p < 0.001$], and several interactions: ethanol access pattern by dose [$F(1,77) = 4.98$, $p = 0.029$]; cycle by line by dose [$F(3,231) = 4.69$, $p = 0.003$]; cycle by ethanol access pattern by dose [$F(3,231) = 7.10$, $p < 0.001$]; and a cycle by line by ethanol access pattern by dose [$F(3,231) = 3.58$, $p = 0.015$; Figure 13]. In general, water intake was highest at baseline (M = 37.46 ml/kg) and lowest during the final cycle (M = 23.37 g/kg). Follow-up 3-way ANOVAs run separately for the two lines revealed that P rats displayed main effects of cycle [$F(3,126) = 40.71$, $p < 0.001$], with intake following the same general pattern as described above, and a significant cycle by dose interaction [$F(3,126) = 3.57$, $p = 0.016$].

Subsequent oneway ANOVAs run separately for P rats injected with vehicle and with yohimbine revealed that both groups varied their water intake as a function of cycle, with vehicle-treated rats drinking more water during baseline (M = 31.69 ml/kg) compared to all other periods and yohimbine-treated rats also drinking more water at baseline (M = 23.63 ml/kg) but also less water at the third cycle (M = 12.76 ml/kg) compared to all other cycles. In HAD-2 rats, a significant main effect of cycle was found [$F(3,108) = 34.81$, $p < 0.001$], as was a cycle by ethanol access pattern by dose interaction [$F(3,108) = 7.45$, $p < 0.001$]. Again, HAD-2 rats drank the most water during baseline (M = 47.25 ml/kg) and the least water during the final cycle (M = 31.67 ml/kg). Subsequent 2-way ANOVAs run for the vehicle and yohimbine groups showed a main effect of cycle in the vehicle group [$F(3,57) = 11.47$, $p < 0.001$], again, with the same pattern of drinking across periods as described above. In the yohimbine group, however, in addition to a main effect of cycle [$F(3,51) = 24.85$, $p < 0.001$], with water intake following the same pattern described above, there was a significant cycle by ethanol access pattern interaction [$F(3,51) = 6.33$, $p = 0.001$]. A follow-up oneway ANOVA showed that only yohimbine-treated HAD-2 rats given continuous ethanol access showed significant changes in water drinking as a function of cycle [$F(3,27) = 44.46$, $p = 0.001$], in which cycle-dependent reductions in water drinking were seen.

Separate factorial ANOVAs with line (P, HAD-2) and dose (yohimbine, vehicle) as between-subjects factors and day as the within-subjects factor during each of the three cycles were performed for rats given continuous ethanol access to determine the effects of yohimbine on the maintenance of water drinking. The water drinking scores were not converted to percent of baseline, as was done for ethanol intake, because there were no

between-treatment differences in water intake on the day before the first injection. During cycle 1, main effects of day [$F(5,190) = 7.84$, $p < 0.001$], line [$F(1,38) = 48.68$, $p < 0.001$], and dose [$F(1,38) = 5.46$, $p = 0.025$] were evident, with HAD-2 rats generally drinking more water (M = 52.03 ml/kg) than P rats (M = 25.22 ml/kg) and yohimbine-treated rats drinking more water (M = 43.10 ml/kg) than those treated with vehicle (M = 34.14 ml/kg; Figure 14). However, multiple significant interactions were found: day by line [$F(5,190) = 5.43$, $p = 0.001$]; day by dose [$F(1,190) = 6.28$, $p < 0.001$]; line by dose [$F(1,38) = 6.54$, $p = 0.015$]; and day by line by dose [$F(1,190) = 2.66$, $p = 0.024$]. Follow-up 2-way ANOVAs run separately for P and HAD-2 rats revealed that P rats showed changes in water drinking as a function of day [$F(5,105) = 5.19$, $p < 0.001$], but these changes were not systematic. In HAD-2 rats, a main effect of day [$F(5,90) = 9.25$, $p < 0.001$] was found, again, with non-systematic changes, as well as a day by dose interaction [$F(5,90) = 4.31$, $p = 0.001$]. Subsequent oneway ANOVAs separated by dose revealed that HAD-2 rats injected with yohimbine altered their water intake as a function of day [$F(5,45) = 10.36$, $p < 0.001$], but again, changes in drinking were not systematic. During cycle 2, similar patterns of water drinking were seen as in cycle 1, with main effects of day [$F(5,190) = 3.94$, $p = 0.002$] and line [$F(1,38) = 62.50$, $p < 0.001$], and several interactions: day by line [$F(5,190) = 3.18$, $p = 0.009$]; day by group [$F(5,190) = 2.41$, $p = 0.038$]; and day by line by group [$F(5,190) = 3.95$, $p = 0.002$]. In general, water drinking only differed on day 1 (M = 39.73 ml/kg) compared to day 4 (M = 34.06 ml/kg), but again, HAD-2 rats drank more water (M = 53.61 ml/kg) than P rats (M = 20.76 ml/kg). Follow-up 2-way ANOVAs run separately for P and HAD-2 rats revealed that while P rats showed changes in drinking as a function of day [$F(5,105) = 2.56$, $p = 0.032$]

and a day by group interaction [$F(5,105) = 5.59$, $p < 0.001$], HAD-2 rats showed only a day by group interaction [$F(5,90) = 2.29$, $p = 0.053$]. Subsequent oneway ANOVAs run separately for treatment group revealed that in P rats, only those treated with yohimbine drank differently as a function of day [$F(5,55) = 6.82$, $p < 0.001$], and drinking was highest on day 2 (M = 30.12 ml/kg), an injection day, compared to all other days. Similarly, in HAD-2 rats, only those treated with yohimbine drank differently as a function of day [$F(5,45) = 2.45$, $p = 0.048$], with the highest (M = 76.18 ml/kg) and lowest (M = 45.97 ml/kg) drinking occurring on days 1 and 4, respectively. During cycle 3, all of the main effects and interactions involving day disappeared; however, a significant main effect of line [$F(1,38) = 52.17$, $p < 0.001$] and a line by group interaction [$F(1,38) = 4.73$, $p = 0.036$] were found. Again, HAD-2 rats drank more water (M = 48.31 ml/kg) than P rats (M = 20 ml/kg). While follow-up oneway ANOVAs run separately by line revealed that neither P nor HAD-2 rats showed significantly different water intake as a function of dose, in general, yohimbine decreased water intake in P rats and increased water intake in HAD-2 rats.

Elevated Plus Maze

A multivariate factorial ANOVA with number of center, open arm, closed, total arm entries, and fecal boli, and duration of time spent in the center, open, closed arms, and the percent of time spent in the closed and open arms as the dependent variables, line (P, HAD-2), ethanol access pattern (continuous, intermittent), yohimbine dose (yohimbine, vehicle) as independent variables, and cohort (1, 2) as a covariate was performed to analyze behavior in the EPM. A significant main effect of line was found

for number of entries into the center [$F(1,70) = 70.5$, $p < 0.001$], open arms [$F(1,70) = 73.41$, $p < 0.001$], closed arms [$F(1,70) = 12$, $p = 0.001$], and total arm entries [$F(1,70) = 73.88$, $p = 0.001$], with P rats showing greater center, open arm, and total entries (Figure 15). Main effects of line were also found for time spent in the center [$F(1,70) = 8.43$, $p = 0.005$] and open arms [$F(1,70) = 19.53$, $p < 0.001$], as well as percent of time spent in the open arms [$F(1,70) = 19.53$, $p < 0.001$] with HAD-2 rats spending significantly more time in the center and P rats spending significantly more time in the open arms of the EPM compared to the other line (Figure 16). However, main effects of cohort were found for number of entries into the center [$F(1,70) = 7.31$, $p = 0.009$] the closed arms [$F(1,70) = 14$, $p < 0.001$], total arm entry [$F(1,70) = 7.74$, $p = 0.007$], and fecal boli [$F(1,70) = 7.98$, $p = 0.006$], as well as time spent in the open [$F(1,70) = 15.56$, $p < 0.001$] and closed arms [$F(1,70) = 13.52$, $p < 0.001$] and percent time spent in the open [$F(1,70) = 15.56$, $p < 0.001$] and closed arms [$F(1,70) = 13.52$, $p < 0.001$]. Rats in cohort 1 entered the center more often, spent more time in the closed arms, and had more total arm entries and fecal boli, while rats in cohort 2 entered the closed arms more often and spent more time in the open arms. No main effects of ethanol access pattern or yohimbine dose were found, nor did these factors interact with any other variable, when both cohorts were analyzed together. In cohort 2 only, a significant main effect of ethanol access pattern emerged, [$F(1,30) = 4.33$, $p = 0.044$], with rats given continuous access spending more time in the center than those given intermittent access.

Discussion

In Experiment 4, ethanol intake increased and water intake decreased as a function of time in all rats. Both lines drank similar amounts of ethanol, but HAD-2 rats drank more water. Yohimbine increased ethanol drinking when analyzed by average weekly intake across the reinstatement cycles. However, increased ethanol drinking during the injection period was not evident in rats maintained on continuous ethanol access. In contrast, yohimbine increased water drinking during the injection period. Rats given intermittent ethanol access displayed increases in ethanol drinking during each cycle compared to baseline, while rats given continuous ethanol access only displayed increased ethanol drinking between baseline and the final reinstatement period. However, experience with either repeated yohimbine injection or repeated ethanol deprivation did not alter anxiety-like behavior in the elevated plus maze, and a challenge injection of yohimbine did not differentially affect ethanol drinking despite differences in stress and ethanol exposure history. These findings do not support the hypothesis that exposure to repeated ethanol deprivation produces increased anxiety-like behavior that may confer an enhanced sensitivity to stress (Baldwin et al. 1991; Becker 2000; Doremus et al. 2003; File et al. 1989; File et al. 1991; File et al. 1992; Holter et al. 1998; Knapp et al. 2004; Moy et al. 1997; Rassnick et al. 1993) evidenced by subsequent ethanol drinking. However, consistent with the findings from Experiment 2 that compared P to HAD-1 rats, P rats were more active and less anxious than HAD-2 rats. Also consistent with Experiment 2 was that cohort had an effect on EPM behavior, as discussed above. Some differences in ethanol drinking were also seen as a function of cohort, but the same was

not true for water drinking. Thus, this underscores that anxiety-like and alcohol drinking behavior are sensitive to environmental factors.

Experiment 5: Effects of yohimbine on appetitive responding for ethanol following intermittent ethanol access

Methods

A total of 36 male P and 25 male HAD-2 rats were used in the present experiment. Rats were given a week to acclimate to the vivarium during which time they were weighed, handled, and habituated to transport. Operant sessions were run seven days a week during the light cycle. Once rats were trained to respond on an RR10 for 10% v/v ethanol, they were given 6-7 weeks to establish baseline intake. During this time, rats were habituated to the injection procedure by receiving once weekly injections of vehicle. Rats were given a single nonreinforced session following a vehicle injection to account for the typically high level of responding during the initial extinction session (EXT 0). Balanced for baseline ethanol intake (g/kg) during the final week of reinforced sessions as well as responding during the initial nonreinforced session, rats were assigned to one of 4 groups: intermittent ethanol access plus yohimbine (INT/YOH); intermittent ethanol access plus vehicle (INT/VEH); continuous ethanol access plus yohimbine (CONT/YOH); and continuous ethanol access plus vehicle (CONT/VEH). Rats in the intermittent access groups were weighed in the vivarium during the one-week ethanol deprivation period but were not tested in the operant chambers. At the end of the deprivation period, all rats were injected with either 1.25 mg/kg yohimbine or vehicle 15 minutes prior to an

extinction session. Reinforced sessions began the following day and lasted for a week before beginning the next deprivation period. The deprivation procedure was repeated three times, and samples for blood ethanol levels were taken at the end of the final week of resumed ethanol self-administration.

Results

Consummatory Responding and Ethanol Intake

One P rat and one HAD-2 rat in the CONT/YOH group did not complete the experiment and were excluded from the analysis. Final sample sizes were as follows: P rats, n = 9/group; HAD-2/CONT/VEH = 6; HAD-2/CONT/YOH = 5; HAD-2/INT/VEH = 6; HAD-2/INT/YOH = 7. A mixed factorial ANOVA with line (P, HAD-2), ethanol access pattern (continuous, intermittent), and yohimbine dose (yohimbine, vehicle) as between-subjects factors, drinking period (baseline, reinstatement 1, reinstatement 2, reinstatement 3) as the within-subjects factor, and cohort (1, 2, 3) as a covariate revealed a significant period by line interaction [$F(3,150) = 5.03$, $p = 0.002$] (Figure 17). Follow-up oneway ANOVAs run separately for P and HAD-2 rats revealed that P rats displayed changes in ethanol drinking as a function of day [$F(3,102) = 6.934$, $p < 0.000$], with the largest amount of drinking occurring during the first cycle, while HAD-2 rats displayed significant increases in drinking [$F(3,69) = 4.34$, $p = 0.007$] during the second cycle. No other main effects or interactions were evident in this analysis.

Subsequent factorial ANOVAs were run with the same between-subjects factors but used the last day of drinking before deprivation (or at the same time point in rats

given continuous access) versus the reinstatement day as the within-subjects factor. Separate comparisons were made for each deprivation/reinstatement period. During the first period, a significant main effect of day was found [$F(1,50) = 7.978$, $p = 0.007$], with rats drinking more ethanol on the day of reinstatement (M = 0.95 g/kg) than on the final day of pre-deprivation drinking (M = 0.79 g/kg) (Figure 18). During the second period, a significant interaction between ethanol access pattern and day [$F(1,50) = 8.35$, $p = 0.006$] (Figure 19). Follow-up oneway ANOVAs run separately for the continuous and intermittent ethanol access groups found that only the latter group displayed differences in intake [$F(1,30) = 17.296$, $p < 0.001$], with higher intake on the reinstatement day (M = 0.98 g/kg) than on the pre-deprivation day (M = 0.73 g/kg). During the third period, a significant main effect of ethanol access pattern [$F(1,50) = 5.36$, $p = 0.025$] and significant interactions between day and ethanol access pattern [$F(1,50) = 4.48$, $p = 0.040$] and line [$F(1,50) = 17.474$, $p < 0.001$] were found (Figure 20). Follow-up oneway ANOVAs run separately for the continuous and intermittent ethanol access groups found that only the latter group displayed differences in intake [$F(1,30) = 21.189$ $p < 0.001$], with higher intake on the reinstatement day (M = 0.90 g/kg) than on the pre-deprivation day (M = 0.70 g/kg). Follow-up oneway ANOVAs run separately for P and HAD-2 rats found that only P rats displayed differences in intake [$F(1,30) = 28.437$, $p < 0.001$], with higher intake on the reinstatement day (M = 0.98 g/kg) than on the pre-deprivation day (M = 0.74 g/kg). Correlational analysis revealed that there was a significant correlation between ethanol intake and blood alcohol level (BAC), $r = 0.338$, $p = 0.009$ (Figure 22c).

A mixed factorial ANOVA with line (P, HAD-2), ethanol access pattern (continuous, intermittent), and yohimbine dose (yohimbine, vehicle) as between-subjects

factors, drinking period (baseline, reinstatement 1, reinstatement 2, reinstatement 3) as the within-subjects factor, and cohort (1, 2, 3) as a covariate was also made for licks during the reinstatement periods. A significant main effect of line [$F(3,150) = 27.75$, $p < 0.001$] revealed that P rats licked more (M = 715) than HAD-2 rats (M = 397) (Figure 21a). A significant day by line interaction [$F(3,150) = 4.50$, $p = 0.005$] was found, and follow-up oneway ANOVAs revealed that P rats altered their lick number as a function of day [$F(3,99) = 10.82$, $p < 0.001$] with the greatest amounts of licks occurring on the baseline extinction session. A trend toward differential lick numbers as a function of day ($p = 0.054$) indicated that licks increased across the four periods. No other main effects or interactions were found for any other variable for number of licks.

Appetitive Responding

One P rat in the intermittent/yohimbine group was determined to be an outlier based on box plots and was therefore eliminated from the analysis. Number of lever presses was subjected to a mixed factorial ANOVA with the same between-subjects groups as above but with extinction day (baseline, 1, 2, and 3) as the within-subjects factor. A significant main effect of line [$F(1,49) = 20.42$, $p < 0.001$] was found, as was a day by line interaction [$F(3,147) = 5.2$, $p = 0.002$] (Figure 21b). In general, P rats pressed significantly more (M = 58.62) than HAD-2 rats (M = 35.27). Follow-up oneway ANOVAs run separately for each line revealed that P rats showed significant differences in responding across extinction session [$F(3,99) = 10.82$, $p < 0.001$], with responding on the baseline extinction session being greater than all others. A trend for ethanol access pattern ($p = 0.064$) showed that rats given continuous ethanol access pressed more during

extinction (M = 52) than those deprived of ethanol (M = 42). It should be noted that four HAD-2 rats failed to lever press during at least one extinction session, and an additional seven HAD-2 rats as well as four P rats responded less than 10 times (the normal response requirement) on at least one extinction session. This effect occurred most frequently in P rats in the continuous/yohimbine group (3/4) and the least frequently in HAD-2 rats in the continuous/vehicle group (2/11; 3-4/11 in the other groups).

Discussion

In Experiment 5, neither intermittent access to ethanol nor yohimbine injection significantly altered binge-like ethanol consumption. However, a significant interaction between ethanol access pattern and pre- versus post-deprivation intake indicated that rats that were given an ethanol deprivation period show greater post-deprivation drinking than those that were never deprived of ethanol. It is possible that the nonreinforced session prior to the reinstatement session elicited an increase in ethanol drinking in both groups of rats, but this effect was enhanced in those given a week-long ethanol deprivation period. During the nonreinforced extinction sessions, yohimbine failed to alter lever pressing, indicating that stress exposure did not increase the motivation to seek ethanol. However, P rats exhibited greater responding during extinction sessions than HAD-2 rats, indicating a greater motivation to respond for ethanol.

GENERAL DISCUSSION

Overall, the results of the present experiments support the hypothesis that stress plays a role in ethanol-self administration and also demonstrate that the P and HAD lines differ in behaviors associated with ethanol reinforcement (see Table 1 for a summary). In Experiment 1, P rats displayed increased binge-like ethanol self-administration following low and moderate doses of yohimbine. Further, yohimbine was able to reinstate ethanol seeking in P rats that were trained to self-administer ethanol using the sipper tube model. However, as HAD-1 rats were unable to acquire the operant response, comparisons between these lines in the ability of yohimbine to alter ethanol seeking and self-administration cannot be made in this experiment. In Experiment 2, acutely administered yohimbine increased water drinking but decreased ethanol drinking when given limited access to the assigned fluid. In the EPM, yohimbine exerted the expected anxiogenic effect, but self-administered ethanol did not reduce anxiety nor did it block yohimbine-induced anxiogenesis. Interestingly, it was found that P rats are less anxious and more active than HAD-1 rats. When the yohimbine pretreatment interval was increased to 8 and 24 hours to minimize the suppressive effects of acute yohimbine injection on ethanol drinking, yohimbine increased ethanol drinking compared to vehicle, and this effect was more pronounced in HAD-1 rats. However, rats injected with vehicle in the 8- and 24-hour pretreatment groups during Phase IIa of the experiment had previously received

yohimbine during Phase I, and this finding may be due to carry-over effects of prior stress experience. In Phase IIb, stress-naïve HAD-1 rats demonstrated transient but significant increases in ethanol drinking following yohimbine treatment. Experiment 3 showed that yohimbine failed to significantly alter the acquisition of ethanol drinking, although there was a trend for decreased ethanol intake in HAD-2 rats treated during the first two weeks of access compared to those in the control group. In Experiment 4, ethanol intake increased across the cycles of ethanol deprivation/yohimbine injection, and intermittent ethanol access and yohimbine each caused small but significant increases in ethanol drinking. However, the acute effects of yohimbine on rats maintained on continuous ethanol access were not evident. Previous experience with stress and/or with repeated ethanol deprivations failed to alter behavior in the EPM, although line differences between P and HAD-2 rats showed P rats to be less anxious and more active than HAD-2 rats. In addition, varying the exposure to yohimbine and to intermittent ethanol access did not significantly alter the ethanol drinking response to a challenge injection of yohimbine. In Experiment 5, neither yohimbine nor ethanol deprivation altered appetitive responding for ethanol, although within-subjects differences comparing pre- and post-deprivation drinking revealed that ethanol intake was increased on the post-deprivation day. However, P rats had greater licks when ethanol was available, and had more active lever presses during nonreinforced trials than HAD-2 rats.

Yohimbine has previously been shown to increase operant self-administration of ethanol in outbred rats at all doses used in the present experiment (Le et al. 2009; Le et al. 2005; Marinelli et al. 2007). While the 2.5 mg/kg dose was shown to increase ethanol self-administration in the early study (Le et al. 2005), subsequent studies showed that this

dose tended to suppress drinking, while the 0.625 mg/kg dose was able to increase drinking (Le et al. 2009). The 1.25 mg/kg dose of yohimbine consistently increased ethanol self-administration and reinstatement of ethanol seeking. Consistent with these findings, ethanol intake was increased at the low (0.625 mg/kg) and medium (1.25 mg/kg) dose in the present experiment compared to both the high (2.5 mg/kg) dose and vehicle. This high dose significantly increases anxiety-like behavior (Johnston and File 1989), and a similar dose (2.0 mg/kg) decreases locomotor activity (Bowes et al. 1992). Thus, it is possible that stress-related suppression in activity or freezing could contribute to the effects of the high dose on ethanol self-administration. However, rats injected with the high dose of yohimbine did not significantly differ from vehicle-injected rats in the amount of time it took to perform the response requirement, indicating that locomotor effects alone cannot account for the changes in ethanol self-administration.

Adding to the long list of studies that have found 1.25 mg/kg yohimbine to elicit reinstatement of ethanol seeking (Cippitelli et al. 2010; Le et al. 2009; Le et al. 2005; Marinelli et al. 2007; Richards et al. 2009; Richards et al. 2008; Simms et al. 2010), P rats in the present experiment also displayed significantly higher responding for ethanol following yohimbine treatment. Of particular interest, reinstatement was evident in rats that had been trained to self-administer ethanol using the sipper tube model. Typically, rats are trained on a fixed-ratio 3 in 30- or 60-minute sessions (Cippitelli et al. 2010; Le et al. 2009; Le et al. 2005; Marinelli et al. 2007; Richards et al. 2009; Richards et al. 2008; Simms et al. 2010) prior to extinction and reinstatement, but these findings show that reinstatement can be evident in rats trained to emit a single response requirement for

uninterrupted access to ethanol. Thus, in addition to replicating previous findings, the present experiment extends the utility of the sipper tube model.

In contrast to the findings from the operant self-administration experiment, yohimbine administered at the same pretreatment interval in rats given the same duration of access to ethanol decreased home cage drinking. One important methodological difference between these studies is that rats trained in the sipper tube model had a longer history with ethanol drinking (~15 sessions) than those given home cage limited access (4 sessions) prior to receiving yohimbine. It could be, therefore, that ethanol acquired aversive properties in the home cage project because ethanol access occurred soon after yohimbine treatment, and the rats had insufficient experience with ethanol to prevent this aversion from developing. In support of this theory, both yohimbine (Myers et al. 2005) and ethanol (Lester et al. 1970) have been shown to produce a conditioned taste aversion (CTA), but a diminished CTA to ethanol is seen in ethanol-experienced P rats (Stewart et al. 1991). These finding are consistent with the conditioned stimulus (CS) pre-exposure effect on the CTA, in which prior exposure to the appetitive CS (such as ethanol) later paired with an aversive US (such as yohimbine; File 1986) diminishes the development of a CTA (Best 1975; Domjan and Wilson 1972; Revusky and Bedarf 1967). To minimize the effects of a potential yohimbine-induced CTA to ethanol, rats in Phase II of Experiment 2 were treated with yohimbine 8- or 24-hours prior to ethanol access following a ten-day washout period. During the baseline drinking period in Phase II, differences between P rats previously injected with yohimbine and those injected with vehicle dissipated, but this effect persisted in HAD-1 rats. As such, the increases in ethanol intake as a function of yohimbine during Phase II in HAD-1 rats is confounded

by carryover effects from previous experience with yohimbine. However, this effect was evident in both the 8- and the 24-hour pretreatment groups, which had previously experienced yohimbine within the context of ethanol and water, respectively. Therefore, an ethanol CTA does not explain why HAD-1 rats in the vehicle and yohimbine groups showed differential baseline drinking in the 24-hour pretreatment experiment. During the post-injection period, drinking tended to decrease overall in both treatment groups, but HAD-1 rats injected with yohimbine maintained higher intakes compared to the other groups. In stress-naïve HAD-1 rats (Phase IIb), yohimbine given at the 8-hour pretreatment interval increased ethanol drinking compared to vehicle. Thus, it appears that yohimbine can have differential effects on altering drinking in HAD-1 rats depending on the timing of exposure.

Another factor that may have played a role in the differential effects of yohimbine on limited access ethanol drinking in the operant and home cage studies was that the former were run during the light cycle and the latter were run during the dark cycle. As nocturnal animals, rats would be expected to be more active during the dark portion of the light/dark cycle, and differences in arousal and activity could impact the effects of stress on alcohol drinking. Fluctuating corticosterone levels are critical in the regulation of circadian rhythms. In rats, corticosterone levels are highest near the onset of the dark cycle at which time they are more active, but lower at the onset of the light cycle (Droste et al. 2008; Ixart et al. 1977). In addition, ultradian rhythms of corticosterone secretion are evident in hourly pulses that are greater in magnitude during the dark cycle (Windle et al. 1998b). The degree to which corticosterone levels fluctuate in response to stress is affected by stress type as well as time of day during which stress is applied. For example,

forced swim stress caused greater increases in corticosterone release in rats that were more disruptive to the ultradian rhythms than exposure to a novel environment (Droste et al. 2008). Footshock, 2- and 6-hour immobilization, and forced swim stress all increased plasma corticosterone during the light cycle, but only 2-hour immobilization and forced swim stress increased corticosterone during the dark cycle (Retana-Marquez et al. 2003). Previous studies had also shown enhanced elevations in corticosterone during the light cycle after immobilization stress (Torrellas et al. 1981). Following chronic administration, footshock and forced swim stress elicited elevated corticosterone release during both phases, but immobilization stress elevated corticosterone levels during the light cycle only (Retana-Marquez et al. 2003). Exposure to acute noise stress elicits similar increases in corticosterone during both phases, but habituation of the stress response was seen after the second presentation of the stressor only during the light cycle (Atkinson et al. 2006). It appears, therefore, that rats show greater stress responses when stressed during the light cycle. While the effects of yohimbine on circadian-related stress responses have not been examined, norepinephrine and $\alpha 2$ adrenoreceptors are involved with the regulation of circadian/ultradian rhythms (Alam and Mallick 1994; Drijfhout et al. 1996; Grass et al. 1996; Mallick and Alam 1992; Mustanoja et al. 2000). Taken together, it is possible that yohimbine led to more consistent elevations in ethanol drinking in Experiment 1 than 2 due to enhanced responsiveness to stress during the light cycle.

The findings from the 24-hour pretreatment group in Experiment 2 suggest that yohimbine can affect the acquisition of limited access ethanol drinking. Unfortunately, this effect did not generalize to continuous, home cage access to ethanol. In Experiment 3, repeated yohimbine injection prior to or during the first two weeks of ethanol access did

not alter the development of drinking, despite a trend for decreased consumption in HAD-2 rats in the acquisition group. Few studies have examined the effects of stress exposure on the acquisition of alcohol drinking. In adult rodents, restraint and footshock stress failed to affect subsequent alcohol intake (Chester et al. 2006; Ng Cheong Ton et al. 1983), while social isolation facilitated drinking (Wolffgramm and Heyne 1991). Therefore, stress type could play an important role in the effects of stress on the acquisition of ethanol consumption. However, when animals are exposed to stress during adolescence, significant increases in later ethanol acquisition are seen (Chester et al. 2008; Schenk et al. 1990). Clearly, adolescence is a critical period to focus on when examining the development of alcohol drinking (DeWit et al. 2000; Grant and Dawson 1998; Spear 2000). As stress during adolescence has been shown to be associated with increased subsequent drinking behavior (Aseltine and Gore 2000; Pohorecky 1991; Schmid et al. 2010; Wagner 1993), it would be of interest to replicate Experiment 3 using a developmental model.

In Experiments 4 and 5, no significant differences were evident between groups of rats given continuous or intermittent ethanol access. However, in Experiment 4, an interaction between ethanol access pattern, cycle, and dose revealed that rats injected with vehicle and deprived of ethanol displayed consistently higher ethanol intakes compared to baseline, while rats given continuous access only showed significantly higher intakes on the final cycle compared to baseline. As such, it could be that intermittent ethanol access led to increases in ethanol drinking as a function of time that were due to an ADE, in contrast to simple increases in ethanol consumption due to acclimation. In Experiment 5, within-groups comparisons showed that rats consistently

drank more ethanol on the post-deprivation day compared to the pre-deprivation day, suggesting that while deprived rats were showing elevated drinking on the day when ethanol was returned, rats given continuous ethanol access are also showing this effect. One possible explanation for the lack of a robust ADE in Experiment 5 is that all rats were given a non-reinforced trial the day before ethanol was returned to rats in the intermittent access group. Thus, rats in the continuous access group could have also been showing deprivation-related increases in drinking and effectively masking between-group differences. In support of this hypothesis is the finding that significant day by access group interactions were evident at the second and third cycles, indicating that rats exposed to intermittent ethanol access showed greater differences in pre- and post-deprivation intake than those given continuous ethanol access. However, this relative increase in drinking was small and not pharmacologically relevant. When deprivation-related changes in drinking in the sipper tube model have been tested previously in outbred rats, no changes in drinking were evident (Samson and Chappell 2001). Thus, the moderate increases in drinking in alcohol-preferring rats suggest that selective breeding may play a role in deprivation-related binge drinking in this paradigm.

The lack of strong effects of ethanol deprivation on drinking in these experiments is in contrast to previous research that shows that P rats readily show the ADE in home cage (Gilpin et al. 2005; Rodd-Henricks et al. 2000b; Sinclair and Li 1989; Vengeliene et al. 2003) and operant self-administration paradigms (McKinzie et al. 1998b; Rodd et al. 2003). Consistent with these studies, rats in the present experiment had at least 6 weeks of ethanol access prior to ethanol deprivation, so it is not likely that ethanol experience played a role in the lack of an effect. Since deprivation periods of two weeks or greater

were used in previous studies, it is possible that the one-week deprivation cycles implemented in Experiments 4 and 5 may not have been sufficient to produce an ADE. Arguing against this possibility is the finding that P rats will show an ADE after only two days of ethanol deprivation (Bertholomey et al. 2011; Breese et al. 2004; Overstreet et al. 2007). In addition, while the ADE is more robust following 14- and 28-day deprivation periods, it is still evident after as few as 5 or 7 days of abstinence (Heyser et al. 1997). Further complicating the findings of the present experiment, the ADE is enhanced following multiple deprivation cycles (Heyser et al. 1997; Overstreet et al. 2007; Rodd et al. 2003; Rodd-Henricks et al. 2000a; Rodd-Henricks et al. 2000b) in terms of the magnitude and duration of the effect in P rats, and an ADE appears in HAD rats that do not normally show an ADE following a single deprivation cycle. Consistent with these studies, P rats tended to show greater drinking following deprivation compared to HAD-2 rats in the present experiment, although these effects were not exacerbated by multiple deprivation periods. However, in a recent study that implemented repeated 4-day cycles of ethanol access and deprivation, HAD-1 and -2 rats demonstrated a robust ADE, while P rats showed only a modest, nonsignificant increase in drinking (Bell et al. 2008). The findings of this study therefore demonstrate the transient nature of the ADE.

Despite the notion that abstinence from ethanol can be stressful and increases anxiety-like behavior (Baldwin et al. 1991; Becker 2000; Doremus et al. 2003; File et al. 1989; File et al. 1991; File et al. 1992; Holter et al. 1998; Knapp et al. 2004; Moy et al. 1997; Overstreet et al. 2004; Rassnick et al. 1993; Valdez et al. 2002; Valdez et al. 2004), yohimbine failed to significantly alter deprivation-related drinking. P rats in Experiment 4 tended to show an ADE and greater drinking following yohimbine, but no interaction

between these factors was evident, nor was there a significant main effect of yohimbine on drinking in general. The lack of an effect of yohimbine on continuous or intermittent operant self-administration in Experiment 5 is not entirely unexpected, since yohimbine was administered roughly 24 hours prior to ethanol reinstatement. This is consistent with the findings from P rats in the 24-hour pretreatment group in Experiment 2 (since HAD-1 rats likely were affected by yohimbine pre-exposure). However, neither acute yohimbine nor ethanol deprivation altered responding for ethanol during non-reinforced sessions in Experiment 5, suggesting that these factors did not increase the reinforcing properties of alcohol. Interactions between stress and alcohol deprivation in P rats given continuous ethanol access were evident in some studies (Breese et al. 2004; Overstreet et al. 2007) but not others (Bertholomey et al. 2011). While ethanol dependence has been shown in P rats given a similar amount of history with ethanol drinking (i.e., 6 weeks; Kampov-Polevoy et al. 2000), presence of withdrawal symptoms during deprivation was not assessed in the present experiment. Therefore, it is not known whether rats in the present experiment were experiencing heightened stress as a function of alcohol withdrawal and a greater sensitivity to yohimbine. When tested in the EPM, experience with intermittent ethanol exposure and with yohimbine failed to alter anxiety-like behavior, indicating that neither repeated ethanol deprivation nor repeated stress exposure had significant anxiogenic effects. Since intermittent ethanol access did not produce a robust ADE or increase appetitive responding, and yohimbine did not alter either of these responses, the findings from this experiment do not support the hypothesis that alcohol deprivation and stress promote “craving” (Koob 2000; Le and Shaham 2002; Li 2000; Mason et al. 2009; Rodd et al. 2004b; Shaham et al. 2003; Weiss 2005).

While EPM behavior was not affected when rats were six days into their third ethanol deprivation and/or 48 hours following yohimbine injection, acute injection of yohimbine produced an anxiogenic effect in Experiment 2, consistent with previous results (Guy and Gardner 1985; Johnston and File 1989; Pellow et al. 1985a). Yohimbine did not alter locomotor activity as measured by total arm entries in the EPM, or as measured by appetitive responding during extinction, inactive lever pressing, or licks on the sipper tube in the operant experiments. Self-administered ethanol did not affect EPM behavior compared to self-administered water, nor did ethanol block the anxiogenic effect of yohimbine. However, given that yohimbine suppressed drinking in this experiment, the ability of ethanol to block yohimbine-related anxiogenesis might have been prevented. Given this possibility, follow-up analyses revealed that self-administered ethanol was positively correlated with number of entries in the open arms, which suggests that ethanol was having an anxiolytic effect.

Perhaps the most interesting differences revealed in the present study were between P and HAD rats. Due to availability issues, P rats were compared to HAD-1 and to HAD-2 rats in different experiments. However, it was consistently found that P rats were more active and less anxious than both replicate HAD lines. Since HAD-1 and HAD-2 rats were not run in the same experiments it is not possible to statistically compare the replicates in terms of EPM behavior; however, looking at the values from these experiments, HAD-1 rats spent relatively more time in the closed arms and less time in the open arms compared to HAD-2 rats (with considerable consistency in P rats tested in the EPM in each of these experiments). This suggests that HAD-1 rats are more anxious than HAD-2 rats. However, findings from a recent study (Roman et al. 2011)

indicate that the behavioral profiles of P, HAD-1, and HAD-2 are remarkably similar when tested in the multivariate concentric square field, open field, and EPM. Still, in the present experiment, P rats were also shown to be more active than HAD-2 rats in their propensity to lick more for ethanol when it was available in the operant chambers and to press more for ethanol during non-reinforced sessions, so line differences in activity are confirmed using several measures. However, differences in lick pattern are likely due to the P rats' larger size. While P and HAD-2 rats drank similar amounts of ethanol when corrected for body weight, the larger P rats needed to drink a greater volume to reach the same gram per kilogram intake. In addition, while P rats pressed more on the active lever during non-reinforced sessions, this effect diminished with repeated sessions, indicating a reduction in motivation to seek ethanol based on learning rather than simply showing increases in locomotor activity.

Yohimbine tended to have a suppressive effect on ethanol drinking in HAD rats when given acutely during limited home cage access. However, when yohimbine was administered 8 hours before limited access or during continuous home cage access, ethanol drinking in HAD rats tended to increase. In contrast, yohimbine more consistently elevated ethanol drinking in P rats, as evidenced by the dose-response and reinstatement studies and to some extent the home cage maintenance and deprivation study. While yohimbine exerted a suppressive effect on limited access drinking in Experiment 2 that was consistent with a taste aversion, this effect extinguished in P rats during the washout and re-acquisition periods, while HAD-1 rats continued to exhibit low drinking. Interestingly, HAD rats drank more water overall than P rats, and yohimbine potentiated this effect, while ethanol intake was consistent between the lines. It is

possible that yohimbine served as a dipsogen in HAD rats when given access to only water acutely or to both ethanol and water chronically, rendering the effects of yohimbine on ethanol drinking in HAD rats difficult to interpret.

The effects of yohimbine on ethanol-related behaviors in these lines could be related to inherent differences in key neurotransmitter systems in the selected lines. For example, P rats have lower levels of metencephalic norepinephrine than NP rats (Murphy et al. 1982), and both P and HAD rats have lower tomoxetine binding sites, indicative of a downregulation of the noradrenergic transporter (Hwang et al. 2000). Consistent with this, desipramine, a noradrenergic transporter inhibitor, significantly reduces alcohol drinking in P rats (McBride et al. 1988; Murphy et al. 1985). However, P and NP rats do not differ in expression of α1, α2, or β-adrenergic receptors (Wong et al., 1998). Compared to NP rats, P rats also have lower amygdalar levels of "anti-stress" peptides such as NPY (Ehlers et al. 1998; Hwang et al. 1999a; Suzuki et al. 2004) and BDNF (Prakash et al. 2008), but higher levels of stress-related peptides such as CRF (Ehlers et al. 1992; Hwang et al. 2004). While HAD rats have lower levels of NPY in the central nucleus of the amygdala than LAD rats (Hwang et al. 1999b), no differences in CRF content are evident between these lines (Hwang et al. 2004). Recently, it has also been shown that prazosin, an α1 receptor antagonist, reduces ethanol drinking in P rats (Rasmussen et al. 2009). Given these neurochemical findings in P and HAD rats, along with suggestions of an "anxious" profile of the P rat, one might predict that P rats would be more sensitive to yohimbine than HAD rats. However, whether sensitivity to yohimbine stress would manifest itself as behavioral inhibition (e.g., HAD rats) or as an increased propensity to drink alcohol (e.g., P rats) is not easily identified. Yohimbine has

been shown to decrease or have no effect on locomotor activity within the dose range and route of administration used in the present study (Bowes et al. 1992; Chopin et al. 1986; Mueller et al. 2009), so yohimbine may have had a greater impact in HAD rats exhibiting lower levels of activity than P rats. However, no main effects of yohimbine were found for activity-related behaviors such as total arm entries or lever presses, nor were interactions between yohimbine and line found.

Recent reviews have recommended that better consilience between alcohol-related phenotypes in humans and in rodents is needed (Crabbe 2010; Sher et al. 2010). In light of this, the line differences evident in the present study suggest that P and HAD rats might represent different typologies proposed to exist in clinical populations of alcoholics. In order to account for the considerable heterogeneity among behavioral profiles of alcoholics, several investigators have developed typologies of alcoholism (Babor et al. 1992; Cardoso et al. 2006; Cloninger 1987; Hauser and Rybakowski 1997; Jellinek 1960; Lesch et al. 1988; Moss et al. 2007; Schuckit et al. 1985; Windle and Scheidt 2004). However, of these, Cloninger's typology is the most widely recognized. Cloninger's Type I alcoholic – the "anxious" type – is characterized by having later onset of dependence, ability to abstain from ethanol (low ethanol seeking), low social problems, loss of control, guilt, low novelty seeking, high harm avoidance, and high reward dependence. In contrast, Cloninger's Type II alcoholic – the "antisocial" type – is characterized by an earlier onset, familial (genetic) contribution, inability to abstain (high ethanol seeking), social/behavioral problems, no loss of control, less guilt, high novelty seeking, low harm avoidance, and low reward dependence (Cloninger 1987). P and HAD rats share many of these characteristics; for example, both lines clearly implicate genetics

in their propensity to drink alcohol, and both show an early onset to ethanol drinking and sensitivity to the stimulating effects of ethanol in adolescence (McKinzie et al. 1998a; Rodd et al. 2004a), which are consistent with a Type II categorization. However, P and HAD rats also differ in other significant ways. For example, P rats endorse the behaviors of a Type II alcoholic in terms of high ethanol seeking, as evidenced by operant self-administration (Ciccocioppo et al. 2001; Czachowski and Samson 2002; Files et al. 1998), and loss of control, as evidenced by robust expression of the ADE (McKinzie et al. 1998b; Rodd et al. 2003; Rodd-Henricks et al. 2001; Rodd-Henricks et al. 2000b; Sinclair and Li 1989) and greater behavioral disinhibition (Blankenship et al. 1998; Steinmetz et al. 2000), while HAD rats do not show these behaviors (Blankenship et al. 2000; Czachowski and Samson 2002; Files et al. 1998; Oster et al. 2006; Rodd-Henricks et al. 2000a). Consistent with this categorization, P rats in the present experiment demonstrated reinstatement of ethanol seeking (HAD rats were not tested) in Experiment 1, and displayed greater licks and non-reinforced lever presses, and a more pronounced ADE than HAD rats in Experiments 4-5. However, HAD rats also endorse the behaviors of a Type II alcoholic in terms of low harm avoidance, evidenced by deficits in acquiring avoidance learning (Blankenship et al. 2000; Rorick et al. 2003), in contrast to P rats that readily learn this task (Blankenship et al. 1998; Stewart et al. 1993). While HAD rats display elevated novelty seeking (Nowak et al. 2000) and exploratory behavior (HAD-1; Roman et al., 2011), P rats have been shown to have both lower (McMillen et al. 1998) and higher (Nowak et al., 2000) levels of novelty seeking. HAD-1 rats also tend to be less risk-taking and HAD-2 rats are less risk assessing and less shelter-seeking, but these differences are confounded with locomotor activity in P rats (Roman et al. 2011). HAD

rats demonstrate steeper delay discounting compared to LAD rats, indicative of greater impulsivity (Wilhelm and Mitchell 2008). Although using a different task, P rats were shown to be less impulsive than Wistar rats on a differential reinforcement of low rates (DRL) procedure (McMillen et al., 1998). Based on these findings, P and HAD rats each show characteristics in common with both Type I and II alcoholics. However, line differences are evident in terms of anxiety-like behavior and stress-related drinking. Currently, more studies support the notion that the P line is not a model of an "anxious alcoholic" (Godfrey et al. 1997; Overstreet et al. 1997; Roman et al. 2011; Viglinskaya et al. 1995), consistent with the findings of the present experiment. However, compared to LAD rats, HAD rats have heretofore not been shown to be "anxious" (Badia-Elder et al. 2003; Hwang et al. 2004; Roman et al. 2011). The findings from the present experiment are more consistent with the HAD replicate lines representing the "anxious" Type I alcoholic. The extent to which stress-related increases in ethanol drinking endorse either type is not easy to determine, since increases in drinking as a function of stress could represent a sensitivity for the need for tension-reduction, loss of control, or greater ethanol seeking, while decreases in drinking could represent a sensitivity to harm avoidance and anxiety-related suppression of activity. Despite these difficulties, it is clear from the findings of this study that the P line differs from the HAD replicate lines, and these differences could be capitalized upon in further studies exploring the motivation to seek and consume ethanol.

An advantage to the approach used in the present study was the use of different paradigms to try to arrive at a consensus on the effects of stress and ethanol deprivation on drinking and other behaviors. There was reasonable consistency between ethanol

intakes when given 20-minute operant access to ethanol and 20-minute home cage access to ethanol, although intakes tended to be higher in the home cage. These differences could arise from methodological differences between the two paradigms. First, since rats had to perform a response requirement for limited access to the sipper tube in the operant chambers, ethanol intake is at least partially dependent on motivation to make said response. Second, the sipper tubes used in the operant experiments were fitted with a drinking spout that had double ball-bearings to prevent spillage. This might also contribute to the greater correlations between ethanol intake and BAC in the operant studies. Third, rats were drinking during the dark cycle in the home cage experiments, but during the light cycle during the operant experiments. As such, greater consumption in the home cage could be due to increased general activity during the dark cycle in these nocturnal animals. However, patterns of intake were consistent when rats were given intermittent access to ethanol in the home cage or the operant chamber. While no strong deprivation effects were evident using either paradigm, rats consistently showed greater drinking when ethanol was returned compared to the final day of drinking before deprivation. These findings are consistent with those of Green and Grahame (2008) who concluded that free-choice ethanol drinking and ethanol-reinforced operant self-administration were positively correlated in animal models in which genes and alcohol drinking had been manipulated (such as P and HAD rats).

One factor that clearly had a strong affect in the present study was cohort. Since testing the number of animals needed in each experiment at once was not feasible, rats were run in smaller cohorts. While every effort was taken to maintain consistency between these groups, cohort differences exerted significant effects on various behaviors

in the home cage drinking studies. In fact, in some cases, effects seen in the overall analysis were generated solely by one cohort, although separate analyses for each cohort were underpowered. The reason for these differences could be genetic, environmental, or a gene by environment interaction. While genetic drift could possibly play a role in inconsistencies in findings within the same selected line in the same behavior across time, it is not likely that within the same generation and within a two-year period such changes would occur. Considerable variability has been demonstrated with inbred mouse lines have been tested in different facilities, despite extraordinary efforts to keep the procedures as similar as possible (Crabbe et al. 1999; Wahlsten et al. 2003), which emphasizes the impact of gene by environment interactions. However, studies that have tested lines of rats selectively bred for anxiety-like behavior (Salome et al. 2002) and alcohol drinking (Roman and Colombo 2009; Roman et al. 2007; Roman et al. 2011) in different countries found remarkable consistency in behavioral profiles. Further, the influence of environment in the mouse studies was primarily due to testing in different facilities; thus, such strong cohort effects on projects run in the same facility with the same principal experimenters are unexpected in the present study. It should be noted, however, that effects of cohort were present in Experiments 2-4, which were conducted in a different facility than Experiments 1 and 5, in which cohort failed to significantly alter behavior. Further, while the pairs of cohorts in Experiments 2-4 were run within 2-5 months of one another, the three cohorts tested in Experiment 5 spanned over a year. It is clear, therefore, that environment can cause extreme differences in behavior and should be controlled for as much as possible.

Given the administration pattern of both yohimbine and ethanol, it is likely that each of these drugs had individual and combined effects on neural systems. For example, yohimbine enhances feelings of intoxication and anxiety in abstinent alcoholics when co-administered with ethanol (McDougle et al. 1995). However, yohimbine failed to substitute for ethanol using subjective ratings, indicating that the effects of yohimbine are discriminately different from those of alcohol (Krystal et al. 1994). In addition, repeated administration of yohimbine and ethanol both alter HPA axis activity. As discussed previously, yohimbine stimulates norepinephrine (McDougle et al. 1995; Tanaka et al. 2000; Tjurmina et al. 1999) and glucocorticoid release (Gurguis et al. 1997; Marinelli et al. 2007; Mattila et al. 1988; Suemaru et al. 1989; Vythilingam et al. 2000). Repeated yohimbine administration blunts responses to an acute yohimbine challenge in its effects on plasma catecholamine levels in humans (Galitzky et al. 1990), and in rodents, reduces conditioned suppression (Davidson and Lucki 1987), minimizes yohimbine- (but not footshock-) induced reinstatement of cocaine seeking (Kupferschmidt et al. 2009), and reduces morphine withdrawal symptoms (El-Kadi and Sharif 1997). These findings suggest a habituation to the effects of yohimbine, and that prior experience with stress reduces its efficacy in its ability to act as a stressor later. However, sensitization to the locomotor activating effects of yohimbine was seen after ten days of daily 2 mg/kg yohimbine injection (Schroeder et al. 2003), indicating that some of the effects of yohimbine sensitize after chronic administration. The yohimbine injection procedure used in the present study was intended to reflect a sub-chronic dosing of stress that was an intermediary between acute and chronic exposure. As yohimbine was able to elicit anxiety-like behavior in the EPM following the sixth injection, it does not appear that rats

in the present experiment habituated to the stress effects of yohimbine. In addition, prior experience with yohimbine during the self-administration portion of Experiment 1 did not reduce the efficacy of yohimbine to reinstate ethanol seeking after two weeks of yohimbine-free sessions. However, while yohimbine increased drinking in rats maintained on continuous ethanol access during the first cycle, this effect appeared to dissipate, potentially signaling habituation to yohimbine's effects on ethanol drinking. Chronic exposure to ethanol also alters stress pathways by increasing ACTH and glucocorticoids (Adinoff et al. 1991; Adinoff et al. 2003; Keedwell et al. 2001; Mendelson et al. 1971; Mendelson and Stein 1966) but producing blunted cortisol responses to stress in humans (Bernardy et al. 1996; Costa et al. 1996; Errico et al. 1993; Lovallo 2006; Lovallo et al. 2000; Margraf et al. 1967; von Bardeleben et al. 1989). Blunted corticosterone responses to ethanol (Lee and Rivier 2003; Richardson et al. 2008; Rivier and Lee 2001) and to stress (Rivier et al. 1990) following chronic ethanol exposure is also found in rats. Thus, stress reactivity appears to be reduced following chronic stress or ethanol exposure, and could account for the inconsistent effects of stress on drinking across the present experiments.

Despite the lack of strong interactions between stress and ethanol deprivation in the present study, there is a vast literature that implicates these two factors, with specific focus on the CRF/H system. Primarily, the CRF system is implicated in studies that target alterations in brain stress systems as a function of alcohol dependence and withdrawal. For example, withdrawal from ethanol increases CRF release and CRH-1 receptor transcripts and mRNA in the amygdala (Merlo Pich et al. 1995; Olive et al. 2002; Weiss et al. 2001; Zorrilla et al. 2001) and the anterior pituitary (Zhou et al. 2010), an effect that

is reversed by subsequent ethanol exposure (Olive et al., 2002). Ethanol withdrawal-related increases in anxiety-like behavior are blocked by CRF-1 receptor antagonists (Baldwin et al. 1991; Breese et al. 2004; Overstreet et al. 2004; Valdez et al. 2002). CRF-1 antagonists also block stress-related anxiety (Breese et al., 2005), behavioral inhibition (Heinrichs et al. 1994; Sommer et al. 2008), and increases in ACTH (Rivier et al. 2003). However, CRF can also be modified by more acute/binge-like administration of ethanol. Acute ethanol administration increases CRF mRNA and gene transcription (Li et al. 2005), CRF and corticosterone levels (Zhou et al. 2000) and CRF-1 receptor mRNA (Lee and Rivier 1997). CRF antagonists block stress-induced reinstatement of ethanol seeking while typically having no effects alone (Le et al. 2000; Liu and Weiss 2003; Marinelli et al. 2007). Gene expression of CRF and CRF-1 receptors also interact with ethanol. For example, mice that overexpress CRF are more anxious, and this anxiety-like behavior is reversed by CRF-1 receptor antagonists (Stenzel-Poore et al. 1994). Paradoxically, CRF-deficient mice drink twice as much ethanol as their wildtype counterparts, do not show ethanol-related increases in locomotor activity, and show a conditioned place preference at moderate (3.0 mg/kg) doses of ethanol (Olive et al. 2003). Other rats selectively bred for high ethanol intake (Marchigian Sardinian Alcohol Preferring Rats) display upregulated *CRHR1* transcript, which may confer the sensitivity to the effects of a selective CRH-1 receptor antagonist on attenuating operant ethanol self-administration and reinstatement of ethanol seeking not apparent in outbred rats (Hansson et al. 2006). Non-human primates that carry a polymorphism in the CRH promotor consumed more ethanol and display greater behavioral and endocrine responses to social stress (Barr et al. 2009). In humans, the polymorphisms in the *CRHR1* gene are associated with greater

stress-related alcohol consumption (Blomeyer et al. 2008; Schmid et al. 2010), and P3 amplitude, a proposed endophenotypic marker for alcoholism (Chen et al. 2010). In general, an inverse relationship between CRF and stress-related alcohol drinking and anxiety suggest that CRF1 receptor antagonists may prove to be useful pharmacotherapies (Lowery and Thiele 2010). As such, it would be of interest in future studies to explore the role of the CRF system in stress-related ethanol seeking and consuming in P, HAD-1, and HAD-2 rats.

The results of the present study indicate that P rats show greater motivation to seek and consume ethanol basally and in response to stress and ethanol deprivation compared to HAD rats. This difference may be due to reduced anxiety-like behavior and greater activity in the P rats, although the degree to which stress should promote ethanol seeking and self-administration based on negative reinforcement or prevent it based on behavioral inhibition/freezing is difficult to determine. Overall, yohimbine proved to be an effective stressor as it increased anxiety-like behavior and reinstated ethanol seeking, consistent with previous research (Chopin et al. 1986; Cippitelli et al. 2010; File 1986; Funk et al. 2006; Le et al. 2009; Le et al. 2005; Marinelli et al. 2007; Pellow et al. 1985a; Pellow et al. 1985b). However, acute ethanol drinking and experience with intermittent exposure to stress and ethanol deprivation did not alter anxiety-like behavior. Future research that systematically varies stress application imposes more prolonged alcohol deprivation periods may provide clearer evidence for an interaction between stress and ethanol drinking and relapse. In addition, taking a developmental approach to examining the effects of stress on the acquisition of ethanol self-administration might reveal more pronounced effects. Also, as the CRF system has been highly implicated on the

interaction between stress and relapse, further examination of this system and its effects on ethanol seeking and self-administration in P and HAD rats could provide more evidence for these lines of rats representing difference sensitivities to the effects of stress on alcohol-related behaviors. Finally, determining the efficacy of stress- and anxiety-reducing compounds on stress-related drinking in different models is an important step in translating potential therapeutic targets into the clinical population of alcoholics. Stress remains an important factor in the development of alcohol drinking and should be targeted in subsequent studies examining the mechanisms of promoting and preventing alcohol dependence.

TABLES

Table 1. Summary of the direction of the effects of yohimbine on ethanol drinking.

	LINE	
PARADIGM		
	P	**HAD**
Limited Access		
Home cage		
~ 15-min Pre-Tx	↓	↓
~ 8-hr Pre-Tx	↔	↑[a]
~ 24-hr Pre-Tx	↔	↑[a]
~ 8-hr Pre-Tx (naïve)	N/A	↑
Operant		
~ Dose-Response	↑[b]	N/A
~ Post-Deprivation	↔	↔
Lever Presses		
Reinstatement	↑	N/A
Appetitive Session	↔	↔
Continuous Access		
Pretreatment	↔	↔
Acquisition	↔	↓[c]
Maintenance	↔	↔
Post-Deprivation	↑	↑

a = may be due to carry-over effects
b = at the 0.625 and 1.25 mg/kg doses
c = trend ($p = 0.08$)

FIGURES

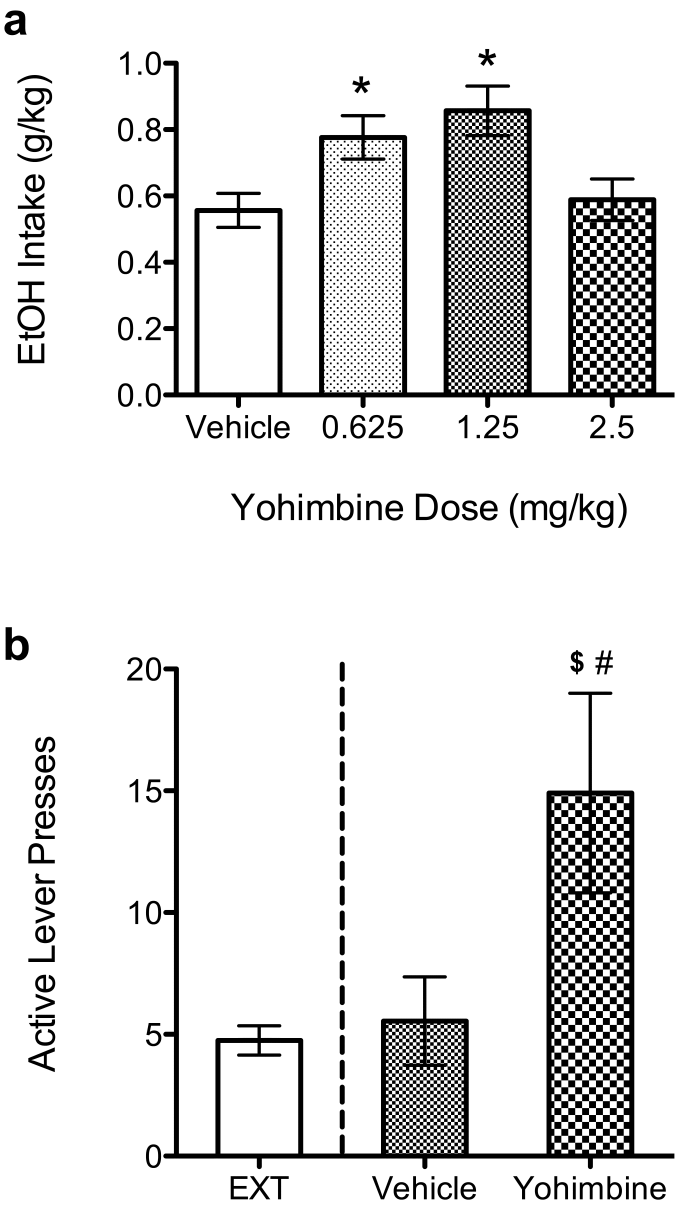

Figure 1. Ethanol self-administration (a) and reinstatement to ethanol seeking (b) following yohimbine injection in P rats. Ethanol intake was increased following injection of the 0.625 and 1.25 mg/kg doses of yohimbine. Yohimbine (1.25 mg/kg) elicited more lever presses compared to vehicle and to extinction responding. * $p < 0.05$ vs. vehicle and 2.5 mg/kg; $^{\#}$ $p < 0.05$ vs. vehicle; $^{\$}$ $p < 0.05$ vs. EXT

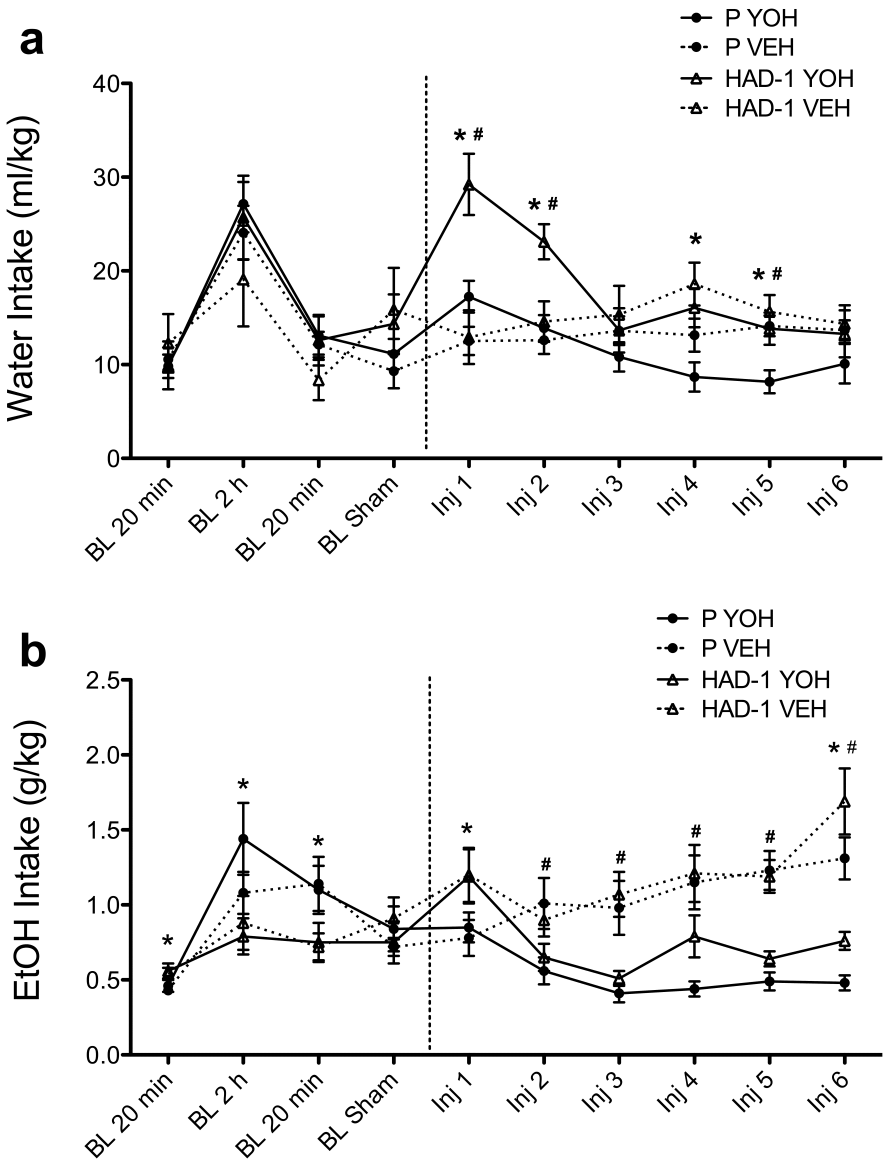

Figure 2. Water (a) and ethanol (b) intake during 20-minute access sessions 15 minutes following yohimbine or vehicle injection in P and HAD-1 rats. Yohimbine increased water drinking compared to vehicle, and HAD-1 rats drank more water than P rats. Yohimbine decreased ethanol drinking compared to vehicle, but P rats drank more ethanol during baseline and HAD-1 rats drank more ethanol during injections. *$p < 0.05$ P vs. HAD-1; $^{\#}$ $p < 0.05$ yohimbine vs. vehicle

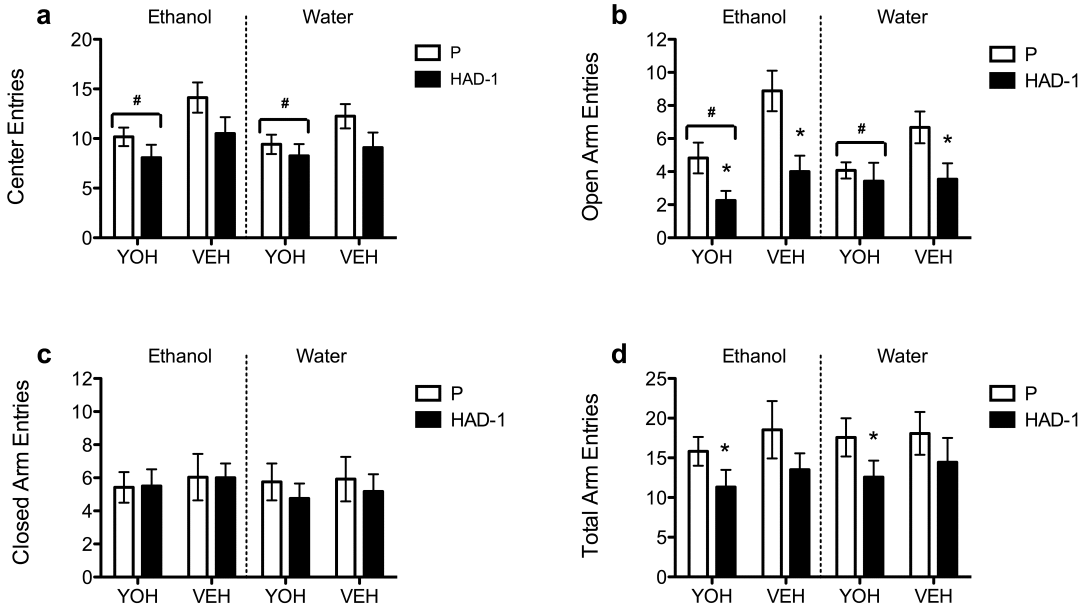

Figure 3. Number of entries in the center (a), open arms (b), and closed arms (c), and total arm entries (d) in the EPM in P and HAD-1 rats given ethanol or water access and injected with yohimbine or vehicle. P rats had greater center, open and total arm entries and spent more time on the open arms than HAD-1 rats. Rats injected with yohimbine entered the center and the open arms less than those injected with vehicle. *$p < 0.05$ P vs. HAD-1; $^{\#}p < 0.05$ yohimbine vs. vehicle

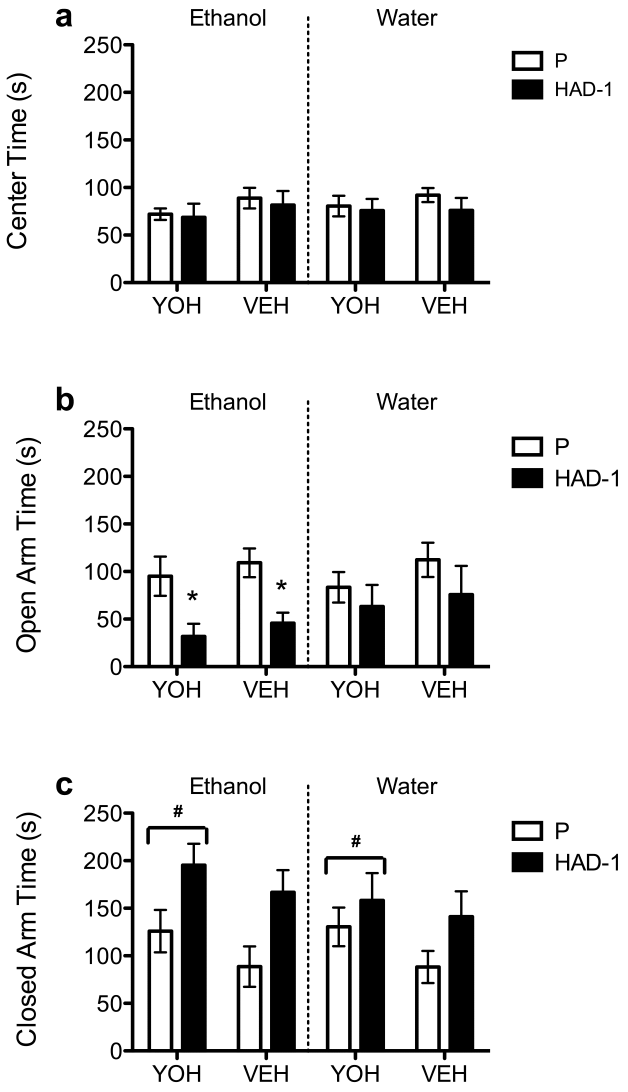

Figure 4. Time spent in the center (a), open arms (b), and closed arms (c) in the EPM in P and HAD-1 rats given ethanol or water access and injected with yohimbine or vehicle. P rats spent more time on the open arms than HAD-1 rats, while HAD-1 rats spent more time in the closed arms than P rats. Rats injected with yohimbine spent more time in the closed arms. *$p < 0.05$ P vs. HAD-1; $^{\#}$ $p < 0.05$ yohimbine vs. vehicle

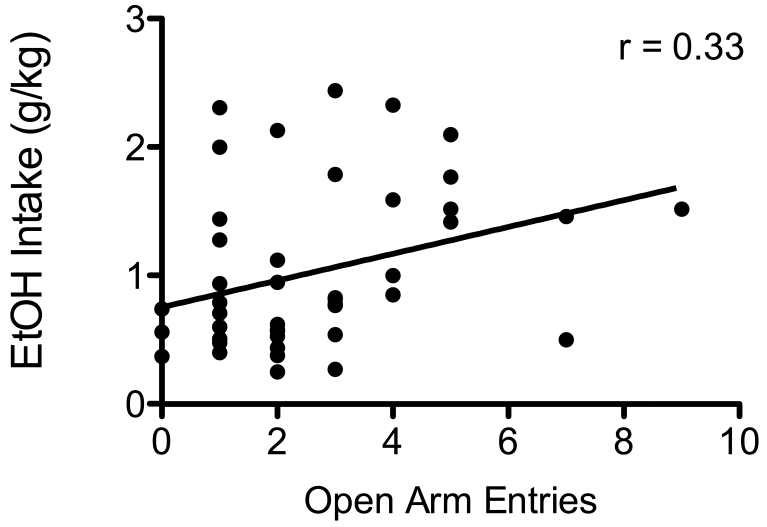

Figure 5. Correlation between ethanol intake (g/kg) and open arm entries in the EPM on the final day of injection/access. A significant positive correlation was found between ethanol intake and open arm entries.

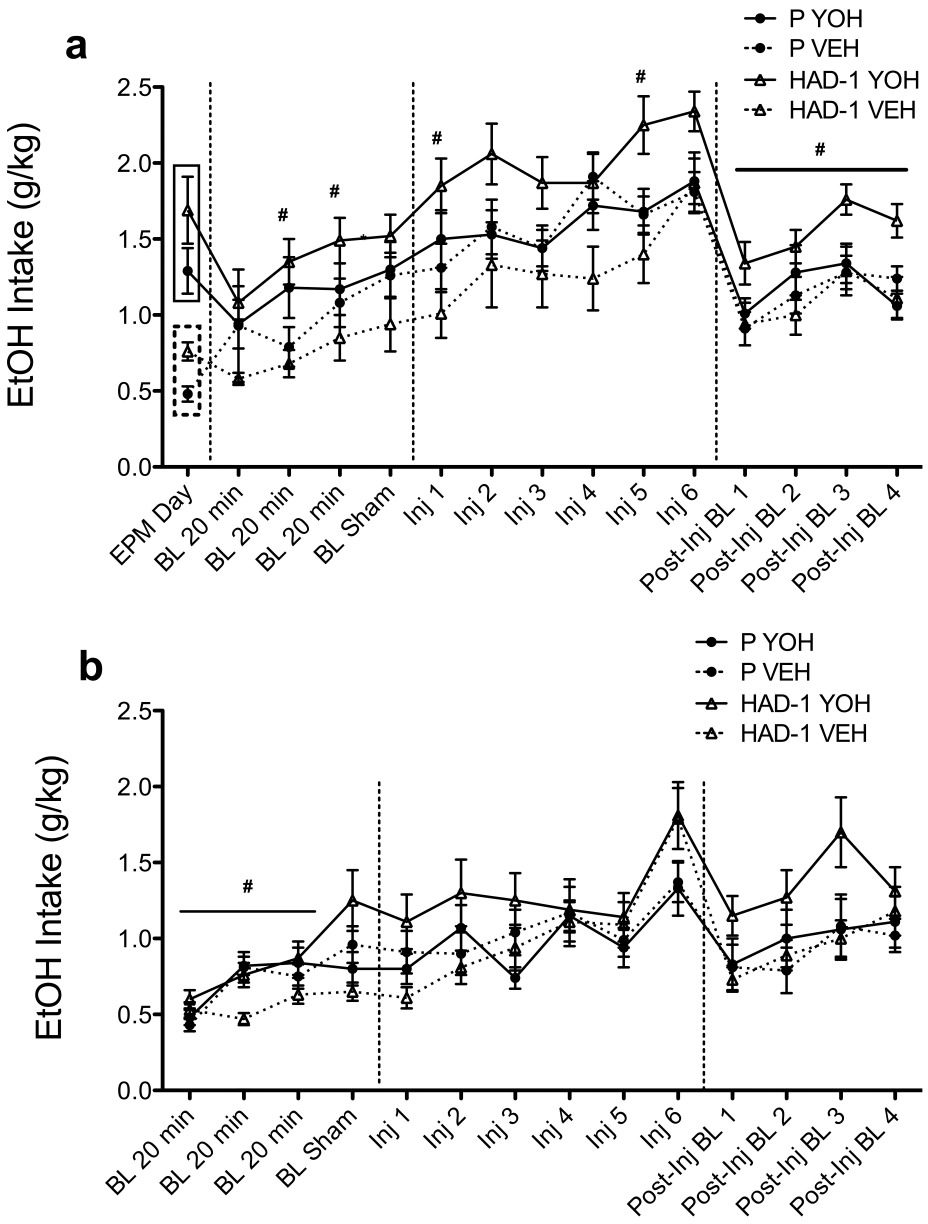

Figure 6. Ethanol intake in P and HAD-1 rats injected with yohimbine or vehicle 8 (a) or 24 hours (b) prior to 20-minute ethanol access. In both groups, rats injected with yohimbine drank more than those injected with vehicle. This difference was greater in HAD-1 rats in the 8-hour pretreatment group, and HAD-1 rats tended to drink more than P rats in the 24-hour pretreatment group. *$p < 0.05$ P vs. HAD-1; $^{\#}$ $p < 0.05$ yohimbine vs. vehicle

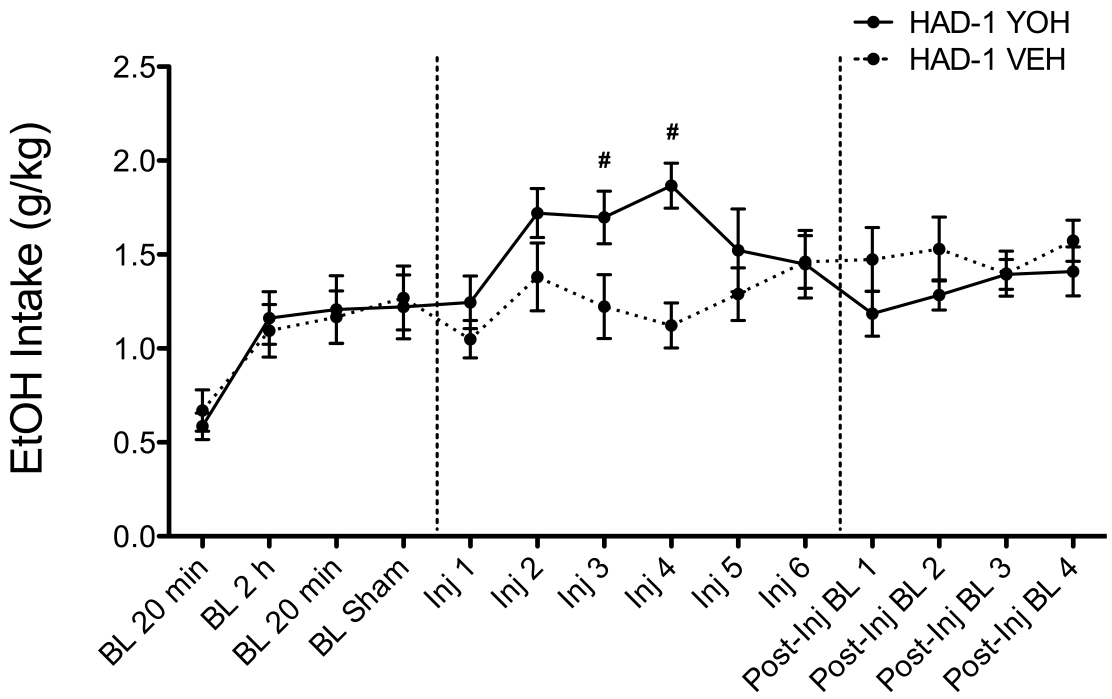

Figure 7. Ethanol intake in HAD-1 rats injected with yohimbine or vehicle 8 hours prior to 20-minute ethanol access. Yohimbine increased ethanol intake relative to vehicle. $^{\#}$ $p <$ 0.05 yohimbine vs. vehicle

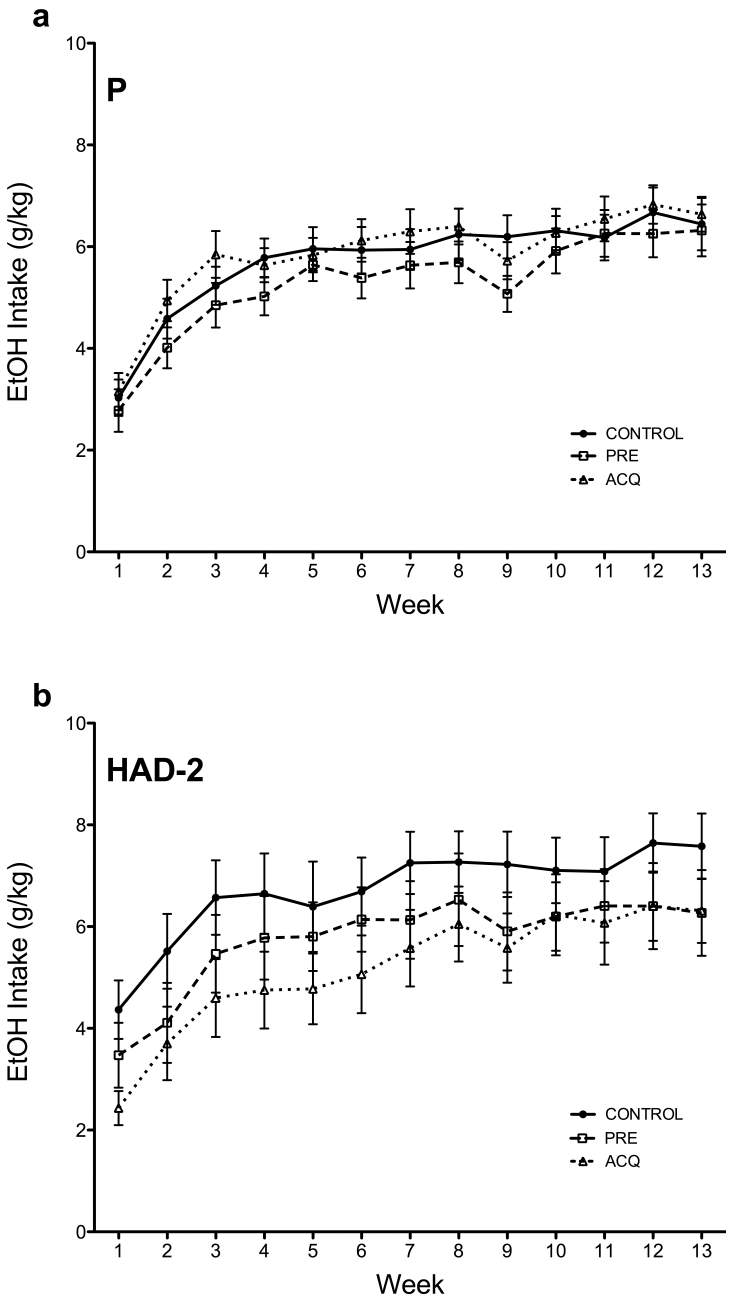

Figure 8. Ethanol intake averaged across week in P (a) and HAD-2 (b) rats injected with vehicle (CONTROL) or with yohimbine prior to ethanol access (PRE) or during the first two weeks of ethanol access (ACQ). Yohimbine treatment failed to significantly alter ethanol intake in either line.

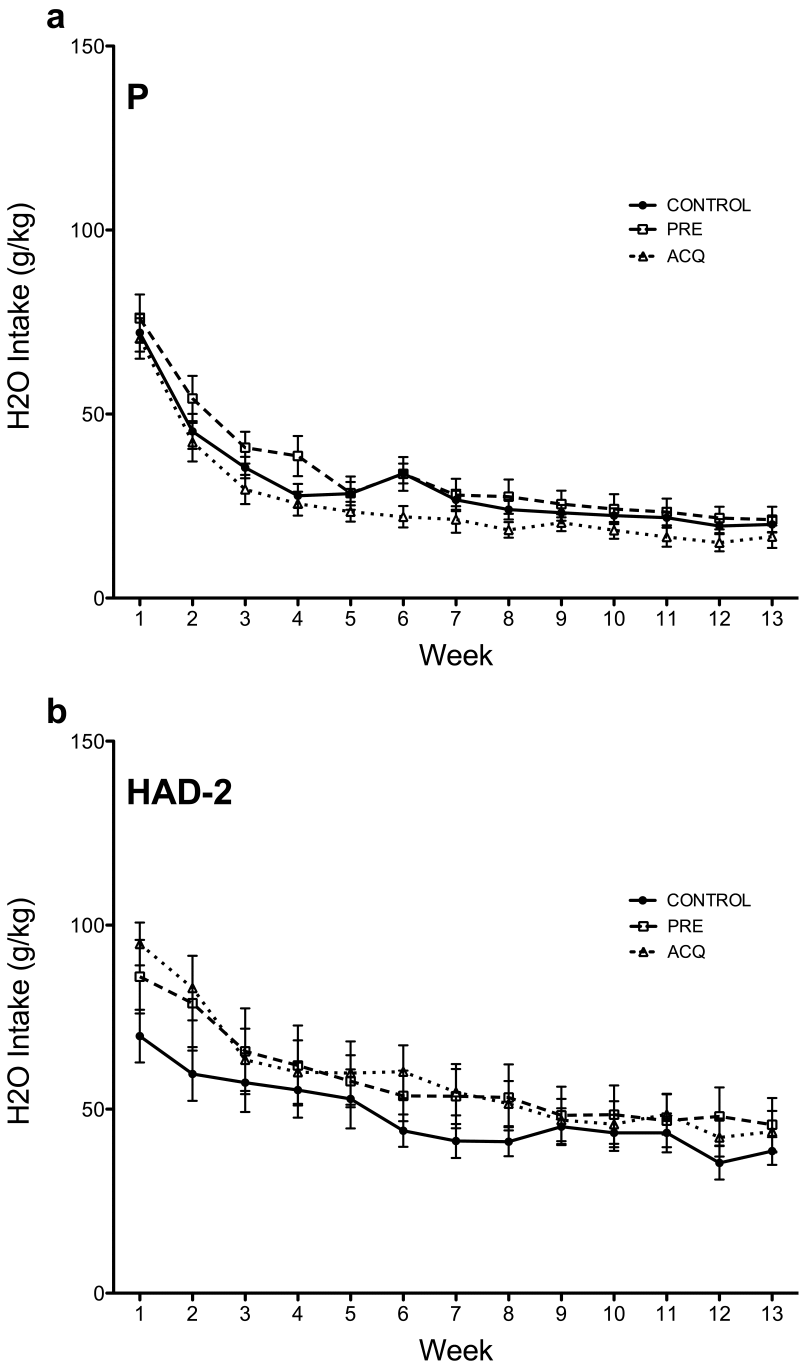

Figure 9. Water intake averaged across week in P (a) and HAD-2 (b) rats injected with vehicle (CONTROL) or with yohimbine prior to ethanol access (PRE) or during the first two weeks of ethanol access (ACQ). Yohimbine treatment failed to significantly alter water intake in either line.

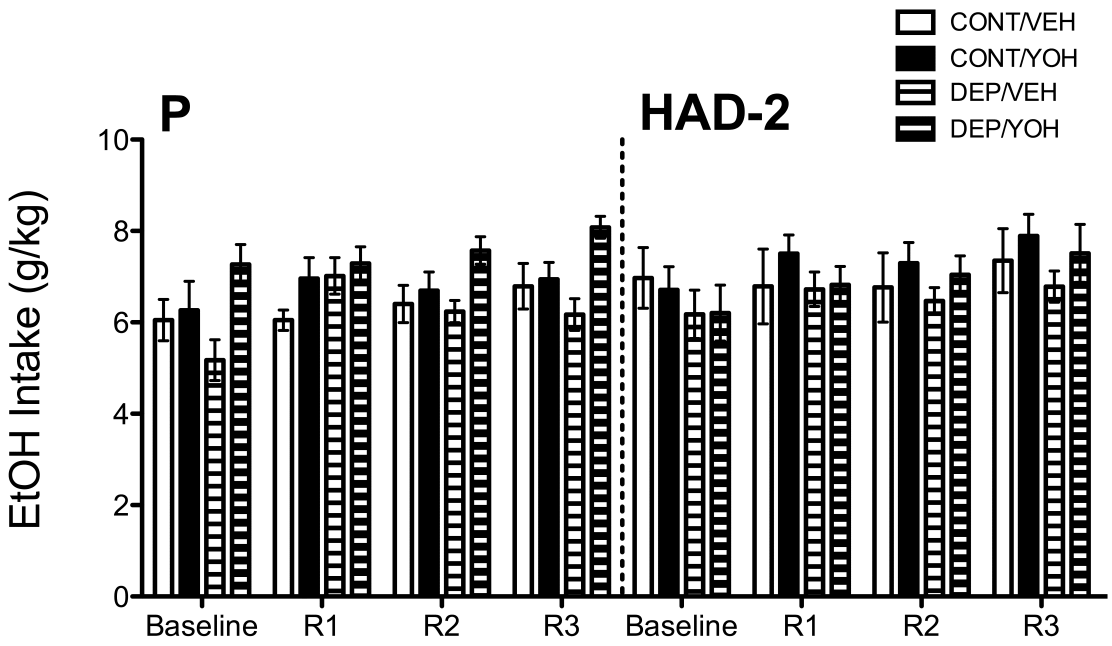

Figure 10. Ethanol intake in P and HAD-2 rats given either continuous or intermittent ethanol access and were injected with either yohimbine or vehicle over three cycles. Rats drank more ethanol during each reinstatement day compared to baseline and the greatest amount of drinking occurred on the final reinstatement day. Overall, rats injected with yohimbine drank more ethanol than those injected with vehicle.

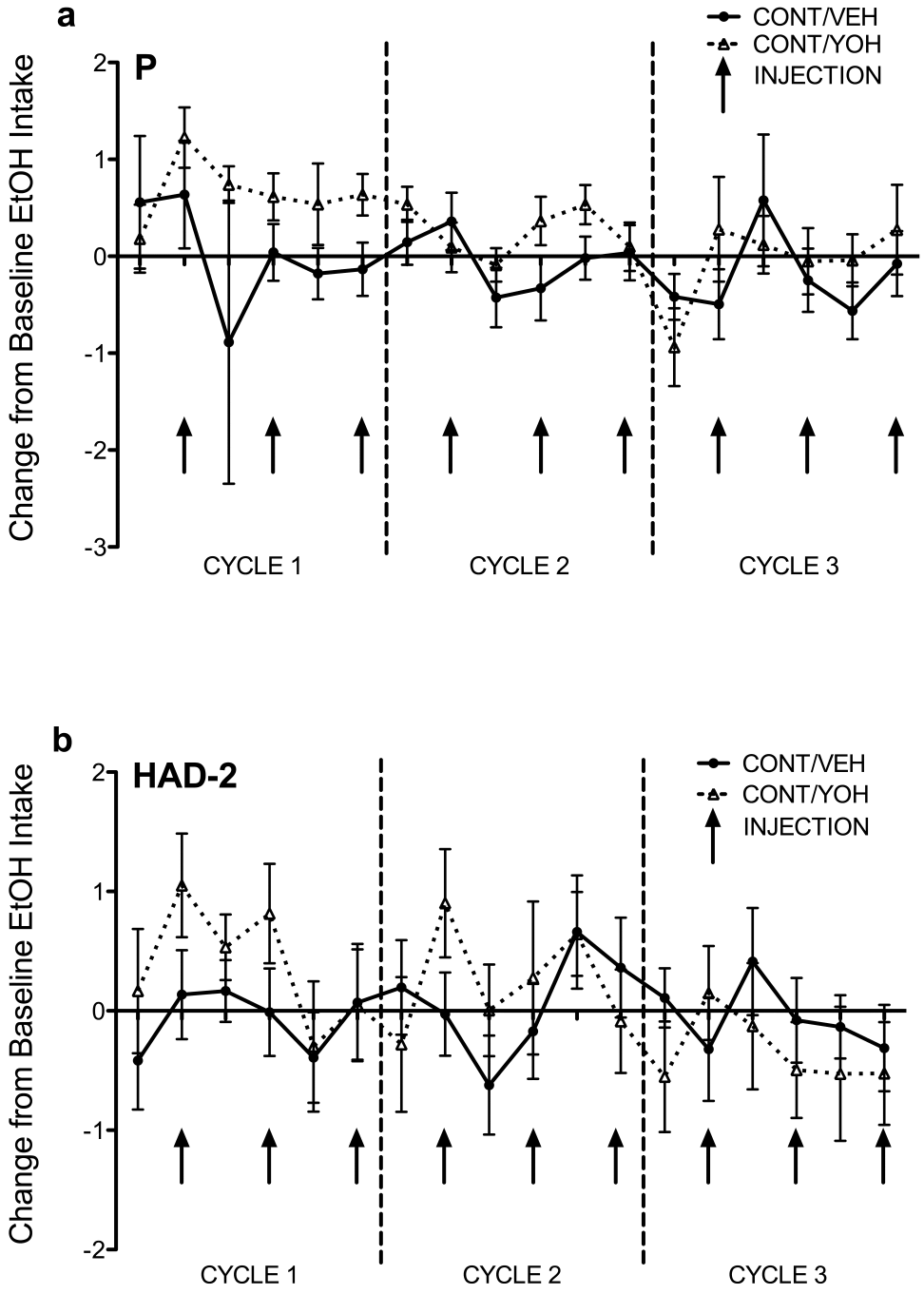

Figure 11. Effects of yohimbine on ethanol intake in P (a) and HAD-2 (b) rats maintained on continuous ethanol access. Yohimbine increased drinking on injection days, but this effect dissipated with successive cycles.

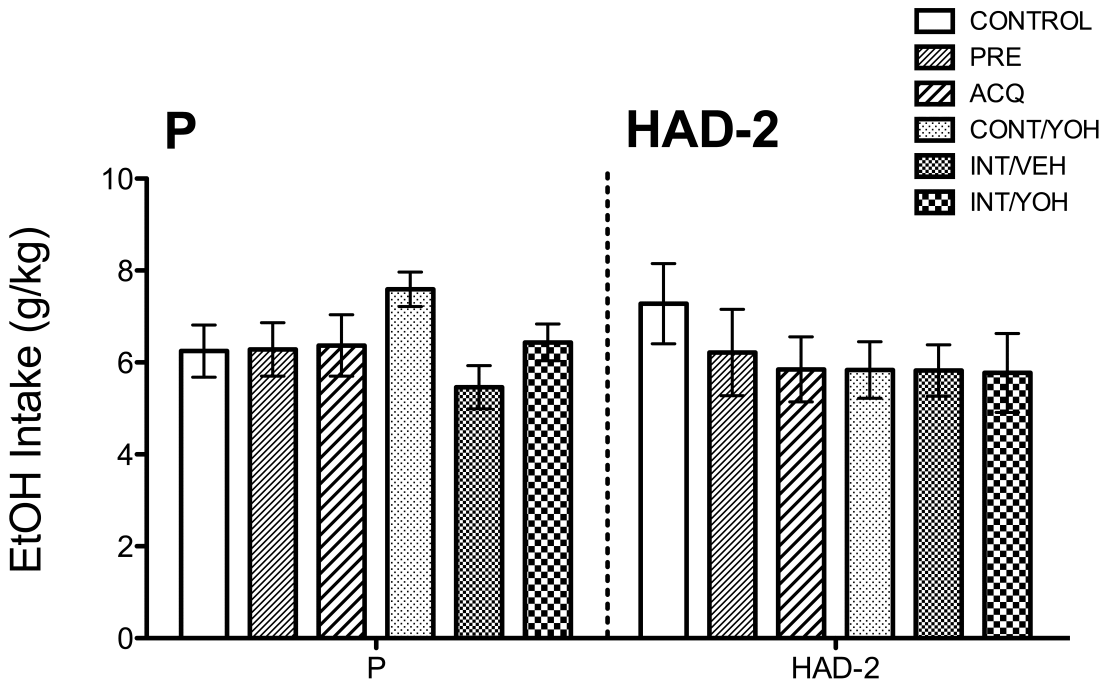

Figure 12. Ethanol intake in P and HAD-2 rats previously receiving vehicle injection only (CONTROL), yohimbine injection prior to ethanol drinking (PRE), yohimbine injection during the first two weeks of ethanol drinking (ACQ), yohimbine and continuous (CONT/YOH) or intermittent access (INT/YOH), and vehicle and intermittent access (INT/VEH) on the yohimbine challenge day. Ethanol drinking and yohimbine injection history failed to alter drinking responses to a yohimbine challenge.

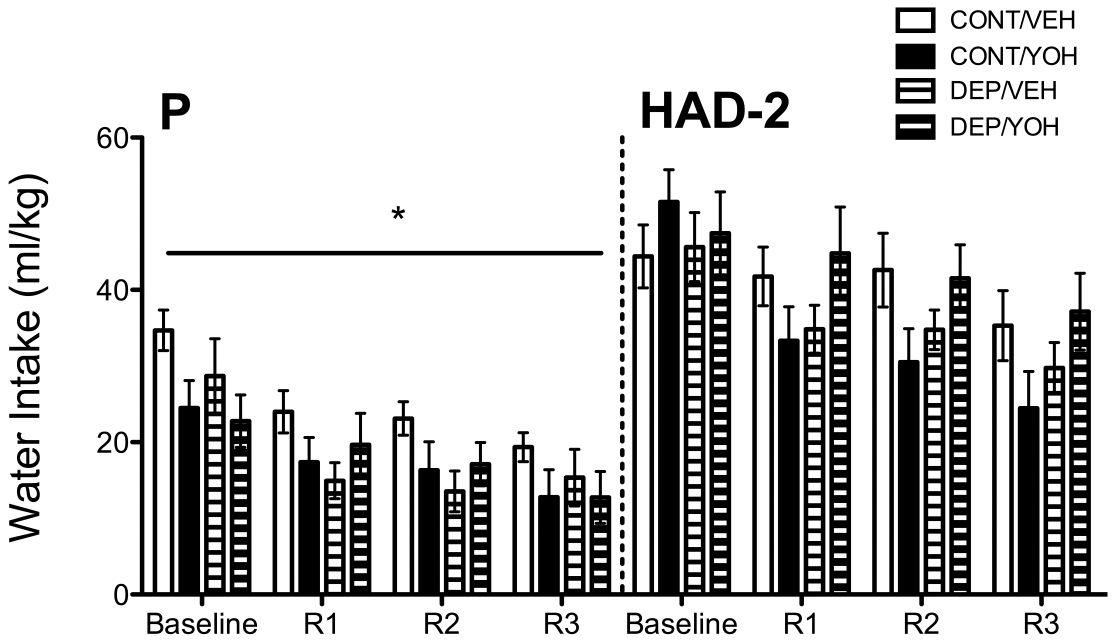

Figure 13. Water intake in P and HAD-2 rats given either continuous or intermittent ethanol access and were injected with either yohimbine or vehicle over three cycles. Rats drank less water during each reinstatement day compared to baseline. Overall, HAD-2 rats drank more water than P rats.

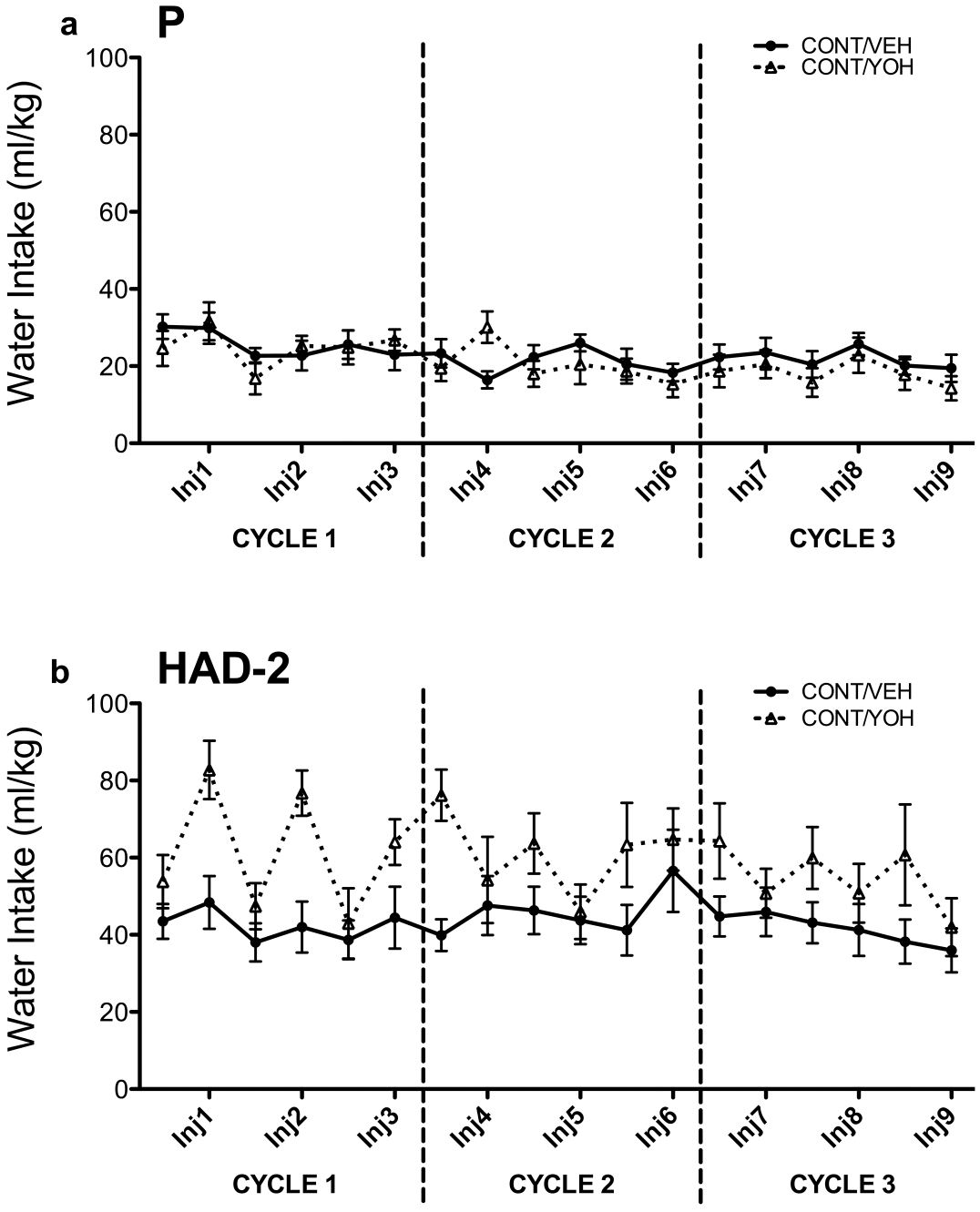

Figure 14. Effects of yohimbine on water intake in P (a) and HAD-2 (b) rats maintained on continuous ethanol access. Yohimbine increased drinking on injection days in HAD-2 rats.

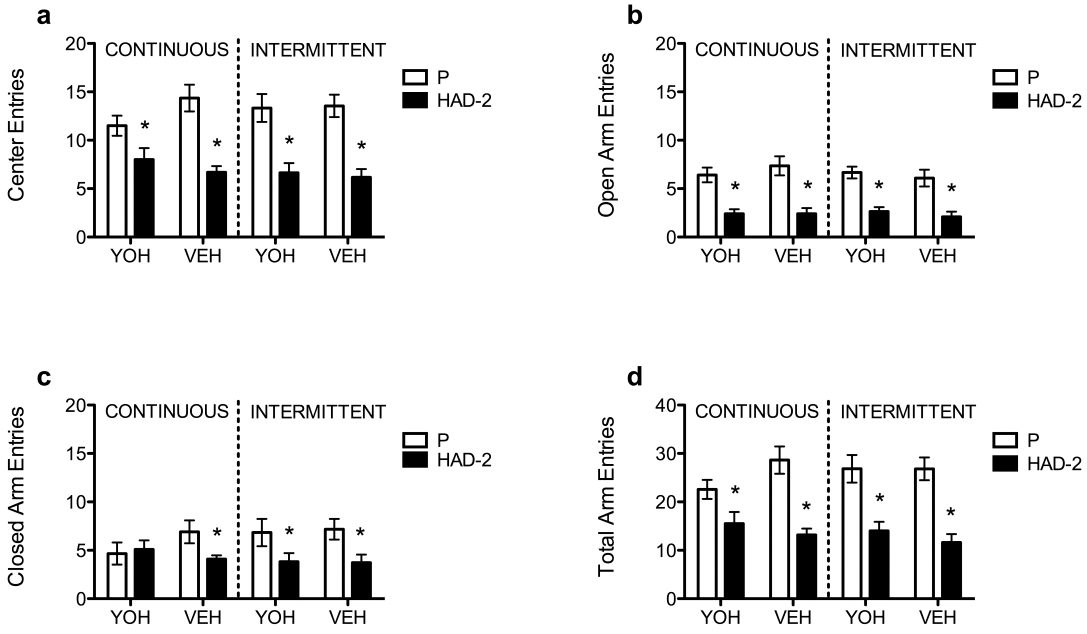

Figure 15. Number of entries in the center (a), open arms (b), and closed arms (c), and total arm entries (d) in the EPM in P and HAD-2 rats given continuous or intermittent ethanol access and injected with yohimbine or vehicle. P rats showed greater center, open arm, and total entries. *$p < 0.05$ P vs. HAD-2

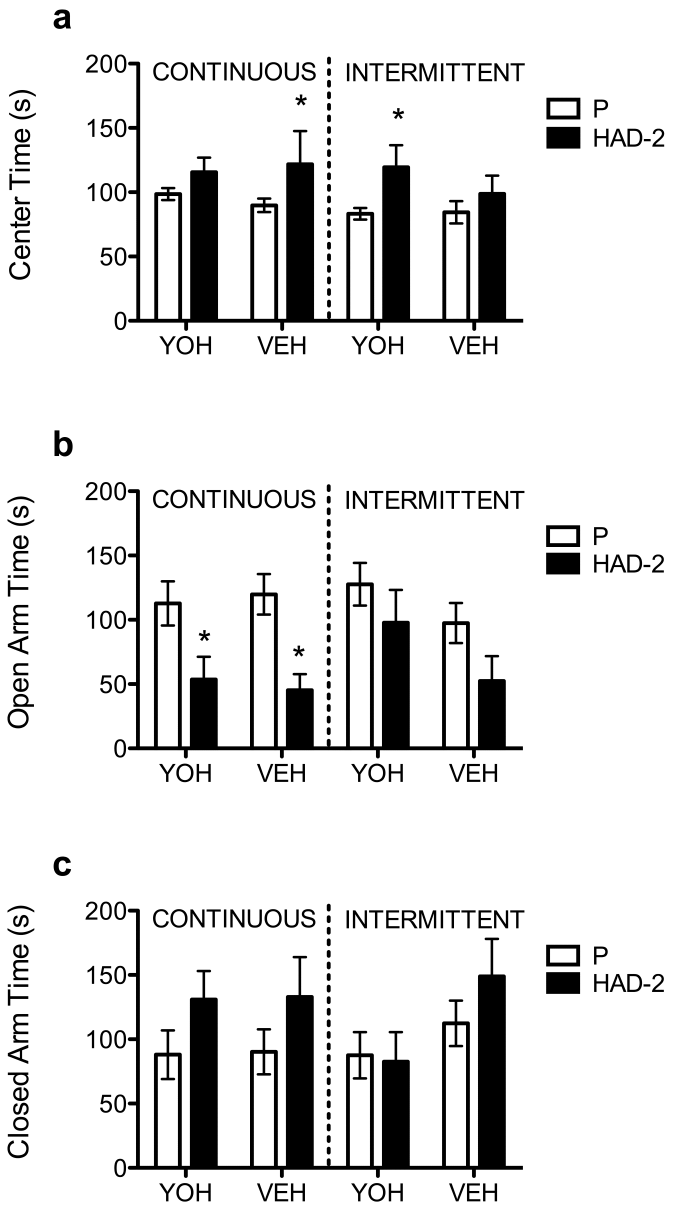

Figure 16. Time spent in the center (a), open arms (b), and closed arms (c) in the EPM in P and HAD-2 rats given continuous or intermittent ethanol access and injected with yohimbine or vehicle. HAD-2 rats spent significantly more time in the center and P rats spent significantly more time in the open arms of the EPM compared to the other line. *$p < 0.05$ P vs. HAD-2

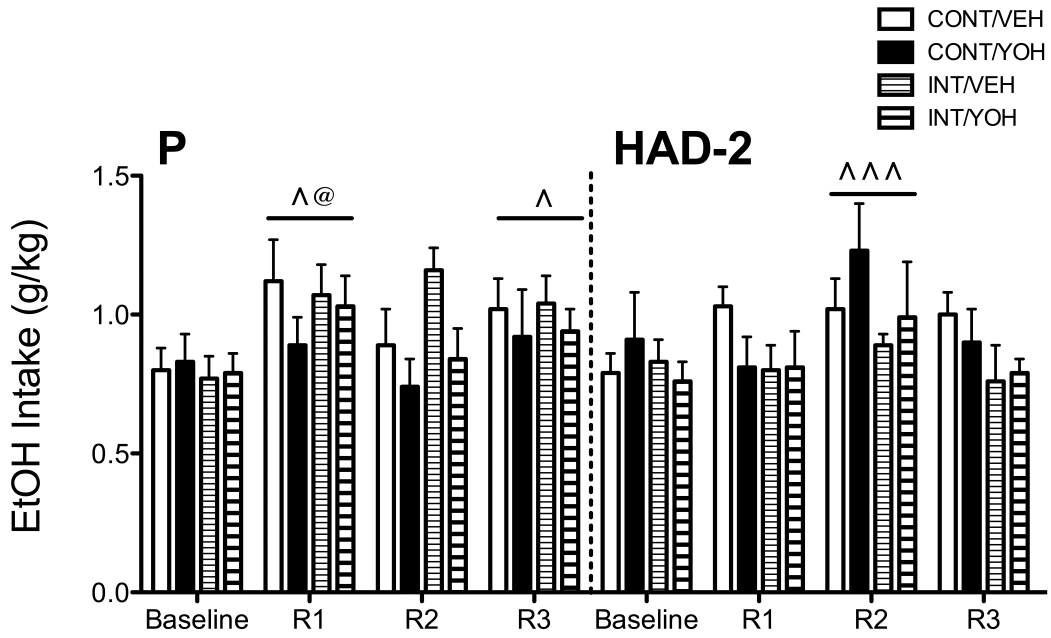

Figure 17. Ethanol intake in P and HAD-2 rats given either continuous or intermittent ethanol access and were injected with either yohimbine or vehicle over three cycles. While HAD-2 rats displayed elevated ethanol drinking after the second reinstatement (R2) compared to all other periods, P rats displayed elevated ethanol drinking following the first reinstatement period (R1) compared to baseline and R2, and third reinstatement period (R3) compared to baseline. ^ $p < 0.05$ vs. baseline; @ $p < 0.05$ vs. R2; ^^^ $p < 0.05$ vs. all other groups

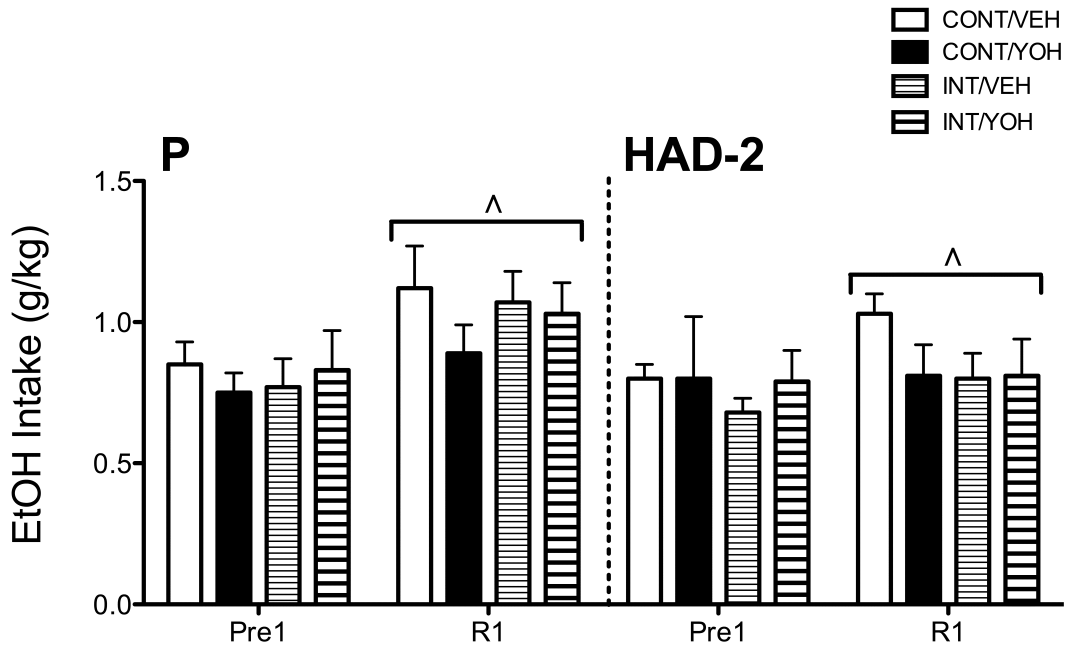

Figure 18. Ethanol intake in P and HAD-2 rats given either continuous or intermittent ethanol access and were injected with either yohimbine or vehicle during the first cycle. Rats drank more ethanol on reinstatement day 1 (R1) than during the pre-deprivation baseline (Pre1). ^ $p < 0.05$ vs. baseline

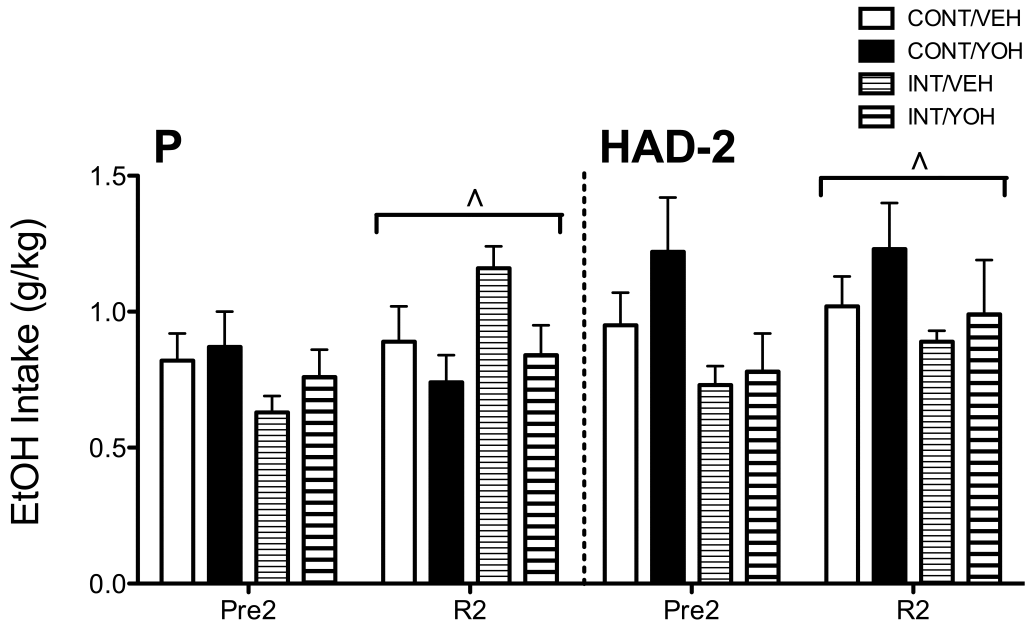

Figure 19. Ethanol intake during the second cycle in P and HAD-2 rats given either continuous or intermittent ethanol access and were injected with either yohimbine or vehicle. Rats drank more ethanol on reinstatement day 2 (R2) than during the pre-deprivation baseline (Pre2). This effect was stronger in rats that had been deprived of ethanol than those that had continuous ethanol access. ˆ $p < 0.05$ vs. baseline

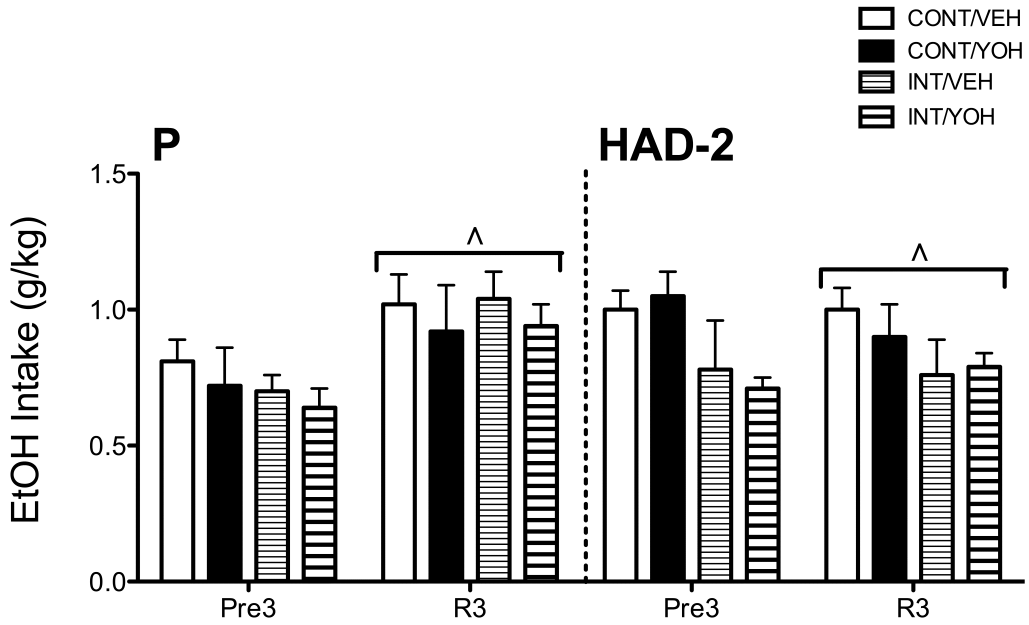

Figure 20. Ethanol intake during the third cycle in P and HAD-2 rats given either continuous or intermittent ethanol access and were injected with either yohimbine or vehicle. Rats drank more ethanol on reinstatement day 3 (R3) than during the pre-deprivation baseline (Pre3). This effect was stronger in rats that had been deprived of ethanol than those that had continuous ethanol access, and in P rats compared to HAD-2 rats. ^ $p < 0.05$ vs. baseline

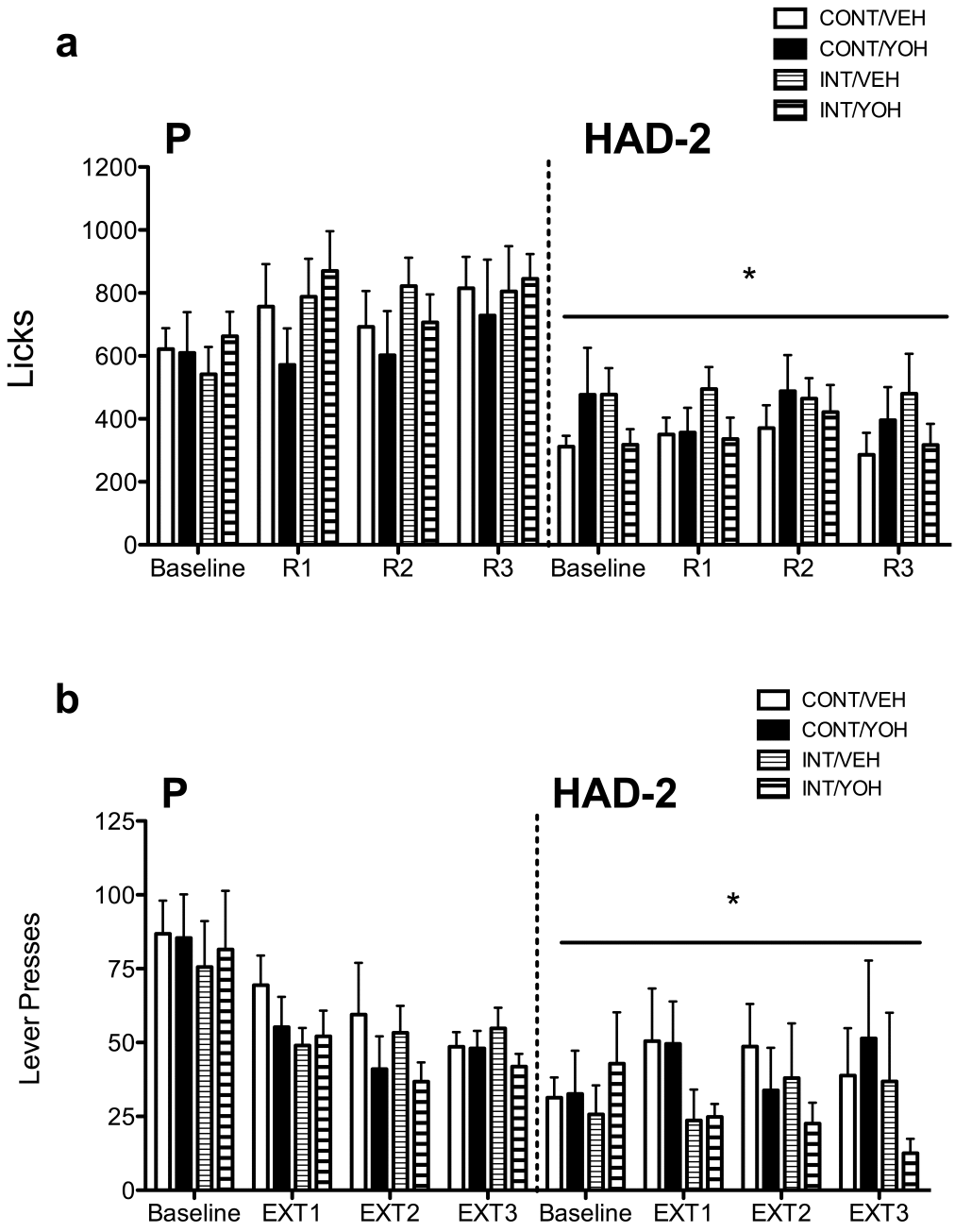

Figure 21. Licks during reinstatement (a) and lever presses during extinction (b) in P and HAD-2 rats given continuous or intermittent ethanol access and injected with yohimbine or vehicle across three cycles. P rats showed more licks and lever presses than HAD-2 rats. *$p < 0.05$ vs. P rats

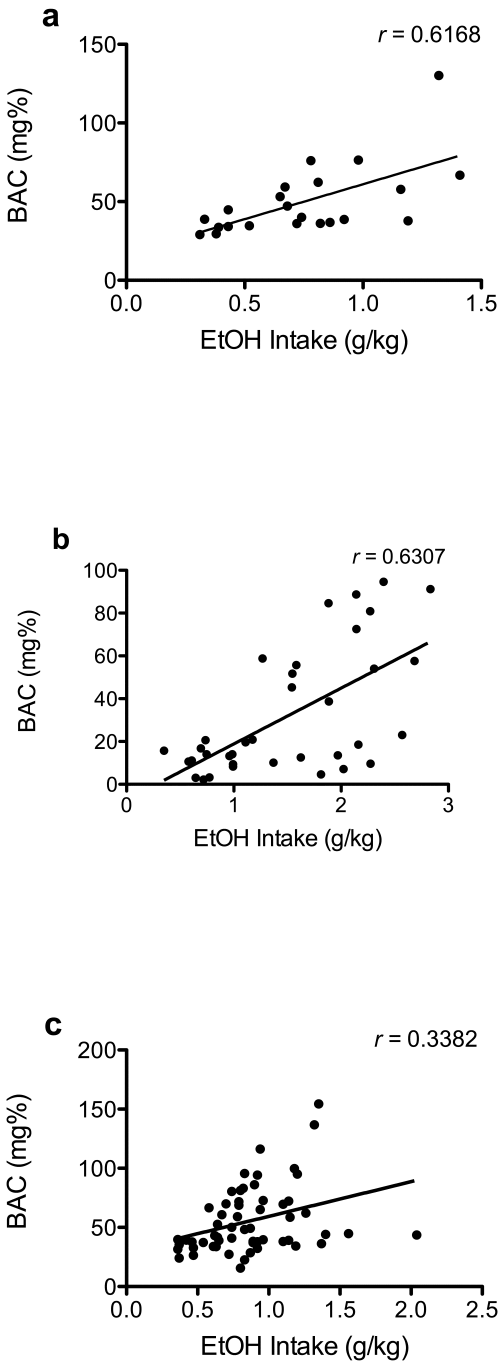

Figure 22. Correlations between ethanol intake and BAC in rats following a dose-response of yohimbine (a), given limited ethanol access in the home cage (b), and following intermittent ethanol exposure and yohimbine (c).

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Education

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1999 - 2003	***Knox College,*** Galesburg, IL B.A. in Studio Art, Minor in Psychology (2003) Cum Laude

Honors & Distinctions

2011	Paul McKinley Memorial Award, Psychobiology of Addictions Program
2011	IUPUI SOS-USG Graduate Student Sponsorship for Travel
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2010	Covance Poster Award, Indianapolis Society for Neuroscience Meeting
2009	Indiana University Department of Psychiatry Diversity Poster Award
2008	Research Society on Alcoholism Student Merit Travel Award
2007 - 2011	NIAAA Training Grant Appointee, IUPUI
2006 - 2007	Research Investment Fund (RIF) Fellowship, IUPUI
2006	Guze Symposium on Alcoholism Meeting Award, Washington University
2005 - 2006	University Fellowship, IUPUI

Published Manuscripts

Roman, E., Stewart, R. B., **Bertholomey, M. L.**, Jensen, M. L., Colombo, G., Hyytiä, P., Badia-Elder, N. E., Grahame, N. J., Li, T. K., Egli, M., & Lumeng, L. Behavioral profiling of multiple pairs of rats selectively bred for high and low alcohol intake using the MSCF test. Accepted 2/1/2011 for publication in *Addiction Biology.*

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Henderson, A. N., **Bertholomey, M. L.**, Stewart, R. B., and Badia-Elder, N. E. (2007). Acquisition and maintenance of ethanol drinking following multiple daily infusions of neuropeptide Y (NPY) in alcohol-preferring (P) rats. *Alcohol Clin Exp Res, 31(s2),* 198A. (Research Society on Alcoholism Meeting, Chicago, IL, June 2007)

Accepted Abstracts/Poster Presentations

Bertholomey, M. L., Lumeng, L. & Stewart, R. B. *Differences in anxiety-like behavior in rats selectively bred for high alcohol intake: Rats in the alcohol-preferring P line are less anxious than rats in the high-alcohol-drinking HAD-1 and HAD-2 replicate lines.* To be presented at the International Behavioural and Neural Genetics Society Meeting in Rome, Italy, May 2011.

Bertholomey, M. L. & Czachowski, C. L. *Yohimbine dose-dependently increases operant self-administration of ethanol in alcohol-preferring (P) rats*. To be presented at the Volterra Conference in Volterra, Italy, May 2011.

Submitted Abstracts

Bertholomey, M. L. & Czachowski, C. L. *Yohimbine dose-dependently increases binge-like ethanol self-administration and reinstates ethanol seeking in alcohol-preferring (P) rats*. Submitted for presentation and for consideration of the Enoch Gordis Award at the Research Society on Alcoholism Meeting in Atlanta, GA, June 2011.

Bertholomey, M. L. & Stewart, R. B. *Differences in anxiety-like behavior in rats selectively bred for high alcohol intake: Rats in the alcohol-preferring P line are less anxious than rats in the high-alcohol-drinking HAD-1 and HAD-2 replicate lines.* Submitted for presentation at the International Behavioural and Neural Genetics Society Meeting in Rome, Italy, May 2011.

Invited Talks

"Yohimbine dose-dependently increases binge-like operant ethanol self-administration and reinstates ethanol seeking in alcohol-preferring (P) rats.*"* Presented as part of the Czachowski lab talk at the Indianapolis Society for Neuroscience Meeting in Indianapolis, IN, 10/29/2010.

"Contribution of Stress to the Escalation of Alcohol Drinking and Relapse Behavior." Presented at the First Annual Ann Daugherty Symposium For Basic Science and Addiction Recovery, Tara Treatment Center, Franklin, IN, 5/14/2010.

"Tolerance to the effects of NPY on ethanol drinking in alcohol-preferring rats following repeated daily NPY infusions." Presented at the 2009 NIAAA Trainee Workshop in New Orleans, LA, 3/14/2009.

"Effects of stress, repeated ethanol deprivation, and neuropeptide Y (NPY) on ethanol intake in alcohol-preferring (P) rats." Presented at the 2007 NIAAA Trainee Workshop in Indianapolis, IN, 9/30/2007.