Stress and Alcohol: The Role of Glucocorticoids in Stress-Escalated Ethanol Consumption in a Murine Model

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Abstract

Rationale

Clinical and preclinical research has identified an association between stress and subsequent substance use. The signaling molecules of HPA axis have been considered critical mediators in this relationship. Glucocorticoids, in particular, have been investigated for their importance in ethanol consumption.

Objectives

The current series of experiments investigates the role of glucocorticoids in social defeat stress-escalated voluntary ethanol drinking in CFW and C57BL/6J mice.

Methods

CFW and C57BL/6J mice were mildly (15 bites) or moderately (30 bites) socially defeated for eight or ten consecutive days. After a 10-day recovery period, animals were allowed intermittent or continuous access to a two-bottle choice of water and ethanol. After 4 to 6 weeks of drinking, animals were pretreated with the 11-β hydroxylase inhibitor metyrapone 30 minutes prior to ethanol access, and drinking was assessed.

Results

Social defeat escalated-drinking was observed in C57BL/6J animals exposed to continuous and intermittent access. Metyrapone dose-dependently suppressed ethanol drinking in both strains and access schedules for up to 24 hours. Metyrapone either enhanced or had no effect on water intake.

Conclusion

Social defeat stress can escalate voluntary ethanol consumption in a murine model. Inhibition glucocorticoid synthesis dose-dependently suppresses ethanol consumption regardless of stress history.

Introduction:

Substance abuse is one of the most damaging biomedical and mental health issues in the modern world. Substance use disorder, as categorized by the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), is an umbrella grouping that contains within it numerous diagnoses revolving around 10 frequently abused substance classifications. One of the most prevalent substance use disorders is alcohol use disorder, which affects 8.5% of adults and 4.6% of children aged 12 - 17 in the United States (DSM-5). Alcohol use in the United States is a significant public health issue, contributing to 88,000 alcohol-associated deaths per year (CDC). In addition to the emotional repercussions of excessive alcohol consumption, such drinking patterns place a severe economic burden of $223.5 billion per year on US citizens (Stahre et al., 2014). The impacts of alcohol use disorder and excessive alcohol consumption have prompted significant efforts from researchers and clinicians alike to develop interventions to stem the damage of alcohol use. Despite decades of research, the development of efficacious pharmacological therapies remains elusive.

Relationship Between Stress and Drug Use:

Of particular interest in the field of substance abuse is the association between stressful experiences and drug use. This relationship has been identified in human populations in the clinic, as well as in preclinical animal models (Sinha, 2001) (Jackson et al., 2010). The role of stress in the development of, maintenance

of, and relapse to substance abuse has been subject of extensive research. While untangling the complicated relationship has proven challenging, researchers have implicated the influence of stress at every stage in the maladaptive cycle toward addiction.

In human populations, a history of stress has been implicated in the development of maladaptive substance use (Sinha, 2001). Retrospective studies have indicated that individuals with a history of physical and/or sexual abuse are more likely to develop substance abuse (Widom et al., 1999) (Harrison et al., 1997). Preclinical studies have supported clinical results, identifying the presence of a connection between stress and substance abuses as well. In a variety of animal models examining various substances of abuse, early life stressors have been implicated in escalated drug administration (Adler et al., 1975) (Schenk et al., 1987) (Alexander et al., 1978) (Higley et al., 1991) (Miczek et al., 2008).

This relationship has been more accurately defined to indicate that drug use, as a coping mechanism for stress, is associated with the development of compulsive drug use (Cooper et al., 1992). One of the most prominent hypotheses, specific to alcohol research, connecting stress and substance use is the "tension reduction' hypothesis. First described in the 1950s by John Conger, the tension reduction hypothesis contends that alcohol drinking is a learned reinforcer due to its action in reducing psychological or physiological tension (Conger, 1951) (Conger, 1956). In his experiments, Conger demonstrated that alcohol was capable of enhancing food-rewarded behavior that was suppressed by concurrent punishment (Conger, 1951). While the tension reduction hypothesis has gathered some support from clinical

studies (Steffen et al., 1974) (Levenson et al., 1980), it remains a theory, and has yet to be fully accepted by the scientific community (Polivy et al., 1976).

Regardless of the motivation to drink, the strength of the association between stress and alcohol use have led to the consideration of the physiological responses to stress, primarily the hypothalamic-pituitary-adrenal (HPA) axis, as the molecular mediator for this relationship. Attempts have been made to uncover how stressful experiences can produce long-lasting physiological changes, and how this changes can continue to influence subsequent drug-taking. Clinical and preclinical studies have demonstrated that early life traumas produce persistent hyperactivation of the HPA axis (Sanchez et al., 2001) (Heim et al., 2008) (Miller et al., 2007) (Plotsky & Meaney, 1993). Additionally, certain parameters of early life maternal separation have been shown to enhance sensitivity to stressors in adult rats (Plotsky & Meaney, 1993). With these observations in mind, one of the most prominent hypotheses in the research community connects these stress-related alterations in HPA axis activity with drug use, suggesting that stress positively modulates the reinforcing effects drugs of abuse (Kalivas & Stewart, 1991). This hypothesis is supported through studies examining the association between basal HPA axis activity and individual propensity to drug self-administration. It has been demonstrated that rats with higher basal corticosterone concentrations are at a higher risk to self-administer psychomotor stimulants (Piazza et al., 1989). Additionally, in humans, individuals diagnosed with mental health disorders associated with HPA axis hyperactivity (Arborelius et al., 1999) are often diagnosed with comorbid substance use disorders (Kushner et al., 2000). The strength of these

associations has led researchers to implicate several HPA axis signaling molecules, such as corticotropin releasing factor (CRF) and glucocorticoids, as modulators of the reinforcing effects of drugs of abuse.

Stress Physiology:

In order to explore how stress can influence drug taking, it is necessary to review the physiological systems associated with stress. The word stress originates from material sciences, in which it applies to the force placed upon a material. However, Hans Selye characterized the physiological stress response in 1936, and subsequently, popularized the concept of stress in biology (Selye, 1956). Since the conception of stress in the physiological realm, it has expanded, and is now widely used in the field of psychology as well. The NIH defines stress as "the brain's response to any demand" (NIMH), however, stress can take many different forms, and most often carries negative connotations with it.

Without stress, we would not be able to live. The physiological pathways connected to the experience of stress are critical to the maintenance of homeostasis in the body, influencing inflammatory response, immune activity, metabolism, and fluid balance (Munck et al., 1984). While stress is critically important in our daily lives, the relationship between stress and physiological well-being is biphasic; acute stress responses are critical to physiological adaptation, but excessive activation can adversely affect physical and mental health (McEwen, 2009).

In addition, the magnitude of the stress response is highly dependent upon the nature of the stress (Sinha, 2001). Factors that have been implicated in stress

response include frequency, interval (De Boer et al., 1990) (Kirschbaum et al., 1995), intensity (Pitman et al., 1990), predictability (Weiss, 1970), and controllability (Seligman et al., 1971). In understanding the influence of stress on the development of substance use disorders, it is crucial to review the physiological pathways associated with the stress response.

Of all physiological systems affected by stress, the most canonical pathway is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is a signal transduction pathway that serves as the slow physiological response to stress, and ultimately results in the release of glucocorticoids from the adrenal cortex. Significant research has allowed for mapping of the individual signaling pathways involved in the HPA axis. Stress results in the release of corticotropin-releasing factor (CRF) and other adrenocorticotropic hormone (ACTH) secretagogues produced in the paraventricular nucleus (PVN) of the hypothalamus and released for the median eminence. These secretagogues travel into the hypophyseal-portal blood system connecting the median eminence and the pituitary gland (Herman & Cullinan, 1997). CRF exerts its action through binding to high-affinity G-protein-coupled receptors throughout the CNS and pituitary (De Souza & Kuhar, 1986) (De Souza et al., 1985). This binding results in the activation of numerous intracellular signal transduction pathways, including adenylyl cyclase and phospholipase-C (Dautzenberg & Hauger, 2002). CRF binding in the pituitary stimulates the release of ACTH from the anterior lobe of the pituitary into general blood circulation. ACTH acts upon the cortex of the adrenal glands, stimulating the secretion of glucocorticoids and mineralocorticoids (Walker et al., 2001) (Herman & Cullinan, 1997).

In addition to the canonical action of CRF in stimulating ACTH release, CRF-releasing and CRF-receptors expressing neurons are located throughout the CNS, including in regions associated with drug use (Olschowka et al., 1982) (De Souza et al., 1985). The wide distribution of CRF-associated neurons in the CNS suggests extrahypothalamic activity of CRF. Indeed, CRF signaling has been implicated in the development of several mental health disorders (Arborelius et al., 1999), and has been associated with use of a variety of substances of abuse (Nie et al., 2004) (Erb et al., 2001).

Glucocorticoids are a classification of steroid molecules, most notably cortisol/corticosterone, synthesized in the adrenal cortex. These molecules exert their physiologic action through binding with glucocorticoid receptors (GR2) (Bamberger et al., 1996). The GR is a member of the nuclear receptor superfamily (Bamberger et al., 1996), a family of ligand-dependent transcription factors (Mangelsdorf et al., 1995). GRs are located in the cytoplasm, however, glucocorticoid binding stimulates nuclear localization and results in promotion of transcriptional activity (Picard & Yamamoto, 1987). Once bound by glucocorticoids, the GR-glucocorticoid complex interacts with DNA sequences known as Glucocorticoid Response Elements (GREs), enhancing transcription of associated DNA regions (Yamamoto, 1985). GR is ubiquitous throughout the body (Lowry, 1989), and as would be expected from the wide distribution, glucocorticoid-GR interactions

influence numerous physiological systems, including inflammatory response, immune activity, metabolism, and fluid balance (Munck et al., 1984).

Like glucocorticoids, mineralocorticoids are adrenal cortex-produced steroids, so termed due to their effects on mineral homeostasis. The effects of mineralocorticoids are mediated by their activity at the mineralocorticoid receptor (MR or GR1), another member of the nuclear receptor superfamily (Mangelsdorf et al., 1995). MR is widely distributed in peripheral tissues and the CNS (Funder et al., 1989) (Arriza et al., 1988). The canonical effects of mineralocorticoids are on kidney function (Mulrow & Forman, 1972), however, mineralocorticoids have also been shown to influence CNS activity (Gould et al., 1991) (De Kloet & Reul, 1987) (McEwen et al., 1992) (Joels et al., 2008).

It must be noted, that while glucocorticoids and mineralocorticoids are traditionally considered to act on their own discrete populations of receptors, similarity between GRs and MRs allows for high-affinity binding of both corticosteroid classifications (Arriza et al., 1987) (Rupprecht et al., 1993) (Funder et al., 1988). In fact, this cross-ligand binding appears to be critical to homeostasis (Funder, 2005) (Funder, 1997).

As with all of the body's physiological systems, regulation of the HPA axis is crucial to maintenance of proper biological function. Regulation of the HPA axis is mediated primarily by negative feedback mechanisms. Glucocorticoids serve as the main regulatory molecule, inhibiting HPA activity at the level of the hypothalamus and pituitary (Widmaier & Dallman, 1983) (Sawchenko, 1987) (Plotsky et al., 1986)

(Keller-Wood & Dallman, 1984). Additional research has suggested a contribution from CRF-binding-protein as a regulatory point, serving to sequester CRF, suppressing CRF-induced ACTH release (Linton et al., 1988) (Westphal & Seasholtz, 2006).

The physiological responses resulting from HPA activation serve as a means of coping with stressful events (Axelrod & Reisine, 1984), and are most frequently adaptive (Munck et al., 1984). However, as with all homeostatic systems, dysregulation of the HPA axis can be damaging. Both excessive and insufficient HPA axis activity has been implicated in the development of numerous pathologies (Munck et al., 1984) (McEwen & Stellar, 1993) (Guilliams & Edwards, 2010).

Manipulations of the HPA Axis:

The influence of the HPA axis on clinical pathologies has led to the investigation of various points of intervention in the HPA axis as a means to control HPA dysregulation. The most dramatic option for intervention is the surgical removal of the adrenal glands, adrenalectomy (ADX), which lowers circulating concentrations of cortisol. However, patients subjected to bilateral ADX require lifelong supplemental adrenal steroid administration (Shen et al., 2006). Additionally, ADXs have been associated with the development of pituitary ACTH-secreting tumors, resulting in the escalation of plasma ACTH concentrations (Nelson et al., 1960). The side-effect profile of ADX has led to its employment primarily as a treatment of Cushing disease (Newell-Price et al., 2006).

Despite the limited application of ADX in the clinic, it has served as a valuable technique in preclinical models for exploring the role of corticosteroids in alcohol consumption. Numerous studies have indicated that ADX is associated with a decrease in alcohol consumption, particularly in high consuming animals (Fahlke et al., 1994) (Lamblin & De Witte, 1996)(Fahlke & Eriksson, 2000). Additionally, corticosterone-replacement treatment has been shown to attenuate ADX-induced suppression of ethanol intake, suggesting a role of corticosterone in maintaining ethanol consumption (Lamblin & De Witte, 1996)(Fahlke & Eriksson, 2000).

Researchers have also developed non-surgical, pharmacological interventions as a means to control the HPA axis. CRF antagonism has been widely explored in preclinical settings. Limited clinical studies have indicated that non-specific CRF antagonism transiently lowers plasma ACTH and cortisol concentrations, most likely through antagonism of pituitary CRF receptors (Baram et al., 1996). Preclinical studies demonstrated that antagonism of CRF-R1 is responsible for sustained suppression of plasma ACTH and corticosterone concentrations (Bornstein et al., 1998).

The success of CRF antagonism in modulating extra-hypothalamic HPA activity has led to the exploration of the effects of CRF antagonism on ethanol consumption. Preclinical research has identified that CRF antagonism, via CRF-R1 knockout, and modulation of CRF-R1, -R2, and -binding-protein activity, suppresses alcohol intake across a variety of models (Chu et al., 2007) (Hwa et al., 2013) (Hwa et al., 2015) (Albrecht-Souza et al., 2015). These preclinical results have proven

promising, however, use of CRF antagonists in the clinic has been hindered by inconclusive results and unacceptable side-effect profile (Zorrilla et al., 2013).

Pharmacological inhibition of ACTH secretion has also served as a point of HPA axis control; however, of the various points of intervention, direct inhibition of ACTH secretion has been the least pursued. Administration of somatostatin (Growth Hormone Inhibiting Hormone) has been shown to lower ACTH release in individuals lacking glucocorticoid feedback mechanisms on HPA axis activity (Tyrell et al., 1975) (Fehm et al., 1976). This inhibition is suspected to occur through direct action on the pituitary (Fehm et al., 1976). While such results are promising, direct action on the pituitary can produce off-target hormonal alterations, resulting in poor side-effect profiles. Indeed, the administration of somatostatin and somatostatin analogues can induce hyperglycemia, diabetes mellitus, and other adverse health effects (Colao et al., 2012). As a result of the associated consequences, the effects of ACTH inhibition on substance use have not been investigated.

Intervention at the level of glucocorticoid synthesis and release has been the most intensely investigated clinical option for HPA axis control. In the clinic, multiple pharmacological agents have been employed for the treatment of diseases of excessive HPA axis activation. The two main classes of inhibitory compounds are synthesis inhibitors and receptor antagonists. Synthesis inhibitors, such as metyrapone and ketoconazole (Table 1), have been shown to suppress plasma cortisol concentrations in clinical and preclinical settings (Verhelst et al., 1991)

(Pont et al., 1982). These compounds inhibit glucocorticoid synthesis through reversible inhibition of 11-β hydroxylase (Engelhardt et al., 1985) (Cheng et al., 1974) (Tucker et al., 1985) (Hays et al., 1984), an enzyme required for the biosynthesis of glucocorticoids (Kawamoto et al., 1992). Inhibition of glucocorticoid synthesis with these compounds has been shown to produce escalations in plasma concentrations of ACTH, as would be expected from removal of glucocorticoid inhibition on ACTH release (Burrin et al., 1986) (Staub et al., 1979).

Preclinical studies have indicated that antagonism of glucocorticoid synthesis by metyrapone suppresses ethanol consumption in high-drinking animals (Fahlke et al., 1994b). However, preliminary clinical studies show no effect of metyrapone treatment on ethanol drinking (Erikkson et al., 2001).

11-β hydroxylase Inhibitor:	Mechanism of Inhibition	K_i (μM)	Excretion
Ketoconazole	Competitive (Lin & Liu, 1998)	0.56μM (Nagai et al., 1985)	0.36 – 0.42 L/hr/kg (6 -7 ml/min/kg) in rats (Lin & Lu, 1998)
Metyrapone	Competitive (Sampath-Kumar et al., 1997)	0.1-0.2μM (Sato et al., 1978)	Plasma $T_{1/2}$= 20-26min (Sprunt et al., 1967)

Table 1. Kinetic profiles of 11-β hydroxylase Inhibitors.

Control of HPA axis activity by glucocorticoid receptor (GR) antagonists relies on inhibiting the physiological effects of glucocorticoid-GR binding, rather than lowering circulating levels of HPA axis intermediates. In fact, application of the GR antagonist mifepristone in the clinic has been shown to produce escalations in plasma ACTH and cortisol (Proulx-Ferland et al., 1982) (Belanoff et al., 2002). These

increases in plasma ACTH and cortisol are a result of disinhibition on the HPA axis; inhibition of GR receptor binding prevents glucocorticoid negative feedback regulation on the HPA axis, increasing secretion of HPA axis molecules. However, despite these escalations in HPA axis signaling molecules, use of GR antagonists has produced efficacious results in the clinical treatment of HPA axis associated disorders, such as Cushing's syndrome and depression (Fleseriu et al., 2012) (Belanoff et al., 2002).

Application of GR antagonists in substance abuse research has produced promising results. GR antagonism via mifepristone treatment has been shown to suppress ethanol intake in rats (Koenig & Olive, 2004). Additional antagonism studies have confirmed a role for GR in response to ethanol (Roberts et al., 1995) (Vendruscolo et al., 2015).

Ethanol Pharmacology:

In understanding the interaction between stress and ethanol, it is critical to review the pharmacology of ethanol in the CNS. Ethanol modulates the activity of numerous receptors in the CNS (Mihic, 1999) (Lovinger, 1999) (Cardoso et al., 1999) (Valenzuela et al., 1998). However, molecular ethanol research has traditionally focused on the activity of $GABA_A$ modulation as the most significant in the neural effects of ethanol (Suzdak et al., 1986). Further research has identified ethanol as a positive allosteric modulator of $GABA_A$ activity (Mihic et al., 1997). The $GABA_A$ receptor is the primary receptor for the inhibitory neurotransmitter gamma-aminobutyric acid, GABA. $GABA_A$ is an ionophoric ligand-gated chloride channel that

allows chloride ions to enter the cell upon binding by the neurotransmitter GABA, or another ligand (Olsen & Tobin, 1990) (Macdonald & Olsen, 1994). Activation of the $GABA_A$ channel increases chloride conductance and stabilizes the resting potential, inhibiting action potentials (Bormann, 1988). The binding of ethanol stabilizes the $GABA_A$ receptor, potentiating its activity (Jung et al., 2005) (Dildy-Mayfield et al., 1996).

Ethanol potentiates the activity of the 5-HT_3 receptor (Lovinger & Zhou, 1994) (Machu & Harris, 1994). The 5-HT_3 receptor is a ligand-gated cation channel, in which activation results in depolarization of the membrane and an excitatory response (Peters & Lambert, 1989) (Derkach et al., 1989) (Maricq et al., 1991). While there has not been extensive exploration of this relationship, antagonism of 5-HT_3 has been shown to suppress voluntary ethanol consumption in preclinical and clinical studies, indicating the significance of ethanol's on the 5-HT_3 receptor (Fadda et al., 1991) (Sellers et al., 1994).

Ethanol also exerts activity on glycine receptors (GlyR) and N-methyl-d-aspartate (NMDA) receptors. The activity of ionophoric chloride-conducting GlyR (Curtis et al., 1968) (Barker & Ransom, 1978) is potentiated by ethanol (Celetano et al., 1988). This potentiation is accomplished through ethanol-GlyR binding (Mihic et al., 1997), resulting in stabilization of the open form of the GlyR (Murail et al., 2011). The NMDA receptor is a ligand-gated calcium-conducting channel (Johnson & Ascher, 1987) (MacDermott et al., 1986) (Furukawa et al., 2005). The activity of the NMDA receptor has been shown to be inhibited by ethanol (Simson et al., 1991)

(Criswell et al., 2003) most likely through activity at the glycine coagonist site (Hoffman et al., 1989) (Rabe & Tabakoff, 1990).

While extensive biochemical research has identified numerous sites of action for ethanol in the CNS, determining which ethanol-receptor associations are responsible for the subjective effects of ethanol remains challenging (Harris, 1990) (Harris et al., 2008)

Additional research has identified peripheral effects of alcohol that are pertinent to the study of stress and alcohol. Excessive alcohol intake has been demonstrated to produce hypercortisolism, termed pseudo-Cushing's syndrome, in some individuals (Rees et al., 1977). It has been suggested that this hypercortisolism is a result of defective cortisol metabolism that may be associated with alcohol intake (Stewart et al., 1993).

Stress and Alcohol:

Despite the strength of the association between stress and many drugs of abuse, the relationship between stress and alcohol remains elusive (Becker et al., 2011). Many studies examining stress and ethanol self-administration have been contradictory, with some suggesting potentiation, while others demonstrate no influence or suppression (Sinha, 2001) (Becker et al., 2011). Despite the inconsistency of these results, researchers have been able to conclusively indicate that the HPA axis activity influences ethanol consumption and neurochemical response to ethanol exposure. Disruption of HPA axis activity suppresses basal ethanol intake (Fahlke et al., 1994a) (Fahlke et al., 1994b), and HPA axis

hyperactivity potentiates ethanol-induced increases in extracellular DA (Yavich & Tiihonen, 2000). Such results suggest that there is an interaction between stress and ethanol consumption, however, producing consistent results in preclinical behavioral experimentation remains challenging.

One explanation for the inconsistency of behavioral results linking stress and ethanol consumption is that the effect is largely dependent on the nature of the stress (Miczek et al., 2008) (Sinha, 2001) (Becker et al., 2011). Amidst the inconsistency of this research, the role of a stress history has been suggested as critical in influencing subsequent ethanol consumption (Becker et al., 2011). In particular, procedures incorporating a history of social stress have been reasonably successful in eliciting stress-escalated alcohol drinking (Higley et al., 1991) (Fahlke et al., 2000). This effect is time-dependent, in that stress concurrent with the presentation of alcohol results in suppression of ethanol intake (van Erp et al., 2001) (van Erp & Miczek, 2001) (Norman et al., 2015). However, recent research has indicated that a history of episodic defeat stress can profoundly elevate future ethanol consumption (Norman et al., 2015). These results beg for further exploration of the relationship between previous experience with episodic social defeat and non-concurrent ethanol access.

Preclinical Models of Stress:

Studying the effects of stress on drug abuse has proven to be a complicated undertaking. Stress is an incredibly broad definition that encompasses every imaginable stressor that can be experienced, from physical to psychological. This

breadth of experiences is reflected in the number of stress models that have been employed in preclinical studies. Some of the most popular of these models include maternal separation, foot-shock, immobilization, and social defeat. Determining the influence of stress on drug abuse is further complicated by two factors: administration of a psychoactive substance can in itself be considered a stressor (Barry & Buckley, 1966) (Ellis, 1966) (Rivier et al., 1984) (Rivier et al., 1990), and not all preclinical models of stress successfully elevate administration of all drugs.

The production of stress methodologies that consistently elevate ethanol consumption has been particularly challenging. The most reliable models for the production of stress-escalated alcohol consumption have relied on a history of social stress (Higley et al., 1991) (Fahlke et al., 2000). In particular, recent research has indicated that a history of social defeat stress can evoke escalations in future ethanol intake (Norman et al., 2014). Such models of social stress are some of the most translatable models from preclinical studies to the intricate social life of human populations (Miczek et al., 2008).

From a physiological perspective, social defeat stress is ideal for studying the long-term effects of stress. Social defeat has been shown to produce persistent hyperactivity of the HPA axis (Raab et al., 1986) (Koolhaas et al., 1997), and animals fail to habituate to social defeat stress after repeated exposure (Raab et al., 1986) (Nikulina et al., 2004) (Covington et al., 2005) (Norman et al., 2015). Additionally, social defeat stress selectively increases extracellular dopamine in the mesocorticolimbic system (Tidey & Miczek, 1996), suggesting an interaction between social defeat and drug use. These results have been supported by other

studies indicating that social defeat stress consistently produces escalations in self-administration of a variety of drugs (Haney et al., 1995), identifying social defeat as an ideal methodology for ethanol abuse research.

Social defeat stress does, however, have downfalls. One criticism of social defeat stress is that most defeat paradigms use the behaviors of the aggressive resident, such as the number of bites, to quantify a uniform defeat experience. Given that there is variability in resistance to social defeat stress (Miczek et al., 1982), applying uniform defeat undoubtedly produces different physiologic and behavioral responses, according to the Yerkes-Dodson Law (Yerkes & Dodson, 1908) (Miczek et al., 2008).

Preclinical Models of Ethanol Exposure:

While the ultimate goal of alcohol use research is to address the issue of alcohol use and abuse in human populations, the empirical examination of alcohol drinking in humans has proven to be challenging. Human studies are limited by ethical concerns and the quantity of data obtained (Tabakoff & Hoffman, 2000). Such obstacles prevent the speedy and comprehensive study of the physiological processes contributing to patterns of excessive alcohol consumption. To overcome these challenges, animal models, specifically rodent models, are frequently employed in preclinical studies (Tabakoff & Hoffman, 2000). By presenting animals with alcohol on different schedules, researchers can attempt to model patterns of drinking observed in human populations. Three of the most frequently used models

of alcohol drinking are continuous access, intermittent access, and drinking-in-the-dark (DID).

Continuous and intermittent access schedules of ethanol exposure are closely related in that they are long-term paradigms, providing ethanol access over several weeks. In both schedules, ethanol solution and water are presented simultaneously (two-bottle choice), allowing for assessment of voluntary ethanol consumption and the ethanol preference. The most appealing characteristic of continuous and intermittent access schedules of ethanol exposure is the voluntary nature of consumption and the ability to more accurately model human alcohol intake, eg. drinking (Cunningham et al., 2000). Historically, many attempts have been made to model excessive ethanol consumption, such as the ethanol vapor chamber (Rijk et al., 1982), liquid diet (DeCarli & Lieber, 1967), and one-bottle presentation (Cicero, 1980). However, these techniques are forced consumption models, and as such, they lack the ability to mirror voluntary alcohol consumption in humans. With two-bottle choice paradigms, the prolonged access to ethanol allows for animals to voluntarily consume large quantities of ethanol, which more closely models the patterns of drinking seen in humans.

Continuous access schedule provides constant access to both ethanol and water, while the intermittent access schedule provides ethanol every other day. Traditionally, continuous access schedules have been used extensively in research to assess ethanol consumption in animal models (Cunningham et al., 2000). Under continuous access conditions, animals will consume the largest amounts of ethanol over the course of their drinking career (Wise, 1973) (Hwa et al., 2011); however,

intermittent access offers several advantages over continuous access. Intermittent access in mice has been shown to produce escalations in daily ethanol drinking and preference when compared to continuous access (Wise, 1973) (Hwa et al., 2011) (Melendez, 2011).

Escalations in drinking, combined with repeated abstinence periods are associated with the amplification of withdrawal symptoms (Overstreet et al., 2002), contributing to the "kindling" of drug withdrawal and the development of dependence (Becker & Hale, 1993). The pattern of drinking associated with intermittent access cultures this "kindling" effect, allowing for the development of dependence in voluntary ethanol-consuming animals (Hwa et al., 2011) as assessed with handling-induced-convulsions (HIC) during withdrawal (Goldstein, 1972) (Crabbe et al., 1985). This quality of intermittent access is invaluable in modeling drug dependence in humans, which is theorized to develop in some instances as a result of neuroadaptations associated with repeated episodes of drug use and abstinence (Koob & Le Moal, 1997).

Another widely employed schedule ethanol access is drinking-in-the-dark (DID), developed by Rhodes et al. in 2005. DID is a limited access paradigm that attempts to model the practice of binge drinking in human populations. One of the most significant criticisms of the two-bottle choice, extended access schedules is the pharmacological relevance of ethanol drinking patterns (Dole & Gentry, 1984). While some rodent strains prefer consuming ethanol solution to water, patterns of drinking remain homeostatic and do not produce prolonged periods of intoxication-inducing blood ethanol concentration (BEC) (Dole & Gentry, 1984). These qualities

fail to reflect the abnormal patterns of drinking that produce long-lasting periods of intoxication characterizing alcohol use disorders (DSM-5). The DID protocol attempts to address these gaps in animal models of drinking through a procedure that results in excessive alcohol consumption that elicits pharmacologically significant levels of intoxication. DID is characterized by the presentation of ethanol solution, in the place of tap water, 3 hours into the dark cycle. After a two-hour period, the ethanol is removed. This procedure is repeated for four consecutive days, on the fourth day, the animals are presented with ethanol for 4, instead of 2 hours (Rhodes et al., 2005). On the day of 4-hour ethanol access, there is a robust increase in the amount of alcohol consumed that mirrors excessive alcohol consumption in humans. This procedure has been widely accepted by the research community as a model for the production of excessive voluntary ethanol consumption against which pharmacological agents can be screened for their therapeutic potential.

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Preclinical Animal Models:

Another critical factor to consider in preclinical ethanol research is the animal model. Rodents, particularly mice, have frequently been used in preclinical research as they are cheap and large amounts of data can be obtained quickly. Mice are ideal for ethanol research, as the extensive breeding of mice has allowed for the production of discrete strains with different drinking patterns (Belknap et al., 1993). The application of these strains allows for the modeling of human populations with

different experiences with ethanol consumption. The most widely used strain of mice is the C57BL/6 strain, which displays a high alcohol preference phenotype (McClearn & Rodgers, 1959). C57BL/6 mice will voluntarily consume dependence-inducing levels of ethanol, which is critical to modeling ethanol consumption in human populations (Hwa et al., 2011). Despite the usefulness of the C57BL/6 strain in alcohol research, it is important to consider that their inbred background is associated with other physiological differences (Griebel et al., 2000) (Lucki et al., 2001) (Beck et al., 2000) (Vivien-Roels et al., 1998). Another strain that has been employed in alcohol research is Swiss Webster (CFW) mice. While CFW mice do not consume as much ethanol as C57BL/6 mice, their genetic diversity is useful in modeling the genetic variation of human populations.

Purpose:

Through the research presented here, we attempt to build upon the growing field connecting glucocorticoids and substance use. Using two mouse strains and two ethanol access schedules, we offer a novel perspective, exploring the role of glucocorticoids in social defeat stress-induced escalations in ethanol consumption. We predict that inhibition of glucocorticoid synthesis via systemic administration of the metyrapone will selectively attenuate social defeat stress-enhanced ethanol consumption in murine models.

Materials and Methods:

Animals:

Swiss Webster mice (CFW) and C57BL/6 mice were used in all experiments. Adult male CFW mice (n = 32 , weight = 23-25g) were obtained from Charles River Laboratories, Wilmington, MA, USA. Male C57BL/6J mice (n = 50, weight = 23-25g) were obtained from Jackson Laboratory, Bar Harbor, ME, USA.

Animals were single-housed in polycarbonate cages (28 x 17 x 12 cm) with stainless steel lid cage tops and pine shaving bedding. Throughout the experiment, animals were provided a diet of Rodent Diet 5001 (LabDiet, St. Louis, MO) *ad libitum*. Prior to ethanol access, animals were provided tap water *ad libitum.* During periods of ethanol exposure, animals were provided fluids according to their access schedule. Animals were housed in vivarium rooms with a 12-hour reversed light/dark schedule (lights off at 0700, lights on at 1900). Throughout experimentation, vivarium rooms were maintained at constant temperature (21±2 °C) and humidity (25%).

All experimental procedures conducted were approved by the Tufts University Institutional Animal Care and Use Committee.

Social Defeat:

The social defeat procedure consisted of three phases: pre-defeat threat, defeat, and post-defeat threat. Experimental animals ("intruders") received between 8 and 10 defeat sessions, depending on experimental conditions. Defeat procedures were conducted between 0900 and 1100 daily.

Resident animals ("residents") used in experimentation were male animals, pair-housed with female animals. To ensure uniform defeat experience in intruders, all residents were screened for aggression prior to their use. Additionally, to prevent habituation of the resident to the intruder, residents were rotated between intruders across defeat sessions.

The resident female was removed from the resident's home-cage and placed in a holding-cage for the duration of the social defeat procedure. Prior to defeat, each intruder was weighed. In the pre-defeat threat phase, the intruder was placed into a protective cage (15 x 7 x 7 cm) which was then placed into the resident's home-cage containing the male resident. The intruder remained in the protective cage for 5 minutes, after which the protective cage was removed and the intruder was placed in the resident's home-cage without protection. This began the defeat phase, during which the intruder and resident were allowed to interact. Defeat lasted until one of four conditions was met: 5 minutes elapsed, the intruder had received 15 or 30 attack bites from the resident (depending on experimental group), the intruder shows visible signs of wounding, or the intruder adopted a defeat posture for three consecutive seconds (Miczek et al., 1982). When one condition was met, the intruder was removed from the resident home-cage, placed back into the protective cage, and then returned to the resident home-cage for the post-defeat threat phase. The intruder remained in the resident home-cage for 5 minutes, after which the intruder was removed and returned to its own home cage. The resident female was then returned to the resident home-cage.

Ethanol:

20% ethanol solutions (w/v) were prepared from 95% Ethyl alcohol (Pharmaco-AAPER, Brookfield, CT) and tap water. Ethanol solution and water were presented in two 50 mL centrifuge tubes (Nalgene), topped with no. 5 rubber stoppers, each containing a stainless steel, double ball bearing nozzle. The bottles were inserted upside-down through the wire lid of the cage, allowing the mice to draw solution from the nozzle.

Drugs:

Metyrapone (an 11-beta hydroxylase inhibitor) was purchased from Tocris Bioscience (Minneapolis, MN). Metyrapone drug solutions were prepared in 0.9% NaCl saline and injected intraperitoneally in a volume of 10ml/kg of body weight.

BEC Measurements:

Blood for BEC measurements was taken at the end of experimentation, after which animals were euthanized. Blood samples were taken after 2 hours of ethanol access via the submandibular vein using disposable lancets. Samples were centrifuged for 10 min at 3000 rpm and 4°C. Plasma was then extracted for analysis with an Alcohol Analyser (Analox Instruments, Lunenberg, MA).

Experimental Design:

After 10 days of habituation to the vivarium and light/dark schedule, animals were randomly assigned to either the experimental or control group. Experimental animals were subjected to a social defeat procedure of the resident-intruder paradigm for 8 or 10 days, while control animals were weighed daily during the social defeat period.

After the social defeat period, animals were allowed 10 days of rest before exposure to alcohol. On day 9 of the rest period, animals were provided two bottles of water from which to drink, in order to habituate them to a two-bottle choice presentation. After the 10-day rest period, animals were presented with 20% ethanol on either a continuous or intermittent access schedule. Bottles were weighed daily to determine fluid intake, and animals were weighed weekly to provide bodyweight-controlled intake values. For every ethanol access day, ethanol and water bottles switched sides to control for side preference. A drip cage, without an animal, accompanied each cohort of animals. The drip cage bottles were weighed daily to provide a baseline level of fluid loss from evaporation or transfer loss that was incorporated into calculations of animals' fluid intake.

Continuous access:

Animals on continuous access were presented with water and 20% ethanol solution during all days of experimentation. Bottles were weighed and refilled between 0930 and 1030.

Intermittent access:

Animals on intermittent access received two-bottle choice of water and 20% ethanol on M, W, and F. On days without ethanol access (T, R, S, U), animals were presented with two sipper tubes filled with water. Bottles were weighed and refilled between 930 and 1030.

Metyrapone Administration:

After 4-6 weeks of 20% ethanol access, animals were habituated to intraperitoneal injections. Throughout habituation, animals continued to receive ethanol on the same schedule as previously used. Habituation was accomplished through injection handling (handling of animals without providing any injection) for three consecutive days, followed by injections of 0.9% NaCl saline administered at 10ml/kg for 2 days. This procedure was repeated until animals no longer displayed acute handling stress-induced suppression of alcohol intake.

Once animals had become habituated, drinking patterns were assessed after systemic metyrapone injection. Treatments were scheduled using a Latin-square design in which each animal received all treatments. All drug injections were separated by at least 48 hours to prevent carry-over of persistent drug effects. Injections were delivered in a volume of 10ml/kg and administered 30 min prior to ethanol access. On drug injection days, bottles were weighed 2, 4, and 24 hours after the beginning of ethanol access.

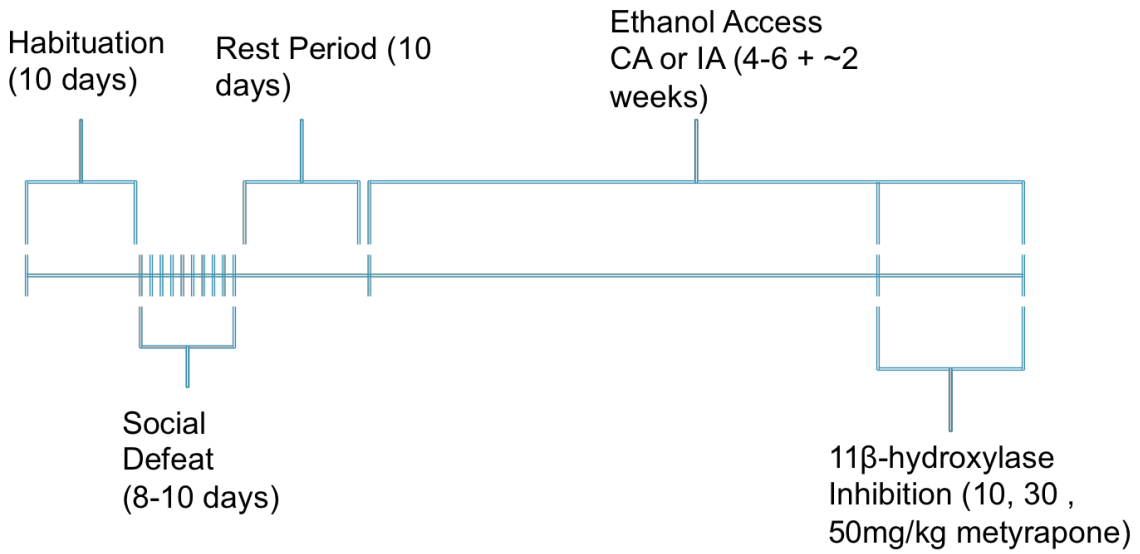

Figure 1. Experimental timeline for social defeat, ethanol drinking on continuous or intermittent access, and metyrapone administration.

Statistical Analyses:

SigmaStat 13.0 software (Systat Software, San Jose, California) was used for statistical analysis of experimental results. Two-way repeated measure (RM) analyses of variance (ANOVA) were conducted to analyze the effects of stress procedures and time on voluntary ethanol consumption and ethanol preference across a variety of access schedules. One-Way ANOVA was used to compare career drinking and preference between groups. Two-Way RM-ANOVA was also used to determine the effects of stress manipulations and metyrapone administration on voluntary ethanol consumption at several time points following metyrapone administration. Holm-Sidak post hoc *t* tests were used to identify statistically significant treatment differences, $p<0.05$. Descriptive statistics for all measurements are indicated as mean ± SEM.

Results:

Experiment 1A: CA CFW Ethanol Intake Following Social Defeat

In this experiment, defeated animals were subjected to eight consecutive days of the social defeat procedure. Following six weeks of CA ethanol presentation, drinking patterns for defeated and control animals were assessed (Fig 2.). During the six weeks of ethanol access, the defeat group consumed more ethanol than the control group (defeat: mean=10.732±0.738; control: mean=7.226±0.691) (Fig.2a). Two-Way RM ANOVA indicated a significant main effect of time [$F(5, 26) = 8.842$, $p<0.001$]. There was not a significant effect of stress on ethanol intake [$F(1, 26)) = 2.061$, $p=0.163$]. For ethanol preference, Two-Way RM ANOVA indicated a significant main effect of time [$F(5, 26) = 8.200$, $p<0.001$]. Again, there was no significant effect of stress ($p=0.179$).

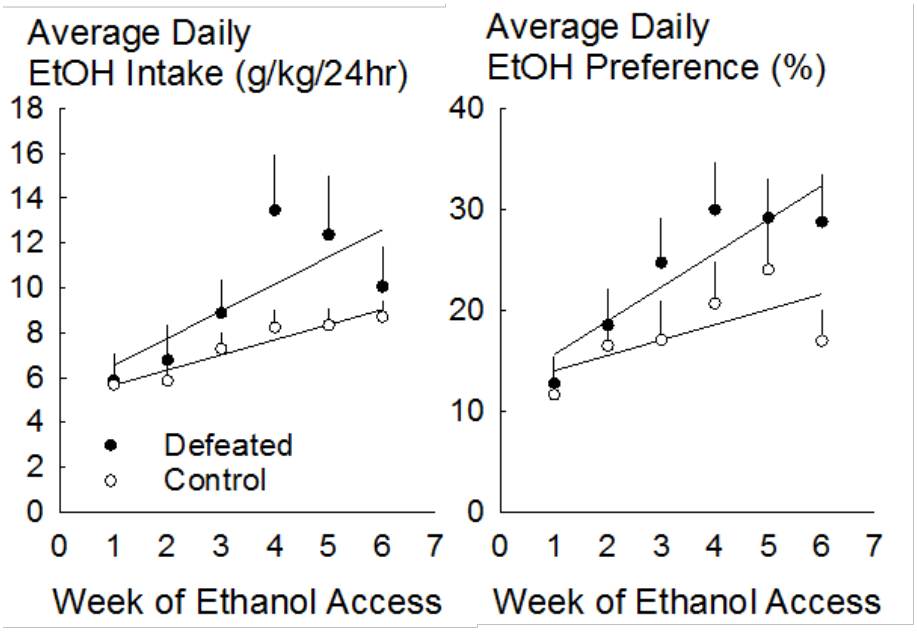

Figure 2. Career ethanol drinking for Continuous Access CFW. Defeated n=20; Controls n=10. a) Daily ethanol intake (g/kg) averaged across weeks; b) Daily ethanol preference (%) averaged across weeks. Data points are weekly averages ± SEM.

Experiment 2A: CA C57BL/6 Ethanol Following After Social Defeat

Following ten days of consecutive social defeat stress and a resting period of ten days, adult male C57BL/6 mice were provided ethanol (20% w/v) on a continuous access schedule for four weeks (Fig.3.). Animals in the defeated group consumed more ethanol than non-defeated controls throughout the four-week access period (defeat: mean= 19.240±0.495; control: mean= 15.865±0.541) (Fig. 4a.). Two-Way RM ANOVA revealed a significant effect of stress on ethanol consumption averaged across weeks [$F(1, 3) = 6.414$, $p<0.019$]. Defeated mice drank significantly more ethanol than control animals ($p<0.019$). Preference data indicated a significant main effect of group on ethanol preference [$F(1, 21) = 5.049$, $p=0.035$] (Fig. 4b.). Defeated animals preferred ethanol to a greater degree than control animals (defeat: mean = 55.064±1.224%; control: mean=47.827±1.756) ($p=0.037$).

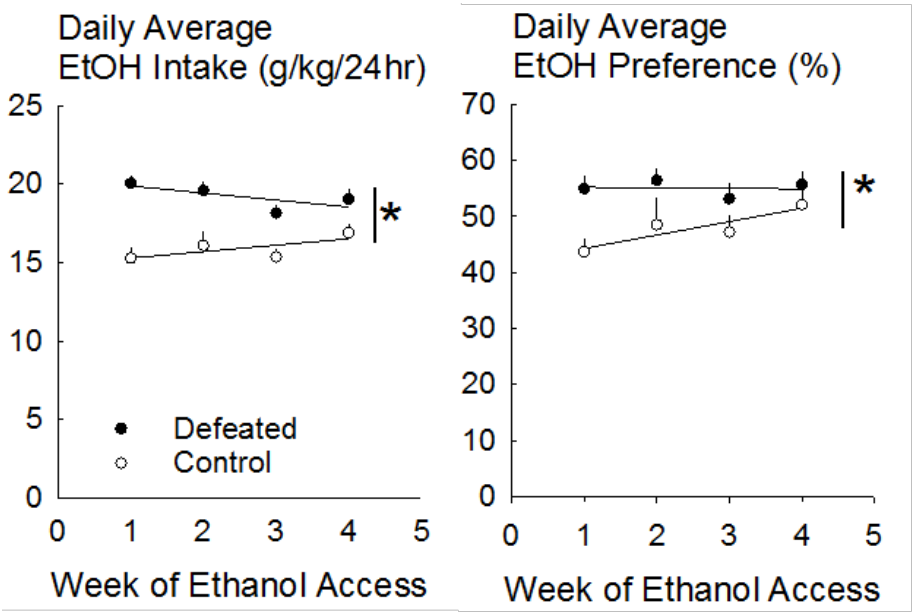

Figure 3. Career ethanol drinking for Continuous Access C57BL/6J. Defeated n=15; Controls n=8. a) Daily ethanol intake (g/kg) averaged across weeks; b) Daily ethanol

preference (%) averaged across weeks. Data points are weekly averages ± SEM; *p<0.05 compared to controls.

Experiment 3A: IA C57BL/6 Ethanol Intake Following Social Defeat

Two intensities of social defeat procedure were used in this experiment: mild (defeat ended following the delivery of 15 bites) and moderate (defeat ended following the delivery of 30 bites). Regardless of defeat intensity, defeated animals were subjected to ten consecutive days of the social defeat procedure. Following a ten-day rest period, animals were provided ethanol on an intermittent access schedule for four weeks (Fig. 4.). During the four-week period, both of the defeated groups consumed more ethanol than non-defeated controls (moderate: mean= 25.807±0.800; mild: mean= 27.088±0.998; control: mean= 23.194±0.912) (Fig. 5a). Two-Way RM ANOVA revealed significant main effects of stress [F(2, 21)= 4.171, p=0.030] and time [F(3, 21) = 59.724, p<0.001] on ethanol consumption. Post hoc analysis with Holm-Sidak indicated a significant difference in ethanol drinking between the mild defeat and control group (p=0.035), while the moderate defeat and control groups were not statistically different (p=0.093). Preference data indicated a main effect of time (F(3, 21) = 39.414, p<0.001), however, there was no significant main effect of group (Fig. 5b).

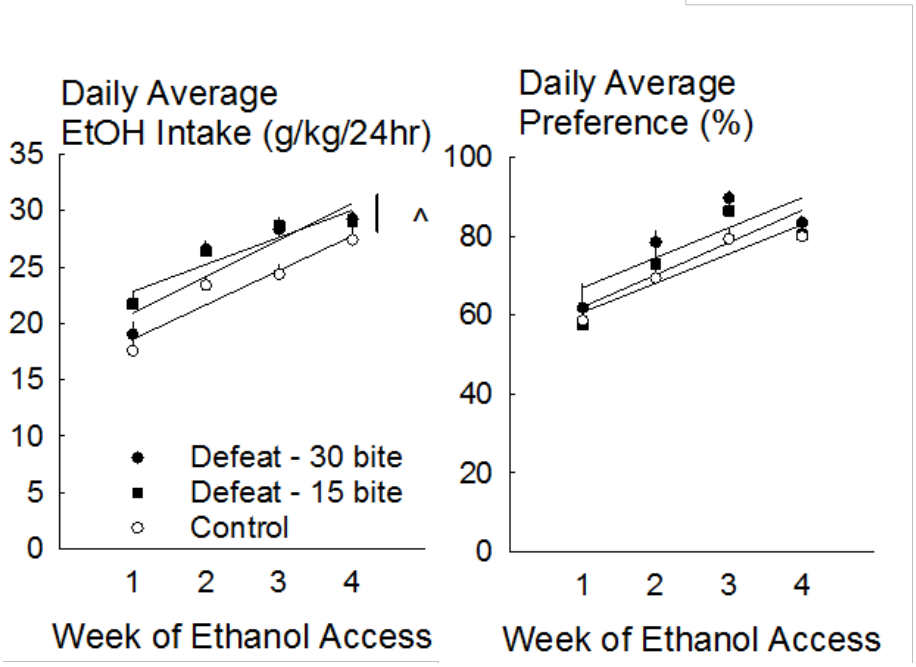

Figure 4. Career ethanol drinking for Intermittent Access C57BL/6J. Defeat – 30 bite n=10; Defeat – 15 bite n=6; Controls n=8. a) Daily ethanol intake (g/kg) averaged across weeks; b) Daily ethanol preference (%) averaged across weeks. Data points are weekly averages ± SEM; ^p<0.05 Defeat - 15 bite compared to controls.

Experiment 1B: Metyrapone Administration in CA CFW

Following six weeks of continuous access to ethanol, animals were habituated to intraperitoneal injections. After habituation, animals were administered three doses of metyrapone on within-subjects design. Ethanol consumption was determined at time-points of 2, 4, and 24 hours following presentation with ethanol (Fig. 5.).

Metyrapone administration produced a dose-dependent decrease in ethanol consumption at the two-hour time-point. Two-Way RM ANOVA indicated a main effect of metyrapone dose on ethanol consumption [$F(3, 28) = 4.329$, p=0.007], and Holm-Sidak post hoc analysis revealed a significant difference between ethanol consumption with saline vehicle and 50mg/kg metyrapone administration ($p<0.05$).

At the 4- and 24-hour measurements, the main effect of metyrapone dose had disappeared. There was no significant effect of group or metyrapone dose on water consumption at any of the time-points.

Experiment 2B: Metyrapone Administration in CA C57BL/6J

After four weeks of continuous access to ethanol, animals were habituated to IP injection, and administered three doses of metyrapone IP on a within-subjects design. Ethanol consumption was recorded 2, 4, and 24 hours after ethanol presentation (Fig. 6.).

At all time-points, a dose-dependent decrease in ethanol consumption was observed following metyrapone administration. A significant effect of metyrapone dose was present at the 2-, 4-, and 24-hour time-points, as determined by Two-Way RM ANOVA (2-hr: [$F(3, 24) = 5.125$, $p=0.003$; 4-hr: $F(3, 24) =11.186$, $p<0.001$; 24-hr: $F(3, 24)=6.891$, $p<0.001$). Post hoc Holm-Sidak analysis indicated a significant difference in ethanol intake between treatments with vehicle or 50mg/kg metyrapone ($p<0.05$).

A significant effect of metyrapone dose on water intake was observed at 2- and 4-hour time-points [2-hr: $F(3, 24) = 11.248$, $p<0.001$; 4-hr: $F(3, 24) = 5.295$, $p=0.002$]. Holm-Sidak analysis indicated a significant difference in water intake between vehicle and 50mg/kg metyrapone dose ($p<0.05$).

Experiment 3B: Metyrapone Administration in IA C57BL/6J

Following four weeks of intermittent access to ethanol, animals were habituated to IP injection, and administered three doses of metyrapone IP on a within-subjects design. Ethanol consumption was recorded 2, 4, and 24 hours after ethanol presentation (Fig. 7.).

Two-Way RM ANOVA revealed significant main effects of metyrapone dose at the two-, four-, and 24-hour measurement [2-hr: $F(3, 21) = 4.545$, $p<0.001$; 4-hr: $F(3, 21) = 7.534$, $p<0.001$; 24-hr: $F(3, 21) = 8.557$, $p<0.001$]. A significant main effect of group was seen at the two-, four-, and 24-hour time-points as well [2-hr: $F(2, 21) = 15.636$, $p<0.001$; 4-hr: $F(2, 21) = 7.082$, $p=0.004$; 24-hr: $F(2,21) = 10.248$, $p<0.001$]. Post hoc analysis via Holm-Sidak indicated significant differences at all time-points between moderately stress animals and controls ($p<0.05$) and differences between consumption with vehicle and 50mg/kg metyrapone administration ($p<0.05$).

There was a significant main effect of metyrapone dose on water intake at the 2-hour time-point [$F(3, 21) = 2.856$, $p<0.044$], and a significant main effect of group on water intake at the 4-hour time-point [$F(2, 21) = 9.506$, $p=0.001$). Post hoc analysis indicated that at the 4-hour measurement, controls drank significantly more water than 30-bite defeat animals ($p<0.05$).

Blood Ethanol Concentration Measurements:

Following conclusion of metyrapone administration, C57BL/6J animals on CA and IA (n=39) were administered either an effective dose of metyrapone (50mg/kg)

or vehicle. 30 minutes after injection, animals were provided ethanol access for two hours. After two hours, animals had blood drawn, and BECs were obtained (Fig. 8.).

Statistics were not run, as there were few animals. Comparison with previous research indicates that ethanol intake and associated BECs were consistent with previous observation (Rhodes et al., 2007). Additionally, there does not appear to be any effect of metyrapone on alcohol metabolism; the relationship between ethanol consumed and BEC does not appear to be different between vehicle and metyrapone treated animals.

Discussion:

This series of experiments provides evidence of the link between stress history and subsequent alcohol consumption in a murine model. These results confirm previous observation that the social defeat procedure employed in these experiments has the potential to elicit escalated voluntary ethanol consumption across a variety ethanol access schedules (Norman et al., 2015) (Hwa et al., 2015). Daily ethanol consumption was highest in intermittent access C57BL6/J animals, with stress-escalated drinking exceeding 25g/kg/24hr, and some animals achieving pharmacologically relevant Blood Ethanol Concentrations (BECs) (80mg/dl) in the two-hour period following presentation with ethanol.

To explore the role of glucocorticoids in this stress-escalated alcohol drinking, after four to six weeks of drinking, animals were systemically administered the glucocorticoid synthesis inhibitor metyrapone prior to ethanol

presentation. Metyrapone dose-dependently suppressed ethanol consumption regardless of stress history, and this effect persisted up to 24 hours following ethanol presentation. Additionally, metyrapone administration either enhanced or had no effect on water intake, indicating specificity for suppression of ethanol intake.

Socially defeated CFW mice failed to show statistically significant escalations in ethanol consumption compared to control animals. While these data contradict previously published data (Norman et al., 2015), the large amount of variability in defeated animals, despite the large sample size, suggests that the genetic variability present in CFW populations is responsible for the lack of an observable stress-escalation in drinking. Clinical and preclinical research has identified that stress-escalated alcohol consumption only manifests itself in a subset of human and rodent populations (Uhart & Wand, 2009) (Clarke et al., 2008) (Becker et al., 2011). Such data suggests that this pattern may emerge in outbred CFW populations. However, the current experiments do not have a sufficient number of animals to accurately define subpopulations of CFW's that are "resistant" and "vulnerable" to stress-escalated drinking.

C57BL6J mice that were subject to intermittent social defeat stress displayed higher ethanol intake than control animals. These data compliment previous research indicating the success of this particular paradigm of social defeat in escalating subsequent ethanol drinking (Norman et al., 2015) (Hwa et al., 2015). Interestingly, in the intermittent access C57BL/6J animals, moderately defeated

animals (30 bites) failed to show significantly higher drinking than controls over the four-week drinking period, while mildly defeat animals (15 bites) did show stress-escalated drinking. It is suspected that this lack of significant stress-escalation is a result of excessive defeat severity. As previously described, stress has a biphasic nature in relation to physiological well-being (McEwen, 2009). This relationship has been shown to hold between stress and ethanol consumption as well (Miczek et al., 2008). In depth assessment of the defeat experience in these animals would be required to make further conclusions.

The reliability of this social stress methodology comes as a boon to the study of the relationship between stress and alcohol use. While there is strong clinical evidence of a relationship between stress and ethanol consumption (Sinha, 2001) (Widom et al., 1999) (Harrison et al., 1997), development of animal methodologies that consistently model this effect has been challenging (Becker et al., 2011). Early attempts at modeling social defeat stress-induced escalations in ethanol intake were unsuccessful, indicating a suppression of alcohol consumption with concurrent exposure to as stressor (van Erp & Miczek, 2001) (van Erp et al., 2001) (Funk et al., 2005). These studies demonstrated that acute stress suppresses ethanol intake. Further research prolonged observation of ethanol intake following social defeat stress, indicating a delayed onset of stress-escalated drinking (Sillaber et al., 2002) (Croft et al., 2005). Such results suggest the presence of time-dependent stress-escalations in ethanol intake. The procedure used in this experimentation confirms this relationship, demonstrating that a history of social defeat stress has the

potential to escalate voluntary ethanol intake across ethanol access schedules (Norman et al., 2015) (Hwa et a., 2015).

In the present series of experiments, glucocorticoid synthesis inhibition via metyrapone administration suppressed ethanol consumption in both C57BL/6J and CFW mice, and in animals with intermittent or continuous access to ethanol. Such results compliment previous research indicating a role of glucocorticoids in the ethanol drinking. Suppression of glucocorticoids has been shown to suppress ethanol consumption in high-drinking animals (Fahlke et al., 1994b), while application of exogenous corticosterone can elicit escalations in ethanol consumption (Fahlke et al., 1995). Taken together, these results provide compelling evidence for the role of glucocorticoids in potentiating ethanol intake in animal models.

We hypothesize that social defeat stress-induced HPA hyperactivity influences the escalation in ethanol consumption observed in these experiments. Previous research has indicated that exposure to stressors can produce persistent HPA hyperactivity (Sanchez et al., 2001) (Heim et al., 2008) (Miller et al., 2007) (Plotsky & Meaney, 1993), suggesting that defeat history can result in HPA hyperactivity weeks after the stressful experience. Additional research has indicated that high plasma corticosterone concentrations have the potential to suppress glucocorticoid receptor expression (Sapolsky et al., 1984a), and as such, could result in hypersecretion of glucocorticoids via disinhibition of feedback mechanisms

(Sapolsky et al., 1984b). The social defeat stress parameter used in our experimentation has been shown to be resistant to HPA habituation, such that the first and last defeat sessions elicit the same intensity of HPA response (Norman et al., 2015). With this in mind, it is reasonable to suspect that the repeated HPA hyperactivity produced by the social defeat stress procedure could result in persistent changes in HPA axis activity. However, without assessment of plasma corticosterone concentrations, definitive conclusions cannot be made.

HPA signaling molecules have been implicated as modulatory molecules for neurochemical systems associated with drugs of abuse. Dopamine (DA) signaling has been of particular interest to drug abuse research. Researchers have identified that DA signaling underlies the natural reward circuitry of the brain (Wise & Rompre, 1989), and that the rewarding effects of drugs of abuse are dependent on and correlated with activation of the mesolimbic dopamine system (Spanagel & Weiss, 1999) (Pierce & Kumaresan, 2005) (Volkow et al., 1999). The mesolimbic system is a series of connected anatomical structures of the basal ganglia, including ventral tegmental area (VTA) and the nucleus accumbens (NAcc) (Pierce & Kumaresan, 2005). As with other drugs of abuse, ethanol has been shown to elicit increases in extracellular mesolimbic DA (Di Chiara & Imperato, 1988) (Weiss et al., 1993), suggesting that modulation of mesolimbic DA activity could influence ethanol drinking.

Stress itself activates dopaminergic systems of the brain, increasing extracellular dopamine concentrations in a variety of brain regions, including the

shell of the NAcc (Abercrombie et al., 1989) (Kalivas & Duffy, 1995). Administration of CRF and corticosterone independently produce increases in dopaminergic activity, suggesting that both HPA axis molecules are responsible for this activity (Kalivas et al., 1987) (Piazza et al., 1996). Additionally, glucocorticoid receptors are present in DA neurons of the mesolimbic system, confirming the possibility of direct modulation of DA activity by glucocorticoids (Harfstrand et al., 1986). Researchers have identified that the presence of glucocorticoids potentiates DA release from dopaminergic neurons (Ronken et al., 1994) (Piazza & Le Moal, 1996). In this way, the presence of higher concentrations of glucocorticoids could influence the neurochemical response to ethanol, contributing the elevated ethanol consumption in stressed animals.

Investigations of the mechanisms underlying this relationship have revealed that stress induces neuroplastic changes in the mesolimbic system to potentiate DA activity. Stress has been shown to upregulate expression of tyrosine hydroxylase (TH), while also suppressing expression of monoamine oxidase (MAO) (Ortiz et al., 1996) (Piazza & Le Moal, 1996). Such effects promote an increase in DA synthesis, while simultaneously antagonizing DA metabolic degradation. In addition to the effects of stress on expression of DA-related proteins, intermittent stress has also been shown to increase expression of brain-derived neurotrophic factor (BDNF), a protein involved in synaptic formation and plasticity (Nikulina et al., 2014). This suggests that stress can promote the proliferation of synaptic connections in the DA system, potentiating DA activity (Nikulina et al., 2014). Of particular interest, mesolimbic upregulation of BDNF after stress has been shown to persist for weeks

following the stressful experience (Fanous et al., 2010). These effects of stress on gene expression suggest that HPA hyperactivity during the social defeat phase could produce long-term changes in mesolimbic activity that could influence subsequent ethanol consumption.

In these experiments, systemic metyrapone administration dose-dependently suppressed ethanol consumption. This effect of metyrapone was observed on both CA and IA schedules, and in C57BL/6J and CFW mice. The metyrapone dosing parameters used in these experiments has been shown to suppress plasma corticosterone concentrations (Goeders & Guerin, 2008). This effect on corticosterone has been attributed to the action of metyrapone as an 11-β hydroxylase inhibitor. We have yet to assess plasma corticosterone in our animals, however, previous research indicates that it reasonable to suspect metyrapone administration at the 50mg/kg dose suppressed plasma corticosterone in our animals (Goeders & Guerin, 2008).

We hypothesize that suppression of ethanol drinking by metyrapone is a result of inhibition of glucocorticoid synthesis. Previous research has identified that escalation of corticosterone via exogenous application results in increased alcohol consumption (Fahlke et al., 1995). Complementary data indicate that suppression of corticosterone concentrations produces lower ethanol intake (Fahlke et al., 1994a) (Fahlke et al., 1994b). As discussed above, glucocorticoids have been shown to potentiate the neurochemical effects of ethanol and other drugs of abuse.

Suppression of plasma glucocorticoid concentration via metyrapone administration would remove this potentiation, contributing to lower ethanol consumption.

In addition to the activity of metyrapone as an 11-β hydroxylase inhibitor, researchers have implicated influence on other pathways in the production of metyrapone's behavioral effects. Metyrapone has been shown to competitively inhibit 11- β- hydroxysteroid dehydrogenase type 1 (11-β-HSD1) at micromolar affinity (Sampath-Kumar et al., 1997). 11-β-HSD1 is an enzyme that regenerates corticosterone from its inactive ketone metabolite 11-dehydrocorticosterone (11-DHC), increasing intracellular concentrations of glucocorticoids (Holmes et al., 2003). In this way, metyrapone administration exerts a second mechanism to suppress glucocorticoid action; antagonizing the restoration of metabolically inactivated intracellular glucocorticoids, effectively lowering glucocorticoid concentrations.

Additional research has suggested a non-glucocorticoid dependent mechanism of metyrapone action. Metyrapone has been shown to increase biosynthesis of $GABA_A$-active neurosteroids (Raven et al., 1996). This activity occurs as a result of modulation of steroidogenesis pathways (Schmoutz et al., 2014). Inhibition of 11-β-hydroxylase results in accumulation of metabolic precursors deoxycorticosterone (11-DOC) and progesterone. The build-up of these precursors results in their funneling toward the synthesis of other steroids, enhancing the synthesis of the neurosteroids tetrahydrodeoxycoricosterone (THDOC) and allopregnanolone (ALLO) (Schmoutz et al., 2014).

While metyrapone has been shown to increase $GABA_A$-active neurosteroid production, the effect that this has on ethanol consumption remains inconclusive. Some studies have indicated a potentiation of ethanol consumption in the presence of neurosteroids (Janak & Gill, 2003) (Nie & Janak, 2003), while others have suggested a suppression of ethanol consumption with $GABA_A$ agonism (Besheer et al., 2010) (Ramaker et al., 2015). Interestingly, this activity may be dependent on previous ethanol history, such that neurosteroids decrease ethanol intake in animals with a history of high consumption (Roberts et al., 1996) (Morrow et al., 2001). Such data suggest that metyrapone-induced increases in neurosteroid production could influence drinking in animals with a history of high consumption. However, research in our lab has also indicated that inhibition of neurosteroid synthesis with finasteride suppresses ethanol drinking in mice with equivalent drinking history to the animals in these experiments (Auld Senior Honors Thesis, unpublished). These contradictory results indicate that further research is necessary before drawing conclusions about the effects of metyrapone-induced neurosteroid enhancement on our paradigms of ethanol drinking.

Interestingly, the administration of metyrapone resulted in strain-specific timelines for the suppression of ethanol consumption. Metyrapone administration in C57BL/6J mice produced suppression of ethanol consumption 24 hours after ethanol presentation. This suppression was present in animals on IA and CA ethanol schedules, suggesting that the efficacy of metyrapone was not dependent on ethanol schedule. Metyrapone administration in CFW mice, however, did not suppress

ethanol consumption at four or 24 hours. These strain-specific differences in the duration of metyrapone efficacy parallel previous data indicating suppression of ethanol drinking with metyrapone in high-preferring animals only (Fahlke et al., 1994b). In high-preferring animals, suppression in drinking with metyrapone administration was observed after six hours of ethanol access, while low-preferring animals showed no such suppression after six hours (Fahlke et al., 1994b). These results indicate that metyrapone has greater effect in high-preferring animals (Fahlke et al., 1994b). In our experiments, C57BL/6J mice can considered a high-alcohol preferring strain (McClearn & Rodgers, 1959), while CFW mice are a low-preferring strain. Suppression of ethanol drinking with metyrapone can be observed up to 24 hours in high-preferring animals, while this effect is absent past two hours in low-preferring animals. No correlation was found between ethanol history and the magnitude of metyrapone effect, however, the duration of metyrapone efficacy was longer for C57BL/6J mice than CFW mice.

Another factor that could influence strain-specific differences in the duration of metyrapone action could be a result of differential metabolic decomposition rates. Like other pharmaceuticals, metyrapone is metabolically inactivated through reactions with cytochrome p450 enzymes present in the liver (Maser & Netter, 1989). It has been identified that cytochrome P450 enzyme expression profiles differ across mouse strains (Zhang et al., 2003). Such differences could result in different rates of metabolic inactivation of metyrapone, accounting for the prolonged duration of metyrapone activity in C57BL/6J animals.

It is intriguing that we fail to see an interaction between stress experience and metyrapone dosing in these experiments. If stressed-escalated ethanol drinking were dependent on higher circulating levels of glucocorticoids, it could be expected that the metyrapone effect would vary depending on stress history. We have yet to assess plasma corticosterone concentrations in stressed and control animals, with and without metyrapone administration. As such, we cannot definitively identify differences in corticosterone concentrations across groups.

If we identify that plasma corticosterone does not differ between stressed and non-stressed animals, it could be the result of a return to baseline HPA axis activity in the stressed animals over the course of the experiment. As previously mentioned, in some instances, stress has been shown to produce persistent HPA hyperactivation (Sanchez et al., 2001) (Heim et al., 2008) (Miller et al., 2007) (Plotsky & Meaney, 1993). However, there are additional studies suggesting a return to baseline, or even suppression of HPA axis following stress (Miller et al., 2007). Additionally, access to ethanol over several weeks could influence circulating glucocorticoids in experimental animals. It has been shown that ethanol activates the HPA axis and results in elevated plasma levels of corticosterone (Ellis, 1966). With chronic exposure, ethanol results in higher basal CRF expression, and higher plasma concentrations of ACTH and corticosterone (Rivier et al., 1990). The influence of ethanol on HPA axis activity could overwhelm any difference in plasma corticosterone between the stressed and non-stressed groups.

Recent preclinical research has identified that glucocorticoids are of particular importance to the development and continuation of alcohol dependence. Such results build off of the previous research identifying the role of glucocorticoids in high-preference drinking (Fahlke et al., 1994b). Antagonism of GR has been shown to prevent escalated alcohol intake in dependent animals (Vendruscolo et al., 2012). Additionally, clinical research has identified that GR antagonism reduces alcohol craving and consumption in alcohol-dependent humans (Vendruscolo et al., 2015). Such results indicate the pivotal role of glucocorticoids in dependence-inducing drinking, and suggest that further exploration of metyrapone's effects should be conducted in dependent animals.

The results of this study and several others indicate that 11-β hydroxylase inhibition suppresses ethanol intake. Interestingly, application of glucocorticoid synthesis inhibitors has been shown to increase plasma ACTH via feedback disinhibition (Burrin et al., 1986) (Staub et al., 1979). Such increases in ACTH suggest the presence of accompanying increases in CRF. As extrahypothalamic CRF has been shown to potentiate drug use (Nie et al., 2004) (Erb et al., 2001), this creates opportunity for future research. If possible, the effect of metyrapone on CRF should be explored. Additionally, the combined application of metyrapone with CRF-receptor antagonists may prove synergistic.

We have confirmed previous experimentation demonstrating that a history of social defeat stress can induce subsequent escalations in ethanol consumption

across ethanol access schedules (Norman et al., 2015) (Hwa et al., 2015). Pretreatment with the 11-β-hydroxylase inhibitor metyrapone dose-dependently suppressed ethanol consumption in C57Bl/6J and CFW mice with intermittent and continuous access to ethanol. This effect of metyrapone persisted up to 24 hours after presentation with ethanol. Previous research has identified that the dosing parameters used in these experiments are sufficient to suppress plasma corticosterone concentrations (Goeders & Guerin, 2008). With this in mind, we suspect that reductions in plasma glucocorticoid concentrations associated with metyrapone treatment are responsible for suppression of ethanol intake. We failed to observe an interaction between social defeat stress experience and treatment with metyrapone. These results suggest that stress-escalated drinking observed in these experiments is to some degree dependent on long-term neuroplastic changes associated with stressful experience, rather than persistent escalations in plasma corticosterone concentrations. Further analysis of plasma corticosterone concentrations in animals in this study will allow for more unambiguous conclusions to be made.

Appendix:

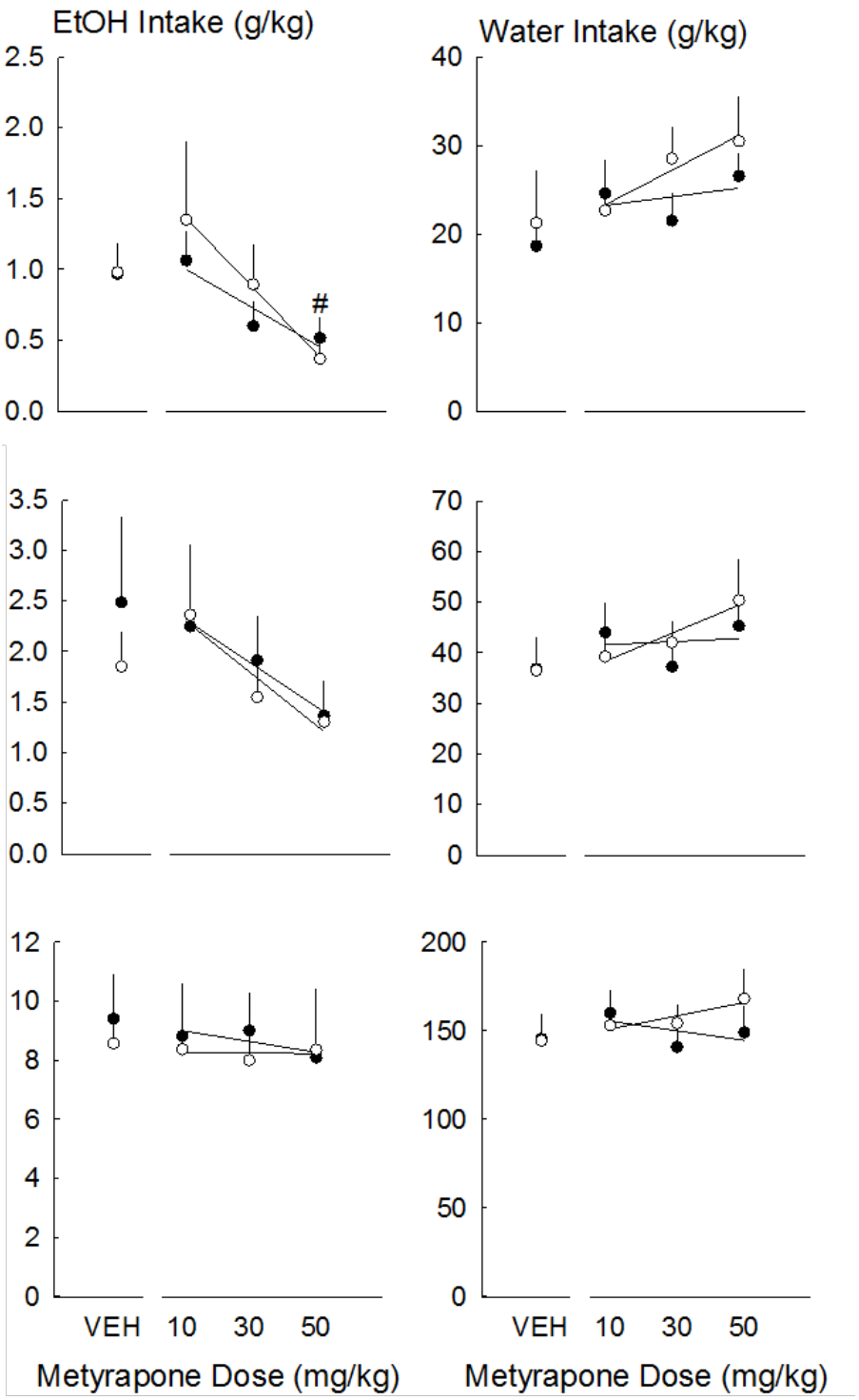

Figure 5. Post-metyrapone ethanol and water drinking for Continuous Access CFW. Defeat – 30 bite n=10; Defeat – 15 bite n=6; Controls n=8. a) 2-hr EtOH; b) 2-hr water; c) 4-hr EtOH; d) 4-hr water; e) 24-hr EtOH; f) 24-hr water. Data points are averages ± SEM; #p<0.05 compared to vehicle.

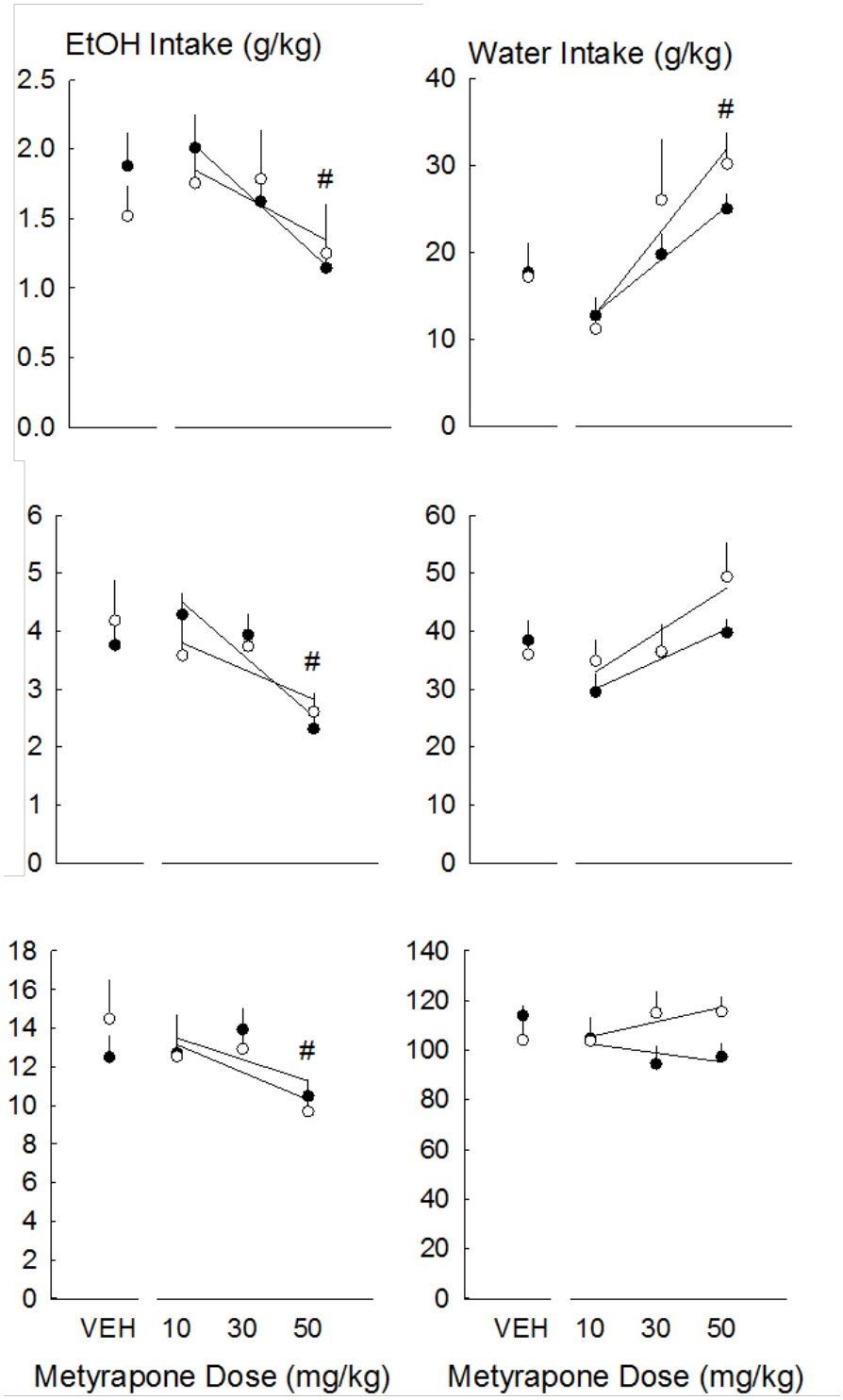

Figure 6. Post-metyrapone ethanol and water drinking for Continuous Access C57BL/6J. Defeat – 30 bite n=10; Defeat – 15 bite n=6; Controls n=8. a) 2-hr EtOH; b) 2-hr water; c) 4-hr EtOH; d) 4-hr water; e) 24-hr EtOH; f) 24-hr water. Data points are averages ± SEM; #p<0.05 compared to vehicle.

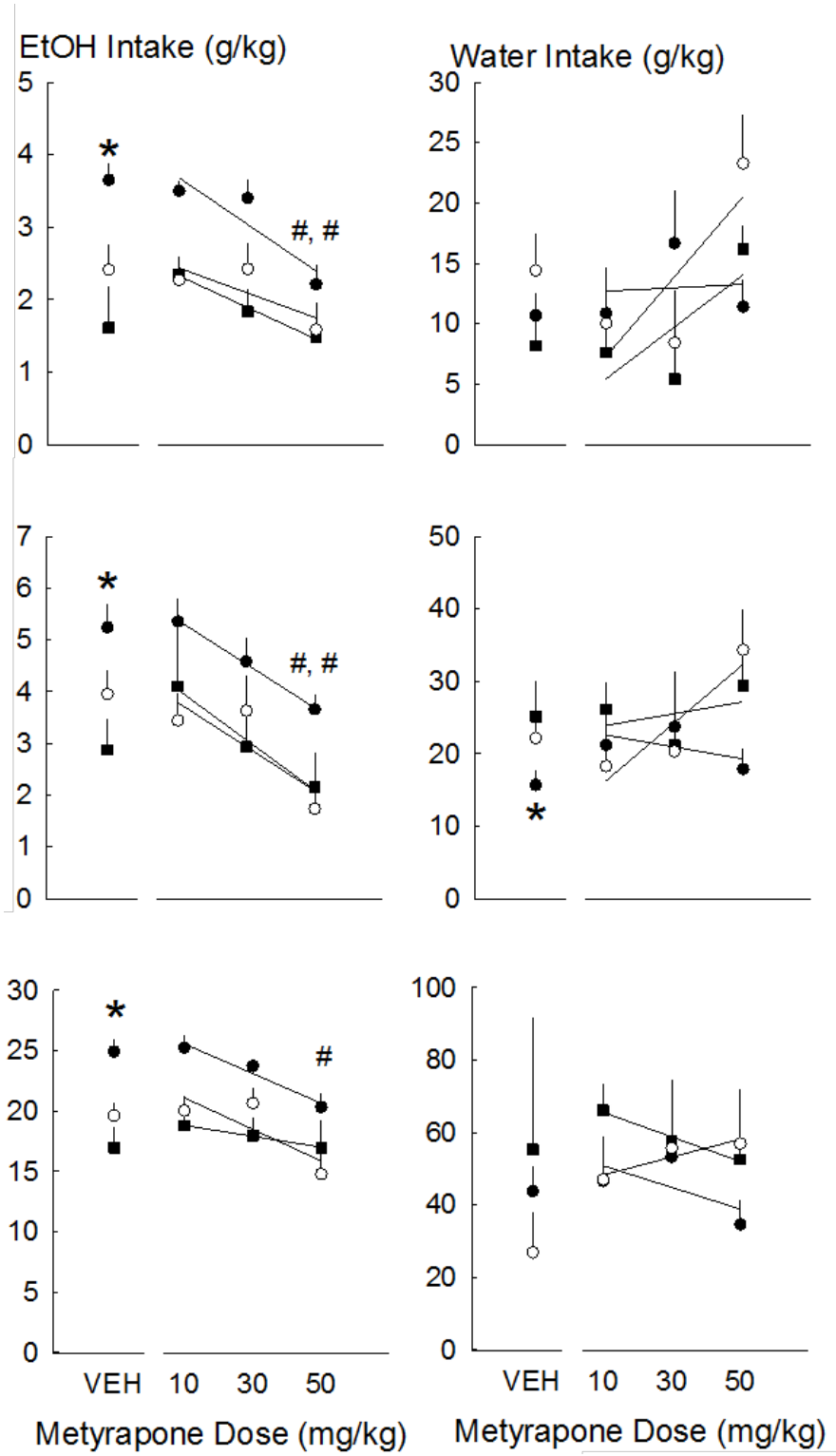

Figure 7. Post-metyrapone ethanol and water drinking for Intermittent Access C57BL/6J. Defeat – 30 bite n=10; Defeat – 15 bite n=6; Controls n=8. a) 2-hr EtOH; b) 2-hr water; c) 4-hr EtOH; d) 4-hr water; e) 24-hr EtOH; f) 24-hr water. Data points are averages ± SEM; *p<0.05 Defeat compared to control, #p<0.05, ##p<0.001 compared to vehicle.

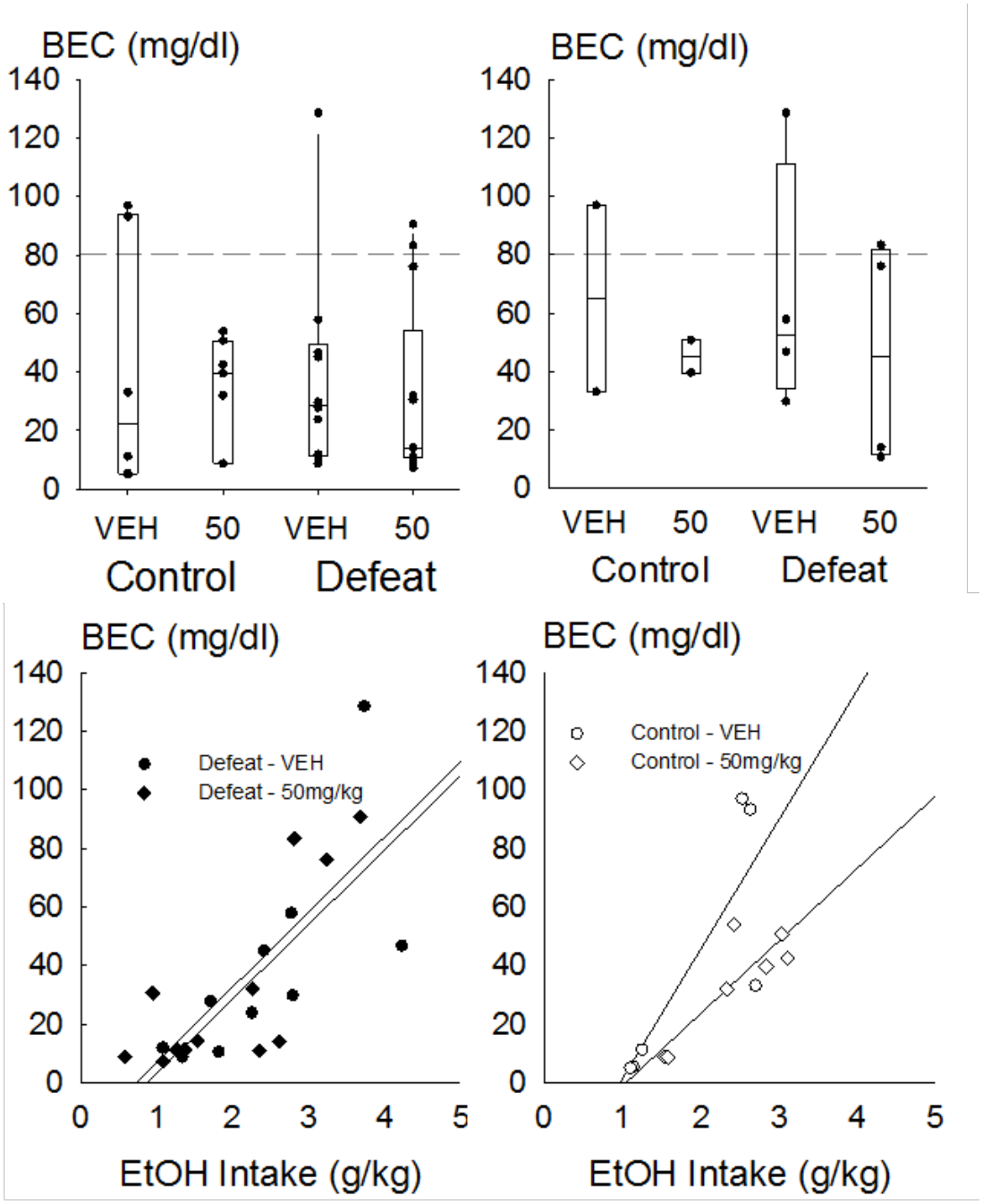

Figure 8. Blood Ethanol Concentrations (BECs) in C57BL/6J mice measured after 2 hours of ethanol access following pretreatment with saline vehicle or 50mg/kg Metyrapone. a) Continuous access; Control-VEH n=4; Control-50 n=5; Defeat-VEH n=6; Defeat-50 n=9. b) Intermittent access: Control-VEH n=2; Control-50 n=2; Defeat-VEH n=4; Defeat-50 n=4. Correlation between ethanol intake (g/kg) and BEC (mg/dl) in vehicle and metyrapone treated c) defeated (vehicle n=10; metyrapone n=13) and d) non-defeated animals (vehicle n=6; metyrapone n=7).

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