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POLYAMINE MODULATION IN ALCOHOLISM: EXAMINATION USING A NOVEL SCREENING PROCEDURE DESIGNED TO PREDICT ANTI-RELAPSE AND NEUROPROTECTIVE EFFICACY

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POLYAMINE MODULATION IN ALCOHOLISM: EXAMINATION USING A
NOVEL SCREENING PROCEDURE DESIGNED TO PREDICT ANTI-RELAPSE
AND NEUROPROTECTIVE EFFICACY

DISSERTATION

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

By

Ben Lewis

Lexington, Kentucky

Director: Dr. Susan Barron, Professor of Psychology

Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

POLYAMINE MODULATION IN ALCOHOLISM: EXAMINATION USING A NOVEL SCREENING PROCEDURE DESIGNED TO PREDICT ANTI-RELAPSE AND NEUROPROTECTIVE EFFICACY

Alcohol dependence is a major public health concern. Despite the FDA's approval of multiple anti-relapse drugs, relapse rates remain unacceptably high. Furthermore, cognitive deficits among chronic drinkers are evident and are suggested to contribute to relapse risk. Current evidence suggests that several critical features of alcoholism and alcohol-associated neurodegeneration are mechanistically linked to glutamatergic actions; specifically, they appear positively affected by glutamatergic inhibition, particularly inhibition via polyamine modulation of a subpopulation of *n*-methyl-*d*-aspartate receptors. The current project was designed to evaluate the performance of two putative polyamine modulators (JR-220 and CP-101,606) in a variety of screens designed to identify the potential to reduce withdrawal severity, neurotoxicity and relapse risk. Screens included a complex organotypic screen designed to assess neuroprotective potential (Experiment 1), a simple behavioral screen designed to assess withdrawal severity (Experiment 2) as well as several more complex behavioral screens designed to examine cue-conditioning during withdrawal (Experiment 3), relapse behavior (Experiment 4), stress-associated consumption (Experiment 5) and binge-like consumption (Experiment 6). An additional open field experiment (Experiment 7) was conducted in order to address interpretational issues concerning activity in Experiments 2-6. Finally, as a first step in moving beyond simple screening, we expanded our binge screen to adhere more closely to an established, validated model of binge consumption (Experiment 8). While some interpretational issues were noted, taken together, the results from these experiments provide strong evidence for both drugs as potential pharmacotherapies for alcoholism and further implicate polyamines and NR2B subunits as critical mechanisms in ETOH consumption and withdrawal.

KEYWORDS: Alcohol, Polyamines, Excitotoxicity, Relapse, NMDAr, Drug Development

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11/15/2011

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*This dissertation is dedicated to Kristen & Megan,
whose shoulders were always there to lean on,
and whose laughter & comfort I miss immeasurably.*

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Chapter 1. Introduction

Alcohol dependence is a major public health concern, with an estimated annual economic cost of over \$180 billion (Harwood, 2000; NIAAA, 2004; Lupton, Burd & Harwood, 2004). In the US, alcohol dependence ranks 3rd in preventable mortality (DHHS, 2000), is estimated to affect approximately 4% of the population (Grant et al., 2004), and contributes to more than 100,000 deaths, annually (McGinnis & Foege, 1999).

Alcohol dependence, herein referred to as alcoholism, is a chronic, relapsing disorder with numerous detrimental health consequences. DSM-IV characterization of dependence includes frequent intoxication, compulsion to consume alcohol, tolerance, loss of control in limiting consumption despite adverse consequences and emergence of a negative emotional state during withdrawal (American Psychiatric Association [*DSM-IV-TR*], 2000). The DSM-IV recognizes both alcohol dependence and abuse as alcohol use disorders (AUDs), however alcohol abuse diagnoses are reserved primarily for individuals not reaching criteria for dependence, but who drink despite alcohol-related problems, or drink in dangerous situations (e.g., driving). Alcohol abuse was the most common problem treated in substance abuse treatment centers from 1994-1999, with 18.2 million people in the US meeting AUD criteria (SAMHSA, 2008).

Ethanol (C₂H₅OH; ETOH), also referred to as ethyl or grain alcohol, is a small straight-chain molecule, and the intoxicating component in alcoholic beverages. Evidence for human ETOH consumption has been noted as far back as the Neolithic period (McGovern et al., 2004), and its contemporary consumption across cultures is nearly ubiquitous. Ethanol is considered a central nervous system (CNS) depressant and psychoactive drug. Dosage is quantified as blood alcohol content (BAC), either as mg/dl, or as % v/v.

Due to the aforementioned economic and health concerns, the development and identification of efficacious treatments for alcoholism remain primary goals in basic research. The current work is focused on the identification and development of known and novel compounds, examining their potential efficacy as neuroprotective and anti-relapse agents.

1.1 Subjective Effects

ETOH has a wide array of subjective effects, dependent on individual differences, dose, and expectations (Sher et al., 2005), which can be generally characterized as anxiolytic, disinhibitive and euphorogenic. Paradoxically, while considered a CNS depressant, ETOH possesses strong stimulatory properties, particularly at lower doses, and during the ascending limb of the BAC curve. At lower doses (.02-.05 mg/dl BAC), ETOH produces feelings of “warmth” and relaxation. Moderate doses (.05-.09 BAC) are associated with reduced inhibitory control, euphoria and mild psychomotor perturbances. Higher doses (.1-.19 BAC) are associated with ataxia, confusion, mood disturbances and drowsiness, with still higher doses inducing amnesia, unconsciousness, respiratory distress and death, with an LD50 of approximately .4 (for review see Doweiko, 2010).

1.2 Absorption

Ethanol is both water and lipid soluble, facilitating its rapid distribution to all blood-rich tissues in the body. Due to its high water solubility, concentrations in brain quickly surpass those in blood, (Kranzler & Ciraulo, 2005). The small intestine provides the main route of ETOH absorption, however 10-25% of absorption occurs via stomach lining (Kaplan, Sadock & Grebb, 1994). Ethanol is detectable in blood within one minute following consumption (Rose, 1988), with its rate of absorption primarily dependent on the presence of food, which limit alcohol’s ability to diffuse through the stomach lining and intestinal walls (Sher et al., 2005; for review see Doweiko, 2010).

1.3 Metabolism & Excretion

The primary metabolism of ETOH occurs in liver via several isoenzymes of alcohol dehydrogenase (ADH). There are five classes of ADH (1-5), but the hepatic form (in humans) is primarily Class 1, and is found in stomach and liver. ADH catalyzes the oxidation of ETOH to acetaldehyde by converting NAD^+ to NADH. A secondary mechanism of ETOH metabolism is the microsomal ethanol oxidizing system (for review see Lieber, 1999). This system involves metabolism by cytochrome P450, particularly CYP2E1 (Koop, 2006; Lieber, 2004), also producing acetaldehyde. Acetaldehyde is unstable, forming free radical structures (discussed in greater depth below) and prolonged acetaldehyde exposure in liver and kidneys is a major health concern in chronic alcoholics. Acetaldehyde is converted to acetic acid by acetaldehyde dehydrogenase. Acetic acid may be excreted in urine, or further reduced to Acetyl-CoA by Acetyl-CoA Synthetase. Acetyl-CoA can then enter the citric acid cycle, where it is converted to CO_2 and H_2O (for review see Holford et al., 1987). 90-95% of ETOH elimination in humans is accounted for by oxidation, with excretion via skin, lungs and kidneys accounting for the remainder (Holford, 1987).

Chapter 2. Pharmacology

In the 1960s, the study of ETOH interactions with ion channels was initiated with the discovery of its suppressant effect on sodium and potassium currents in squid axons (Armstrong & Binstock, 1964; Moore, Ulbricht & Takata, 1964). Since that time, it is difficult to name a receptor/transmitter system whose interaction with ETOH has not been examined. While a complete review of these interactions is beyond the scope of the current work, ETOH effects on a number of neurotransmitter systems, with attention to neuropharmacology thought to relate to its neurotoxic and/or reinforcing effects, will be discussed.

2.1 ETOH & Dopamine

Although a number of transmitter systems and brain structures contribute to ETOH's reinforcing action, alterations in mesolimbic dopamine (DA) is a common downstream effect. This system is comprised of DAergic projections extending from the ventral tegmental area (VTA) of the midbrain to the nucleus accumbens (NAcc). Systemic ETOH injections increase extracellular DA in rodent NAcc (e.g., Blomqvist, Engel & Nissbrandt, 1993; Diana, Pistis & Carboni, 1993; Di Chiara & Imperato, 1985), as does voluntary ETOH consumption (Howard et al., 2008), with DA and BAC levels highly correlated. Additionally, DA increases in human ventral striatum following ETOH administration, which correlate with subjective euphoria, have been reported (Boileau, et al., 2003). That mesolimbic DA is involved in ETOH reinforcement is supported by reductions in ETOH self-administration following DA antagonism in NAcc (Rassnick, Pulvirenti & Koob, 1992). Activation of mesolimbic circuitry is induced by a number of ETOH effects, including interaction with glutamate (GLU), nicotinic acetylcholine (nACh), opiate, serotonin (5-HT) receptors, and gamma-aminobutyric acid (GABA) receptors (for review see Vengeliene, 2009).

However, while mesolimbic DA activation is necessary for the reinforcing

properties of many drugs of abuse, ablation of mesolimbic and nigrostriatal systems does not abolish ETOH consumption in rodents (Kiiianmaa et al., 1979; Shoemaker et al., 2002). Similarly, selective ablation of NAcc fails to disrupt consumption in both heterogenous (Fahlke et al., 1994; Myers & Quarfordt, 1991) and alcohol preferring (Ikemoto et al., 1997; Koistinen et al., 2001) rats. These findings do not suggest that ethanol's activation of DAergic systems is unimportant, but rather highlight the varied contribution of numerous systems to consummatory behavior (for review see Gonzales, Job & Doyon, 2004), in a manner which seems somewhat unique relative to other substances of abuse.

2.2 ETOH & 5-HT

ETOH potentiates 5-HT receptor function (Lovinger & Zhou, 1994) and increases 5-HT at numerous nuclei throughout brain (McBride et al., 1993; LeMarquand et al., 1994; for review see Kirby et al., 2011). 5-HT is known to interact with DAergic systems, modulating mesolimbic DA output. While this interaction is not well understood, microdialysis studies suggest that 5-HT exposure in the NAcc and striatum stimulate DA release (Kelland & Chiodo, 1996). Direct synaptic contact between 5-HT terminals and DA cells in the midbrain supports such an interaction, and provide an additional mechanism through which ETOH is suggested to exert a reinforcing effect. However, while it is clear that altering 5-HT function can modulate ETOH self-administration, contrasting findings in the literature somewhat obscure the mechanistic contributions to this interaction; increasing 5-HT bioavailability appears to reduce ETOH intake (Fadda et al., 1991; Hodge et al., 1993), although 5-HT₁ antagonism of several receptor subtypes, including 5-HT₃ and 5-HT₂, also reduces intake (Roberts et al., 1998).

2.3 ETOH & Opiate Systems

The opioid receptor system is composed of metabotropic receptors of at least three types (μ , δ , κ). Endogenous opioid peptides are known to play a role in the rewarding

effects of several drugs of abuse, primarily (though not exclusively) through their modulation of mesolimbic DAergic activity, although such action appears generally limited to peptides activating the μ and δ receptors. It is now clear that significant interaction with the opioid system occurs following ETOH exposure (for review see Hertz, 1997), however the details of much of this interaction remain equivocal. ETOH appears to increase the density of both μ and δ receptors in animal models (Charness et al., 1993; for review see Tabakoff et al., 1996). Low to moderate concentrations of ETOH increase the release of the endogenous μ receptor agonist, beta endorphin, in the VTA (Jarjour, Bai & Gianoulakis, 2009). Such findings provide another mechanism through which ETOH exerts reinforcing effects, as activation of VTA-associated μ receptors enhances DA efflux in NAcc. Additionally, powerful evidence for the significance of ETOH's interaction with this system has been elucidated via opioid-specific pharmacological manipulation (reviewed below).

2.4 ETOH & Acetylcholine

ETOH alters the function of neuronal nicotinic acetylcholine receptors (nAChRs). ETOH enhances the response to nicotine *in vivo* (Breese et al., 1993) and appears to interact directly with nAChRs (Aistrup et al., 1999); it is suggested to act as a co-agonist to ACh at some receptor subtypes, and may function allosterically, increasing agonist affinity for the receptor (Forman, Righi & Miller, 1989). For example, ETOH increases responsiveness to nicotine in $\alpha 2b2$, $\alpha 3b2$, and $\alpha 4b2$ receptor subtypes (de Fiebre, Papke & Meyer, 1995), and potentiates inward currents at $\alpha 3b4$ nAChR subtypes. ACh agonism in VTA stimulates DA release in NAcc (Li et al., 2008) providing a possible mechanism of ACh-dependent ETOH reinforcement. However, inhibitory effects have also been noted (e.g., Covernton & Connolly, 1997), highlighting the complexity of ETOH-nAChR interactions. Of particular interest, ETOH has been shown to inhibit $\alpha 7$ nAChR subtypes (e.g., de Fiebre & de Fiebre 2005; Narahashi et al., 1999), with further examinations suggesting that this $\alpha 7$ subtype is particularly important in modulating several behavioral and physiological responses to ETOH (Bowers et al., 2005). Additional evidence for

cholinergic involvement is provided by nAChR antagonists, which attenuate consummatory responses in a variety of models and species (e.g., Ericson et al., 1998; Blomqvist et al. 1996).

2.5 ETOH & GABA

GABA is the major inhibitory neurotransmitter in mammalian CNS. ETOH allosterically modulates the ionotropic GABA-A receptor complex and potentiates chloride flux through the membrane, resulting in hyperpolarization produced by GABA. Many behavioral effects of ETOH mimic those of GABA-A receptor agonists, suggesting the direct involvement of GABA-A receptors in its action. GABA-A receptors are co-localized on DA neurons and GABAergic interneurons in VTA (Xi & Stein, 1998), facilitating a close interaction between these systems, with mesolimbic DA release regulated by GABA interneurons (Maldonado et al., 1997). Inhibition of these interneurons, thought to occur following ETOH exposure, increases NAcc DA release (Kalivas et al., 1990). GABA appears involved in ETOH's behavioral effects (e.g., ataxia, anesthesia), as GABA-mimetics enhance ETOH-associated behaviors, whereas functional antagonists decrease them (for review see Deitrich et al., 1989). Interestingly, such GABA antagonists also appear to reduce consummatory behaviors (Lister & Linnoila, 1991; Mihic & Harris, 1996; for review see Grobin et al., 1998). While these findings provide only surface detail concerning the interactions of ETOH in the GABA system, ample evidence suggests GABA receptor subunit composition and concentration of ETOH in the system can alter such interactions in a complex manner (for review see Lobo & Harris, 2008).

2.6 ETOH & Glutamate

Glutamate is the major excitatory transmitter in the mammalian brain. To date, three groups of G-protein coupled metabotropic GLU receptors (mGluRs) have been identified; Group I (mGluR1 & mGluR5) activates phospholipase C, producing

diacylglycerol and inositol triphosphate as secondary messengers, while Groups II (mGluR2 & mGluR3) and III (mGluR4, mGluR6, mGluR7 & mGluR8) are negatively coupled to adenylyl cyclase. The mGluRs are responsible for slow glutamate-mediated neurotransmission and modulation of transmitter release. They couple with G-proteins and are located throughout the limbic and cortical brain regions implicated in alcoholism, and in particular, group I mGluRs (mGluR1 and mGluR5) appear important in regulating the effects of drugs of abuse (Kenny & Markou, 2004).

While mGluRs are almost undoubtedly involved in ETOH action, the ionotropic GLURs have received far more attention; three basic receptors families have been identified, including the *N*-methyl-D-aspartate receptor (NMDAr), the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr) and the kainic acid receptor (KAr). All three are tetrameric complexes, and are inhibited by ETOH at physiologically relevant concentrations (Lovinger et al., 1989; Weiner, Dunwiddie & Valenzuela, 1999; Simson et al., 1991). Fast synaptic transmission within this system is mediated by AMPAr & KAr, while NMDAr appear to mediate slower synaptic kinetics, involving Ca^{2+} and Ka^{+} flux (for review see Hoffman, 2003).

AMPA receptors are composed of subunits GLUR1-4, which all contain a GLU binding site. AMPAr are generally permeable to Ca^{2+} , Na^{+} and K^{+} , although many contain the Ca^{2+} -impermeable GLUR2 subunit. ETOH inhibition of AMPARs is well noted (Dildy-Mayfield & Harris, 1992; Lovinger, 1993a; Wirkner et al., 2000) and occurs at a wide range of concentrations (10-100mM). AMPAr undergo strong desensitization following agonist exposure (Trussell et al., 1988; Tang et al., 1989), although comparably weaker and more rapid desensitization following AMPA or GLU exposure (Tang et al., 1989; Trussell & Fischbach, 1989; Hestrin, 1992; Barbour et al., 1994). Emerging evidence suggests that ETOH inhibits AMPARs by stabilizing this desensitized state (Moykkynen et al., 2009). Such inhibition is thought to be noncompetitive, distinct from channel blockade (Peoples et al., 1997; Wirkner et al., 2000), although interestingly,

AMPAr do not appear to undergo adaptation following chronic ETOH (for review see Davis & Wu, 2001).

KA receptors are composed of subunits GLUr5-7 and KA1-2, and can form homomeric (GLUr only) or heterometric (GLUr5 and KA1-2) stoichiometries (for review see Dingledine et al., 1999). While initial reports indicated preferential sensitivity of NMDAr to ETOH, compared to AMPA/KAr (Hoffman et al., 1989; Lovinger et al., 1989), such sensitivity appears to rely heavily on receptor localization. For instance, KAr in hippocampal CA3 neurons appear strongly affected by ETOH exposure (Weiner, Dunwiddie & Valenzuela, 1999). Postsynaptic KAr appear to share a primary cellular function with AMPAr, both enabling the voltage-dependent functioning of the NMDAr.

2.6.1 NMDAr

The NMDAr are critically involved in the synaptic plasticity associated with neuronal development, learning & memory (Collingridge & Singer, 1990; Collingridge & Lester, 1989). It is generally agreed (see Hawkins et al., 1999; Premkumar & Auerback; 1997 for evidence to the contrary) that they are constructed as tetrameric ligand-gated cation channels, composed of two NR1 and two NR2 subunits (McBain & Mayer, 1994; Schorge and Colquhoun 2003). Although recent findings have also identified NR3 subunits, these appear to render receptors insensitive to GLU or NMDA and may assemble with NR1 subunits to form excitatory glycine receptors (Chatterton et al., 2002), however will not be discussed here. The receptor contains six major binding domains. The GLU binding site, which also binds NMDA, is located on NR2 subunits; the Mg^{2+} binding site is located within the channel, which it blocks under resting conditions; the MK-801 binding site, which binds other phencyclidine-like compounds is also found in the channel; the glycine (GLY) binding site is located on NR1 subunits; polyamine binding sites are located densely on, but not confined to, NR2B subunits (discussed in the following section); the ifenprodil binding site, which also appears to bind several structurally related compounds (e.g. eliprodil), is also found on NR2B.

NMDARs are unique compared to other ionotropic receptors in that ion flux through the channel relies on three events. Firstly, GLY must bind NR1, co-activating the receptor. Secondly, GLU (or NMDA) must bind NR2. Finally, GLU must bind AMPARs or KARs, inducing partial depolarization of the membrane. This partial depolarization releases Mg^{2+} from the pore, allowing Ca^{2+} and Na^{+} flux through the channel. The requirement of both GLU binding and partial depolarization of the resting potential allow the NMDAR to act as a molecular “coincidence detector”, facilitating its roles in long-term potentiation and synaptic plasticity.

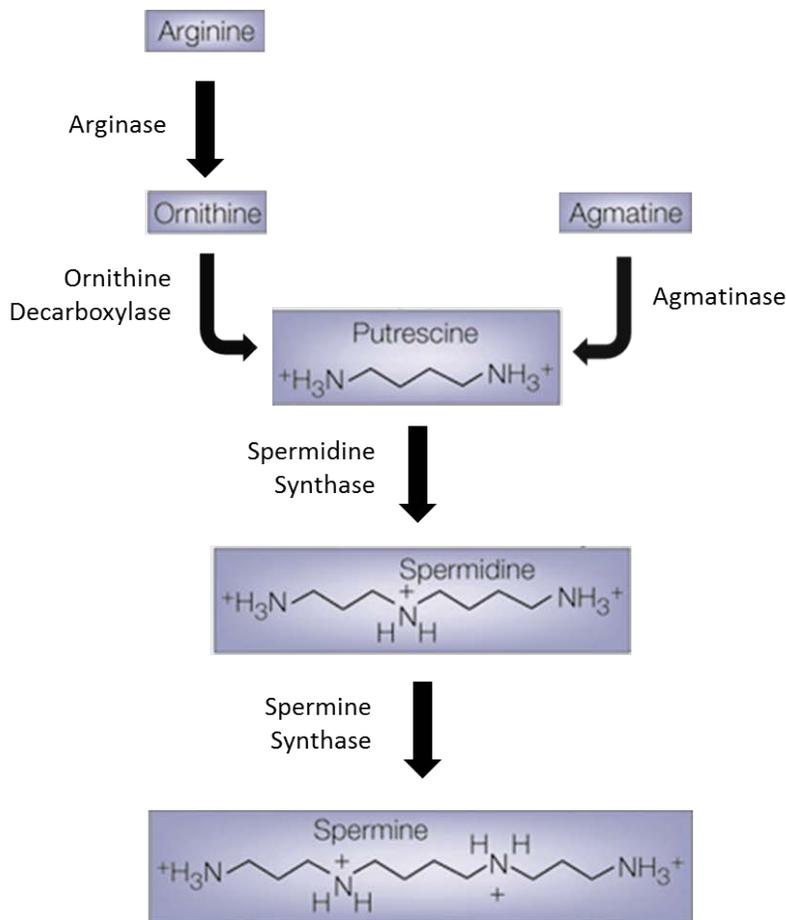
Crystallization studies have demonstrated that NR1/NR2 dimers form within the receptor complex, providing allosteric modulation of gating activity. NR1 subunits are ubiquitous in brain, and expressed as at least eight splice variants (Nakanishi et al., 1992). Four NR2 transcripts have been identified (NR2A-D), and are implicated in the pharmacologic specificity of the receptor, such that various subunit combinations can differ widely in their pharmacology (Wafford et al., 1993; Monaghan et al., 1998; Cull-Candy et al., 2001). NR2 subunits appear to determine the synaptic localization and function of the receptor, as NR2 C-terminal deletion disrupts receptor localization, activity, and plasticity (Chung et al., 2004). While receptor stoichiometries are clearly localization-dependent in brain, the degree to which ternary versus binary compositions are found is a matter of some debate. It has been suggested that NR1/NR2A/NR2B composition is the most abundant, with binary species present at much lower levels, however the opposite has also been suggested (Chazot & Stephenson, 1997; Blahos & Wenthold, 1996). Of particular interest to the current project is the NR2B subunit, for a variety of reasons; NR2B appear highly plastic, are concentrated in areas known to be particularly sensitive to ETOH and contain a major binding domain for polyamines.

2.6.2 Polyamines

Polyamines are simple cation compounds, derived from the amino acid arginine. Arginine can be converted into ornithine, which is then further converted to putrescine

via ornithine decarboxylase (ODC) the rate limiting step in the synthesis of polyamines. Alternatively, arginine can be converted to agmatine and then further converted to putrescine, however this pathway appears to account for only a small portion of polyamine production. Putrescine is the precursor for two other major polyamines, spermidine and spermine, converted via spermidine synthase and spermine synthase, respectively (see Figure 1). These polyamines are ubiquitous in brain, and are involved in cell proliferation, differentiation, growth and apoptosis (Slotkin & Bartolome, 1986; Slotkin et al., 2000).

Figure 1. Simplified Polyamine Biosynthesis



Polyamines bind to NMDARs at several sites (Williams et al., 1995; Kashiwagi et al., 1996; Zheng et al., 1999; Sharma and Reynolds, 1999), and spermine and spermidine

potentiate GLU-mediated responses by binding to the receptor and allosterically increasing GLU binding affinity (Williams, 1997a). Putrescine appears to act differently, exhibiting some antagonistic effects (Romano et al., 1992). Due to the diminished implication of putrescine in NMDAr activity, and its significantly lower concentration in brain (4-70 nM/g vs. 50-1400 nM/g for spermine/spermidine; Seiler and Schmidt-Glenewinkel, 1975; Shimizu et al., 1964), for the purposes of this dissertation 'polyamines' will refer primarily to spermine and spermidine. Polyamines exert this effect even in the presence of saturating concentrations of GLY ('GLY-independent stimulation'; Benveniste & Mayer, 1993; Lerma, 1992; Rock & Macdonald, 1992), however a GLY dependent mechanism has also been noted, with polyamines increasing the affinity of GLY binding (GLY-dependent stimulation; Benveniste & Mayer, 1993; McGurk, Bennett & Zukin, 1990). Furthermore, voltage-dependent inhibition, possibly involving polyamine interaction within the channel has also been noted (for reviews see Dodd, 2000; Williams, 1997b). Subunit composition appears to strongly influence the aforementioned effects. For instance, GLY-dependent stimulation appears to occur regardless of composition (in NR2A/2A, 2B/2B and 2A/2B stoichiometries), while GLY-independent stimulation is favored by a composition including NR2B subunits (for review see Williams, 1997). While polyamines are known to affect NR2A-containing receptors, it is the presence of NR2B which appears to confer polyamine sensitivity (Williams et al., 1994; Gallagher et al., 1997; Sharma and Reynolds, 1999).

2.6.3 NMDARs and ETOH

Behavioral and molecular examinations strongly implicate the NMDAr in the acute actions of ETOH. Early *in vitro* studies have demonstrated ETOH's inhibition of NMDAr-mediated transmission (Lovinger, et al., 1989; Hoffman et al., 1989). Since then, such observations have been confirmed and elaborated upon by examining slices in cortex (Wright, Peoples & Weight, 1996; Wirkner et al., 2000), amygdala (Calton, Wilson & Moore, 1998), NAcc (Malde et al., 2002; Nie, Madamba & Siggins, 1994), dorsal striatum (Yin et al., 2007; Wang et al., 2007; Popp et al., 1998) and hippocampus (Wright, Peoples & Weight, 1996; Lovinger, White & Weight, 1990; Morrisett, 1991;

Kolb, Trettel & Levine, 2005). While the literature has focused on post-synaptic mechanisms of inhibition, more recent evidence suggests ETOH interactions with the receptor may also involve a presynaptic component (Hendricson et al., 2004; Zhu et al., 2007). It remains unclear exactly how and where ETOH binds the receptor; it does not appear to block the channel (Reynolds & Rush, 1990), to compete with GLU binding (Abdollah & Brien, 1995), polyamine binding (Matsumoto et al, 1993), Mg^{2+} or Zn^{2+} binding (Morrisett et al., 1991; Chu et al., 1995). However, evidence for ETOH occupation of hydrophobic pockets (Peoples & Weight, 1992; Ren et al., 2003; Ronald et al., 2001) has been observed, which suggests ETOH functions allosterically to alter channel-gating properties and is consistent with observations of its wide-ranging pharmacology.

NMDARs appear involved in the discriminatory stimuli involved in ETOH exposure. NMDAR antagonists substitute for ETOH in two-choice discrimination (Bienkowski et al., 1996; Kotlinska and Liljequist, 1997; Grant, 1999; Holter et al., 2000). While such discrimination studies clearly implicate the NMDAR as a primary target of ETOH, the recognition that GABA-A or 5-HT agonists can also substitute for ETOH in drug discrimination highlight both the complexity of its CNS action, and its lack of selectivity for any single transmitter system (Hodge & Cox, 1998; Grant, 1999; Hodge et al., 2001). It is known that the training dose of ETOH used in discrimination studies alters the degree to which NMDAR antagonists can act as substitutes, with higher concentrations involving a stronger NMDAR component (Colombo & Grant, 1992; Green & Grant, 1998). This is particularly interesting, suggesting that at higher doses (associated with AUDs), the NMDAR may become a more important target for ETOH, and thus perhaps a more important target for pharmacological intervention in treating alcoholism.

ETOH inhibition of NMDAR function appears to depend, at least in part, on subunit composition. Receptors containing NR2A and/or NR2B subunits appear to display much

greater sensitivity to alcohol than those including NR2C or NR2D (Kuner et al., 1993; Mirshahi & Woodward, 1995; Masood et al., 1994; for reviews see Allgaier, 2002; Sucher et al., 1996). However, whether NR2A or NR2B is the critical subunit for ETOH interactions remains a matter of some debate. Early studies suggested ETOH targeted NR2B-containing NMDARs (Lovinger, 1995; Fink & Gothert, 1996), however such claims were at least partially refuted with demonstrations in the cerebellum, where NR2C is abundantly expressed (Popp et al., 1999). Furthermore, examinations in hippocampus have produced divergent results, suggesting both NR2A (Suvarna et al., 2005) and NR2B-specific (Izumi et al., 2005) action. Such examinations are further complicated by the lack of NR2A-specific NMDAR antagonists, the suggestion that NR1 splice-variant expression affects sensitivity (Jin & Woodward, 2006) and noted differences in sensitivity depending on expression system (Smothers et al., 2001). A recent study utilized NR2A knockout mice to demonstrate equivalent ETOH inhibition of both knockout and wild types, suggesting the presence of NR2A is not a requirement for NMDAR inhibition (Kash et al., 2008), however, this demonstration was conducted in the ventral bed nucleus of the stria terminalis, and while supported by other examinations (Roberto et al., 2004; Izumi et al., 2005), these results may be unique to the regions studied. *While NR2A-specific interactions are still being explored, the current literature suggests a substantial interaction with NR2B-containing NMDARs, which is further described and explored in the current project.*

Exposure to ETOH is known to change NMDAR function and expression, particularly following chronic and/or binge-like exposure, and during withdrawal (WD) from ETOH. NMDAR upregulation appears to occur following chronic treatment (for reviews see Tabakoff & Hoffman, 1996; Kumari & Ticku, 2000). Ligand binding and autoradiography has demonstrated increases in receptor number following chronic exposure (Grant et al., 1990; Gulya et al., 1991; Snell et al., 1993), however examinations of subunit alterations reveal some inconsistencies. It seems that following chronic exposure, NR2B may be upregulated in cortex (Follesa & Ticku, 1995; Kalluri et al., 1998) and hippocampus (Hardy et al., 1999; Follesa & Ticku, 1995; Kalluri et al., 1998),

however some examinations failed to detect this upregulation (Chandler et al., 1997). Importantly, while much of this literature utilizes rodents, ETOH-associated increases in NR2B have been noted in humans (Biermann et al., 2007; Biermann et al., 2009).

ETOH-related NR2A increases in hippocampus are found consistently (Kalluri et al., 1998; Follesa & Ticku, 1995; Snell et al., 1996). However, cortical increases were detected by some groups (Follesa & Ticku, 1995; Chandler, 1999), but not others (Snell et al., 1996; Hu et al., 1996; Follesa & Ticku, 1996a; 1996b; Chandler et al., 1997). NR1 examinations have been similarly inconsistent, with upregulation detected in hippocampus by some (Snell et al., 1996; Trevisan et al., 1994; Kalluri et al., 1998), but not others (Follesa & Ticku, 1995), and in cortex by some (e.g., Chandler et al., 1999; Kalluri et al., 1998) but not others (e.g., Snell et al., 1996; Hu et al., 1996). While the variables contributing to such inconsistent results have not yet been fully elucidated, the basic finding that ETOH increases subunit expression, resulting in enhanced NMDAr agonist sensitivity, is well supported. *Furthermore, polyamines appear to play a complex role in mediating effects of ETOH on the NMDAr (Davidson and Wilce, 1998; for review see Littleton et al., 2001), and are increased following chronic exposure. The implications of both subunit and polyamine increases are thought to be far-reaching for dependence, relapse, and ETOH-associated neurotoxicity, areas which are primary foci of the current work, and which are discussed at length in their respective sections, below.*

Chapter 3. ETOH-Associated Brain Damage

AUDs are associated with cognitive deficits and neurodegeneration, although even moderate drinking has been associated with reduced cognitive function (Evert & Oscar-Berman, 1995). A subset of alcoholics display deficits in intellectual functioning, with the most prevalent impairments including visuo-spatial ability and higher cognitive function (Oscar-Berman et al., 1997). Imaging studies have revealed reductions in brain size and density (Pfefferbaum, et al., 1988; Pfefferbaum et al., 1992), most noticeable in cortex and cerebellum, regions in which brain activity also appears altered in heavy drinkers (Eberling & Jagust, 1995). Fortunately, many such deficits in structure and function appear at least partially reversible during abstinence (e.g., Sullivan et al., 2000; Sullivan, Rosenbloom & Pfefferbaum, 2000). Of particular relevance to the current work, cognitive impairments appear most severe during and immediately following acute WD (Allen, Goldstein & Seaton, 1997).

3.1 Mechanisms of Damage

The proposed mechanisms underlying deficits originating from either adulthood or fetal exposure are numerous: oxidative stress, non-oxidative metabolites (e.g., fatty acid ethyl esters), malnutrition, HPA-axis dysfunction, intracellular microtubule disruption and excitotoxic cellular damage have all been implicated (Butterworth, 1995). While multiple mechanisms undoubtedly contribute to ETOH-induced damage, the current work focuses on GLU and disrupted GLUr function, which appear to play primary roles. While ubiquitous, and crucially important to homeostatic function, GLU can also be toxic; it is implicated in the pathogenesis of a wide variety of disorders, including ischemia (Hammerman & Kaplan, 1998), amyotrophic lateral sclerosis (for review see Bogaert et al., 2010), AIDS and/or HIV-associated dementia (Prendergast et al., 2002; Self et al., 2004), neuropathic pain (for review see Brown & Krupp, 2006), Parkinson's disease (Bonuccelli and Del Dotto, 2006), epilepsy (Tzschentke, 2002) and Huntington's disease (Fan et al., 2007). GLU over-activation of NMDArs, leading to excessive Ca^{2+} entry, can induce several intracellular cascades which lead to both necrotic and apoptotic cell death.

This pathological excitation of the receptor is termed ‘excitotoxicity’ and appears to occur, at least in part, due to increases in NMDAr expression and sensitivity following chronic ETOH exposure (e.g., Hoffman et al., 1995; for review see Lovinger., 1993). *A major underlying hypothesis of the current project is that ETOH-associated cell death and neurodegeneration involves this hyper-excitabile state, resulting in excitotoxic injury during withdrawal.*

While the toxicity of GLU has been recognized for some time (Lucas & Newhouse, 1957), this was followed by the identification of Ca^{2+} increases as a major contributor (Choi et al., 1985; Choi et al., 1987). Increases in intracellular Ca^{2+} may be achieved via multiple mechanisms, however, NMDAr-mediated Ca^{2+} entry has been critically implicated; higher concentrations of Ca^{2+} from alternate sources have been shown to be less toxic than lower concentrations whose source was NMDAr-mediated (Tymianski et al., 1993; Sattler, Charlton & Hafner, 1998). One proposed mechanism for the NMDAr-specificity of excitotoxicity involves its intracellular coupling to neuronal nitric oxide synthase (nNOS; Sattler, Xiong & Lu, 1999); increased NO is a hallmark of excitotoxic events and major contributor to damage. NOS inhibitors are known to prevent cell death *in vitro* (Dawson et al., 1991), and resistance to NMDAr-mediated excitotoxicity is conferred by nNOS knockout (Dawson, Kizushi & Huang, 1996). The structural link between NMDAr and nNOS appears to involve a postsynaptic density protein associated with the C-terminus of the NR2B subunit and N-terminus of nNOS, creating a “microenvironment” wherein Ca^{2+} entering the cell via NR2B-containing NMDAr preferentially activate nNOS via calmodulin (Sattler, Xiong & Lu, 1999). NO appears to directly interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to initiate apoptotic cascades (Hara et al., 2005), as well as the free radical superoxide, forming toxic peroxynitrite. Free radicals are highly reactive molecules formed during mitochondrial reactions with oxygen during energy production. Free radicals and peroxides are collectively referred to as reactive oxygen species (ROS) and when overproduced under pathological conditions, damage several intracellular structures including proteins, lipid membranes (Sun et al., 1997) and nucleic acids (Navasumrit et

al., 2000). “Oxidative stress” generally refers to both increases in ROS-associated intracellular damage (Reinke et al., 1987; Henderson et al., 1995; Davis et al., 1990), and reductions in the cell’s ability to compensate (Bailey et al., 2001). Excitotoxicity and oxidative stress are frequently dissociated in the literature as differential pathways of cell death, however the role of NO in excitotoxicity illustrates the interconnected nature of these pathways. ROS production following excitotoxicity is well-established (Dugan, Sensi & Canzoniero, 1995; Reynolds & Hastings, 1995) and appears dose-dependent with increased NMDAr activation (Lafon-Cazal et al., 1993). In sum, the literature suggests a model wherein excessive intracellular Ca^{2+} accumulation via NMDAr pathologically increases NO production and results in mitochondrial failure. ROS produced by such failure interact with NO to form peroxynitrite, directly damaging DNA and leading to the release of apoptosis-inducing factors from mitochondria, resulting in cell death (for review see Lau & Tymianski, 2010).

3.2 Excitotoxicity & Polyamines

Polyamines may play a critical role in ETOH-associated excitotoxicity; increased polyamine expression has been reported in hippocampus, striatum, cortex, and cerebellum during periods of ETOH WD (Davidson & Wilce, 1998; Gibson et al., 2003). Increases in ODC expression (indexing increases in polyamine concentration) are noted in these areas following chronic ETOH exposure (Koenig et al., 1990; Shibley et al., 1995; Davidson & Wilce, 1998). Additionally, polyamine activity seems to correlate positively with behaviors indexing the severity of WD-associated damage, including WD-induced tremor and seizure in dependent animals (Davidson & Wilce, 1998). Additionally, reductions in ODC activity correlate with reductions in severity of WD symptoms (Davidson & Wilce, 1998). *In vitro* examinations of WD-induced excitotoxic damage suggest that exogenous application of polyamines exacerbates damage (Butler et al., 2010; Mayer et al., 2002a), while polyamine antagonism (Gibson et al., 2003) attenuates damage. Furthermore, inhibition of polyamine synthesis via difluoromethyl ornithine, an ODC inhibitor, inhibits WD-induced seizure, improves WD-associated outcomes *in vivo* (Davidson & Wilce, 1998), and attenuates cell death *in vitro* (Gibson et

al., 2003). Taken together, this evidence suggests that the withdrawal state can be characterized by increases in polyamine-sensitive (NR2B-containing) NMDArs as well as increases in polyamine synthesis and accumulation. This suggests that polyamine modulation during WD may provide a useful substrate for pharmacotherapeutic intervention. Further evidence for this hypothesis is garnered from examinations of compounds that inhibit polyamine-sensitive NMDArs (reviewed below in section 5).

Chapter 4. ETOH Dependence

The aforementioned evidence implicates the GLU system in ETOH-associated neurotoxicity, however the selection of this system as a pharmacological target in the current project is also due to its contribution to ETOH dependence and withdrawal. While numerous neurotransmitter systems are involved, GLUergic intervention seems uniquely capable of reducing relapse risk through multiple mechanisms, including positive and negative reinforcement, as well as alterations in cue conditioning and salience.

4.1 Acute & Protracted Withdrawal

Cessation of chronic ETOH consumption is associated with the alcohol withdrawal syndrome, characterized by autonomic hyperactivity, increased tremor, nausea/vomiting, anxiety, hallucination, psychomotor agitation and seizure (APA, 2000). Recent estimates suggest up to 2 million Americans experience symptoms of alcohol withdrawal per year (Bayard et al., 2004). Although acute withdrawal is potentially life-threatening, detoxification has become a routine medical procedure which rarely results in mortality (for review see Romach & Sellers, 1991). Current detoxification practices involve GABAergic manipulation via benzodiazepines, and are extremely effective at controlling withdrawal symptoms and morbidity, however there is no evidence for reductions in neurotoxicity or relapse risk following treatment. *The current project focuses on developing compounds with the capability of reducing both toxicity and relapse.*

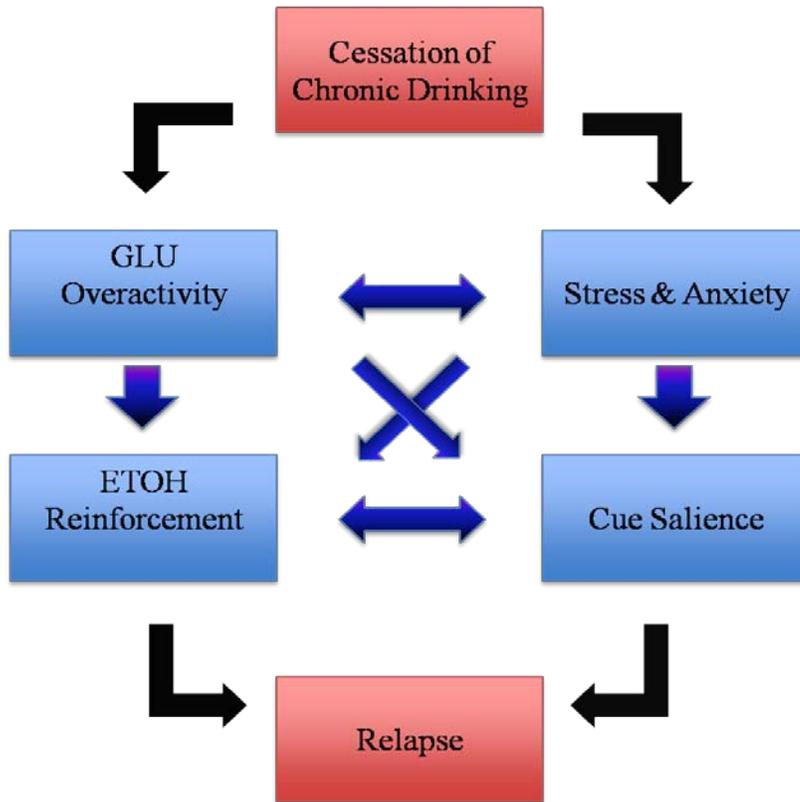
Withdrawal can be characterized by increased glutamatergic activity and increased stress/anxiety. While the most severe withdrawal symptoms generally dissipate within 1-2 weeks, symptomology and underlying altered brain function can persist for months, and possibly much longer. It is hypothesized that such altered glutamatergic function and negative affect during both acute and protracted withdrawal contribute to increased relapse risk.

4.1.1 Glutamatergic Hyperactivity

Extracellular concentrations of GLU appear increased following withdrawal, and are highly correlated with withdrawal symptomology (Gonzales et al., 1996; Rossetti & Carboni, 1995). The hyperexcitability during withdrawal appears mediated by NMDARs, whose upregulation following chronic exposure is well-established; a similar syndrome of hyperexcitability is noted following withdrawal from chronic NMDAR antagonism (Ripley & Little, 1995). Furthermore, GLU inhibition during withdrawal reduces symptom severity (Grant, 1990; Liljequist, 1991; Morrisett et al., 1990; Rossetti & Carboni, 1995) while agonism worsens it (Danysz et al., 1992; Davidson et al., 1995; Morrisett et al., 1990; Sanna et al., 1993). As noted above, elevations in polyamines appear to contribute to this hyperactivity, and polyamine inhibition is also capable of reducing WD symptoms (Davidson & Wilce, 1998).

GLU hyperactivity also appears to contribute to ETOH dependence and relapse (Tsai & Coyle, 1998; Pulvirenti & Diana, 2001; Siggins et al., 2003; Heinz et al., 2003; Krystal et al., 2003). Although the exact mechanisms are still being investigated, GLU-mediated increases in cue salience and incentive motivation for ETOH during withdrawal are implicated. Additionally, GLU hyperactivity may exacerbate negative affect during withdrawal, indirectly increasing cue salience and ETOH reinforcement further. Though discussed in greater detail below, a simple schematic of these relationships is presented in Figure 2.

Figure 2. Contributions of ETOH Withdrawal to Reinforcement and Cue Salience



4.1.2 Stress, Anxiety & Negative Affect

Withdrawal from chronic ETOH increases anxiety (Rassnick et al., 1993; Valdez et al., 2002). Stress & anxiety during withdrawal appear closely related to resumption of drinking in humans (Hershon, 1977). Stress responses are controlled by the hypothalamic-pituitary-adrenal (HPA) axis; basic functioning of this system involves CNS stimulation of corticotropin-releasing hormone from hypothalamus, inducing pituitary secretion of adrenocorticotropin, resulting in adrenal glucocorticoid (GC; cortisol, in humans) production. Alcohol intoxication and withdrawal are known to increase GCs (Adinoff et al., 1991; Keedwell et al., 2001; Mendelson et al., 1971), with a number of implications. Hippocampal GCrs, via negative feedback, are responsible for regulating HPA-axis activity and recovery. Chronically elevated GC levels and chronic

ETOH binge/WD cycles are known to induce hippocampal damage, disrupting negative feedback control and dysregulating the HPA system (for review see Adinoff et al., 1998).

Both GLU hyperactivity and disrupted HPA-axis activity are thought to contribute to relapse behaviors by altering ETOH reinforcement and ETOH-associated cue salience, as indicated in Figure 2. However, these systems also appear to interact with one another dynamically during withdrawal. GC exposure upregulates polyamine-sensitive NR2B subunit expression (Lu et al., 2003), as well as increasing the production of polyamines. (Weiland, Orchinik & Tanapat, 1997). Finally, GC elevations upregulate excitatory amino acid activity, increasing Ca^{2+} flux via NMDAr (for review see, Reagan & McEwen, 1997; Armanini et al., 1990). However, inhibition of GLUrs is anxiolytic (Dunn et al., 1989; Faiman et al., 1994), protects from GC-associated hippocampal neurotoxicity (Mulholland et al., 2005), and reduces WD-associated behaviors indexing stress (Cagetti et al., 2004).

Taken together, these interactions suggest that GLU inhibition during withdrawal may serve to balance GLU activity both directly, as well as indirectly, by preventing increased GC activity via neuroprotection to critical (hippocampal) stress-regulation circuitry. Thus, reductions in GLU activity are likely to reduce negative affect and HPA-axis dysregulation, both critical contributors to relapse.

4.2 Relapse & ETOH Reinforcement

Following cessation of chronic drinking, the majority of dependent individuals will experience a relapse, despite efforts to maintain sobriety (Hayashida et al., 1989; Prochaska, DiClemente, & Norcross 1992). Preventing relapse is a (if not *the*) major therapeutic challenge in treating ETOH dependence. Contemporary theories of relapse hold that following chronic consumption and cessation, drug-associated alterations in

neurochemistry contribute to a state in which the reinforcing value of the drug is increased during sobriety.

Positive reinforcement is indicated when a stimulus (alcohol) increases the probability of a particular response (consumption or ETOH-seeking behavior). Negative reinforcement is indicated when the probability of the response is increased when it allows for escape or alleviation from an aversive stimulus (e.g., anxiety). It is now generally agreed that the pathogenesis of alcoholism can be described as a transition from motivation to consume ETOH based on its positive reinforcement, to compulsive use associated with its alleviation of the negative affective state created during WD. This conceptualization of addiction, wherein use becomes driven by homeostatic maintenance has been described as ‘allostasis’ originated with Himmelsbach (1943) following his work in opiate dependence, and was not described as ‘allostasis’ until much later (Sterling and Eyer, 1988). Allostasis describes the dynamic adaptation of multiple systems to achieve (or restore) homeostatic function, making it a particularly apt description of the varied physiological adaptations to drugs of abuse noted in dependence & addiction. Allostasis has become a primary, driving conceptual model in the field (e.g., Koob & Le Moal, 2001; Koob & Volkow, 2010).

While progression from positive to negative reinforcement as a basic concept has been popularized, it potentially ignores a subset of individuals with high stress/anxiety prior to initiation of use, whose primary initial motivation to consume may involve ETOH’s anxiolytic properties. Regardless of initial motivation, following the cessation of chronic consumption, ETOH-exacerbated negative affect is thought to persist for months or longer, is referred to as “protracted withdrawal” and is characterized by stress, anxiety, depression and hyperalgesia (Koob, 2003). Thus, heightened negative reinforcement for consumption of ETOH appears to remain persistent despite the absence of acute withdrawal symptoms. However, sensitization to the positive reinforcing value of ETOH may also contribute to its enhanced reinforcing value during withdrawal.

Conceptualizations of conditioning and relapse based on positive vs. negative reinforcement can be referred to as ‘incentive salience’ vs. ‘opponent-process’ models, respectively. Incentive salience models suggest that drug cues will produce conditioned drug-like responses (euphoria), which enhance the incentive motivation to consume the drug. Opponent-process models suggest that with prolonged drug use, physiological ‘drug-opposite’ responses to conditioned cues develop to maintain homeostasis. In a drug-free state, exposure to such cues produces a dysphoric state in which the negative reinforcing value of the drug is increased. Importantly, both of these views hold that relapse involves a state wherein the increased reinforcing value of drugs is “triggered” by environmental cues.

It is clear that ETOH has both positive (euphoric, stimulating) and negative (anxiolytic) reinforcing effects. DA efflux in NAcc has received the most attention as a biological requirement for positive reinforcement, however as noted above, mesolimbic dopamine is modulated by input from a number of transmitter systems (including GLU). Pharmacological interventions which reduce DA efflux or blunt its effects are noted for their ability to disrupt ethanol consumption in humans and animals (discussed in greater depth below). Glutamate tone, NAcc DA levels, ETOH consumption and ETOH’s positive reinforcing value all appear closely related; NAcc DA levels increase in anticipation of ETOH consumption, suggesting an important role for cue-associated increases in positive reinforcement during relapse (Katner, Kerr & Weiss, 1996; Melendez et al., 2002). Furthermore, manipulation of NAcc GLU via microinjection of GLUr inhibitors (including NMDAr antagonists) reduces drinking (Rassnick, 1992; Besheer et al., 2010), emphasizing the GLUergic component of ETOH’s reinforcing effects.

In one particularly interesting study, Spanagel and colleagues (2005) genetically altered astrocytic GLU transporter expression to create mutants with persistently high synaptic GLU activity (as is known to occur in ETOH withdrawal). These mutants

demonstrated increased ETOH intake and increased operant responding for ETOH. Further demonstrating GLUergic control of the behavior, Spanagel's group pharmacologically inhibited the GLU hyperactivity and observed normalized consumption. However, while this work appears to implicate GLUergic mechanisms in positive reinforcement (since the animals received no aversive stimuli or previous ETOH exposure) an alternative interpretation suggests that such persistently high synaptic GLU activity may produce a negative affective state, potentially driving consumption. This interpretation is consistent with observations that the negative reinforcing properties of ETOH are also mediated via GLUs.

That ETOH reinforcement can also be driven by negative components is emphasized by the finding that ablation of the mesolimbic DA system fails to extinguish ETOH consumption (Rassnick et al., 1993). A major underlying hypothesis of the current project is that inhibition of GLUergic activity during acute and protracted withdrawal can mitigate negative affect, thereby reducing ETOH's negative reinforcing value. Supporting evidence includes that GLU inhibition can reverse or attenuate dysregulation in HPA-axis function (described above) and that numerous compounds with GLUergic action which are shown to reduce consumption and relapse behavior (clinically and preclinically) possess anxiolytic action. Finally, amygdalar nuclei which regulate negative affect, particularly anxiety and fear responses, are implicated as key modulators of ETOH reinforcement and appear sensitive to GLU imbalance. The basolateral amygdala is rich in GLU receptors (Farb et al., 1995; for review see Davis et al, 1994;) as is one of its major efferents, the central nucleus (CeA; Nose et al., 1991; Davis et al., 1994). GLU levels in these areas are persistently higher in animals chronically exposed to ETOH (Roberto et al., 2004), contributing to the anxiogenic properties of WD. Similarly, reductions in GLUergic transmission in these areas, whether due to ETOH or NMDAr-specific antagonism, produce anxiolytic effects (Miserendino et al., 1990; Kim et al., 1993). Of particular importance to the current project, amygdalar neuroadaptations to chronic ETOH appear mediated by NR2B-containing NMDARs; CeA NR2B upregulation is persistent (> 4 weeks; Obara et al., 2009) following chronic exposure and the

heightened sensitivity of CeA NMDARs to ETOH observed following such exposure is attenuated via NR2B-specific antagonism (Roberto et al., 2004).

Taken together, the aforementioned evidence suggests that (i) ETOH consumption is under the control of both positive and negative components of reinforcement; (ii) that both positive and negative components are mediated at least in part by GLU; (iii) and that GLU inhibition, specifically NMDAR inhibition, appears capable of reducing both types of reinforcement.

4.2.1 Conditioned Cues

Changes in the reinforcing value of ETOH during withdrawal increase relapse risk, serving to prime the system for relapse, however cues or “triggers” which reinstate the conditioned behavior of alcohol seeking are also critically involved. In traditional conditioning models, environmental cues (including ETOH itself, referred to as ‘priming’ cues) become associated with alcohol use, after which they function as classically conditioned stimuli, evoking conditioned physiological responses. These appear to induce alcohol-related effects that can include feelings of euphoria (drug-similar responses) and/or feelings of withdrawal (drug-opposite responses referred to as “conditioned pseudo-withdrawal” (Solomon, 1980; O’Brien et al., 1998), both of which may result in ‘craving’ or the desire to experience alcohol’s effects. As mentioned previously, divergent models of relapse/reinforcement conceptualize craving as being driven by either euphoric or dysphoric feelings, however contributions are likely to vary on an individual basis and it is suggested that even within individuals craving and relapse may be precipitated by a variety of environments and/or internal states (Connors et al., 1996). Regardless of reinforcement mechanism, extinction of these conditioned cues appears to improve relapse measures (Glautier & Drummond, 1994).

Protracted relapse constitutes a period of heightened stress and anxiety, during which acute stressors are known to trigger conditioned relapse (Annis, 1998; Sinha et al., 2000; for review see Sinha, 2001). Interestingly, acute stress also appears to have a permissive function, enhancing the salience of other conditioned ETOH cues (Field & Powell, 1997). This may be a bi-directional relationship, since (as opponent-process theory would predict), exposure to alcohol cues via guided imagery in abstinent alcoholics increases negative affect (Cooney et al. 1991, Sinha et al., 2000). However, stress-induced relapse appears to operate via dissociable neural mechanisms from cue-induced relapse; Liu & Weiss (2002) demonstrated that opioid antagonism could attenuate cue-induced, but not stress-induced reinstatement of drinking, while a CRF antagonist suppressed stress-induced, but not cue-induced consumption. When animals were simultaneously exposed to both conditioned cues (a situation with greater face validity to human behavior), both the CRF and opioid antagonist were only capable of partial mediation. Their experiment highlights the complexity of relapse/reinstatement behavior, and suggests that an ideal pharmacological intervention would be capable of attenuating both stress and cue-induced reinstatement.

As suggested in Figure 2, in addition to impacting reinforcement, the GLUergic system is critically involved in the conditioning and expression of ETOH-associated cues. ETOH cue-induced reinstatement of self-administration has been demonstrated and attenuated in dependent animals via GLU inhibition (e.g., Backstrom & Hyytia, 2004). Additionally, GLU inhibition reduces responding for ETOH-associated cues in conditioned place preference studies (McGeehan & Olive, 2003; Brown, Lee & Sorg, 2008; Gremel & Cunningham, 2009). More recent examinations have suggested specific GLUr subpopulations, particularly NMDAr containing polyamine-sensitive subunits, may be critical for drug-associated conditioning. In general, polyamines are known to enhance learning and memory processes (Rubin et al., 2000; 2001; Mikolajczak et al., 2002), including cue-associated conditioning (Rubin et al., 2004). However, inhibiting NMDAr via polyamine modulation reduces drug-associated responding while leaving responding for natural reinforcers intact (Ma et al., 2006). Although not yet examined

directly, these findings suggest that known increases in polyamine expression and sensitivity during ETOH withdrawal may enhance conditioned responding to alcohol-associated cues.

Taken together, evidence presented in the preceding sections suggests that chronic ETOH exposure is associated with neurotoxicity and pathological alterations in both GLU and HPA-axis function. These changes are associated with increases in the reinforcing value of ETOH, and increased sensitivity to ETOH-conditioned cues, both of which increase relapse risk. The complexity and range of the involved mechanisms suggests numerous substrates for pharmacological intervention (discussed below), however based on evidence discussed above, *inhibition of polyamine-sensitive NMDARs may provide a unique opportunity to simultaneously reduce excitotoxicity, GLU hyperactivity and negative affect during WD, the downstream effects of which include reductions in reinforcing value (positive and negative) of ETOH and sensitivity to ETOH-conditioned cues.* Additional evidence for this hypothesis is garnered from examinations of a number of pharmacological interventions, presented below.

Chapter 5. Pharmacotherapy

While the current work is focused on mediation of ETOH-associated alterations in physiology and behavior via NMDAr-targeted pharmacotherapy, several FDA-approved pharmacological interventions already exist, as do a number of potentially useful strategies which do not target the NMDAr. Disulfiram and naltrexone are discussed here, while the third FDA-approved compound (acamprosate) will be discussed with other GLUergic compounds, below.

5.1 Disulfiram

Disulfiram (Antabuse) has been FDA-approved since 1951. Disulfiram inhibits the enzyme acetaldehyde dehydrogenase (ADH), stimulating a number of negative symptoms normally associated with WD (“hangover”) from ETOH (e.g., nausea, vomiting). It is unique in that it acts indirectly, providing a punishing stimulus when ETOH is consumed. Early studies reported high efficacy, while later results have been more mixed. Regardless, the most recent data indicate that with supervised use, which greatly improves patient compliance, disulfiram has clinical utility (for review see Fuller & Gordis, 2004).

5.2 Naltrexone

Naltrexone (ReVia, Depade) was FDA-approved in 1995; its extended release formula (Vivitrol) was approved in 2006. Both naltrexone and its active metabolite, 6- β -naltrexol, competitively antagonize opioid receptors, particularly μ receptors, leading to reductions in NAcc DA levels (Benjamin & Grant, 1993; Gonzales & Weiss, 1998; Middaugh et al., 2003). Such reductions are associated with reduced ETOH consumption, as has been shown in several clinical studies (Latt et al., 2002; Krystal et al., 2001), as well as demonstrated by the multi-center COMBINE study, which suggested efficacy when administered apart from adjunct psychotherapeutic intervention (Anton et al., 2006). Such recognized efficacy has been demonstrated by a number of meta-analyses

(e.g., Kranzler & Van Kirk, 2001; Streeton & Whelan, 2001; for review see Anton, 2008; Bouza et al., 2004). Naltrexone is recognized to reduce craving associated with environmental cues, reducing conditioned anticipation of ETOH (Monti et al., 1999; Middaugh & Bandy, 2000), however such reductions are likely to result from blunting of incentive motivation associated with ETOH, rather than weakened cue conditioning.

5.3 Current Promising Strategies

While not yet FDA-approved, a number of additional pharmacotherapeutic agents are currently being investigated for abstinence maintenance and relapse prevention (for review see Johnson et al, 2008). Several do not employ GLUergic manipulation; for instance, specific serotonin reuptake inhibitors (SSRIs) have been employed in a number of preclinical models, with mixed results. It appears that SSRI treatment is capable of reducing ETOH consumption and operant responding for ETOH (Haraguchi, Samson & Tolliver, 1990; Murphy et al., 1988). However, evidence suggests that such attenuations may be non-specific, resulting in suppressions of food and fluid intake (Simpson et al., 1981; for review see Gill & Amit, 1989). Furthermore, it is suggested that while SSRIs are capable of initial reductions in consummatory behavior, rebound to baseline levels are noted (Gulley et al., 1995). Clinical investigations appear to produce short-term decreases in consumption, but many have limited longevity: there is evidence that initial decreases do not persist after the first month of treatment (Gorelick & Paredes 1992; for review see Kenna, 2010). However, it is recognized that SSRIs may have unique utility among ETOH-dependent populations with comorbid depressive symptoms (Pettinati et al., 2010).

While restoring homeostatic GLU tone during and after WD is a focus of the current work, agonism of GABA_A to restore GLU/GABA balance has also been explored. For instance, the GABA-B agonist, baclofen, decreases consumption (Colombo et al., 2000; Colombo et al., 2002), operant responding for ETOH (Colombo et al., 2003a) and reduces relapse-behavior (Colombo et al., 2003b), preclinically. In humans, baclofen

shows efficacy in reducing cravings and improving abstinence (Addolorato et al., 2000; for review see Leggio et al., 2010), effects which may be related to reductions in WD-associated anxiety and negative affect. While promising, clinical examinations of baclofen are limited, and its noted sedative properties may interact negatively with acute ETOH intoxication.

Given the aforementioned involvement of the HPA axis in WD, protracted withdrawal, and the negative reinforcing effects of ETOH, it is not surprising that direct manipulation of this system has been investigated as a pharmacotherapeutic strategy. Antagonists of corticotropin-releasing factor (CRF) have received significant attention; it is known that ETOH WD increases CRF levels (Merlo Pich et al., 1995; Olive et al., 2002), and that CRF antagonists attenuate stress and anxiety responses (Britton et al., 1986). Thus, CRF antagonists appear capable of attenuating ETOH WD-induced stress responses (Baldwin et al., 1991). Interestingly, CRF antagonists, while anxiolytic in nature, appear capable of reducing ETOH consumption in models that other anxiolytics (e.g., diazepam) do not, perhaps suggesting the specific modulation of HPA-axis function is necessary, as opposed to general reduction in anxiety/negative affect (Lodge & Lawrence, 2003).

Additional investigations include cannabinoid receptor antagonists, DAr antagonists (e.g., aripiprazole), DAr agonists (e.g., bromocriptine), 5-HT antagonists (e.g. ritanserin), 5-HT agonists (e.g., buspirone), nAChR antagonists (e.g., varenicline), and others. While all have demonstrated some clinical potential, many have significant side effects or show efficacy only in limited clinical populations. Furthermore, and perhaps most importantly for our strategic approach, neither of the aforementioned FDA-approved compounds, nor these experimental approaches, are noted for significant neuroprotection.

5.4 Acamprosate

The current project focuses on GLUergic manipulation, a key characteristic of which may be the ability to alter relapse trajectories while offering neuroprotection during acute and protracted withdrawal (an ability which may synergize with anti-relapse mechanisms). However, this is not a novel area of investigation. A number of GLUergic manipulations have been explored, both clinically and preclinically, and the third FDA-approved medication, acamprosate, is thought exert its effects primarily via GLUergic action.

Acamprosate (Campral) is structurally related to a number of amino acids thought be to critically important to ETOH's CNS actions, including GLU, GABA and GLY (Spanagel & Zieglgansberger, 1997). These similarities contribute to its varied pharmacology (for reviews see Littleton, 1995; Littleton et al., 2001; De Witte et al., 2005; Mann et al., 2008); it is recognized as an anti-oxidant (Dahchour et al., 2005), GABA-A agonist (Pierrefiche et al., 2004), NMDAr antagonist (Zeise et al., 1993), polyamine modulator (Al Qatari et al., 1998; Naassila et al., 1998; Popp & Lovinger, 2000), and Group I mGLUr antagonist (Harris et al., 2002). Despite FDA approval, the mechanism of action contributing to its anti-relapse efficacy is equivocal. However, it appears capable of normalizing elevated extracellular GLU following repeated binge-WD cycles (Dahchour & De Witte, 2003; Ulrichsen et al., 1998), and it is generally agreed that this action contributes to reductions in the negative reinforcement of ETOH during WD and protracted withdrawal. In animal models, acamprosate inhibits WD-associated behaviors (Spanagel & Zieglgansberger, 1997), reduces relapse behavior (Spanagel et al., 1996), suppresses conditioned pseudo-withdrawal behavior (Cole, Littleton & Little, 1999) and attenuates cue-induced ETOH-seeking behaviors (Bachteler et al., 2005). Clinically, acamprosate appears effective at reducing a number of behavioral endpoints associated with relapse-risk and abstinence maintenance (Mason et al., 2006; Feeney et al., 2006).

Additionally, acamprosate appears to confer neuroprotection, reducing toxicity and Ca^{2+} entry in neocortical cultures (Al Qatari et al., 2001). In hippocampal cultures, which are used in the current project, acamprosate attenuates WD-associated Ca^{2+} entry and neurotoxicity (Mayer et al., 2002a;b), however acamprosate appears to have a unique neuroprotective profile, thought to include mGLUR manipulation, as it is noted to inhibit ETOH WD-associated toxicity but not NMDA-associated toxicity (Harris et al., 2003).

The varied GABAergic and GLUergic interactions of acamprosate are similar in nature to topiramate (Topamax). In animal models, topiramate reduces stress-induced relapse behaviors (Farook et al., 2009) and ETOH cue-conditioned behaviors during WD (Farook et al., 2007). Clinically, the drug appears efficacious (Johnson et al., 2003;2007), and may share acamprosate's neuroprotective potential (Rustembegovic et al., 2002).

5.5 Memantine

A number of GLUergic approaches have utilized NMDAR-specific antagonists. Classic channel-blockers are noted for their lack of clinical potential, however, low-affinity, non-competitive antagonists appear to induce rapidly dissociating channel blockade and may have anti-relapse efficacy. One such compound in particular, memantine, has received attention. Due to its rapid dissociating kinetics, memantine is thought to interfere less with homeostatic NMDAR transmission, compared to classic channel blockade (Chen & Lipton, 1997; Bleich et al., 2003; Johnson & Kotermanski, 2006; Lipton, 2006; for review see Kohr, 2007). It has shown anti-relapse potential (Krupitsky et al., 2007a) in humans, and appears to reduce WD symptoms (Krupitsky et al., 2007b). Memantine is also recognized for its neuroprotective potential in models of excitotoxicity (Lipton, 2007) including ETOH WD (Stepanyan et al., 2008). One criticism of an NMDAR-blockade strategy involves the potential disruption of learning & memory; MK-801 disrupts responding for natural rewards (Lee & Everitt, 2008; Bevins & Bardo, 1999), as does memantine (Popik et al., 2003). However, as noted previously, NR2B-specific antagonism may provide a solution, disrupting drug-associated cues while

leaving cues for natural reinforcers unperturbed (Ma et al., 2006).

5.6 Ifenprodil & Eliprodil

Due to the recognized contribution of the NR2B in ETOH-associated WD, cue-conditioning and neurotoxicity, investigation of compounds capable of targeting NR2B-containing subpopulations has been substantial. One such compound is ifenprodil, which is characterized as a non-competitive allosteric inhibitor of the spermine and Mg^{2+} binding sites, whose action exhibits use-dependence, such that bound GLU increases the receptor's affinity for ifenprodil (Kew et al., 1998). A secondary mechanism of inhibition, shared by ifenprodil and its analogue CP-101,606 (described below), involves proton inhibition. NMDARs are inhibited by protons with an IC_{50} value that corresponds to pH 7.3 (e.g., Giffard et al., 1990; Tang et al., 1990; Traynelis & Cull-Dandy, 1990). While this mechanism is largely understudied, a major function of such proton sensitivity appears related to its neuroprotective capacity during acidification of the extracellular space, noted during seizures and ischemia, during which pH can fall by up to 1 pH unit (e.g., Chesler & Kaila, 1992). Under conditions of excessive extracellular GLU, such decreases in pH appear to limit NMDAR-mediated excitotoxicity (Kaku et al., 1993). In the presence of ifenprodil (and CP-101,606), NMDAR sensitivity to protons is increased, providing an additional mechanism of antagonistic activity (Mott et al., 1998). It is likely that proton inhibition is a mechanism shared by phenylethanamines in general (i.e., eliprodil and others), however this has yet to be demonstrated empirically.

Ifenprodil has shown promise as a neuroprotectant during excitotoxic events, reducing edema and infarct volume in ischemia models (for review see Wang & Shuaib, 2005), improving outcomes in reserpine & MPTP models of Parkinson's (Nash et al., 1999; Nash et al., 2000) and models of neuropathic pain (for review see Chizh & Headley, 2005). Such neuroprotection extends to *in vitro* ETOH WD-associated neurotoxicity (Mayer et al., 2002a), and it appears protective *in vivo* as well, based on attenuation of WD-associated seizure behaviors (Pawlak et al., 2005; unpublished data).

Eliprodil is an ifenprodil analogue, maintaining high specificity for NMDARs containing the NR2B subunit (Avenet et al., 1997), and has shown similar efficacy as a neuroprotectant in several models of excitotoxic injury (Toulmond et al., 1993; Bath et al., 1996; Reyes et al., 1998). Eliprodil's neuroprotective action has been investigated in developmental models of insult; administration during acute WD rescues adulthood behavioral deficits in rodent FAS models (Thomas et al., 2004).

Ifenprodil & eliprodil appear to have more modest inhibitory effects (compared with non-subunit selective compounds) synaptically, where diverse populations of NMDARs are found, including NR1/NR2A and NR1/NR2B diheteromers as well as NR1/NR2A/NR2B triheteromers (Tovar & Westbrook, 1999). However, extrasynaptic populations of NMDARs appear more homogenous, composed primarily of NR1/NR2B diheteromers (Tovar & Westbrook, 1999), and thus are strongly inhibited by ifenprodil. The synaptic localization of NMDARs appears to determine their opposing effects on gene regulation and neuronal survival; synaptic receptors induce anti-apoptotic effects, while Ca^{2+} entry via extra-synaptic NMDARs elicits loss of CREB function, reduced BDNF expression and ultimately mitochondrial dysfunction and cell death (Hardingham et al., 2002). Thus, NR2B-specific antagonism may exert its neuroprotection via extrasynaptic NMDAR inhibition, while largely preserving synaptic function.

Additionally, ifenprodil appears to reduce ETOH consumption in a relapse model examining binge-like consumption following periods of forced abstinence, known as the alcohol-deprivation effect (ADE), a procedure also utilized in the current project (Sinclair & Senter, 1967). Furthermore, ifenprodil has been shown to block ETOH's stimulatory effects (Broadbent, Kampmueller & Koonse, 2003), suppress ETOH WD effects in dependent rodents (Narita et al., 2000, Malinowska et al., 1999) and attenuate ETOH's amnestic effects (Napiórkowska-Pawlak et al., 2000). Interestingly, NR2B-containing NMDARs appear to play an important role in behaviors related to other drugs of abuse. For instance, ifenprodil is known to block both CPP induction and expression for

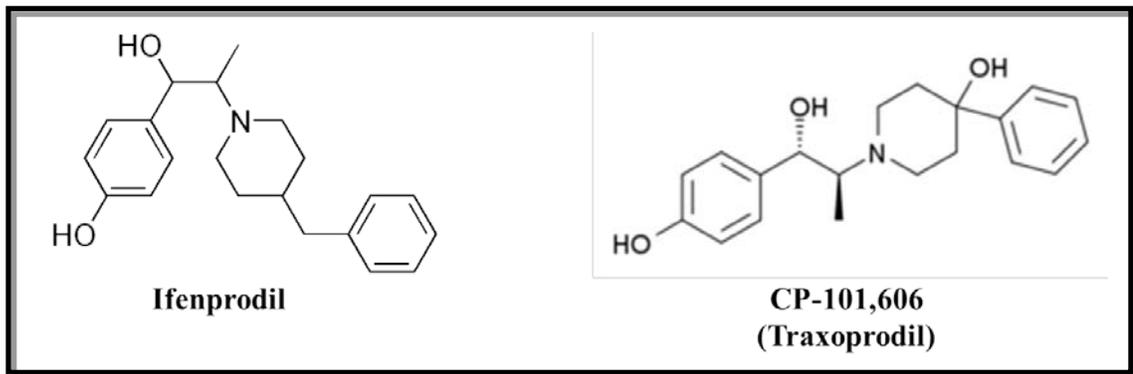
morphine (Suzuki et al., 1999; Ma et al., 2006; Ma et al., 2007). Similarly, treatment with an antibody against NR2B subunits abolishes morphine-induced place preference, whereas antibodies against NR2A do not (Narita et al., 2000). These results suggest that examination of NR2B-specific compounds as anti-relapse agents for alcohol may inform research examining other drugs of abuse.

While examinations of eliprodil and ifenprodil have returned promising results, and further implicated NR2B involvement in ETOH action, their clinical development has been slowed due to secondary effects including calcium channel blockade as well as alpha-adrenergic, 5HT_{1A}, 5HT₂, 5HT₃ and sigma receptor inhibition (Chenard et al., 1991; McCool & Lovinger, 1995; Biton et al., 1994). Although additional receptor system activation does not necessarily preclude clinical advancement, the aforementioned pattern of activation suggests complex cardiovascular and affective complications.

5.7 CP-101,606

Recent medication development efforts altered the composition of ifenprodil at several sites, producing a similar compound (see Figure 3), CP-101,606 (*1S,2S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol (Pfizer). Radioligand binding studies have determined that CP-101,606 (CP) represents a distinct class of NR2B specific antagonists, which bind NR2B with high affinity only if the receptor is an NR2B/NR2B dimer (Chazot et al., 2002). This high degree of specificity is thought to limit its side effect profile in humans, suggesting its potential for clinical use in alcoholism. *Additionally, this NR2B/NR2B specificity provides a unique pharmacological tool with which to investigate NR2B involvement in ETOH-associated relapse and neurotoxicity, and was thus selected as one of the compounds examined in the current project.*

Figure 3. Molecular Structure of Ifenprodil and its Analogue, CP-101,606



Thus far, CP has received limited attention in alcohol research, but has been used in several other fields. CP has shown efficacy as an antinociceptive agent (Boyce et al., 1999), an anticonvulsant (Brackett et al., 2000) and has demonstrated anti-parkinsonian action (Nash et al., 2004; Steece-Collier et al., 2000). CP attenuates the effects of traumatic brain injury and focal ischemia in animal models (Okiyama et al., 1998; Okiyama et al., 1997; Tsuchida et al., 1997; Di et al., 1997; Kundrotiene et al., 2004) and protects hippocampal neurons from glutamate toxicity *in vitro* (Menniti et al., 1997), and of particular relevance to the current project, CP was used in a cortical cell culture model of ETOH WD, and demonstrated the ability to reduce excitotoxic effects *in vitro* (Nagy et al., 2004).

Furthermore, our own work has demonstrated CP's neuroprotective potential. Following chronic, neonatal ETOH exposure, a single administration of CP during withdrawal was able to attenuate a number of behavioral deficits reliably produced by this FAS model, including balance deficits, hyperactivity and learning & memory dysfunction (Lewis et al., submitted).

In experimental mammals, CP seems to have few side effects; it neither impedes

motor performance (Boyce et al., 1999) or learning and memory as measured in Morris water maze (Guscott et al., 2003). In contrast to other NMDAr antagonists, it may enhance performance in cognitive tasks (Higgins et al., 2005). Importantly, CP has been used in several clinical studies, where it appeared well-tolerated (Johnson et al., 2003; Merchant et al., 1999); two in particular report positive reduction in the behavioral consequences of stroke and traumatic brain injury (Yurkewicz et al., 2005; Bullock et al., 1999). Due to the mechanistic contributions of polyamine-sensitive NMDAr in neurotoxicity and relapse, and due to the noted safety and potential of CP-101,606, it was selected for use in the current project.

5.8 Agmatine & Our Current Development Strategy: Novel Polyamine Modulators

Agmatine (decarboxylated L-arginine) was recognized approximately a century ago (Kossel, 1910) but identified in mammalian brain only within the last few decades (Li et al., 1994). The accumulated evidence suggests agmatine meets the criteria of a central modulator, and is currently considered a putative neurotransmitter (for review see Halaris & Plietz, 2007). Agmatine is synthesized in neurons (Reis & Regunathan, 2000; Iyo et al., 2006); is released presynaptically in a Ca⁺ dependent manner (Goracke-Postle et al., 2006); is disrupted by exogenous drugs (11r antagonists; Wu, Su & Liu, 2006); is distributed in specific neuronal populations (i.e., hippocampal; Reis, Yang & Milner et al., 1998); has specific reuptake processes (Reis & Regunathan, 1998; Sastre, Regunathan & Reis, 1997), is enzymatically degraded (i.e., agmatinase; Sastre et al., 1996; Iyer et al., 2002); and acts on receptors (e.g., Olmos et al., 1999; Piletz et al., 1995; Askalany et al., 2005), effecting specific neuronal processes (e.g., nNOS inhibition; Demady et al., 2001).

The action of agmatine most relevant to the design of the current project involves its ability to act as an endogenous modulator of polyamine activity, functioning as a low potency NMDAr inhibitor, however it appears unique for several reasons, including its ability to inhibit or inactivate nNOS by up to 50%, at physiologically relevant concentrations (Auguet et al., 1995; Galea et al., 1996; Demady et al., 2001). NMDAr

and nNOS are both recognized for their contribution to glutamatergic neuroplasticity, suggesting agmatine may provide unique modulation of such neuroplasticity, unattainable via either NMDAr or nNOS-selective manipulation. The observation that agmatine is increased in hippocampus (~85%) following training of learning-dependent tasks (Morris maze) supports this suggestion, however the functional significance of increased agmatine is unknown. Furthermore, its unique NMDAr/nNOS profile suggests its prophylactic potential for excitotoxic damage (given the closely related involvement of both NMDAr and nNOS). Initially, reports of agmatine's physiological concentration in CNS were low (pM-nM), however it is now recognized that acute stress or trauma may induce rapid agmatine synthesis, such that >10 μ M concentrations have been noted following insult (Aricioglu, Regunathan & Piletz, 2003).

Of further significance, particularly in terms of its consideration as a therapeutic agent or lead compound, agmatine appears only a very weak channel inhibitor, with an IC₅₀ of approximately 1 mM, significantly lower than other polyamine modulators ifenprodil or arcaïne (IC₅₀s of 500 nM and 5 μ M, respectively). However, agmatine exhibits a K_i of ~15 μ M for MK-801 binding in the presence of spermidine (100 μ M; Ring, 2006), suggesting its ability to inhibit the NMDAr is polyamine-specific, at relevant concentrations.

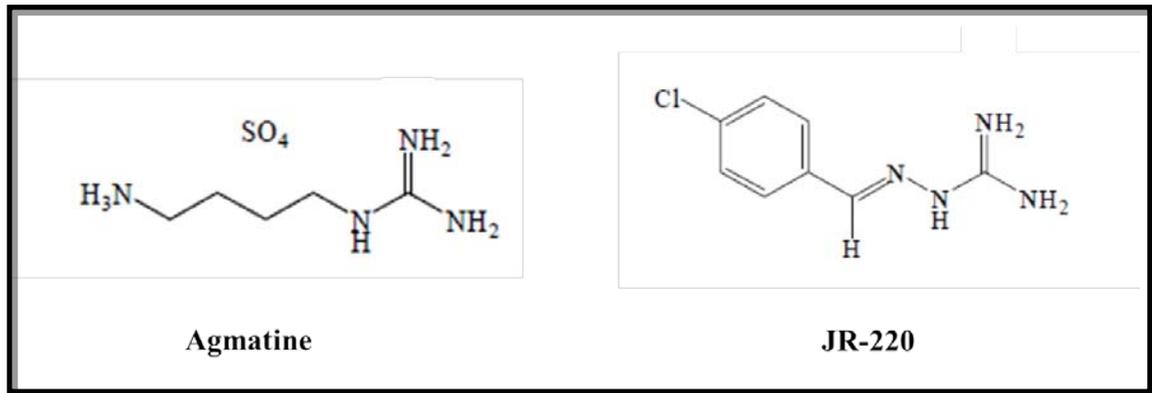
Agmatine's noted neuroprotective potential (e.g., Gibson et al., 2003; Gilad et al., 1996; Lewis et al., 2007), anxiolytic effects (Lavinsky, Arteni, & Netto, 2003), attenuation of drug-associated conditioning (Wei et al., 2005), and reduction of ETOH WD symptoms (Uzbay et al., 2000) have generated interest in its clinical development for a range of diseases including schizophrenia (Uzbay et al., 2009), neuropathic pain (Onal et al., 2003) and substance abuse disorders. Recently, agmatine's clinical efficacy as an antinociceptive agent was demonstrated in subjects suffering herniated lumbar disc-associated radiculopathy (Keynan et al., 2010). While Kenyan & colleagues observed only mild-to-moderate side effects, concerns about uraemia (Piletz et al., 2003) and toxic

interactions with elevated potassium levels (Abe, Abe & Saito, 2003) are noted. One alternative to direct agmatine administration involves agmatinase inhibition (e.g., piperazine carboxamidine), a solution being explored currently (Huang et al., 2003). The current project has selected another strategy, using agmatine as a lead structure for the development of novel, higher potency, agmatine analogues.

To this end, we established a medications development screening project at the University of Kentucky, wherein novel molecules were synthesized in the Pharmaceutical Sciences Division of the College of Pharmacy and examined in molecular screens for polyamine inhibition. Compounds testing positive were then examined in simple cellular screens, followed by more complex tissue screens, simple behavioral screens and finally complex behavioral screens, all contingent on success in the previous tier of screening.

The primary molecular screen involved detection of [3H]MK-801 binding in membrane preparations, both in the presence and absence of spermidine. MK-801 is an open-channel ligand, whose B_{max} is enhanced by approximately 75-100% when spermidine is added to the membrane preparation (Gibson et al., 2002). In this screen, compounds acting via competitive inhibition or steric hindrance display a monophasic binding curve, reducing [3H]MK-801 binding regardless of the presence or absence of spermidine. Compounds which display biphasic curves, such that at low concentrations [3H]MK-801 binding is only reduced in the presence of spermidine, are thought to display selectivity for polyamine inhibition. Over 1500 compounds have been screened in this manner, including a group of aryliminoguanidines. One member of this group, JR-220 ([4-Chlorobenzylideneamino]-guanidine Hydrochloride; see Figure 4), was highly potent, with an IC_{50} of 3.6 μ M.

Figure 4. Molecular Structure of Agmatine and its Analogue, JR-220



The neuroblastoma cell line SH-SY5Y expresses functional GLUergic cells and receptors, and is used in the current project to screen novel compounds for their ability to inhibit NMDA-induced $^{45}\text{Ca}^{2+}$ entry. JR-220 (JR) was shown to inhibit calcium entry in a concentration-dependent manner with greater potency (10-100 μM) than agmatine (100-500 μM). Neuroprotection of SH-SY5Y cells from NMDA insult (500 μM) at saturating concentrations of glycine and spermidine (10 μM and 100 μM , respectively), was assessed using the colorimetric assay, MTT. JR-220 significantly and concentration-dependently (10-100 μM) reduced toxicity. This cell line was further used to screen for protection from ETOH withdrawal-associated damage (with identical concentrations of glycine and spermidine) after a 4-day exposure (180 mM). JR-220 (10-50 μM) demonstrated the highest neuroprotective capability in this screen. Importantly, when these SH-SY5Y experiments were conducted without adding exogenous spermidine, JR-220 failed to show efficacy at aforementioned doses, consistent with the hypothesis that JR-220's mechanism of NMDAr inhibition is polyamine-dependent.

Given its success in the aforementioned molecular and cellular experiments, JR-220 was selected to continue advancement through our screening procedure and is the focus of the current work.

Chapter 6. Hypotheses & Project Summary

The hypothetical framework for the current project conceptualizes the behavioral and cellular alterations associated with chronic ethanol consumption as being driven in large part by persistent alterations in glutamatergic tone and function, and being reversible via NMDAr antagonism.

HYPOTHESIS 1: Compounds which selectively inhibit polyamine stimulation of the NMDAr will perform positively in predictive screens for attenuation of a wide range of ETOH-associated effects and behaviors. Specifically:

Hypothesis 1a) *JR-220 will reduce both in vitro and in vivo (convulsion) indications of neurotoxicity*

Hypothesis 1b) *JR-220 will reduce cue-conditioned withdrawal behavior*

Hypothesis 1c) *JR-220 will reduce drinking behaviors associated with both positive and negative reinforcement, including stress, relapse, and binge screens.*

HYPOTHESIS 2: Compounds which selectively inhibit polyamine stimulation in a subset of NMDARs containing NR2B/2B diheteromers will be sufficient for positive performance in our predictive screens. Specifically:

Hypothesis 2a) *CP-101,606 will reduce both in vitro and in vivo (convulsion) indications of neurotoxicity.*

Hypothesis 2b) *CP-101,606 will reduce drinking behaviors associated with both positive and negative reinforcement, including stress, relapse, and binge screens.*

HYPOTHESIS 3: Selective polyamine inhibitors which perform positively in our predictive screens will demonstrate efficacy in standardized, established models of pathological ETOH consumption. Specifically:

Hypothesis 3a) *JR-220 will reduce consumption in the 'Drinking in the Dark' model.*

Hypothesis 3b) *CP-101,606 will reduce consumption in the 'Drinking in the Dark' model.*

Experiments presented here were conducted in the context of a drug discovery project, designed to identify novel compounds with the potential to reduce withdrawal severity, neurotoxicity and relapse risk. The current work presents a subset of screens from this project. The included screens have been validated with relevant drugs (i.e., acamprosate, topiramate, memantine), with one notable exception (see below). Screens include a complex organotypic hippocampal culture screen designed to assess neuroprotective potential (Experiment 1), a simple behavioral screen designed to assess withdrawal severity (Experiment 2) as well as several more complex behavioral screens designed to examine cue-conditioning during withdrawal (Experiment 3), relapse-like drinking behavior (Experiment 4), stress-associated consumption (Experiment 5) and binge-like drinking behavior (Experiment 6). Though not directly examining drinking behavior, a final behavioral screen (Experiment 7) was included to assess potential anxiety-related and/or activity-related effects of the compounds tested. Finally, a model of binge-like drinking was utilized to further examine screening results (Experiment 8).

The primary compound of interest for the current project is JR-220, a modulator of polyamine enhancement of NMDA_R function. ***Data generated from the examination of JR-220 in Experiments 1-6 will directly test Hypothesis 1.*** CP-101 was examined both due to its therapeutic potential as well as its unique pharmacological profile, which may help illuminate the contribution of NR2B-containing NMDA_Rs to the behaviors examined here. ***Although only examined in Experiments 1, 2, and 6, data generated from these experiments will directly test Hypothesis 2.*** Based on the results of these screens, we utilized a well-recognized model of binge-like consumption (Experiment 8) to further explore these compounds. While ***Experiment 8 will directly test Hypothesis 3,*** it does not represent a complete examination, but rather the first in a series of planned explorations, the entirety of which are beyond the scope of this dissertation.

Chapter 7. Methods, Results & Discussion (By Experiment)

Experiments presented here represent screens which were developed based on several well-established models of ETOH intake behavior, as well as some lesser-known paradigms (e.g., Experiment 3). We use Sprague-Dawley rats as well as Swiss Webster and C57BL6J mice. Species/strains were selected based on either established literatures suggesting the appropriateness of our chosen species/strains (Experiments 1,2,5 and 6) or the experiences of our group in establishing these paradigms (Experiment 3). The exception is Experiment 4; Wistar rats are the most common strain used (P rats are also common) in the model from which this screen was established; Sprague-Dawley rats were selected for pragmatic reasons (our group maintains an established Sprague-Dawley breeding program and has extensive experience with this strain). However, using well-established methods (discussed below) we have repeatedly demonstrated the paradigm's utility with this strain. The information above is described in Table 1.

Table 1. Species/Strain Use by Experiment

Exp.	Screen/Model	Species/Strain	(Strain-specific) References
1	Hippocampal Slice Culture (OHSC)	Rat: Sprague-Dawley (<i>In vitro</i>)	(e.g., Stoppini et al., 1991; Gibson et al., 2003; Prendergast et al., 2000; Stepanyan et al., 2008; Norberg and Zimmer, 1998)
2	Handling-Induced Convulsion (HIC)	Mouse: Swiss Webster	(e.g., Farook et al., 2007; Stepanyan et al., 2008; Crabbe et al., 1980)
3	Conditioned Withdrawal in Elevated Plus Maze (EPM)	Mouse: Swiss Webster	(Farook et al., 2007; Wilson et al., 1998*; Cole et al., 1999*; Cole et al., 2000*) * used TO Mice, derived from Swiss Websters.
4	Alcohol Deprivation Effect (ADE)	Rat: Sprague-Dawley	* The use of Sprague-Dawleys is atypical; Wistars and P rats are used most commonly.
5	Stress-Associated Drinking	Mouse: C57BL/6J	(Farook et al., 2009; Lopez et al., 2011)
6/8	Drinking in the Dark (DID)	Mouse: C57BL/6J	(e.g., Rhodes et al., 2005; Rhodes et al., 2007; Moore & Boehm, 2009; Gupta et al., 2008)
7	Open Field (OF)	All species/strains	(e.g., Rubin et al., 2008; Wellmann et al., 2011)

Preclinical models (both *in vivo* and *in vitro*) have three basic measures of validity. Construct validity describes the extent to which the physiological mechanisms or underlying pathophysiologies are similar between the cellular/animal models and humans. Face validity describes the extent to which the model mimics the behaviors or symptoms exhibited in the human disease. Construct and face validities are historically the models more heavily weighted when considering the usefulness of a particular model. However, for the purposes of drug discovery, predictive validity, or the ability to predict a drug's action and/or efficacy in humans, is considered critical. The challenge for establishing predictive validity is generally the need for an efficacious pharmacotherapy in humans to use as a benchmark with which screen results may be compared. While these data are not shown, Experiments 1-5 were previously validated with several relevant compounds, including ifenprodil, memantine, and most importantly, acamprosate, which was considered a 'best-in-class' reference drug both for its efficacy in treating human alcoholics as well as its putative mechanisms of action, which most closely resemble (compared to other FDA-approved drugs) those of our compounds.

In the development of our screens and screening procedure, predictive validity was the most strongly considered, since high throughput screening with strong predictive validity should have the greatest power to identify potential compounds of interest, which can then be tested more thoroughly in models with face and construct validity (this idea is well represented in our transition from simple screens to Experiment 8). Designations of 'screen' and 'model' are particularly challenging in the current work, which utilizes both. In the context of this project, 'screen' is used to describe an experiment whose primary function involves prediction of, rather than direct evidence for, efficacy. Experiment 2 (described in detail below) illustrates this concept; the elicitation of convulsions during artificially exacerbated ETOH withdrawal fails to resemble clinically-manifested behaviors, but predicts modulatory actions at the receptor level which resemble current NMDAR-directed pharmacotherapies. It is thusly considered a screen, rather than a model. While Experiments 2 and 8 provide clear examples of screens and models, respectively, other experiments are less easily dissociated, and although selected for their predictive validity, offer significant face and construct validities.

Experiment 1: Organotypic Hippocampal Slice Culture (OHSC)

Although numerous *in vitro* models have been utilized to examine neuroprotection from excitotoxic insult, our complex *in vitro* screen required a paradigm which could efficiently characterize the effect of pharmacological agents in live brain tissue. The organotypic hippocampal slice (OHSC) procedure uses a brain area known to be particularly vulnerable to ETOH, and is conducive to the type of screening procedure used here. The procedures described here were originally developed by Stoppini and colleagues (1991), and produce hippocampal slices containing live, intact neurons and glia, maintaining a high level of complex neuronal connection and surviving in an apparently healthy state for extended periods of time (Stoppini et al., 1991). This method has been used extensively by our group (e.g., Gibson et al., 2003; Prendergast et al., 2000; Stepanyan et al., 2008) and others (e.g., Noraberg and Zimmer, 1998) to investigate chronic ETOH exposure and WD. Importantly, the OHSC screen has been validated with a number of highly relevant compounds. Ifenprodil (Gibson et al., 2003), memantine (Stepanyan et al., 2008) and acamprosate (Mayer et al., 2002a) have all been shown to reduce excitotoxic insult during/following ETOH withdrawal in this model. Furthermore, the contribution of polyamines to damage in this paradigm is established, as evidenced by attenuated damage following DFMO treatment (Gibson et al., 2003) and exacerbated damage following exogenous polyamine application (Mayer et al., 2002b), suggesting its particular sensitivity as a screening tool for polyamine modulators.

While described in detail below, the procedure involves extracting hippocampi from rat pups, plating the slices in hormone/nutrient-rich media and chronically exposing the slices to ETOH. Upon removal of the ETOH, significant CA1 toxicity occurs. In the studies presented here, various concentrations of JR-220 and CP-101,606 were examined for their ability to prevent this withdrawal-associated excitotoxicity. In all conditions slices were exposed to exogenous NMDA, either a toxic dose (20 μM) or a mild dose (5 μM), which does not induce significant toxicity (unpublished data). This mild dose was used to model excessive GLU influx from hippocampal afferents during WD. The competitive NMDAR antagonist MK-801 was also included as a treatment. [5 μM

NMDA] alone was used as a negative control, while [20 μ M NMDA] and [5 μ M NMDA + MK-801] provided positive controls (indicating maximal toxicity and protection, respectively). Cell death was indexed by fluorescence of propidium iodide (PI), which binds DNA, entering via compromised cell membranes (Zimmer et al., 2000). This index of cell damage has been validated by several other markers (Wilkins et al., 2006).

Specifically, we hypothesized that both CP and JR would reduce ETOH WD-associated PI fluorescence in CA1, a direct measure of cellular damage.

Subjects

Sprague-Dawley rats used in the current project were born in our breeding facility at the University of Kentucky (UK) Psychology Department. Parent animals were obtained from Harlan Labs (Indianapolis, IN). Animals were mated nightly, and the presence of a seminal plug the following morning indicated copulation had occurred. Pregnant females were individually housed in plastic cages in a temperature controlled nursery (70 \pm 3 $^{\circ}$ F) on a 12:12 light:dark cycle (on at 0700h, off at 1900h) with food and water provided *ad libitum*. On the day following birth (PND1) litters were culled to 10 animals, maintaining a 1:1 sex ratio when possible. All research was conducted in accordance with the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996) and approved by the University of Kentucky Institutional Animal Use and Care Committee.

Hippocampal Extraction & ETOH Exposure

PND8 Sprague-Dawley rat pups were used in all OHSC experiments. Pups from 11 litters were sacrificed (3 males/3 females, per litter) via rapid decapitation, brains aseptically removed, and transferred to ice-cold dissecting media [*Minimum Essential Medium (MEM; Gibco BRL, Gaithersburg, MD)*, 25 mM HEPES (ATCC, Manassas, VA), 200 mM L-glutamine (Invitrogen, Carlsbad, CA), 50 μ M streptomycin/penicillin (ATCC, Manassas, VA)]. Following removal of the meninges, hippocampi were removed, sliced

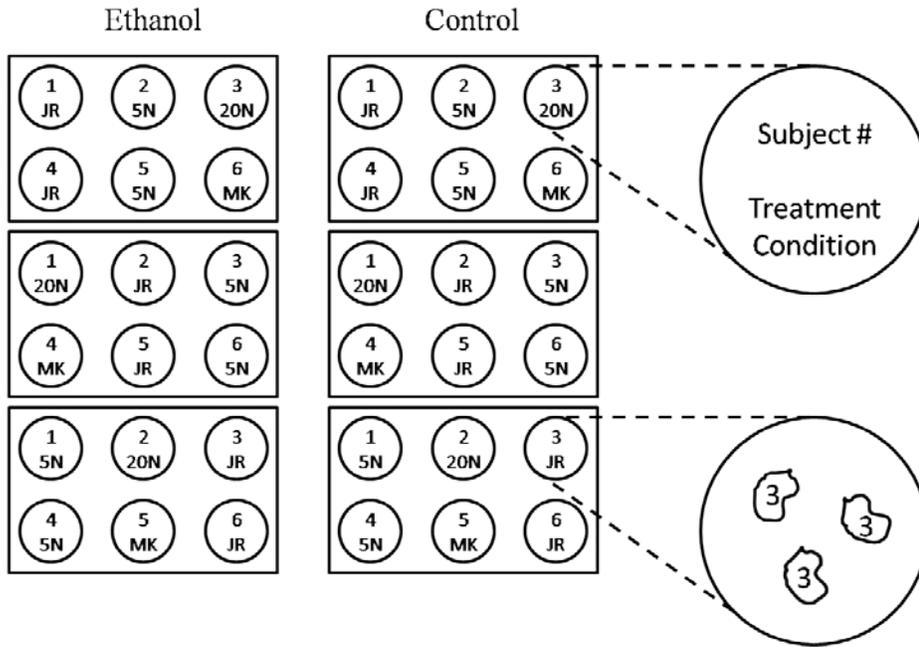
coronally at 200 μm (McIllwain Tissue Chopper; Campden Instruments Ltd., Lafayette, ID) and plated in triplicate onto 0.4 μm Biopore membranes (Millipore, Marlborough, MA). Membranes were suspended in 1 ml culture media [*dissecting media*, 36 mM glucose, 25% Hanks' balanced salt solution (HBSS; Gibco BRL, Gaithersburg, MD), 25% heat-inactivated horse serum (HIHS; Sigma, St. Louis, MO)] using six-well plates.

Plates were incubated at 37 °C in a 5% CO₂/21% O₂/74% N₂ medical grade gas composition for 5 days *in vitro* (DIV) to allow affixture to the membrane. On the fifth day *in vitro* (DIV 5) the plates were treated with new culture medium, with half exposed to 100mM ETOH. This media change was repeated on DIV 10. Between media changes, plates were housed in airtight plastic chambers with humidity and ethanol vapor concentrations maintained by placing 50 ml of 100 mM ETOH into the chambers; control chambers received 50 ml of water instead of ETOH. The chamber environment was filled with an identical composition of medical grade gas before being sealed and placed back into the incubator.

NMDA Challenge & ETOH Withdrawal

On DIV 15, a final media change was conducted, with ETOH-free media containing PI (2.5 $\mu\text{g/ml}$, Molecular Probes, Eugene, OR) added to each well. Well treatments included 5 μM NMDA (5N), 20 μM NMDA (20N; used as a negative control), 5 μM NMDA + MK-801 (20 μM ; used as a positive control) and 5 μM NMDA + Drug (e.g., JR). In order to reduce litter effects and facilitate comparison between groups, treatments were divided across subjects such that no treatment was repeated within a single subject in either the ETOH or control condition. Figure 5 illustrates an example of assigned treatments in a particular culture.

Figure 5. Treatment Distribution Within a Single Culture



Imaging & Analysis

PI uptake was visualized at 5x objective using a Leica DMBIRM microscope (W. Nuhsbaum Inc.; McHenry, IL) fitted for fluorescent detection (Mercury-arc lamp), and imaging software (SPOT Advanced, version 4.0.2, W. Nuhsbaum Inc.; McHenry, IL). PI fluorescence was analyzed using Image J, v1.29x (National Institutes of Health, Bethesda, MD). Dentate gyrus (DG), and cornu ammonis regions 1 (CA1) and 3 (CA3) were all analyzed. For each slice, background fluorescence was subtracted from the region of interest prior to analysis. Fluorescence was recorded in arbitrary units, then converted to percent of control, facilitating comparison across multiple cultures and controlling for variation between litters. Two-way analyses of variance (ANOVAs) were conducted (Sex x Treatment) on a regional basis. If no effect of sex was detected, sex was collapsed in further analyses. If the initial ANOVA was significant, planned comparisons (Fisher's protected *t*) were conducted to compare treated slices to their appropriate controls (ETOH-exposed or ETOH-naïve). Experiments with JR utilized six litters ($N=217$), distributed across 20 treatment groups, as depicted in Table 2. Experiments with CP utilized five litters ($N=177$), distributed across 16 treatment groups, as depicted in Table 3.

Table 2. JR-220 Treatments & Subjects

Treatment	Control	ETOH
No Tx	<i>n</i> =23	<i>n</i> =22
20 μ M NMDA	<i>n</i> =18	<i>n</i> =19
MK-801	<i>n</i> =18	<i>n</i> =20
50 μ M JR	<i>n</i> =8	<i>n</i> =7
60 μ M JR	<i>n</i> =7	<i>n</i> =8
75 μ M JR	<i>n</i> =16	<i>n</i> =18
80 μ M JR	<i>n</i> =4	<i>n</i> =3
90 μ M JR	<i>n</i> =3	<i>n</i> =4
100 μ M JR	<i>n</i> =4	<i>n</i> =4
125 μ M JR	<i>n</i> =4	<i>n</i> =6

Table 3. CP-101 Treatments & Subjects

Treatment	Control	ETOH
No Tx	<i>n</i> =21	<i>n</i> =21
20 μ M NMDA	<i>n</i> =12	<i>n</i> =12
MK-801	<i>n</i> =12	<i>n</i> =12
5 nM CP	<i>n</i> =6	<i>n</i> =6
10 nM CP	<i>n</i> =10	<i>n</i> =9
25 nM CP	<i>n</i> =12	<i>n</i> =12
50 nM CP	<i>n</i> =12	<i>n</i> =12
75 nM CP	<i>n</i> =4	<i>n</i> =4

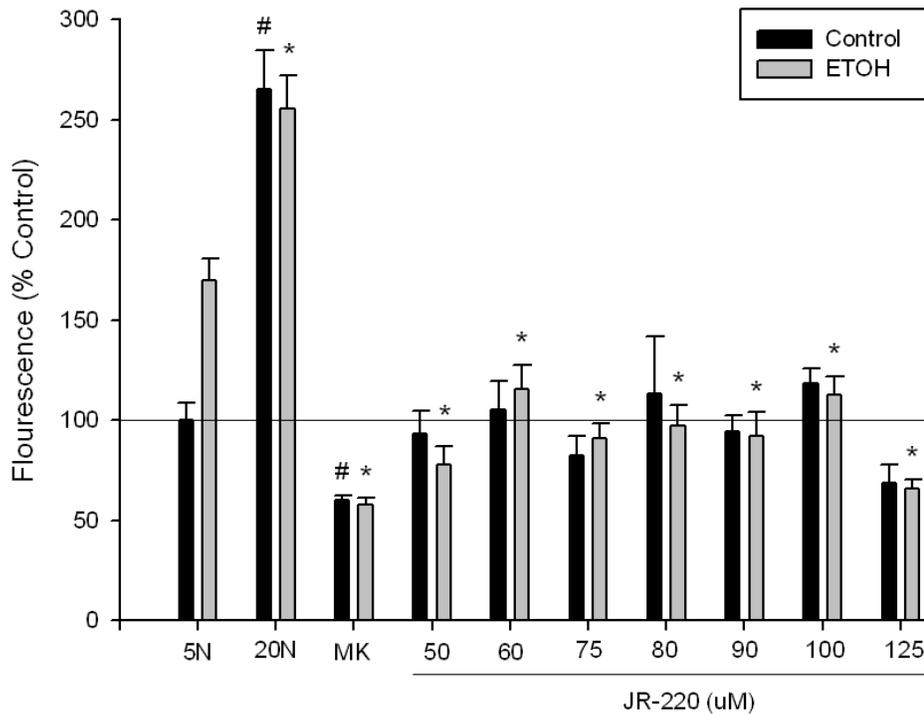
JR-220 Results

Images of representative slices are presented in Figure 12, following these results.

CA1

In CA1, administration of JR reduced fluorescence in ETOH-treated slices at all concentrations. No toxicity in ETOH-naïve slices was noted. The initial overall analysis revealed an effect of TREATMENT, $F(18, 193)=27.633$, $p<.001$, in CA1. No effect of sex was detected. Planned comparisons (Fisher's protected t) revealed that in ETOH-treated groups, fluorescence was significantly reduced by MK and all concentrations of JR ($p<.001$, $p<.001$, $p=.003$, $p<.001$, $p=.001$, $p=.010$, $p=.002$, $p=.021$, $p<.001$, respectively). Fluorescence was increased following 20 μM NMDA treatment ($p<.001$), as expected. A second set of planned comparisons conducted among ETOH-naïve slices indicated differences only in the positive and negative control conditions; increased fluorescence following 20 μM NMDA treatment ($p<.001$) and decreases following MK-801 treatment ($p=.004$) were noted. Figure 6 depicts PI uptake in the CA1 region relative to controls.

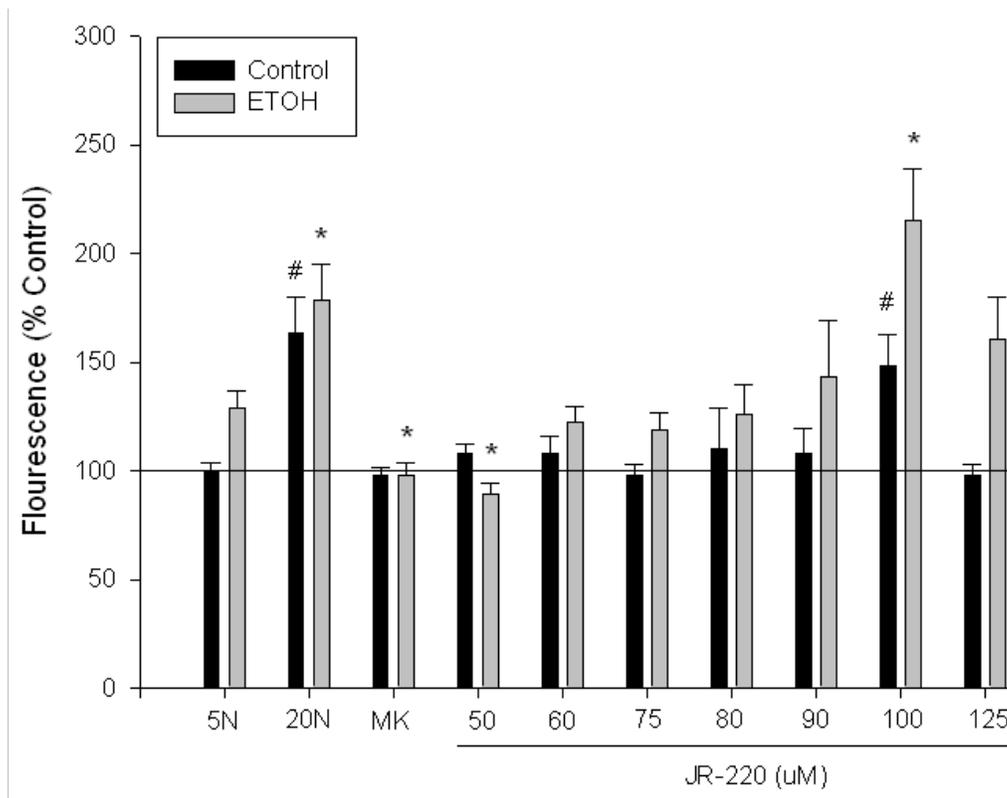
Figure 6. JR-220 in CA1



CA3

In CA3, administration of JR reduced fluorescence at the lowest dose, while appearing toxic at 100 μM . An overall ANOVA revealed a main effect of TREATMENT, $F(18, 193)= 6.301, p<.001$, in CA3. No effect of sex was detected. Planned comparisons (Fisher's protected t) revealed that fluorescence differed between the ETOH control group and four other ETOH-exposed groups. In the case of MK-801 ($p=.013$) and 50 μM JR ($p=.023$) this difference reflected reduced PI uptake, while in the cases of 20 μM NMDA ($p<.001$) and 100 μM JR ($p=.024$), increased uptake was observed. Among ETOH-naïve slices, 20 μM NMDA increased uptake ($p<.001$). Figure 7 depicts PI uptake in the CA3, relative to controls.

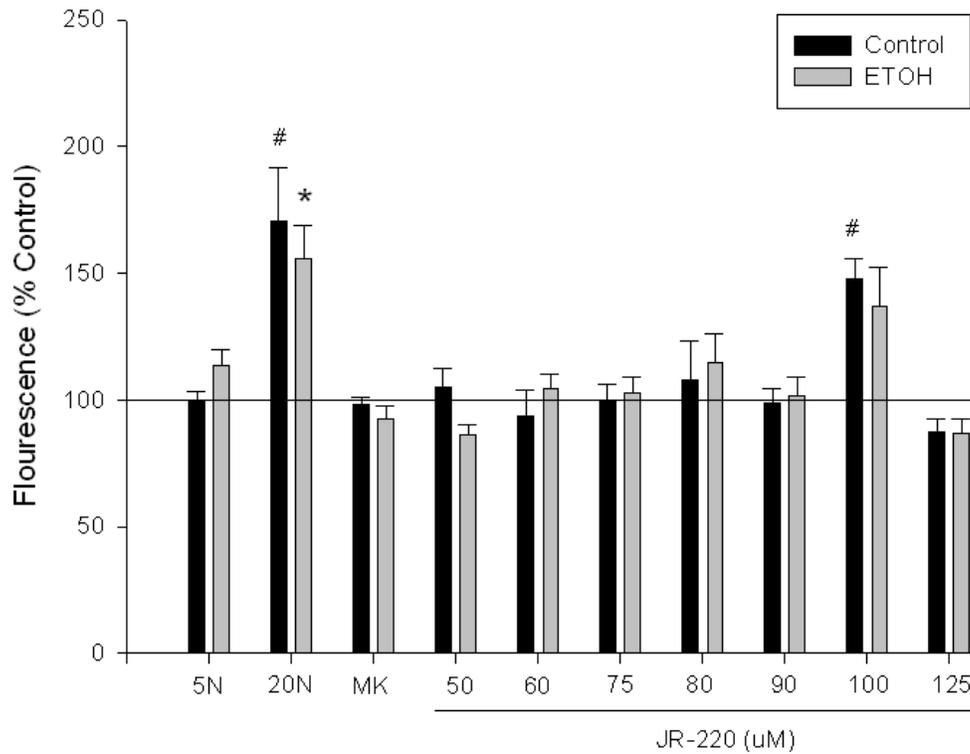
Figure 7. JR-220 in CA3



DG

In DG, 100 μM JR was associated with increased fluorescence, no other effects were noted. An overall ANOVA revealed a main effect of TREATMENT, $F(18, 193)=4.982, p<.001$, in DG. No effect of sex was detected. Planned comparisons (Fisher's protected t) revealed only a difference between ETOH-exposed 20 μM NMDA slices and controls ($p=.001$). Among ETOH-naïve slices 100 μM JR was associated with increased fluorescence ($p=.018$), as was 20 μM NMDA ($p<.001$). Figure 8 depicts PI uptake in the DG, relative to controls.

Figure 8. JR-220 in DG

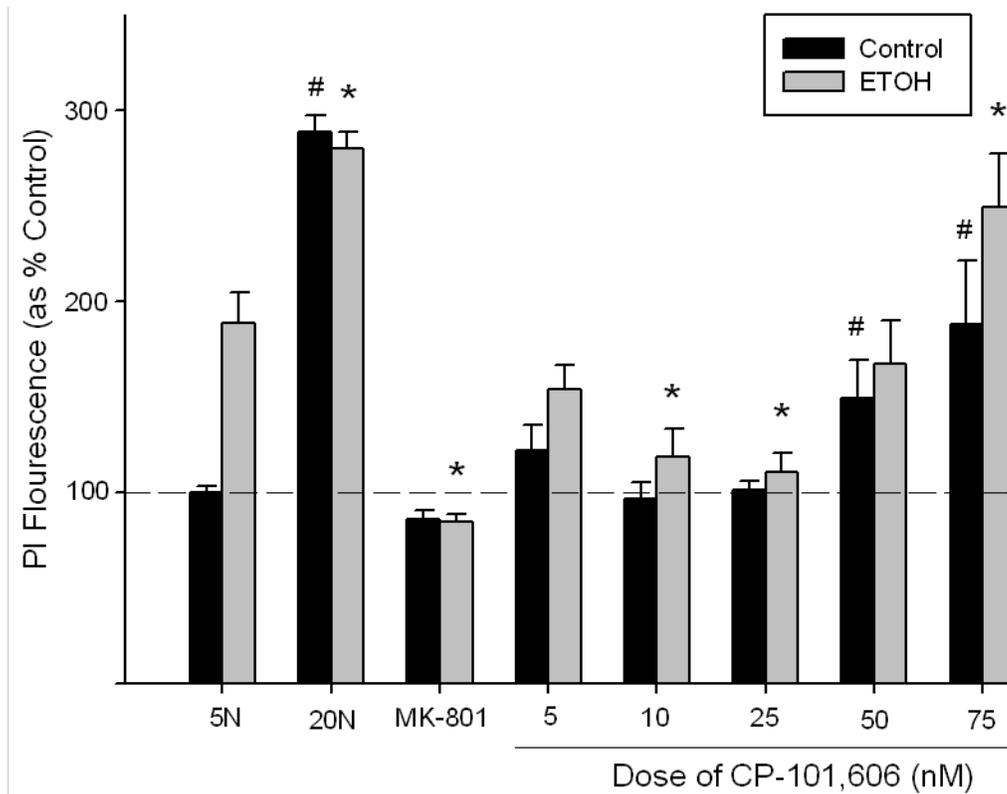


CP-101,606 Results

CA1

In CA1, administration of CP reduced fluorescence in ETOH-treated slices at low concentrations, but was associated with toxicity at higher concentrations in both ETOH and ETOH-naïve groups. The overall ANOVA revealed a main effect of TREATMENT, $F(14, 155)=21.338, p<.001$, in the CA1. No effect of sex was detected. Planned comparisons (Fisher's protected t) revealed decreased PI uptake in 10 nM CP ($p<.001$), 25 nM CP ($p<.001$) and MK-801 ($p<.001$) treated slices, relative to ETOH controls, and increased PI uptake in 75 nM CP ($p=.022$) and 20 μ M NMDA-treated slices ($p<.001$). Among ETOH-naïve controls, increased uptake among 20 μ M NMDA-treated ($p<.001$), 50 nM CP-treated ($p=.003$) and 75 nM CP-treated ($p<.001$) slices was noted. Figure 9 depicts PI uptake in the CA1, relative to controls.

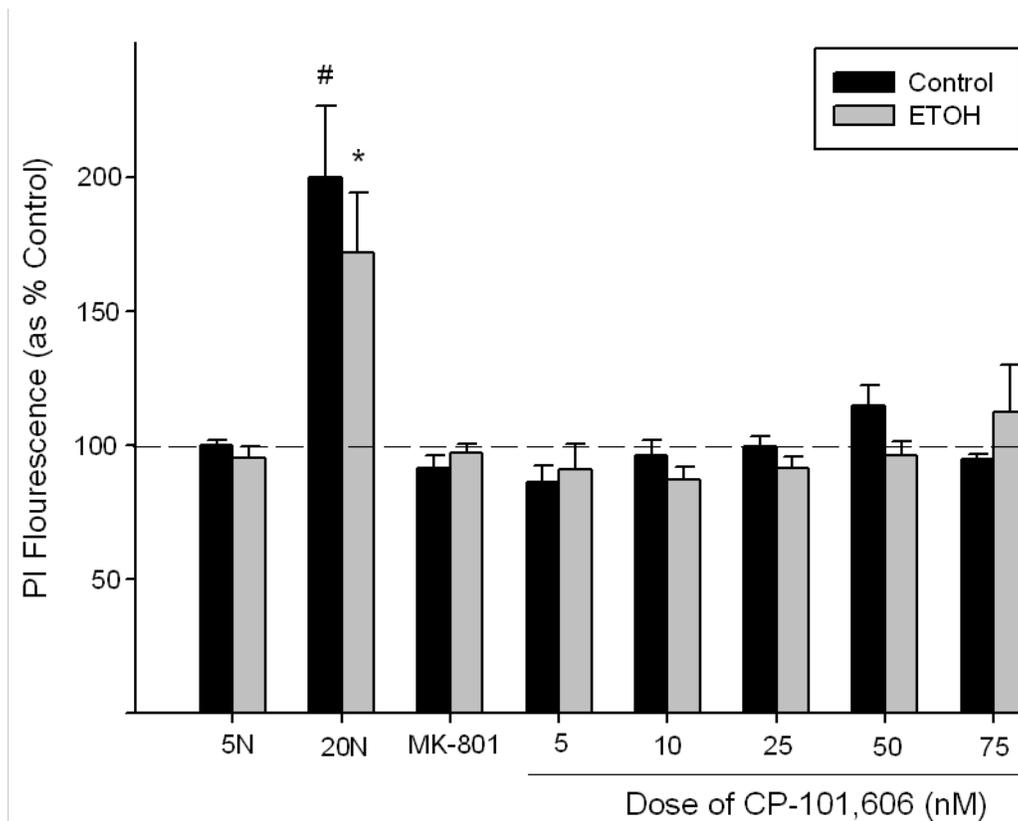
Figure 9. CP-101,606 in CA1



CA3

No effects of CP were noted in CA3. An overall ANOVA revealed a main effect of TREATMENT, $F(18, 193)= 8.864, p<.001$, in CA3. No effect of sex was detected. Planned comparisons (Fisher's protected t) revealed fluorescence differences between ETOH controls and ETOH+20 μM NMDA ($p<.001$), only. Among ETOH-naïve controls, only increased uptake among 20 μM NMDA-treated slices ($p<.001$) was noted. These results are depicted in Figure 10, below.

Figure 10. CP-101 in CA3



DG

No effects of CP were noted in DG. An overall ANOVA revealed a main effect of TREATMENT, $F(18, 193)= 8.290, p<.001$, in DG. No effect of sex was detected.

Planned comparisons (Fisher's protected t) revealed fluorescence differences between ETOH controls and ETOH+20 μM NMDA ($p<.001$), only. Among ETOH-naïve controls, only increased uptake among 20 μM NMDA-treated slices ($p<.001$) was noted. These results are depicted in Figure 11, below.

Figure 11. CP-101 in DG

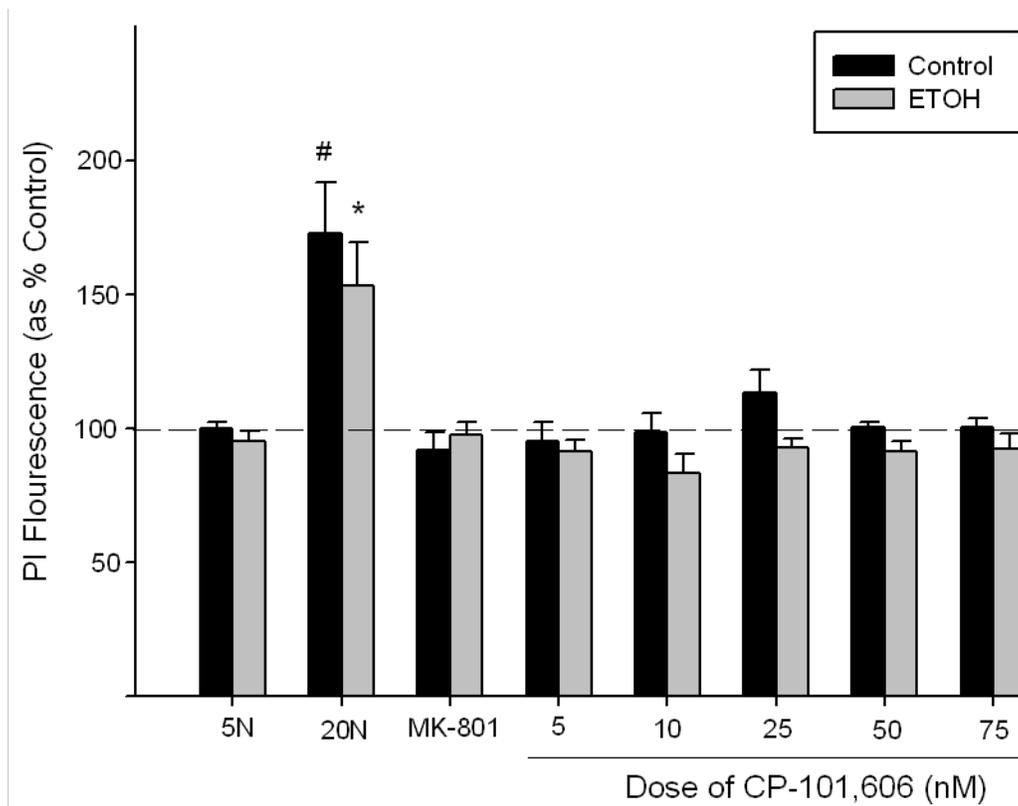
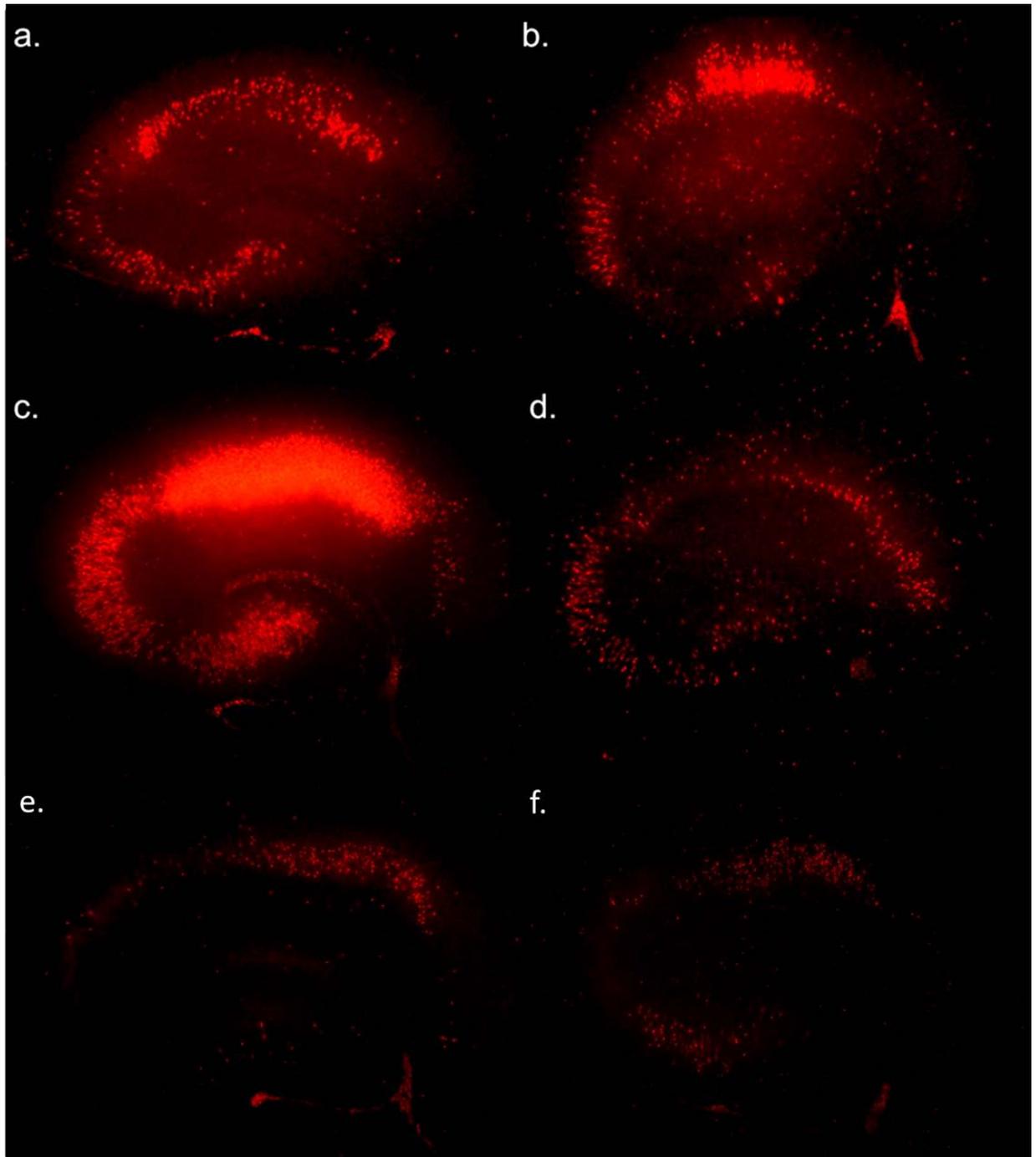


Figure 12. Representative Slices From Select Treatment Groups

including a) Control, b) ETOH, c) 20 μ M NMDA, d) 50 μ M JR-220 , e) 25 nM CP-101, and f) 20 μ M MK-801 .



OHSC Discussion

Results from the OHSC screen demonstrated the neuroprotective potential of both compounds. Among cultures examined in the JR experiments, the untreated, ETOH-exposed slices demonstrated increases in PI fluorescence of approximately 70% in CA1. At all concentrations examined, JR-220 reduced fluorescence. Comparison among ETOH-naïve slices indicated no associated toxicity in controls at any concentration of JR. Results from the CA3 region were slightly perplexing; ETOH was associated with a moderate increase in PI fluorescence. The lowest concentration of JR (50 μM) appeared to reduce ETOH-associated toxicity; while 100 μM significantly increased it. This higher dose was also associated with toxicity in ETOH-naïve cultures. A similar pattern of effects were noted in DG, however only the increased toxicity among ETOH-naïve slices treated with 100 μM JR retained significance. These results are somewhat perplexing, in that JR did not appear toxic in the CA1, considered particularly sensitive to polyamines and polyamine manipulation (Butler et al., 2010), but did appear toxic in less sensitive regions. While these findings should be interpreted cautiously (given the low number of subjects exposed to the 100 μM concentration), they may suggest additional mechanisms of JR-220 action, and potentially an unanticipated toxic mechanism (discussed in greater depth in the final discussion section).

Among cultures examined in the CP-101 experiments, the untreated ETOH-exposed slices demonstrated increases in PI fluorescence of approximately 90% in CA1. The results from CA1 were suggestive of a U-shaped curve; low doses (5 nM) were ineffective at reducing toxicity, moderate doses (10 and 25 nM) were efficacious, while higher doses (50 and 75 nM) indicated an exacerbation of toxicity. These effects were mirrored in ETOH-naïve slices (50 and 75 nM concentrations increased fluorescence). While the high selectivity of CP-101 is suggested to limit its inherent toxicity and side effect profile, the NR2B class of NMDAR antagonists has been associated with toxic effects in NR2B-rich nuclei (Parsons, Danysz & Quack, 1998). In contrast to those cultures in which JR was examined, no ETOH-associated increases in CA3 or DG were noted among control slices in these experiments. Furthermore, no evidence of CP-

associated toxicity was noted among either ETOH-exposed or naïve slices in either CA3 or DG. The contrast between marked CA1 toxicity is likely due to the noted concentration of NR2B subunits in CA1, and suggests low, if any, expression of NR2B/2B stoichiometries in either DG or CA3.

These results are consistent with our hypotheses that inhibitory polyamine modulation, whether NR2B-specific or not, would attenuate excitotoxic injury. The success of both compounds in this screen (at multiple doses) indicated their potential for neuroprotection, thus both compounds were advanced to simple behavioral screening.

Experiment 2: Handling-Induced Convulsion (HICs)

Examining seizure-susceptibility in ETOH-exposed rodents via handling has been used as a behavioral model of withdrawal severity for some time. The HIC procedure was first developed by Goldstein & Pal (1971), and has been utilized by a variety of groups since (e.g., Crabbe et al., 1980; Grant et al., 1990; Wilson & Little, 1998). HICs are precipitated by removing the animal from its home cage by holding the tail, then gently spinning it. HIC seizures are graded in their severity, producing a scorable response, facilitating their use as both an indicator of CNS hyper-excitability and a sensitive assessment tool. However, many HIC studies have used either extended periods of exposure or ETOH inhalation chambers. The current experiment utilizes an abbreviated procedure, designed to more efficiently screen compounds for anticonvulsant effects. Instead of prolonged or inhalation exposure, our procedure utilizes an ETOH dehydrogenase inhibitor (4-methylpyrazole; 4MP), administered concurrently with ETOH. While pyrazole has been used in this manner previously (e.g., Terdal & Crabbe, 1994), our lab has demonstrated that with its use, convulsant activity may be precipitated after only three days of *i.p.* ETOH exposure (Farook et al., 2007).

NMDAr antagonism induces known anticonvulsant effects during ETOH WD (Rossetti & Carboni, 1995). Our abbreviated screening procedure has been validated with numerous relevant compounds; reductions in HICs associated with acamprosate (Farook et al., 2008), topiramate (Farook et al., 2007) memantine (Stepanyan et al., 2008) and ifenprodil (unpublished data) have all been demonstrated. Moreover, while our screen uses Swiss Webster mice, reductions in WD-associated seizures appear across other strains/species (e.g., memantine; Bienkowski et al., 2001; Kotlinska, 2001).

Specifically, we hypothesized that following chronic ETOH+4MP treatments, JR and CP would reduce withdrawal-associated handling-induced convulsion.

Subjects

Male Swiss Webster mice (Harlan, IN) weighing 29 ± 3 g (approximately 15-18 weeks) were housed 3/cage. A 16/8 light/dark cycle (on at 0600h, off at 2100h) and *ad libitum* chow and water were maintained throughout the experiment. Thirty-five mice were used per experimental replication, divided into 7 groups of 5 animals, each. Three experimental replications were conducted for each drug tested.

ETOH & Drug Treatments

A 2.5 g/kg ETOH solution was administered (*i.p.*) daily (1300 h), using an injection volume of 10 ml/kg, for three consecutive days. 4MP (9 mg/kg; Sigma-Aldrich, USA), an ETOH dehydrogenase inhibitor, was co-administered with saline or ETOH. 10 h following the third and final administration of ETOH, animals were administered treatment dissolved in saline and injected at 10 ml/kg (*i.p.*). Thirty minutes following drug administration, animals were assessed for convulsive activity.

A range of JR-220 doses was examined in this paradigm; the first replication utilized low doses (1-15 mg/kg), and when no obvious behavioral perturbances were noted the dose was increased (10-40 mg/kg) for subsequent replications. Since we had greater confidence in our selection of active doses for CP based on available animal literature, all three replications utilized the same 1-20 mg/kg range.

HIC Scoring

Assessment of seizure susceptibility was performed by gently lifting each mouse by the tail and observing convulsive behavior; if no convulsions were present, they were precipitated by gently spinning the animals 360 degrees clockwise, then counterclockwise, using the thumb and forefinger, for a period of 5 sec, after which convulsions were assessed. Scoring was based on a modified scale (Wilson & Little,

1998) and is described in Table 4. Scores indicated severity, with 0 indicating no convulsive activity, 3 indicating clonic convulsion, and 4 indicating convulsion (prior to spin) upon tail-lift. All scoring was conducted by an experimenter blind to treatment condition.

Table 4. Handling-Induced Convulsion Assessment Scale

Score	Behavior
0	No activity on tail lift or 360° spin
1	Facial grimace only after 360° spin
2	Only tonic convulsion after 360° spin
3	Tonic/clonic convulsion after 360° spin
4	Tonic/clonic convulsion upon tail lift

Blood Sampling

Blood samples were collected following the 3rd day of alcohol exposure and analyzed using the Analox AM1 Alcohol Analyzer (Lunenburg, MA). Animals received either ETOH alone or in combination with 4MP. Approximately 8 animals were used for each treatment condition, divided into two groups, such that each group was sampled at three exclusive time points (i.e. group 1 sampled at 30, 120, 480 min; group 2 sampled at 60, 240, 600 min). Blood was gathered by removing the tip of the tail (1 mm) and collecting approximately 40 µL of tail blood in heparinized tubes. Tubes were centrifuged and plasma frozen for analysis. Blood was collected at 30, 60, 120, 240, 480 and 600 min following injection, and again at 24 h. A separate group of animals were used for the BEC analyses.

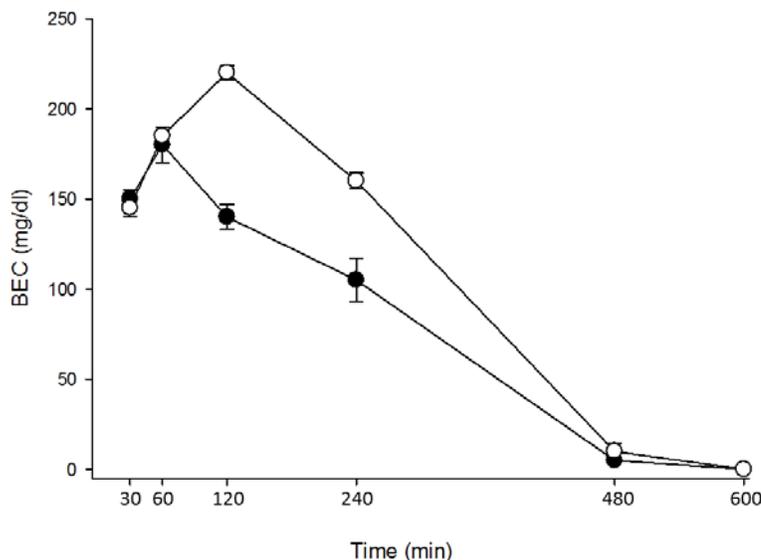
Statistical Analysis

Each compound was analyzed in three experimental replications ($N's=105$). HIC scores were converted to a percentage of the ETOH-naïve group average, to reduce variability and allow for comparison across replications. Scores were analyzed using one-way ANOVA (treatment). Post-hoc comparisons were conducted using Dunnett's test, comparing each group to the ETOH controls. BEC analyses were conducted using repeated measures ANOVA, with 4MP exposure as the between-subjects variable.

Results: BECs

4MP increased BECs at two time-points. An initial repeated measures ANOVA (4-MP x TIME) revealed an interaction of time and treatment. *T*-tests conducted at each time point indicated significantly higher blood ethanol at 120 and 240 minutes following injection ($p's <.001$) showing the differential ethanol exposure caused by the addition of 4MP (or something to provide the reader with what it means in a simple sentence or two). Blood ethanol in both groups was reduced to minimal levels by eight hours, post-injection. These results are depicted in Figure 13.

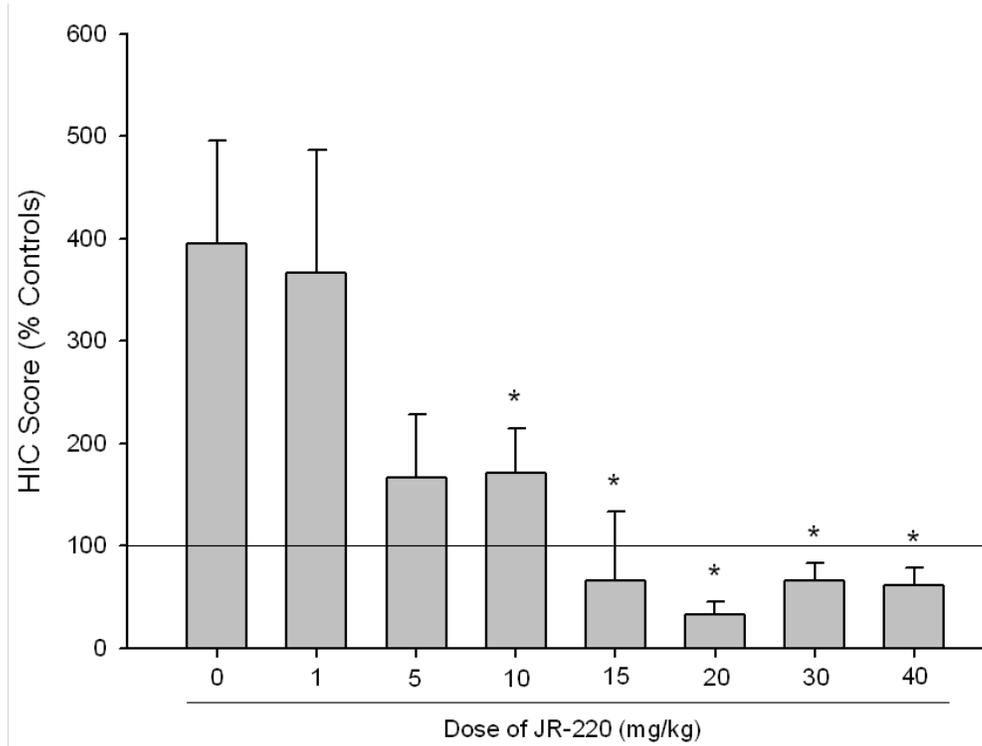
Figure 13. BEC as a Function of Time and 4MP Treatment



JR-220 HICs

JR-220 reduced HIC behaviors at doses ranging from 10-40 mg/kg. HIC scores from all three JR-220 replications were converted to percentages of the average control group's score, and combined into a single dataset. The ANOVA indicated a significant effect of treatment, $F(7, 79) = 4.297, p = .001$. Dunnett's tests detected differences between ETOH controls and 10 ($p = .019$), 15 ($p = .010$), 20 ($p = .001$), 30 ($p = .002$) and 40 ($p = .001$) mg/kg doses of JR-220. These results are depicted in Figure 14.

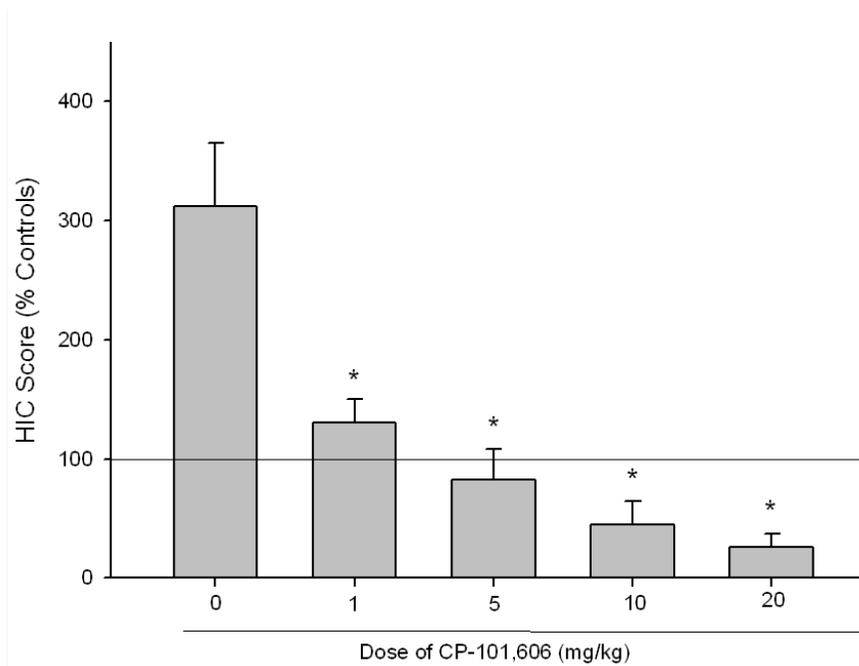
Figure 14. JR-220 HICs, Percent Control Values



CP-101,606 HICs

CP-101 reduced convulsions at all doses tested. The ANOVA indicated an effect of treatment, $F(4, 90) = 15.444, p < .001$. Dunnett's tests indicated significant reductions in HICs for all doses of CP tested, including 1 ($p < .001$), 5 ($p < .001$), 10 ($p < .001$) and 20 ($p < .001$) mg/kg. Results are depicted in Figure 15.

Figure 15. CP-101,606 in HICs, Percent Control Values



HIC Discussion

In the HIC tests, both compounds were found to be effective at reducing convulsive behavior during ETOH withdrawal, across a range of doses. JR-220's attenuation of HICs was dose-dependent, with 1 and 5 mg/kg doses failing to significantly reduce the behavior. However, the trend for reduction noted at 5 mg/kg and the striking similarity in the means and SEs between the 5 and 10 mg/kg groups suggest that the 5 mg/kg dose would have achieved significance had the subject representation been equivalent across groups (5 mg/kg $n=5$; 10 mg/kg $n=15$).

Prior to analyzing compounds in this screen, BEC curves were generated to establish 4-MP's elevation and potentiation of ETOH in blood. While a clear increase in peak BEC was noted following 4MP, as was an increase at the 4 hr measurement, increases in the duration of detectable ETOH in blood may be inferred due to the linear metabolism of ETOH (but are not directly demonstrated in this experiment); BECs returned to 0 between our 4 and 8 hr measurements. Importantly, 4MP has been tested in ETOH-naïve animals, with no discernable effect on HIC scores (Farook et al., 2007).

CP-101,606 appeared to dose-dependently reduce HICs at every dose tested, demonstrating slightly higher potency relative to JR. This is not surprising, when efficacious concentrations in the OHSC experiment are considered (10 & 25 nM CP vs. 50-125 mM JR). The magnitude of CP effects at equivalent doses also appeared greater. However, NR2B-containing NMDARs are thought to uniquely contribute to seizure/convulsive behaviors, and the implication of NR2B involvement in excitotoxicity (thought to underlie convulsive behavior) is well established. Thus, CP's potency should again be unsurprising. While the data are not presented in the current work, CP's potency appeared approximately equivalent to that of the NR2B-specific antagonist ifenprodil.

A parsimonious interpretation of these results suggests that ETOH WD-associated seizure susceptibility is under at least partial control by NR2B subunits. Speculatively, the observed reductions in convulsive behavior, which at higher doses of both compounds were well below that of the ETOH-naïve animals, corroborates evidence that NR2B antagonists have potent anticonvulsant potential (e.g., Wlaz et al., 1999), and suggests that JR-220 shares agmatine's noted anticonvulsant properties (Feng et al., 2005). The success of both compounds in this screen provide further (albeit indirect) evidence for neuroprotection. Taken together, OHSC and HIC results suggested that both compounds possessed the potential to blunt withdrawal effects and protect from withdrawal-associated damage.

These results support the hypothesis that inhibitory polyamine modulation will reduce withdrawal-associated handling-induced convulsion.

Experiment 3: Conditioned Pseudo-Withdrawal in the Elevated Plus Maze (EPM)

Opponent-process theory, mentioned in previous sections, conceptualizes drug effects (*a*-processes) as being mediated by compensatory physiological responses designed to maintain homeostasis (*b*-processes). Following chronic use, it is hypothesized that *b*-processes are induced by drug-associated cues and are thought to trigger drug-opposite effects similar to withdrawal, increasing the negative reinforcing value of the drug and potentially leading to resumption of use. One important capability of potential anti-relapse treatments, therefore, is the ability to blunt responses to drug-associated cues during acute and protracted withdrawal.

While several relapse models have utilized ETOH-associated conditioned cues, the behavioral responses of interest are generally consummatory in nature, and therefore unable to answer questions about inhibition of ETOH-conditioned behavior, since the reinforcing effects of ETOH confound interpretation. The current screen was developed as a solution, and involves repeated pairing of ETOH with an ETOH-associated environment (an elevated-plus maze; Cole et al., 1999). Following repeated ETOH-maze pairings, a vehicle injection appears to induce conditioned abstinence behavior. This behavior is not elicited when the vehicle injection is delivered in a non-conditioned environment, when ETOH is on board, or when previously ETOH-naïve animals are administered ETOH in the plus maze. The primary abstinence behavior is termed ‘stretched-attend’ posture, and involves extension of the forepaws and upper body (“stretch”), without forward movement. An alternate interpretation of this behavior is that it could indicate cued behavior resulting from positive interoceptive or internally rewarding feelings (an ‘incentive salience’ interpretation, rather than an ‘opponent-process’ interpretation), indicating pleasure derived from the conditioned stimulus. However, when naltrexone was examined in this model, no attenuation of the stretched-attend behavior was noted (Cole et al., 2000). In contrast, acamprosate (thought to relieve negative affect) was effective in reducing these behaviors (Cole et al., 2000), suggesting their association with negative affect or conditioned withdrawal. The demonstration of acamprosate’s efficacy in this model suggests strong predictive validity for its use as an

anti-relapse screen. Furthermore, while potentially any novel environment could be conditioned in this manner, the use of the elevated plus maze (EPM) allows for the additional examination of secondary effects of the compounds, including the examination of exploratory and anxiety-related behaviors.

Specifically, we hypothesized that JR would reduce stretched-attend responses to an ETOH-conditioned environment (the elevated plus maze).

Apparatus

The EPM was constructed from black Plexiglas, with two arms enclosed by walls (30 x 17 x 6 cm) and two open (unprotected) arms (30 x 6 cm). The maze was elevated 44.5 cm above the floor.

Procedure

Mice were divided into 4 groups (n 's=5-6) in which 2 groups received saline and 2 received *i.p.* ETOH injections (1.75 g/kg). Animals were injected daily for 9 days, between 1200h and 1300h. 10 min after injections, they were placed on the center of the plus maze test apparatus for a 5 min conditioning session (Cole et al., 2000; Farook et al., 2007). On day 10, subjects received either saline or a 20 mg/kg dose of JR. A 40 mg/kg group was also included, however the generated data were not interpretable due to sedative effects and thus were removed from the dataset; see discussion for further details. Thirty minutes following injection they were then placed in the center of the maze. On day 10, behavior in the maze was recorded for a 5 min test session. The dependent variables included time spent in the open vs. closed arms, total number of arm entrances and number of stretched-attend postures (defined as when the subject stretches forward with its forepaws without actually moving its hind paws) (Cole et al., 1999, 2000; Farook et al., 2007). An arm visit was defined as all four paws in one arm. Behavior was recorded using a tripod-mounted video recorder (SONY). An experimenter remained in the room but was screened from the animals' view.

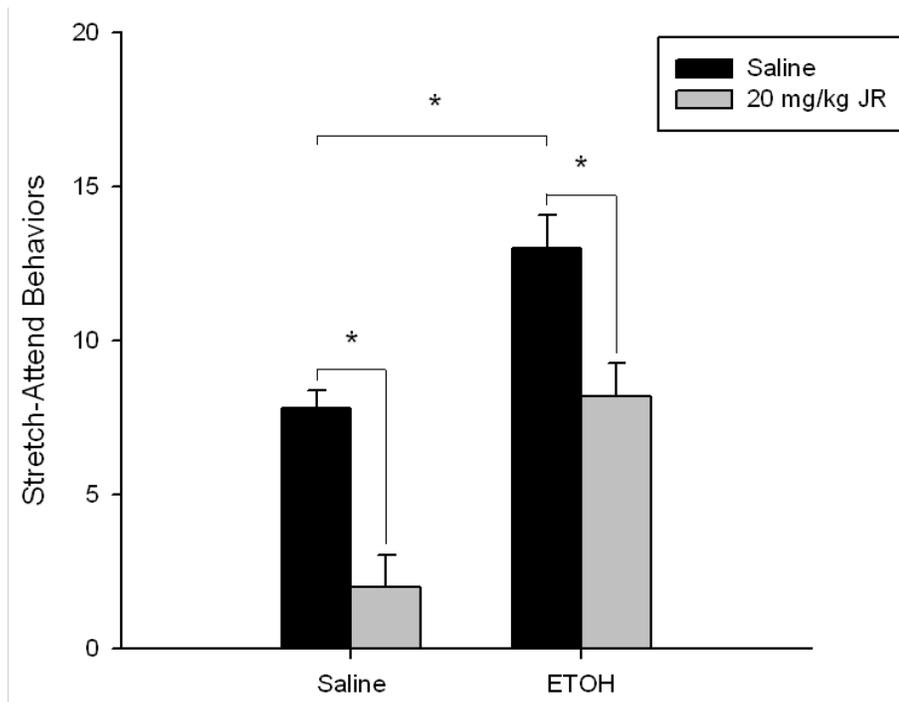
Analysis

For each dependent variable a 2 x 2 ANOVA (ETOH Exposure x Treatment) was conducted. *Post hoc* tests (Fisher's protected *t*) were conducted to further explore significant effects.

Results

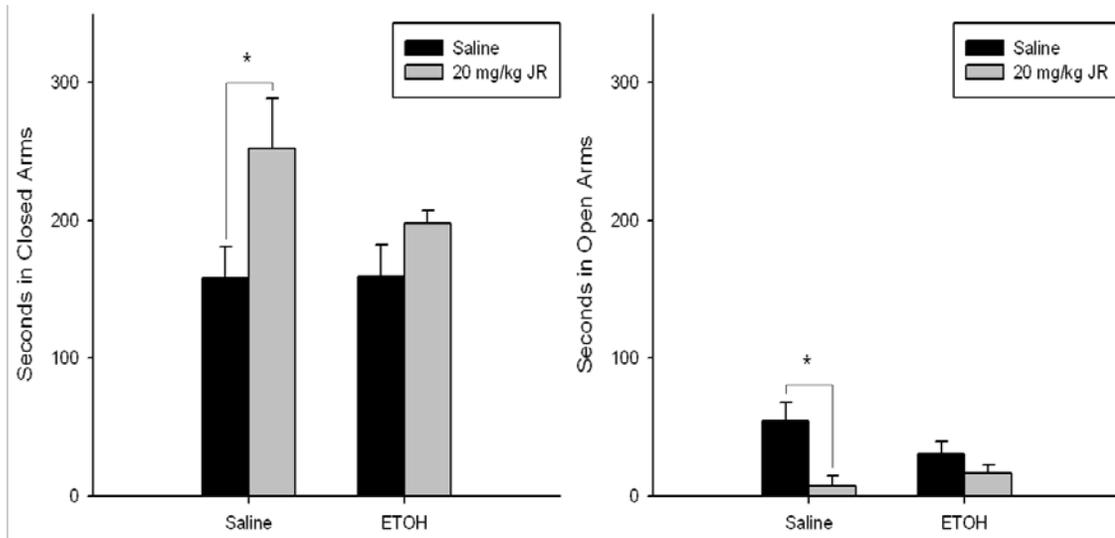
JR-220 reduced stretched-attend posture in ETOH and saline-treated animals. A 2 x 2 ANOVA for stretched-attend behaviors revealed main effects of both pretreatment and JR treatment. Pretreatment with ETOH was associated with increased stretched-attends, $F(1,20)=33.665$ $p=.029$, while JR treatment was associated with reductions in the behavior, $F(1,20)=29.106$ $p=.002$. *Post hoc*s revealed that among JR-naïve animals, ETOH pre-exposure was associated with increases in stretched-attends, relative to saline ($p=.001$). JR-associated reductions were evident among both saline ($p<.001$) and ETOH-pretreated controls ($p<.001$). These differences are depicted in Figure 16.

Figure 16. Stretched-Attend Behaviors



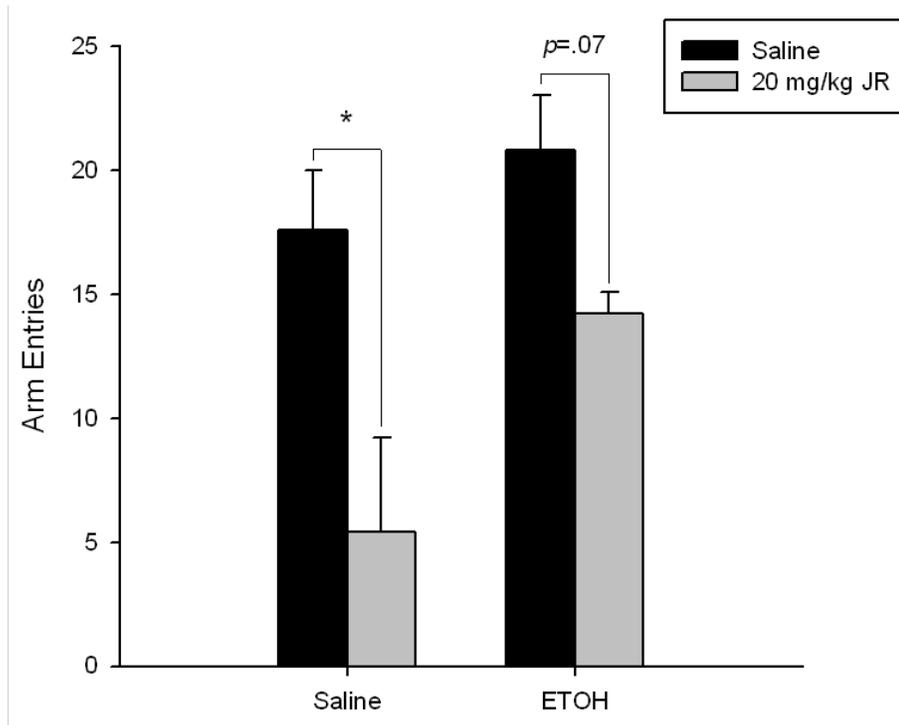
JR-220 was associated with increases in closed-arm occupancy and reduction in open-arm occupancy. A 2 x 2 ANOVA conducted for time spent in closed arms detected a main effect of JR, such groups receiving JR spent greater time in the closed arms of the maze, $F(1,20)=7.425$ $p=.014$. *Post hoc* tests revealed that the significance of this effect persisted for saline pretreated animals ($p=.016$), but was not significant for the ETOH-pretreated animals. Identical analyses were conducted for time spent in open arms; a significant main effect of JR, such that JR treatment reduced time spent in open arms, was detected, $F(1,20)=10.282$ $p=.005$. Again, this effect was significant among saline-pretreated animals, but not ETOH animals. These results are depicted in Figure 17, with the scales being made equal to facilitate direct comparison between the variables.

Figure 17. Time Spent in Closed vs. Open arms



JR-220 was associated with hypoactivity in saline-treated animals. A 2 x 2 ANOVA for total arm entries revealed main effects for both pretreatment and JR treatment. Pretreatment with ETOH was associated with increased arm entries in the maze, $F(1,20)=5.672$ $p=.029$, while JR treatment was associated with reductions in entries, $F(1,20)=13.859$ $p=.002$. *Post hoc*s revealed that JR-associated reductions in activity persisted only among saline-treated animals ($p=.004$), although the reduction approached significance among ETOH-treated animals ($p=.07$). These results are depicted in Figure 18.

Figure 18. Total Arm Entries



EPM Summary

The primary measure of interest in the cue-conditioned EPM screen was stretched-attend postures, thought to index conditioned withdrawal behavior. ETOH-exposed animals produced a greater number of stretched-attends in the conditioned environment, relative to controls. Stretched-attends in this group were significantly reduced following a 20 mg/kg dose of JR-220. While this action is consistent with our hypothesis, JR's action in ETOH-naïve animals provided some interpretational difficulty; JR reduced stretched-attends significantly, and with a similar magnitude as was noted for ETOH-exposed animals.

One possible interpretation would indicate a generalized anxiolytic effect. Agmatine (of which JR-220 is an analogue), as well as a number of compounds which would be hypothesized to produce agmatine-like action (including L-arginine, DFMO,

aminoguanidine and arcaine) are known to reduce ETOH-associated withdrawal anxiety in the EPM (Taksande et al., 2010), consistent with the hypothesis of a generalized anxiolytic effect. However this anxiolytic action would be atypical, as open-arm dwell-time was reduced by JR (and closed-arm dwell-time increased) among the ETOH-naïve group. Generally, this closed-arm preference would be interpreted as evidence of an anxiogenic action.

It is also possible that reduction in stretched-attends was due to a generalized hypoactivity induced by JR, an interpretation consistent with the noted reduction in arm entries among ETOH-naïve animals and the trend ($p=.07$) toward a reduction in ETOH-exposed animals. This interpretation is also supported by the effects of the 40 mg/kg JR dose in this paradigm (data not shown). The 40 mg/kg dose resulted in complete immobility in a subset of animals, precluding any interpretation of stretched-attend behaviors, and thus this data was discarded.

While JR's effects in this screen were equivocal, reductions in stretched-attend behavior among ETOH-exposed animals at least suggested the potential to reduce ETOH-associated cues. Thus, when considered in light of its efficacy in previous experiments, JR was advanced to the more complex behavioral screens. However, we decided that analyzing JR's effects on activity in a more direct manner would be prudent (activity data are presented below in Experiment 7).

Although results are consistent with the hypothesis that JR would reduce stretched-attends, however they add little additional support, considering noted interpretational issues.

Experiment 4: Alcohol Deprivation Effect

The alcohol deprivation effect (ADE) refers to temporary increases in ETOH consumption, compared with baseline conditions, when ETOH is reinstated following a period of forced abstinence (Sinclair & Senter, 1967). The ADE is thought to indicate relapse-like behavior (Spanagel et al., 1996). The phenomenon is noted across several species, including rats (e.g., Vengeliene, 2003), mice (e.g., Salimov et al., 1993), non-human primates (e.g., Kornet et al., 1990) and humans (Burish et al., 1981; Mello & Mendelson, 1972). Adding to its face validity to human relapse, the ADE phenomenon is noted to occur following short deprivations (e.g., 12 h; Sinclair & Li, 1989) as well as following an extended abstinence period (75 d; Sinclair et al., 1973).

Interestingly, alcohol preference among rodents appears relatively unrelated to their propensity to display an ADE (for review see Rodd et al., 2004). While the alcohol-preferring (“P”) rats display robust ADE behaviors, the Alko Alcohol (“AA” line) rats only display short term (12 and 24 h) ADEs (Sinclair & Li, 1989); Sardinian alcohol-preferring (“sP” line) rats do not display ADE behavior (Agabio et al., 2000); and high alcohol drinking (“HAD” line) rats display ADE only under certain conditions (Rodd-Henricks et al., 2000). Vengeliene and colleagues examined these preferring lines with Wistar rats (2003) and found ADE behavior only in the P and Wistar animals. However, the majority of this work examines ADE following only the first deprivation period. When repeated deprivation periods are used, ADE behaviors in preferring lines (e.g., HAD rats) which are not immediately evident initially, can emerge (Rodd-Hendricks et al., 2000). Similarly, in animals which do display initial ADE behaviors, repeated deprivation cycles can markedly increase intake; one study demonstrated 16 g/kg consumption during the 24 hr following reinstatement in P-rats (Li et al., 1993).

Taken together, these findings suggest that relapse-like behavior thought to be modeled in the ADE paradigm may reflect pathophysiological changes occurring during chronic consumption and abstinence, rather than the positive reinforcing value of ETOH

or initial ETOH preference. However, the noted ability of naltrexone to reduce ADE behavior (Heyser, Moc & Koob, 2003; McBride, Le & Noronha, 2002) suggests that the behavior is still under at least partial control of the positively reinforcing value of ETOH following reinstatement. While the mGLUR antagonist, MPEP, reduces ADE behavior (Backstrom et al., 2004), the efficacy of acamprosate in this paradigm (Vengeliene et al., 2010; McBride, Le & Noronha, 2002) is of greatest relevance to the current work, and suggests the predictive validity of our ADE procedure.

Although described in detail below, our modified ADE screen uses Sprague-Dawley rats, which do not readily acquire significant ETOH self-administration. Thus, we employ a sucrose-fading procedure to train drinking. Animals undergo at least three reinstatement periods after deprivation. The first is an untreated period to verify that the deprivation effect is detectable. The second and third reinstatements are conducted following treatment with either the compound of interest, or vehicle, in a within-subject manner (with treatment order balanced across groups). This procedure allows us to efficiently and reliably produce ADE behavior in Sprague-Dawleys following less than one month of ETOH exposure.

Specifically, we hypothesized JR-220 would attenuate increases in consumption following ETOH reinstatement, relative to baseline levels.

Subjects

Subjects were male Sprague-Dawley rats individually housed in plastic cages in a temperature-controlled colony room (70°±2 F) on a 12h light-dark cycle, with food and water provided *ad libitum*.

ADE Procedure

Male Sprague-Dawley rats were trained to consume ETOH using a sucrose-fading procedure, which entailed gradually replacing the sucrose in a 10% solution with ETOH, until animals were consuming a sucrose-free 12% ETOH solution. Free access to water was maintained throughout the experiment in one drinking tube, while varying solutions of sucrose and ETOH were provided in the other. The sucrose fading schedule is presented in Table 5.

Table 5. Sucrose Fading Schedule

Phase	Solution
Days 1-3	Water in both bottles
Days 4-6	One bottle replaced with 10% sucrose
Days 7-9	10% sucrose / 3% ETOH
Days 10-12	10% sucrose / 6% ETOH
Days 13-15	10% sucrose / 9% ETOH
Days 16-18	10% sucrose / 12% ETOH
Days 19-21	5% sucrose / 12% ETOH
Days 22-27	12% ETOH

Ethanol consumption during the last 3 days of 12% ETOH access was averaged and used as a baseline for comparison with post-deprivation consumption. Following the sucrose-fading procedure, ETOH deprivation/reinstatement cycles began. Each reinstatement period lasted for 72 hours. Deprivation periods were varied at 4 ± 1 days, to insure that the animals did not adapt to a schedule. All animals received a single (non-drug treated) reinstatement cycle to assess elevations in drinking compared to baseline. During the following reinstatement period animals were randomly divided into treatment groups, receiving either drug or saline. Two experiments were conducted (each examining a different dose of JR); separate animals were used for each. An initial

experiment examined a 20 mg/kg dose ($n=54$), followed by a smaller-scale experiment examining 10 mg/kg ($n=13$) designed to alleviate interpretational issues concerning general fluid consumption. Injections were administered (*i.p.*) 30 minutes prior to ETOH reinstatement, and then repeated daily for the duration of the treatment phase. The dependent measure was ETOH consumption (g/kg BW).

Analysis

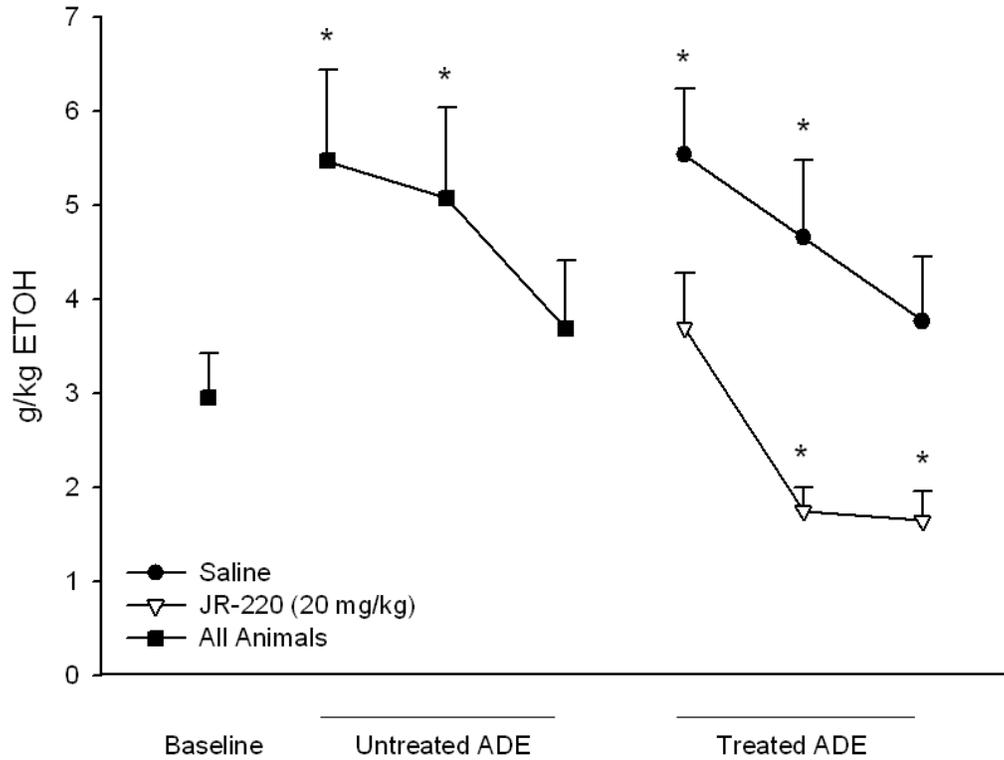
Paired-samples *t*-tests were used to compare g/kg ETOH consumption during each day of the ADE phase with baseline drinking. Total fluid consumption was also analyzed in this manner. Paired-samples *t*-tests were further utilized to disambiguate the direct effects of JR, as compared to saline, on water consumption.

Results

20 mg/kg JR

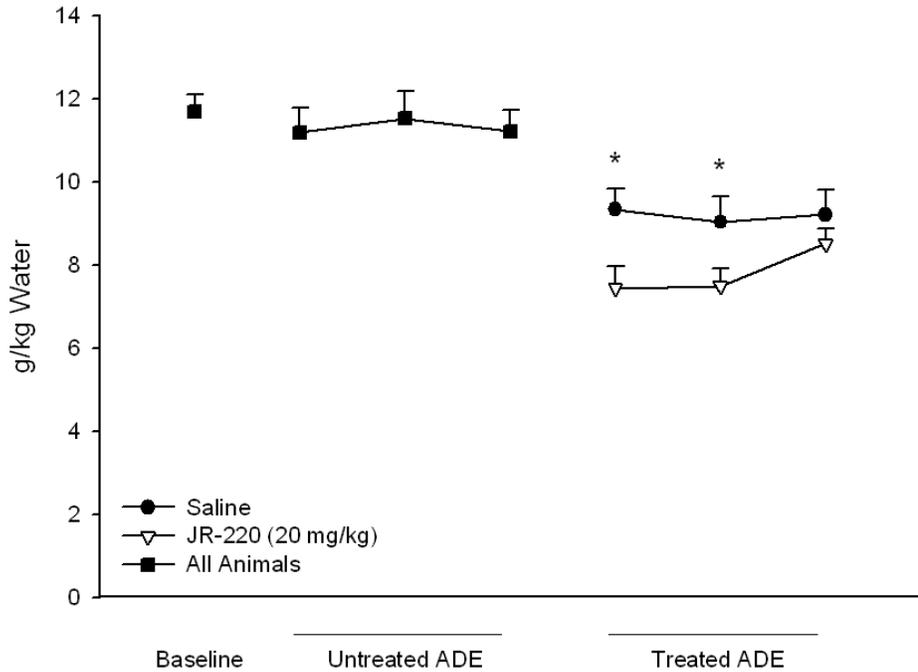
20 mg/kg JR appeared to both attenuate ADE behavior, and reduce consumption on days 2 and 3. Paired sample *t*-tests revealed that following a deprivation period, the animals consumed significantly more ETOH following reinstatement, compared to their baseline drinking. A similar pattern as described above was observed, with increased consumption on day 1, $t(53)=3.890$, $p<.001$, and day 2, $t(53)=3.087$, $p=.003$. Following treatment with JR-220, day 1 consumption did not differ from baseline, however consumption was reduced on day 2, $t(53)=2.693$, $p=.010$, and day 3, $t(53)=4.164$, $p>.001$. Animals which received saline consumed significantly more on day 1, $t(53)=5.170$, $p>.001$ and 2, $t(53)=2.643$, $p=.011$, relative to baseline. ADE phases compared with baseline drinking are depicted in Figure 19.

Figure 19. 20 mg/kg JR in ADE



No differences in water consumption were associated with the untreated reinstatement phase, however both JR-220 and saline injections were associated with reductions in water consumption during all injection days. In order to examine whether JR-220 was associated with reductions in water consumption compared to saline controls, a secondary set of paired-samples t-test was employed to compare these groups. Water consumption was reduced by JR during day 1 $t(53)=3.673, p=.001$, and day 2, $t(53)=2.423, p=.019$, but not day 3 of reinstatement. These results are depicted in Figure 20.

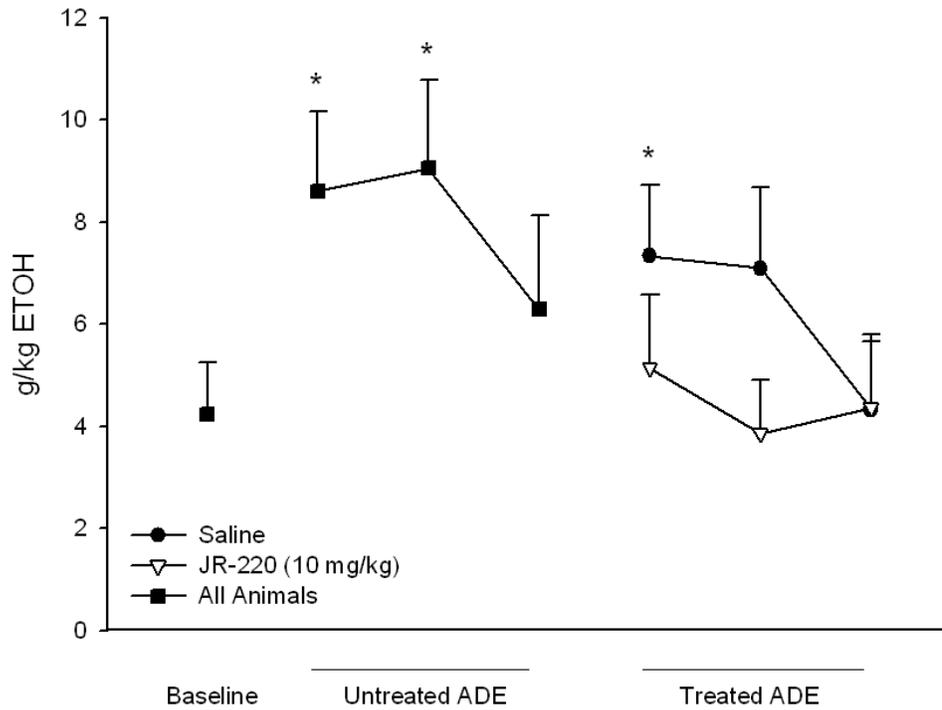
Figure 20. Water Consumption in ADE Reinstatement Phases (20 mg/kg).



10 mg/kg JR

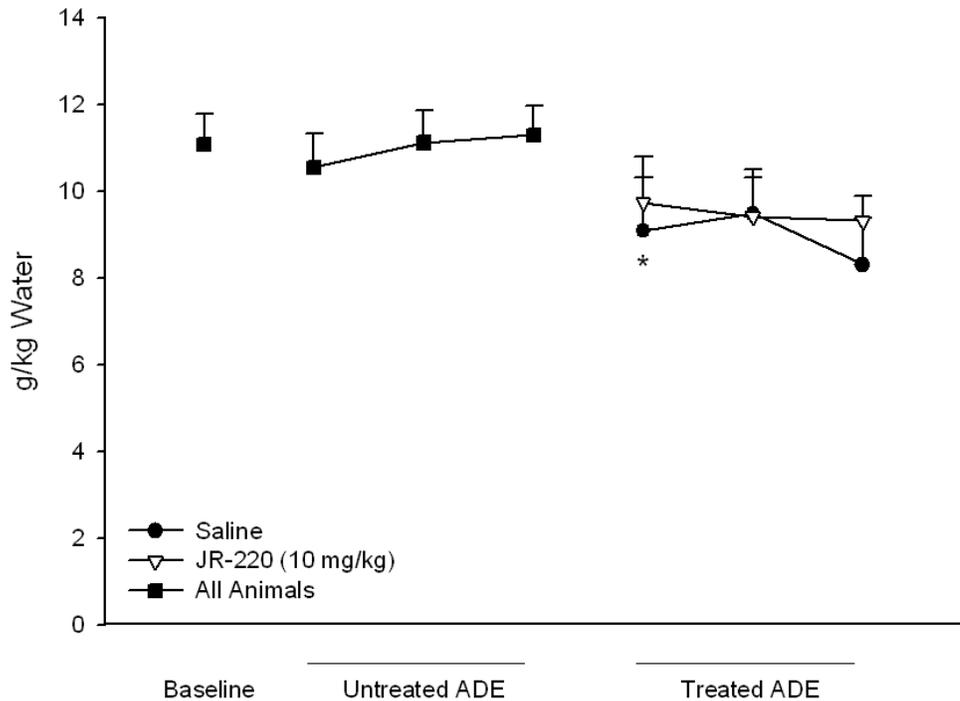
10 mg/kg JR appeared to attenuate ADE behavior. Paired samples t-tests revealed that following a deprivation period, the animals consumed significantly more ETOH following reinstatement, compared to their baseline drinking. This increase persisted for both day 1, $t(12)=3.084$, $p=.006$, and day 2, $t(12)=2.397$, $p=.034$. Animals which received saline consumed significantly more on day 1 of the drinking phase, $t(12)=3.841$, $p=.002$. During the treated ADE phases, 10 mg/kg JR treatment appeared to attenuate increases in consumption. ADE phases compared with baseline drinking are depicted in Figure 21.

Figure 21. 10 mg/kg JR-220 in ADE



A second set of *t*-tests were performed to examine the effects of JR on water consumption during reinstatement. No differences in water consumption were associated with the untreated reinstatement phase, or treatment with JR-220. However, water consumption on the first day of reinstatement was significantly lower among animals administered saline, relative to baseline drinking. These results are depicted in Figure 22.

Figure 22. Water Consumption During ADE Reinstatement Phases (10 mg/kg)



ADE Summary

Using a modified procedure designed to facilitate efficient drug screening, we demonstrated that Sprague-Dawley rats with less than a month of exposure to ETOH-containing solutions will substantially increase ETOH drinking behaviors following a period of forced abstinence. This is notable both for the relatively short pre-deprivation drinking period, as well as the rat strain used, as Wistar or various selectively bred lines are used most commonly in this procedure. Additionally, one advantage of our screening procedure is the relatively high levels of consumption it produces, ranging from 6-9 g/kg during the first 24h following reinstatement, which contributes to the face validity of the screen. Our observed levels of consumption are greater than is generally found in other works (with several notable exceptions involving P-rats), however, the inclusion of a sucrose fading procedure to accelerate ETOH consummatory behavior also raises the possibility that consumption is being partially driven by previous associations with sweetness/calorie content. While this complicates interpretation somewhat, it does not

necessarily subtract from the face validity of the screen, as initiation of ETOH consumption in humans rarely involves high ETOH/low carbohydrate solutions.

JR-220 administration at both 10 and 20 mg/kg doses attenuated the ADE, reducing post-reinstatement consumption to control levels (or below). Paired-samples *t*-tests indicated that following the 10 mg/kg dose, consumption levels during all treatment days were indistinguishable from control levels, compared to saline treatment, in which a significant day 1 increase was noted. Furthermore, the 10 mg/kg dose was not associated with any reduction in water consumption. Saline-treated animals displayed reduced water consumption during day 1, however this was most likely do to their preference for the ETOH solution.

20 mg/kg administration of JR was also associated with a reduction in ADE behavior. Following reinstatement, no increases in ETOH consumption relative to control were noted in the first 24 h of access, in contrast to the significant increase in day 1 consumption among vehicle-treated animals. Interestingly, while animals treated with 10 mg/kg JR were no different from controls on any of the treatment days, 20 mg/kg JR was associated with reductions in drinking on days 2 and 3. However, this is consistent with the hypothesis that JR is capable of reducing a number of ETOH-associated behaviors, including relapse (ADE day 1 consumption), and general reductions in consumption, reflecting reduced reinforcing value. Reductions in water consumption during the treated ADE phase were noted. While some reduction is expected due to the hydrating capability of the injections (injections contained 2.5-5 mL saline, depending on body weight), direct comparison of the saline and JR-220 treatments indicated a significant reduction in water consumption associated with JR. Since saline treated animals demonstrated greater preference for the ETOH solution, yet still consumed more water than JR-treated animals, this is unlikely to be an artifact. The most likely possibilities for this noted reduction in water include general reductions in the reinforcing value of natural rewards or a general reduction in activity levels (including normal drinking behavior). The former possibility

is unlikely, as JR has been directly examined in other drinking paradigms and found not to affect water consumption (Justin Rhodes, personal communication). Furthermore, the 10 mg/kg dose was associated with reductions in ETOH but not water consumption, arguing against a generalized reduction in reinforcement. Thus, JR's potential effects on activity remained a concern and were consequently examined in Experiment 7.

CP was not examined in this screen due in part to limited resources, and in part because NR2B antagonism has been examined previously in the ADE paradigm; the NR2B antagonist ifenprodil dose dependently reduces ADE behavior (Vengeliene et al., 2005). Thus, while CP's efficacy would have been noteworthy due to the noted differences in its binding profile, the recognition of NR2B antagonist efficacy in this paradigm influenced our decision to prioritize its examination in behavioral paradigms in which NR2B involvement is less well-established (i.e., binge drinking).

Taken together, these results supported the hypothesis that inhibitory polyamine modulation has the ability to attenuate reinstatement-associated increases in ETOH consumption. Attenuation of relapse behavior in this experiment was encouraging, however this paradigm lacks a major component associated with human relapse: stress. Thus, we advanced JR to Experiment 5, examining its effects in chronically stressed animals.

Experiment 5: Stress-Associated Drinking

It is well known that stress increases alcohol consumption in a variety of species, and is thought to be a primary contributor to relapse behavior in humans. Thus, we included a stress-related paradigm into our screening hierarchy. A variety of stressors are known to increase drinking behaviors in rodents, including foot-shock (e.g., Matthews et al., 2008), social isolation (e.g., Nunez et al., 2002) and restraint/immobilization (e.g., Farook et al., 2009). C57BL/6J mice are known to readily consume ETOH, and their consumption is both increased following stress challenge (e.g., Boyce-Rustay, 2008) and sensitive to such factors as quality, severity, duration and frequency of stress. We therefore used this strain in a simple restraint-stress procedure to screen for the ability of our compounds to reduce stress-associated consumption.

Unfortunately, the literature involving glutamatergic manipulations of stress-associated drinking is limited. There is some evidence suggesting acamprosate has the ability to reduce stress-associated increases ETOH consumption (Füllgrabe, Vengeliene & Spanagel, 2007), however these findings were age and sex-specific. Clinically, acamprosate appears anxiolytic (Schwartz et al., 2010), and in a sample of patients comorbid for anxiety and alcohol misuse disorders, reduced both anxiety symptoms and alcohol use (Schwartz, Chilton & Aneja, 2007). These findings suggest compounds with ‘acamprosate-like’ action will positively modulate alterations in drinking behavior resulting from stress, despite the paucity of available preclinical examinations involving NMDA receptor manipulation.

While acamprosate has not yet been examined in this restraint-stress procedure, a previous study conducted by our group using topiramate (Farook et al., 2009) suggests that restraint elevates drinking behavior, and that such elevation may be moderated by the ‘acamprosate-like’ action of topiramate. While the screen presented below was designed in a highly similar manner to that described by Farook (2009), the size of the unrestrained

group was substantially reduced in order to increase efficiency and throughput, and only saline and a single dose of JR was examined in controls.

Specifically, we hypothesized that stress-induced increases in ETOH consumption would be attenuated following JR-220 administration.

Subjects

Male C57BL/6J ($n=28$) mice weighing 29 ± 2 g (15-18 weeks old; Jackson Laboratory, Bar Harbor, ME) were singly housed in standard polycarbonate cages with corncob bedding. Chow and water were provided *ad libitum*.

Procedure

Prior to the start of an experiment, C57BL/6J mice were allowed to adjust to the colony room for a period of 7 days, following shipment. Afterwards, water and ETOH were available in feeding tubes (Dyets Inc., PA) inserted directly into the animal cage. During the course of 7 days, the concentration of ETOH was gradually increased from 3% (days 1-2) to 6% (day 3-5) to 12% (days 6-7). Drinking tube position was alternated daily in order to avoid any positional bias. Water and ETOH consumption were measured daily, throughout the experiment.

At the start of the restraint phase, mice were divided into groups; 20 animals received restraint, with 8 controls. Restraint was performed daily, for 5 days, by placing mice into transparent plexiglass restraint chambers (10 cm x 4.5 cm x 3.2cm) for one hour. Unrestrained animals were handled briefly, then transported in their home cage to the restraint room and left for one hour. Restraint was ceased after 5 days (prior to the first day of treatment). Animals exposed to the restraint stress were subdivided into four groups ($n's=5$), each receiving 0, 10, 20 or 30 mg/kg JR-220. Unrestrained animals were divided into two groups ($n's=4$), receiving saline injections or the high dose (30 mg/kg) of JR-220. Injections were repeated, daily, for five days, post-restraint.

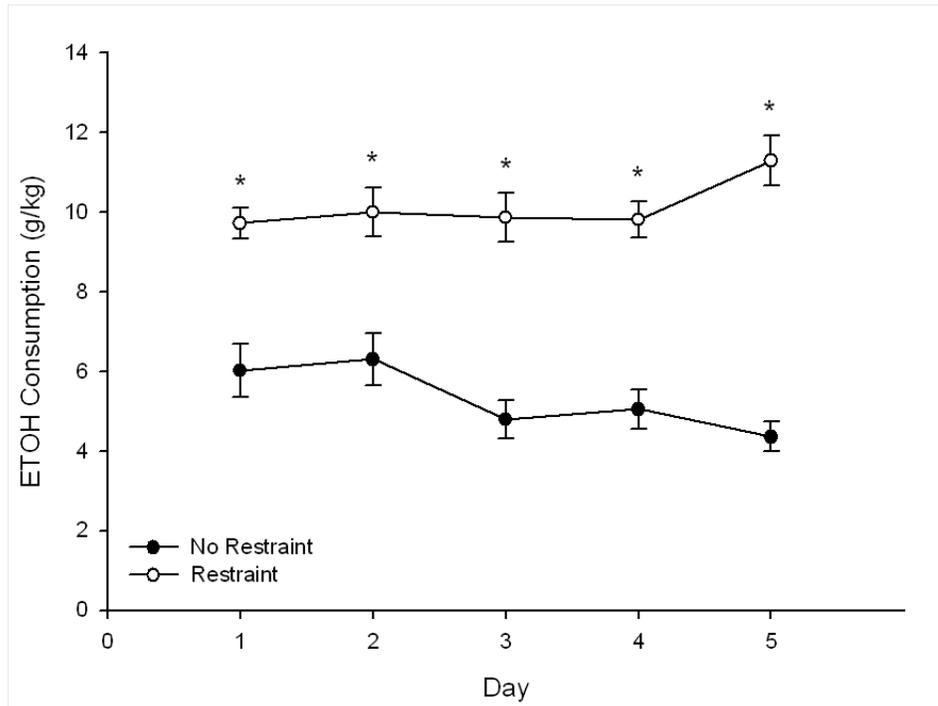
Statistics

An initial repeated measures ANOVA (group x day) was performed between unrestrained controls and restrained animals, to assess the effects of daily restraint on ETOH consumption. Following restraint, the multiple doses of JR were analyzed using a two-way repeated measures ANOVA (Dose x Day). Additional ANOVAs, and subsequent *post hoc*s (Fisher's protected *t*) were conducted to further examine significant effects, where appropriate.

Results

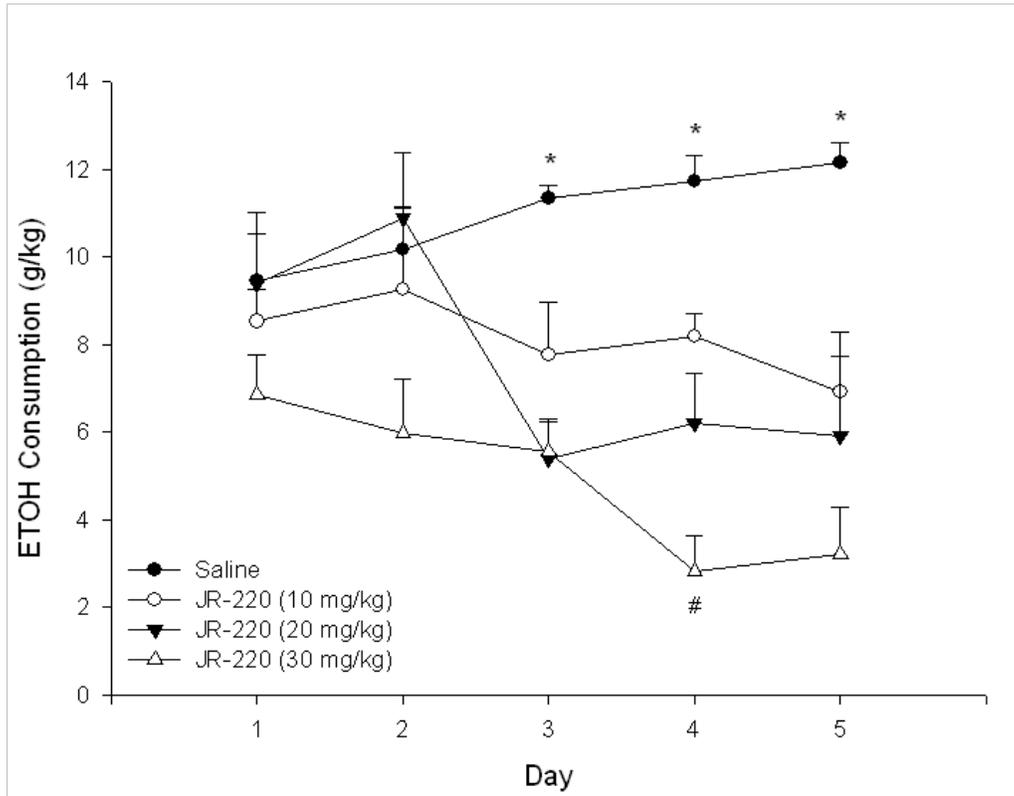
Restraint stress was associated with increases in ETOH consumption. A group x day RM ANOVA detected a main effect of group, revealing that ETOH consumption was increased among animals undergoing restraint, $F(1, 26)=122.479$, $p<.001$. Additionally, a significant interaction between group and day was noted $F(1, 26)=6.773$, $p=.015$. Thus, *t*-tests were conducted between groups for each day, individually. In all cases a significant difference between groups was detected ($p<.001$, $p=.001$, $p<.001$, $p<.001$, $p<.001$, for days 1-5, respectively). Graphing the interaction (Figure 23) suggests divergence of ETOH consumption over time, in a group-dependent manner. Consumption among restrained animals increased over time, while unrestrained animals' consumption decreased over time.

Figure 23. Restrained vs. Unrestrained Drinking



Following the 5-day restraint phase, restrained animals were administered one of four doses of JR (0, 10, 20, 30 mg/kg), daily, and allowed to consume ETOH freely for an additional 5 days. JR administration reduced drinking in a time-dependent manner. An initial RM ANOVA detected an interaction between day and dose, $F(12, 64)=2.186$, $p=.023$, thus, individual ANOVAs were conducted for each day, individually. No effect of dose was noted for days 1 or 2, however by day 3 dose emerged as significant, $F(3,19)=10.958$, $p<.001$, and remained significant during day 4, $F(3,19)=21.722$, $p<.001$, and day 5, $F(3,1)=8.636$, $p<.001$. Fisher's *post hoc*s were performed for days 3-5. During day 3, saline treated animals consumed significantly more ETOH than 10, 20, or 30 mg/kg JR-treated animals ($p=.008$, $p<.001$, $p<.001$, respectively). On day 4, saline-treated animals again consumed significantly more ETOH ($p=.006$, $p<.001$, $p<.001$, respectively), however the animals exposed to 30 mg/kg also consumed less than animals receiving either 10 or 20 mg/kg JR ($p<.001$, $p=.008$, respectively). On day 5 JR-treated animals again consumed less than saline-treated ($p=.010$, $p=.003$, $p<.001$, respectively), although among JR-treated animals consumption did not differ as a function of dose. These results are depicted in Figure 24, below.

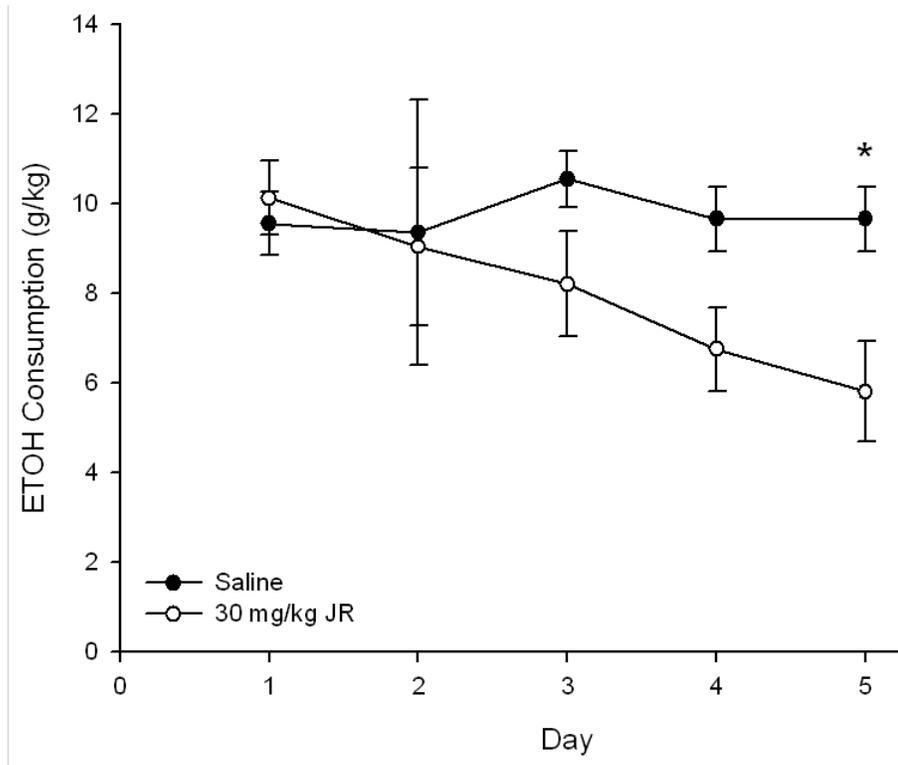
Figure 24. JR-220 Treatment (Following 5-day Restraint Period)



Additional analyses were conducted on animals which received no exposure to the restraint paradigm, but were treated with either saline or the highest (30 mg/kg) dose of JR-220. An RM ANOVA (treatment x day) was conducted, however no main effects or interactions were noted.

Since the investigation of JR in unrestrained animals was not a primary focus of this experiment, sample size for this analysis was low ($n's=4$). Thus, we were concerned that the (treatment x day) RM ANOVA lacked sufficient power to detect differences on individual days. We therefore chose to examine the final day of treatment (Day 5), using a t -test. 30 mg/kg JR appeared to reduce Day 5 consumption, $t(6)=2.900$, $p=.033$, despite the lack of an overall (treatment x day) interaction (depicted in Figure 25). While the t value for this analysis was modest, the low n 's suggested a sizeable effect. The effect size for the 5th day was found to be a "large" effect (Cohen's $d=2.37$).

Figure 25. Unrestrained Animals, 0 vs. 30 mg/kg JR



Restraint Stress Summary

Prior to drug treatment, daily restraint stress induced elevations in ETOH self-administration. The primary measure of interest in this screen was alterations in ETOH self-administration following chronic stress. At all three doses examined (10, 20, 30 mg/kg) JR-220 was capable of reducing consumption. However, the temporal pattern of this reduction was particularly interesting; during the first 2 days of JR administration no differences were detectable, and it was only during the 3rd, 4th and 5th days of ETOH consumption that such differences emerged. However, this is not a unique pattern. Previous use of this procedure (Farook et al., 2009) produced a similar pattern of results, with significant differences between treated and un-treated groups not emerging until the last half of the treatment phase. One interpretation of such delayed efficacy would suggest that both compounds require several days for concentrations to reach maximally

effective levels. While the 21 h half-life of topiramate (Bialer, 1993) is consistent with this interpretation, no data is currently available concerning metabolism of JR-220. An alternative interpretation is that dysregulation of HPA function and resultant negative affect are not immediately reversible, but rather need some time to ‘balance’ or ‘down-regulate’.

However, while patterns of slow-onset efficacy in restrained animals are similar between studies, the patterns of ETOH consumption during the restraint phase, and the magnitudes of consumption in both restrained and non-restrained animals appear markedly different when the current project is contrasted with the topiramate study. Consumption among restrained vs. unrestrained animals in the topiramate study increased from ~10 to ~19 g/kg and ~7.5 to ~12.5 g/kg in restrained and unrestrained animals, respectively; the current study observed only a slight increase associated with restraint (~9 to ~11.5 g/kg), and a decrease (~6 to ~4 k/kg) in unrestrained animals. Since both sets of experiments employed nearly identical methodologies, such differences are puzzling. However, the overall findings from both studies, that restraint-associated increases in consumption may be attenuated in a time-dependent manner via GLUergic modulation, are consistent.

The inclusion of unrestrained controls in this paradigm was primarily to insure that restraint stress resulted in increased consumption, however following the restraint phase this group allowed for the additional examination of JR in unrestrained animals. A JR-associated reduction was noted, however only after several days of treatment (similar to the restrained animals). Finally, the large effect size of the Day 5 difference suggests the reliability of this finding, even with unfortunately low n 's.

These results were consistent with our hypothesis that inhibitory polyamine modulation would reduce stress-associated ETOH consumption.

Experiment 6: Drinking in the Dark (Screen)

As noted previously, the C57BL/6J mouse is considered an alcohol-preferring strain. However, among C57s, other mouse strains, or other species, it has been (historically) difficult to produce binge-like self-administration to the point of behavioral intoxication. For instance, while C57s will take nearly all daily fluid intake from a 10% ETOH solution in a 2-bottle choice, 24 h access paradigm, measurable effects on physiology and/or behavior are lacking and pharmacologically significant BEC levels are not maintained (Dole & Gentry, 1984). Although some noted exceptions exist (e.g., Middaugh et al., 1999; Ryabinin et al., 2003), these and other binge models generally involve extended periods of previous administration, stress (or other environmental manipulation, e.g., food deprivation) or previous forced administration (e.g. ETOH vapor inhalation chambers). While operant techniques are able to avoid many of these pitfalls (e.g., Roberts, Heyser & Koob, 1999), the procedures require significant individualized training over time, and are thus ill-suited for high-throughput screening.

Fortunately, a limited-access procedure which involves neither food nor water restriction, and leads to pharmacologically significant levels of consumption, has recently been established. Rhodes and colleagues (2005), in a methodologically rigorous examination, described the tendency of C57BL/6J mice to consume significant quantities of alcohol under limited-access conditions (2-4 hours) during their active (dark) cycle, termed 'Drinking in the Dark'. Under these conditions the mice are known to consume high concentrations (20%) of ETOH to the point of intoxication with BECs as high as .16 g/dl (Rhodes et al., 2005). Furthermore, several behavioral indices of intoxication are noted, including impaired performance on the accelerating rotarod and balance beam tests (Rhodes et al., 2007) and gait abnormalities (Rhodes et al., 2005). Finally, the establishment of such high levels of consumption requires few days of ETOH experience, and may be generated within 1-2 weeks. This DID procedure thus provides a simple binge model with high face validity which is particularly well suited to genetic and pharmacological investigations.

The predictive validity of this model has been established using a number of compounds. The binge-like nature of DID behavior and the short amount of time required to establish it, suggest that in this particular model consumption may be driven largely by positive reinforcement. The effects of VTA micro-injection of baclofen (Moore & Boehm, 2009), as well as systemically administered naltrexone (Kamdar et al., 2007), are consistent with this hypothesis. However, GLUergic manipulations also appear to reduce consumption; both MPEP (an mGLUR inhibitor) and acamprosate significantly and dose-dependently reduce the behavior (Gupta et al., 2008). Interpretation of such efficacy is equivocal; these GLUergic compounds may serve to reduce positive reinforcement, or, despite short onset of the binge-like behavior, it may still be partially driven by the negatively reinforcing value of ETOH.

The experiments presented below are DID screens which were adapted from Rhodes and colleagues (2005). While the basic methodology was retained, treatment schedules differed (allowing us to assess prolonged effects of treatment on ETOH consumption the day following treatment) and dose ranges were more limited (to facilitate higher throughput). Unlike many of the aforementioned screens, this procedure was novel for our lab at the time of JR and CP testing, thus we included acamprosate in these initial experiments to further the establishment of the screen's predictive validity.

We hypothesized that the high levels of ETOH consumption noted during brief periods of dark-cycle access to ETOH would be reduced by inhibitory polyamine modulation via JR and CP.

DID (Screen) Subjects

Male C57BL/6J ($N=20$) mice weighing 30 ± 3 g (15-18 weeks old; Jackson Laboratory, Bar Harbor, ME) were singly housed, in a room maintained on a 12:12 reversed light/dark cycle, with lights turned off at 0600 h and on at 1800 h EST. Red incandescent lamps were used during feeding, cage-changing and testing to avoid circadian disruption. Animals were housed in standard polycarbonate cages with corncob

bedding. Rat chow and water were provided *ad libitum*, except when ETOH was substituted for water.

DID (Screen) Procedure

Upon arrival, animals were habituated to the colony room for seven days. Twenty percent ETOH solutions were prepared daily. ETOH solutions were delivered in 10 ml graduated cylinders, fitted with double ball bearing sipper tubes (to prevent leakage). ETOH access was initiated 4 hr into the dark cycle. This was done in the home cage, where animals were singly housed. The cylinders remained in place for 4 hrs, after which cylinders were removed and water bottles re-introduced.

A three week (5 days/week) period ETOH exposure was conducted prior to drug treatment. Each animal received all compounds tested, in a counterbalanced, latin-square design (Figure 26). ETOH consumption was allowed each day preceding and following a treatment. Screen 1 examined saline, 10 mg/kg JR and 10 mg/kg CP. Screen 2 examined saline, 300 mg/kg acamprosate, and 20 mg/kg JR. Doses were selected based on efficacy in previous experiments and/or selected from the literature. Figures 27 and 28 depict the treatment schedule and consumption prior to treatment exposure, respectively.

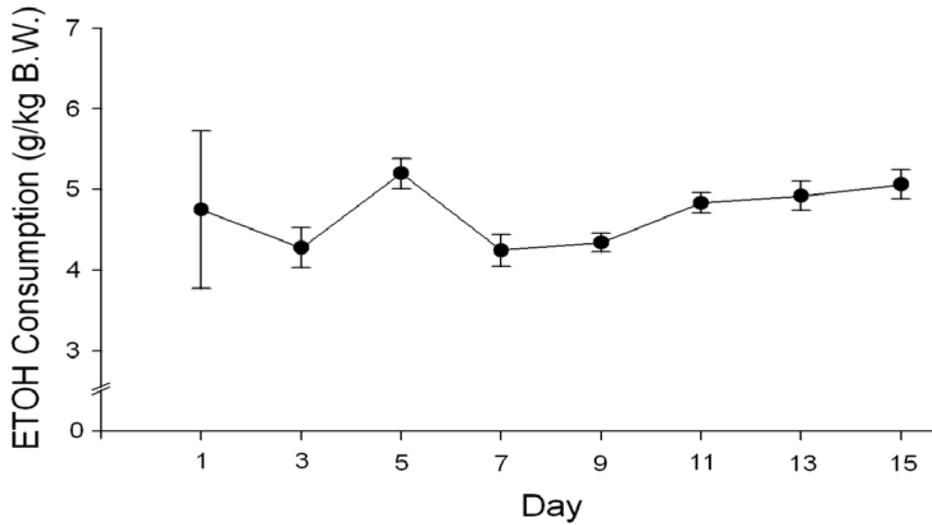
Figure 26. Latin-Square Design for Screen 1 and Screen 2

	Phase 1			Phase 2		
Group 1 (n=7)	Saline	JR	CP	Saline	JR	Acamp
Group 2 (n=7)	CP	Saline	JR	Acamp	Saline	JR
Group 3 (n=6)	JR	CP	Saline	JR	Acamp	Saline

Figure 27. DID ETOH Access Schedule (Single Phase; Dark/Light Boxes Indicate ETOH/Water, Respectively)



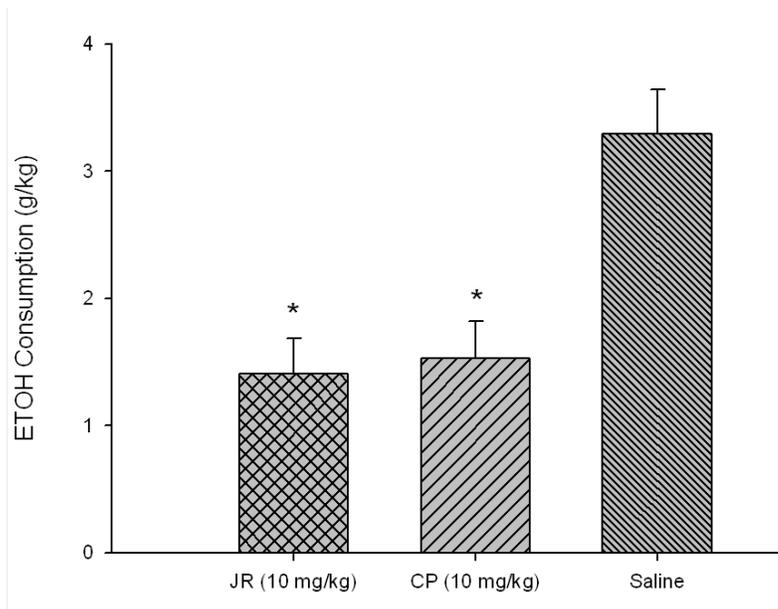
Figure 28. Daily DID Consumption (4 hr/day) Prior to Treatment Phase



DID (Screen) Results

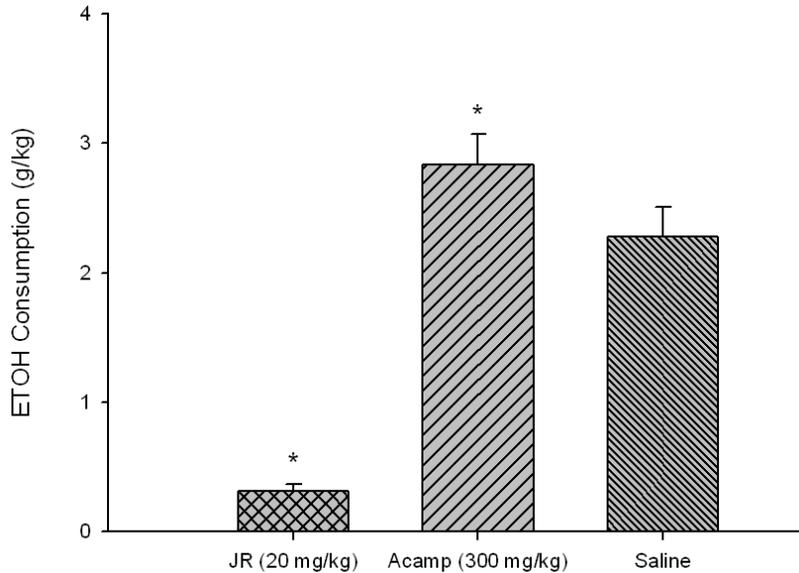
Data were analyzed with paired-samples *t*-tests, comparing treatment groups to controls. An alpha of .05 was used for both phases of the experiment. In screen 1, ETOH consumption was significantly reduced following CP treatment, compared to saline, $t(19)=3.483$, $p=.002$. Likewise, JR-220 treatment significantly reduced consumption, $t(19)=4.041$, $p=.001$. These results are presented in Figure 29.

Figure 29. CP-101,606 (10 mg/kg) and JR-220 (10 mg/kg) in DID



In screen 2, 20 mg/kg JR reduced ETOH consumption relative to saline $t(18)=9.074$, $p<.001$. Acamprosate was associated with increased consumption, $t(18)=2.161$, $p=.044$ (an unexpected finding, discussed in detail below). These results are presented in Figure 30.

Figure 30. JR-220 (20 mg/kg) and Acamprosate (300 mg/kg) in DID



DID (Screen) Summary

Results from both DID experiments indicated the abilities of CP and JR to reduce binge-like consumption in this limited-access drinking paradigm. In the first experimental screen, due to concerns about potential secondary effects of JR-220 (i.e., sedation), we selected the lowest dose (10 mg/kg) of JR which demonstrated efficacy in previous drinking screens. Due to the lack of data concerning CP-101 in ETOH consumption paradigms, we selected a moderate dose (10 mg/kg) based on other animal studies which demonstrated positive behavior effects (e.g., Kundrotiene et al., 2004). Both compounds reduced consumption by a magnitude of ~50%. In the subsequent screen, a higher dose of JR was selected in order to establish (or at least begin to provide evidence for) a dose-dependent effect. While such an effect would have been interesting to examine for CP-101 as well, we prioritized the examination of acamprosate in order to demonstrate the predictive validity of the screen under our particular experimental methods. The higher

dose of JR (20 mg/kg) reduced consumption by an even greater proportion, relative to saline-treated animals.

Acamprosate was unexpectedly associated with increases in consumption. Exactly what this indicates is unclear, however it should be noted that consumption following saline injection during screen 2 was unusually low (~2.25 g/kg) compared with screen 1 (~3.25 g/kg), and may have either driven or contributed to this effect. Vehicle-associated consumption in the DID literature resembles our screen 1 results, suggesting untreated consumption in screen 2 was abnormally low. The nature of our screening procedure is such that to maximize the number of compounds examined, lower subject sizes than might otherwise be expected in more rigorous experiments, are utilized. Thus, although not explicitly noted above, our *post hoc* analyses and treatment of family-wise error throughout this project are generally liberal. While advantageous in some respects, this increases the probability of type 1 errors. Considering this liberality, the low magnitude of the observed effect (~.5 g/kg), the abnormality of screen 2 vehicle-associated consumption and the inconsistency with Dr. Rhodes' examinations of acamprosate in a similar model, we felt drawing conclusion from this result would be premature.

While acamprosate effects in our DID screen should be investigated further, results from Experiments 1-5 suggested that more rigorous exploration using standardized models with larger *n*'s was warranted. While any numbers of well-used models in the preclinical literature might have been selected from, DID was attractive for two major reasons. Firstly, relative to relapse and cue-conditioning models, NMDAR antagonism has remained largely unexplored in models of binge-like consumption, thus DID allowed for both a pointed examination of the potential of these two compounds, as well as an opportunity to contribute to an emerging literature. Secondly, while screening results from JR and CP appeared quite promising, it was concerning that in our hands acamprosate was ineffective; DID remained the only paradigm in which JR/CP results

were inconsistent with acamprosate results, which seemed to warrant further investigation. Both compounds were tested in the standard DID model (Experiment 8).

These results supported the hypothesis that inhibitory polyamine modulation would reduce binge-like consumption in a limited access DID screen.

Experiment 7: Open Field Activity

While the positive activity of both JR and CP in the screens presented here is encouraging, one interpretational concern involved the effects of these compounds on general activity levels. Additionally, the hypoactive effect of JR noted in the EPM, as well as the general reduction in fluid consumption noted in ADE, raised suspicion that JR may be altering activity. For these reasons alone, examining activity seemed prudent, however the examination of these compounds also afforded their comparison to other GLUergic manipulations, as well as secondary analyses of center-zone activity; entries into the center are generally associated with reductions in anxiety and are negatively correlated with behavioral and physiological measures of fear (Archer, 1973), however may also evidence deficits in inhibitory control.

Open field results are presented by species, using the same order in which the behavioral experiments are presented, including Swiss Webster mice (Experiments 2 & 3), Sprague-Dawley rats (Experiment 4) and C57BL/6J mice (Experiments 5 & 6). All open field subjects were drug-naïve (i.e., not used in any previous experiments).

Analysis

While methods varied depending on species/strain, activity was analyzed similarly, using RM ANOVAs (time x treatment), with either Fisher's protected *t* or Dunnett's *post hoc* tests, as appropriate. Center-dwell time was analyzed with one-way ANOVA (treatment), with Fisher's protected *t* tests where appropriate.

Open Field Methods (Swiss Webster)

The activity of adult male Swiss Webster mice (30 ± 2 g; $N=20$; $n's=4$) was analyzed following the administration of both CP and JR. The apparatus was a square, black, plexiglass chamber (64 x 64 x 64 cm). Subjects were transported to a darkened

testing room and habituated for 10 min. Each subject was then individually placed in the open field and recorded for 10 min using a tripod-mounted video recorder (SONY). For analysis, the open field was divided into quadrants, each comprising 25% of the total area. Additionally, a smaller square (25% of the total area) was super-imposed over the center of the open field. The dependent variables included number of quadrant crosses and time spent in the center zone.

Results (Swiss Webster)

High-dose CP (20 mg/kg) was associated with hyperactivity, while high-dose JR-220 induced hypoactivity. The initial RM ANOVA (time x treatment) indicated main effects of both time $F(4,60)=29.622, p<.001$, and treatment, $F(4,15)=10.562, p<.001$. To further examine the treatment effect, a one-way ANOVA was conducted using total quadrant crosses over the 10-minute activity period. Again, a significant effect of treatment was noted, $F(4,19)=10.692, p<.001$, with Dunnett's tests indicating significant increases in crosses associated with 20 mg/kg CP ($p=.054$) and decreases associated with 40 mg/kg JR ($p=.007$). These results are depicted across time (by block) in Figure 31a, as well as summed, in Figure 31b.

Figure 31a. Open Field Quadrant Crosses (By Block) Following Saline, JR and CP

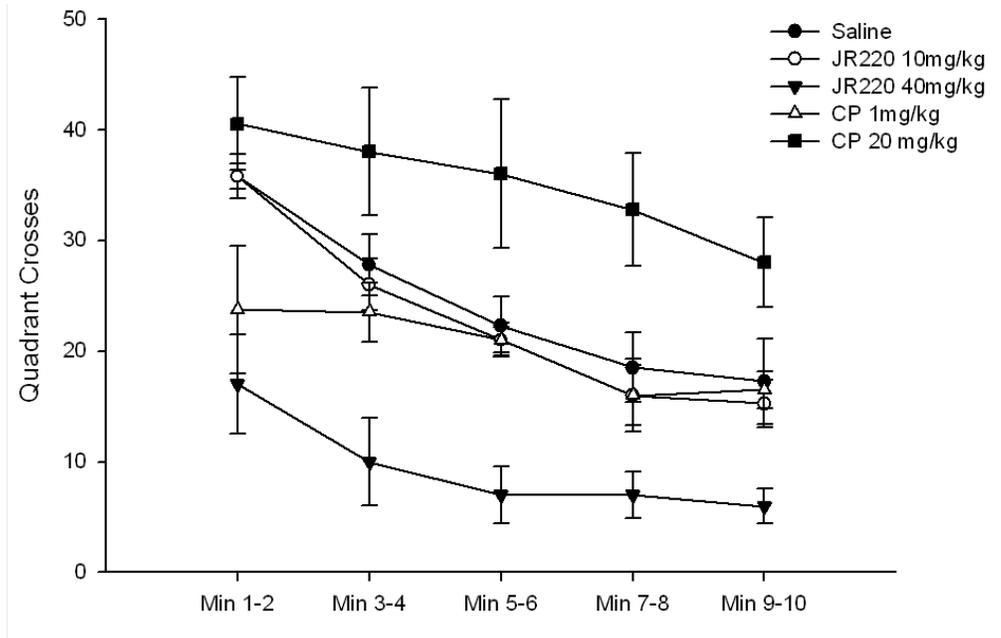
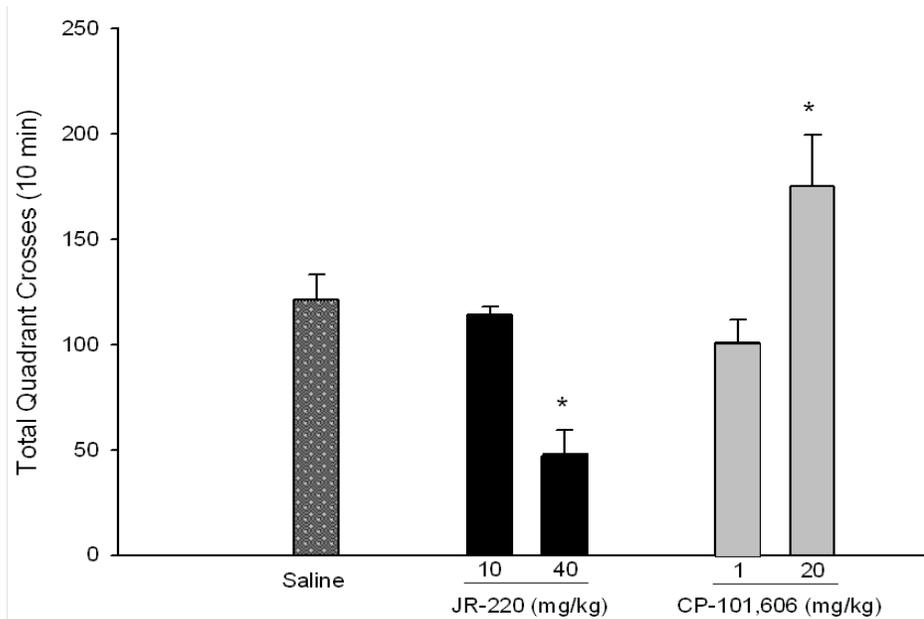
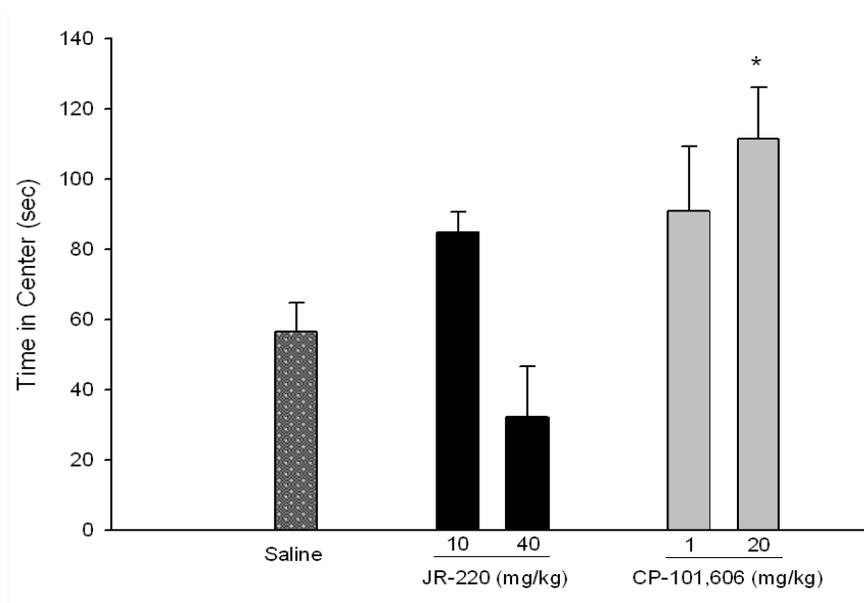


Figure 31b. Open Field Quadrant Crosses (Total) Following Saline, JR and CP



Finally, time spent in the 25% center zone was analyzed using a one-way ANOVA (treatment). A significant effect of treatment was noted $F(4,19)=5.594, p=.006$. Dunnett's tests indicated this effect was driven by increased center dwell-time of 20 mg/kg CP-treated animals ($p=.032$). Time spent in the center zone is depicted in Figure 32, below.

Figure 32. Open Field Center Dwell-Time Following Saline, JR and CP



Open Field Methods (Sprague-Dawley)

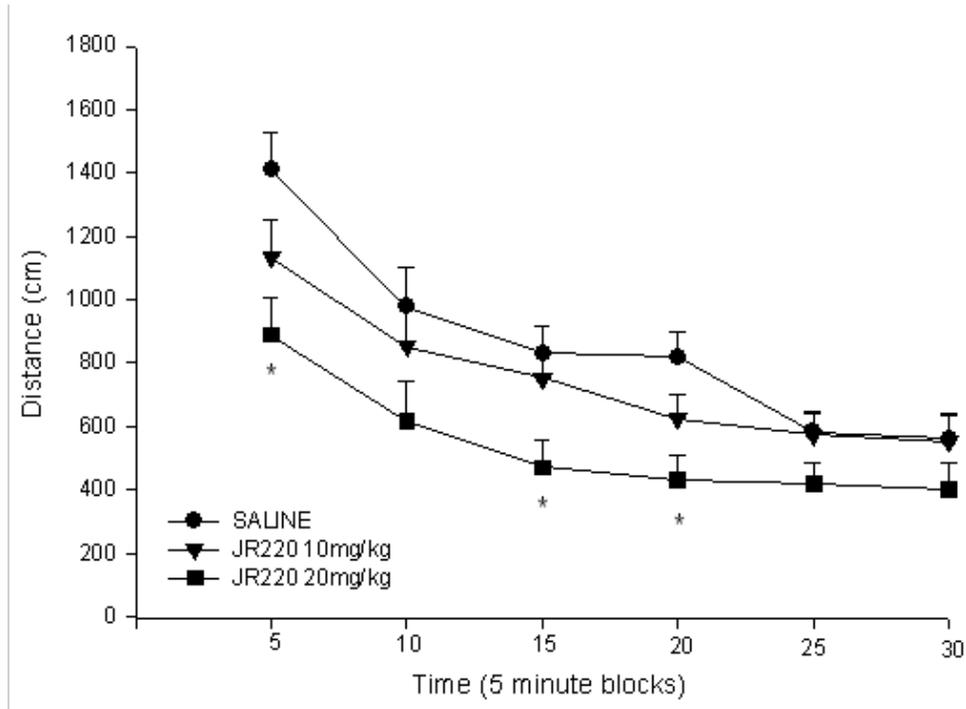
The activity of (PND45) male Sprague-Dawley rats ($N=18$) was analyzed following administration of JR-220. The apparatus was a circular chamber (36 cm height; 58 cm diameter) designed to prevent thigmotaxis or excessive time in corners. Subjects were injected with IP Saline, 10 or 20 mg/kg JR 30 min prior to activity testing. 10 min prior to testing, subjects were habituated to the darkened test room. Each subject was then individually placed in the open field and recorded for 30 min using a video tracking system (SMART program; Panlab, S.L.). Due to anecdotal observation that acute sedative effects of JR were transient, an additional 30 min session was performed, beginning 3 hours following the completion of the first session (4-4.5 hr following JR injection). For analysis, the open field was divided into center and outer zones,

comprising 25% and 75% of the total area, respectively. The dependent variables included distance traveled (in 5 min blocks) and the ratio of activity in the center vs. total area of the field.

Results (Sprague-Dawley)

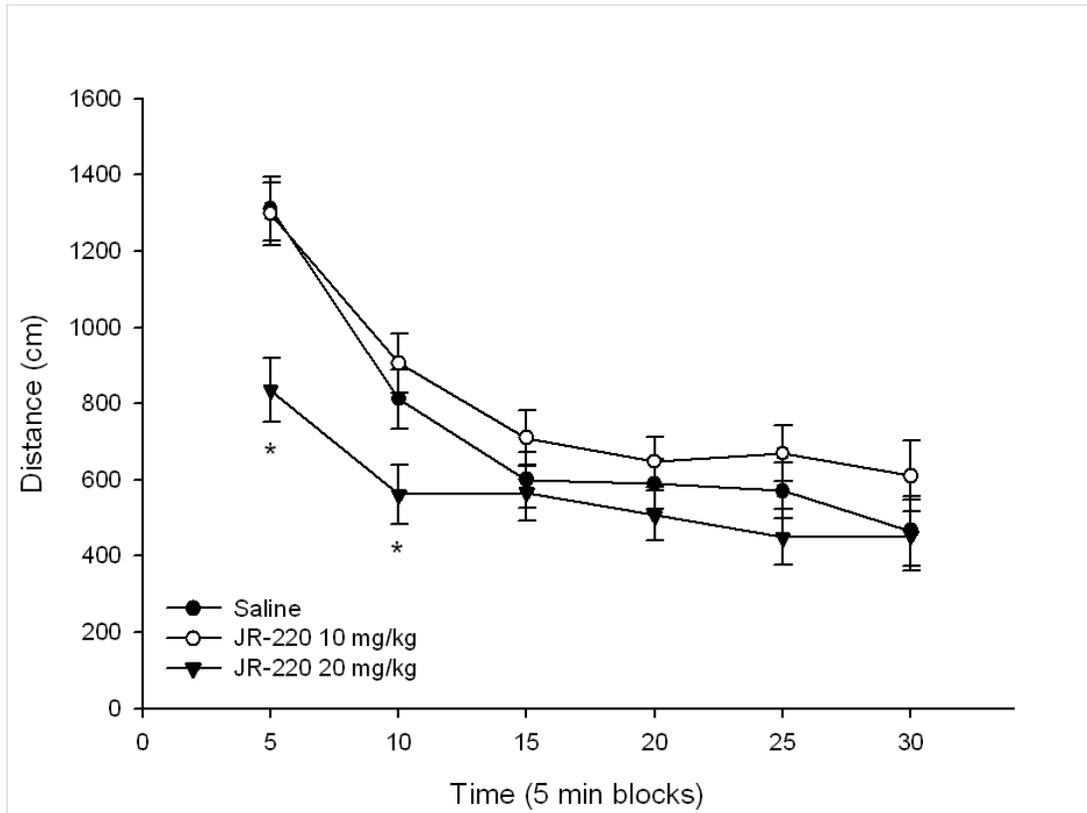
20 mg/kg JR was associated with reductions in activity. An initial RM ANOVA for the first 30-min activity session indicated an interaction of treatment and time, $F(2,15)=4.252, p=.034$, as well as a main effect of JR treatment, $F(2,15)=4.055, p=.039$, thus individual one-way ANOVAs (treatment) were performed for each 5 min block. Significant effects of treatment were noted during block 1, $F(2,17)=4.731, p=.026$; 3 $F(2,17)=4.669, p=.027$; and 4 $F(2,17)=6.128, p=.003$. During block 1 Fisher's protected *t post hoc* tests indicated a significant reduction in activity associated with 20 mg/kg JR treatment, relative to saline ($p=.008$). During block 3, the 20 mg/kg dose reduced activity relative to saline ($p=.011$) as well as the 10 mg/kg treatment ($p=.038$). Finally, during block 4 the 20 mg/kg dose reduced activity relative to saline treatment, only ($p=.003$). These results are depicted in Figure 33. ANOVA performed for center activity failed to reveal any significant effect of treatment.

Figure 33. Activity 30-60 min Following JR-220 Administration (Sprague Dawleys)



At four hours post-injection, activity reductions in 20 mg/kg JR-treated animals remained apparent. An initial RM ANOVA for the second 30-min activity session indicated an interaction of treatment and time, $F(2,15)=9.112$, $p=.003$, as well as a main effect of JR treatment, $F(2,15)=3.748$, $p=.048$, thus individual one-way ANOVAs (treatment) were performed for each 5 min block. Significant effects of treatment were noted during blocks 1 [$F(2,17)=10.650$, $p=.001$], and 2 [$F(2,17)=5.129$, $p=.019$]. During block 1 post hoc tests indicated the 20 mg/kg dose reduced activity relative to saline ($p=.001$) as well as the 10 mg/kg treatment ($p=.001$). During block 2 significant activity reductions were again noted for 20 mg/kg JR-treated animals, relative to both saline ($p=.039$) and 10 mg/kg ($p=.007$) treatment. These results are depicted in Figure 34. ANOVA performed for center activity failed to reveal any significant effect of treatment.

Figure 34. Activity 4-4.5 hr Following JR-220 Administration (Sprague Dawleys)



Open Field Methods (C57BL/6J)

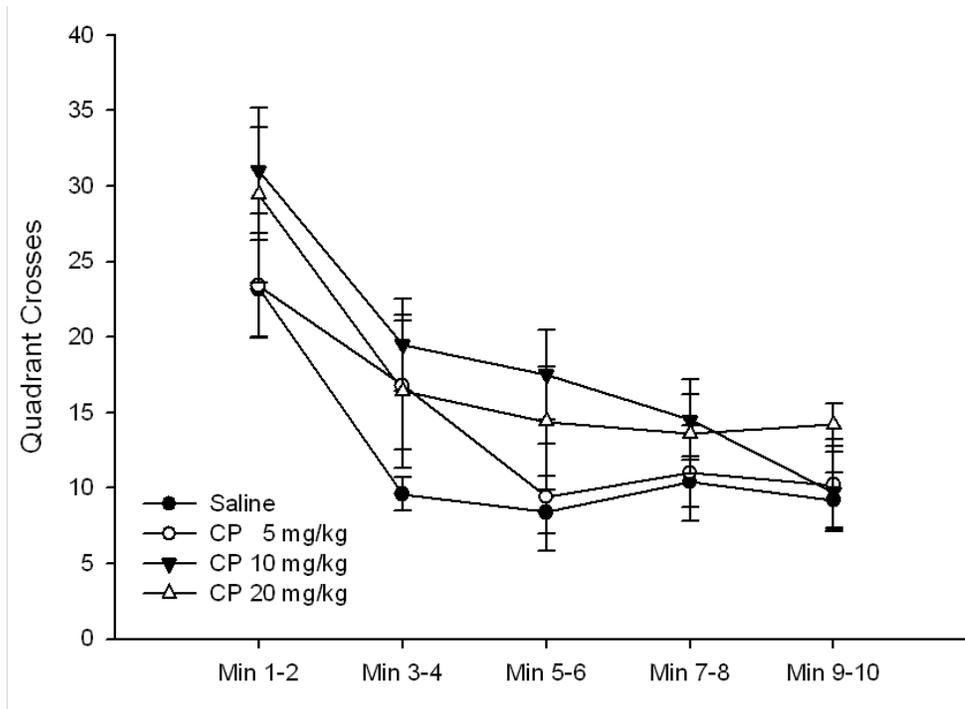
The activity of male C57 mice (30±2 g) was analyzed following the administration of both CP and JR. Nineteen male C57 mice (30±2 grams) were administered 0, 5, 10 or 20 mg/kg CP-101,606 (n’s=4-5/group). In a separate experiment, 24 additional C57s were administered 0, 10, 20 or 30 mg/kg JR-220 (n’s=6/group). Otherwise, methods were exactly as described for Swiss Webster testing, above.

Results (C57BL/6J)

An RM ANOVA (time x treatment) conducted on CP-treated animals yielded neither a main effect of, nor interaction with, treatment. As conducted previously, a one-way ANOVA (treatment) using total quadrant crosses similarly failed to detect any effect

of CP treatment. Quadrant crossings by block are depicted in Figure 35, below. No difference in center dwell-time was detected between treatment conditions.

Figure 35. Open Field Quadrant Crosses Following Saline or CP(C57 Mice)



The highest dose of JR (30 mg/kg) was associated with marked reductions in activity. A RM ANOVA (time x treatment) conducted on JR-treated animals indicated both a time by treatment interaction, $F(12,80)=3.700, p<.001$, and a main effect of treatment $F(3,20)=23.635, p<.001$. To further examine these effects, one-way ANOVAs (treatment) were conducted for each block, individually. Significant treatment effects for block 1, $F(3,23)=12.852, p<.001$, block 2, $F(3,23)=15.292, p<.001$ and block 3, $F(3,23)=60.056, p<.001$ were noted, such that 30 mg/kg JR reduced activity relative to all other groups (all p 's<.001). The same effect was noted for block 4, $F(3,23)=4.159, p=.019$, wherein 30 mg/kg reduced activity compared to saline ($p=.015$), 10 ($p=.006$) and 20 mg/kg ($p=.010$), and block 5 $F(3,23)=8.516, p=.001$, wherein 30 mg/kg reduced activity compared to saline ($p<.001$), 10 ($p=.009$) and 20 mg/kg JR ($p=.001$). Additionally, a one-way ANOVA (treatment) was conducted using total quadrant crosses

as the dependent variable. Again, a significant effect of treatment was detected, $F(3,23)=23.748, p<.001$, such that 30 mg/kg JR-treated animals were hypoactive compared to all others (all p 's<.001). These results are depicted across time in Figure 36, and summed in Figure 37, below. No difference in center dwell-time was detected between treatment conditions.

Figure 36. Open Field Quadrant Crosses Following Saline or JR (C57 Mice)

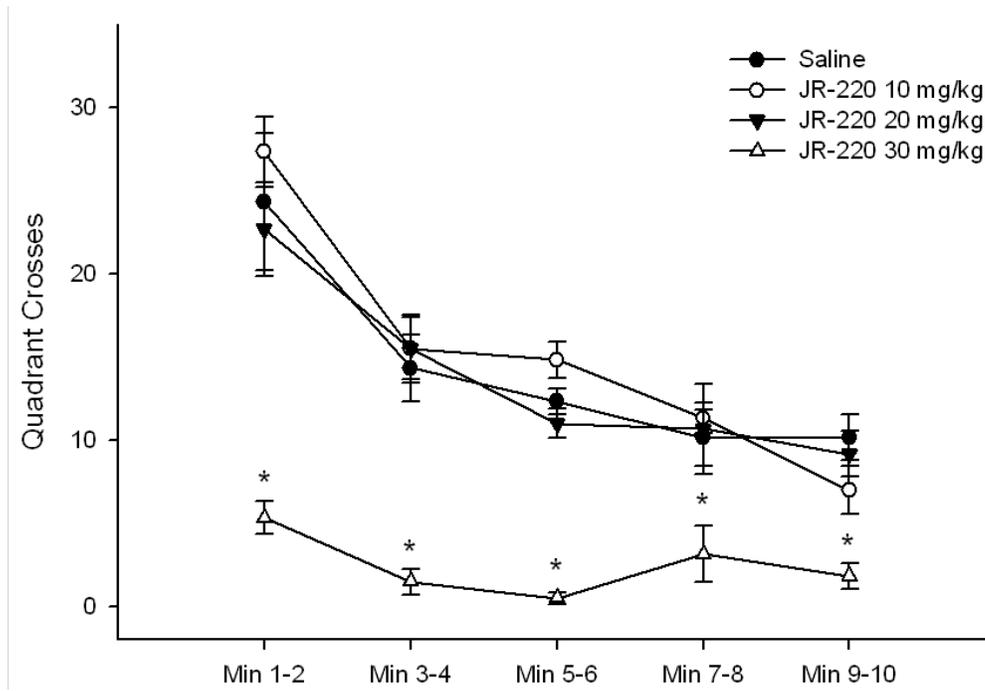
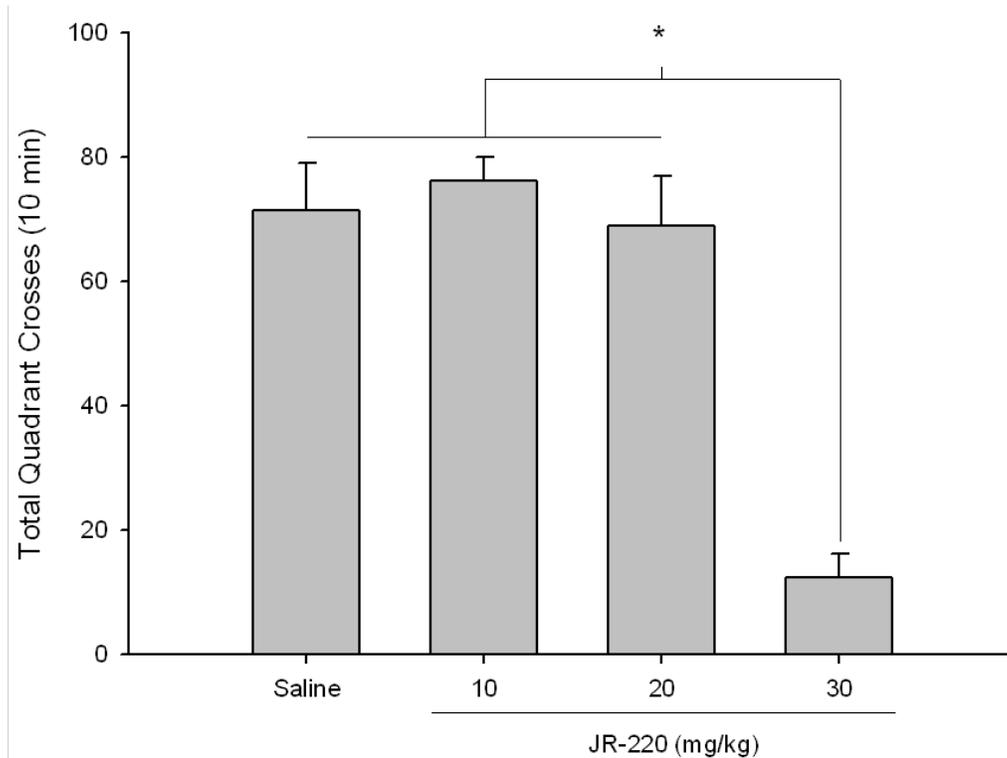


Figure 37. Open Field Quadrant Crosses (Total) Following Saline, 10, 20 or 30 mg/kg JR (C57 Mice)



Open Field Summary

This final experiment was somewhat atypical compared with those preceding it; while it can be considered a screen, its main purpose was to facilitate interpretation of experiments 1-6. Taken together, the activity data presented above indicate that while JR and CP are both capable of behavioral disruption at higher doses (including doses analyzed in previous experiments), several lower doses which tested positively throughout the screening procedure showed no evidence of such disruption.

The first open field analysis included Swiss Webster mice, and was designed to analyze the highest and lowest doses of JR and CP which demonstrated efficacy in the behavioral experiments (10 and 40 mg/kg and 1 and 20 mg/kg doses, respectively). Importantly, at doses which significantly reduced seizure behaviors, CP and JR-treated

animals were indistinguishable from controls. However, an interesting difference emerged at the high doses. 40 mg/kg JR was associated with an approximate decrease in activity of 60%, while 20 mg/kg CP was associated with an approximately 40% increase.

The second open field analysis included Sprague-Dawley rats, and analyzed only JR-220, and examined doses used in the ADE experiment. Anecdotally, JR appeared to induce transient sedation in our Sprague-Dawley rats, however observable in-cage evidence of sedation disappeared quickly. Therefore in addition to analyzing initial (and possibly transient) alterations in activity, 30 min following administration, we re-tested the animals 4.5 h following administration. Results were remarkably similar across testing periods. The 10 mg/kg dose produced activity which was indistinguishable from controls; while the 20 mg/kg dose appeared to reduce activity at both time points, producing nearly identical patterns/magnitudes of activity. While activity was certainly reduced among 20 mg/kg animals, it should be noted that animals remained active throughout both 30 min testing periods, traveling similar distances as the 10 mg/kg and saline groups during the last 10 min of the initial test and last 20 min of the final test, suggesting the sedation was mild. Importantly, the 10 mg/kg dose reduced relapse-like behavior, was not associated with reductions in water consumption and did not induce hypoactive behaviors, either initially or several hours post-injection.

The final open field analysis included C57 mice, and the full range of JR doses examined in the restraint stress and DID experiments: 10, 20 and 30 mg/kg doses. Additionally, CP was analyzed in 5, 10 and 20 mg/kg doses. While only the 10 mg/kg dose was analyzed in experiment 6, this dose ranged was analyzed in anticipation of a more thorough DID analysis (described below). While activity among saline and CP-treated animals was statistically indistinguishable, the lack of an effect was likely due to high variance and relatively low power. The trend toward higher activity among animals receiving 10 and/or 20 mg/kg doses was consistent with the observed increase in activity displayed by Swiss Webster mice. At the two lower JR doses examined (10 and 20

mg/kg), no evidence of activity disturbance was observed, however the 30 mg/kg dose was associated with a substantial reduction in activity. Unlike activity data generated in rats, the observed activity deficit persisted across all blocks.

While the additional examination of potential anxiolytic actions of these drugs was an advantage of including an open-field assessment, alterations in center-zone activity were only evident in the Swiss Webster mice, and the pattern of center-zone activity was nearly identical to the general pattern of activity, likely suggesting that periods of immobility in these animals largely occurred in the more protected periphery. While it is possible that center-zone patterns reflected actions on anxiety-related behaviors, the interpretation would include anxiogenic actions of JR-220, which is inconsistent with the examinations of compounds thought to have similar mechanisms of action (including CP-101).

Taken together these results suggest that neither JR nor CP disrupt activity at lower doses, but each is associated with disruption at higher doses, albeit in opposing directions.

Experiment 8: Drinking in the Dark (Model)

The medications development project, of which this dissertation represents only a fraction, was specifically designed for the screening of novel compounds, with the intention that “hits” would be subjected to a further testing with more established models, using more rigorous methodology, more appropriate controls and/or greater n 's. Experiment 8 was designed and included in this dissertation as the first, albeit modest, step toward such testing. The DID model was specifically selected as the first post-screening experiment for several reasons. The paucity of NMDAr-associated manipulations in the paradigm suggested a gap in the literature, allowing this experiment both to advance our compounds of interest (potentially) as well as contribute to the rapidly growing base of DID knowledge. Furthermore, since acamprosate has demonstrated efficacy under standardized DID conditions (Gupta et al., 2008), the model appears to possess predictive validity which was notably lacking from our initial DID screens. Experiment 8 analyzed a range of doses for both compounds, and adhered more rigidly to established DID methodologies.

DID (Model) Subjects

Mice ($n=28$; Jackson Laboratory, Bar Harbor, ME) arrived at 15 wks of age, and were left undisturbed for 7 days prior to testing. A reverse light:dark cycle was again employed, with lights turned on at 2100 h and off at 900 h.

DID (Model) Procedure

Animals were habituated, housed and handled as previously detailed. Procedures for Experiment 8 differed in that only one week (4 days, total) of ETOH access was given prior to the first week of treatments. Additionally, the treatment schedule was varied, however as in Experiment 6, all animals received all doses of each compounds, in a within-subjects, latin-square design (see Figures 38 & 39). Finally, a 3 hr delay (Rhodes

et al., 2005) between ETOH access and initiation of the dark cycle was used (vs. 4 hr in screening).

Figure 38. Latin-Square Treatment Design (Weeks 1-2: 0, 5, 10, 20 mg/kg CP; Weeks 3-4: 0, 5, 10, 20 mg/kg JR)

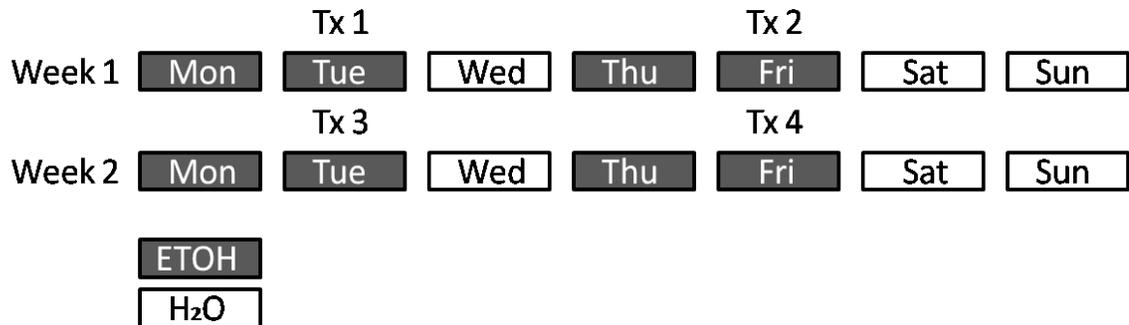
	Week 1		Week 2	
Group	Tx 1	Tx 2	Tx 3	Tx 4
Group 1 (n=7)	Saline	20 mg/kg CP	10 mg/kg CP	5 mg/kg CP
Group 2 (n=7)	5 mg/kg CP	Saline	20 mg/kg CP	10 mg/kg CP
Group 3 (n=7)	10 mg/kg CP	5 mg/kg CP	Saline	20 mg/kg CP
Group 4 (n=7)	20 mg/kg CP	10 mg/kg CP	5 mg/kg CP	Saline
	Week 3		Week 4	
Group	Tx 1	Tx 2	Tx 3	Tx 4
Group 1 (n=7)	Saline	20 mg/kg JR	10 mg/kg JR	5 mg/kg JR
Group 2 (n=7)	5 mg/kg JR	Saline	20 mg/kg JR	10 mg/kg JR
Group 3 (n=7)	10 mg/kg JR	5 mg/kg JR	Saline	20 mg/kg JR
Group 4 (n=7)	20 mg/kg JR	10 mg/kg JR	5 mg/kg JR	Saline

Pre-treatment Drinking: The water bottles were replaced with cylinders containing 20% ethanol, which remained undisturbed for 4 hours, after which consumption was recorded and water bottles returned. Habituation lasted for one week (4 days of ETOH access).

Week 1 & 2: Methods were identical to habituation drinking, with the addition of injections (0, 5, 10, 20 mg/kg, ip) of CP-101,606 on treatment days, given immediately prior to ETOH access.

Week 3 & 4: Methods were identical to habituation drinking, with the addition of injections (0, 5, 10, 20 mg/kg, ip) of JR-220 on treatment days, given immediately prior to ETOH access.

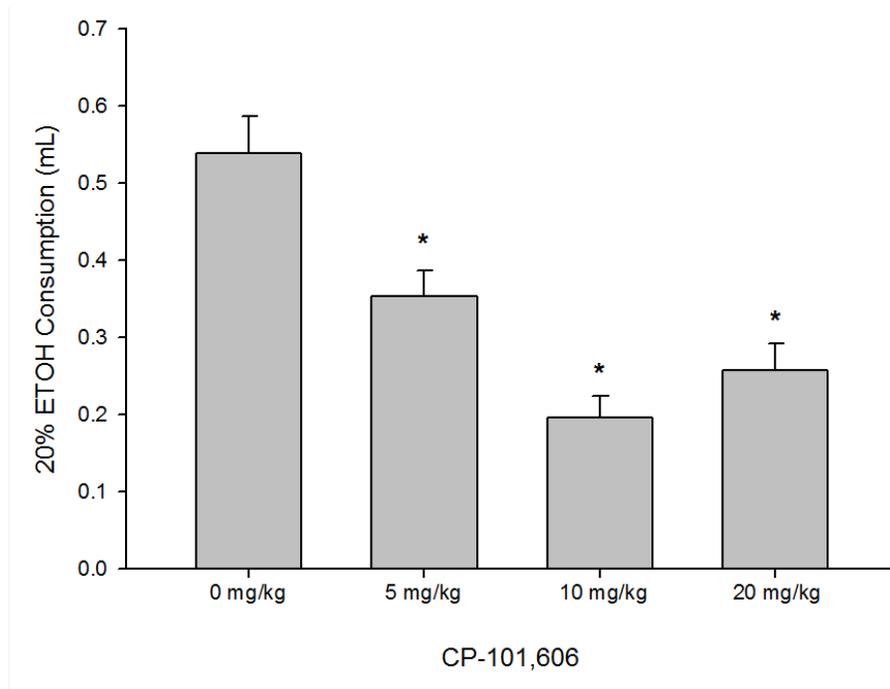
Figure 39. DID ETOH Access Schedule (Single Drug Treatment; Dark/Light Boxes Indicate ETOH/Water, Respectively)



DID (Model) Results

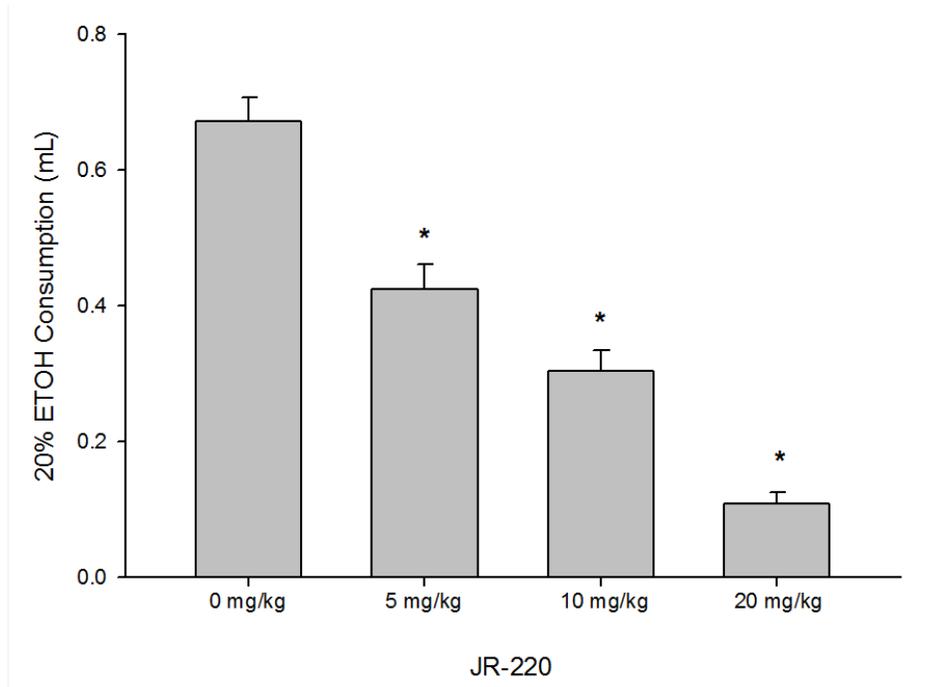
CP significantly reduced ETOH intake, $F(1,27)=59.627$, $p<.001$. Paired-samples t -tests revealed all doses differed from saline, including 5 $t(27)=3.243$, $p=.003$, 10, $t(27)=7.444$, $p<.001$, and 20 mg/kg, $t(27)=6.065$, $p<.001$. These results are depicted in Figure 40.

Figure 40. Ethanol Consumption Following CP-101,606 Administration



JR significantly reduced ETOH intake, $F(1,26)=232.346$, $p<.001$. Paired-samples t -tests revealed all doses differed from saline, including 5 $t(26)=6.206$, $p=.001$, 10, $t(26)=8.986$, $p<.001$, and 20 mg/kg, $t(26)=16.103$, $p<.001$. These results are depicted in Figure 41.

Figure 41. Ethanol Consumption Following JR-220 Administration



DID (Model) Discussion

Results of this experiment were consistent with screening results; CP appeared to reduce consumption relative to vehicle at all doses, however, these reductions did not appear dose-dependent. JR also reduced consumption, however, consistent with the screening results, such reduction appeared dose-dependent, with the 20 mg/kg dose drastically reducing intake. These results are also consistent with the observations of Rhodes and colleagues (*Rhodes, personal communication*), who examined JR-220 at these doses as well, however this group observed significant reductions in ETOH consumption only at the highest (20 mg/kg) dose.

The relevance of these results is supported by the relatively recent recognition of DID as a powerful preclinical tool for medications development, with a number of promising compounds having recently demonstrated reduction in binge-like intake,

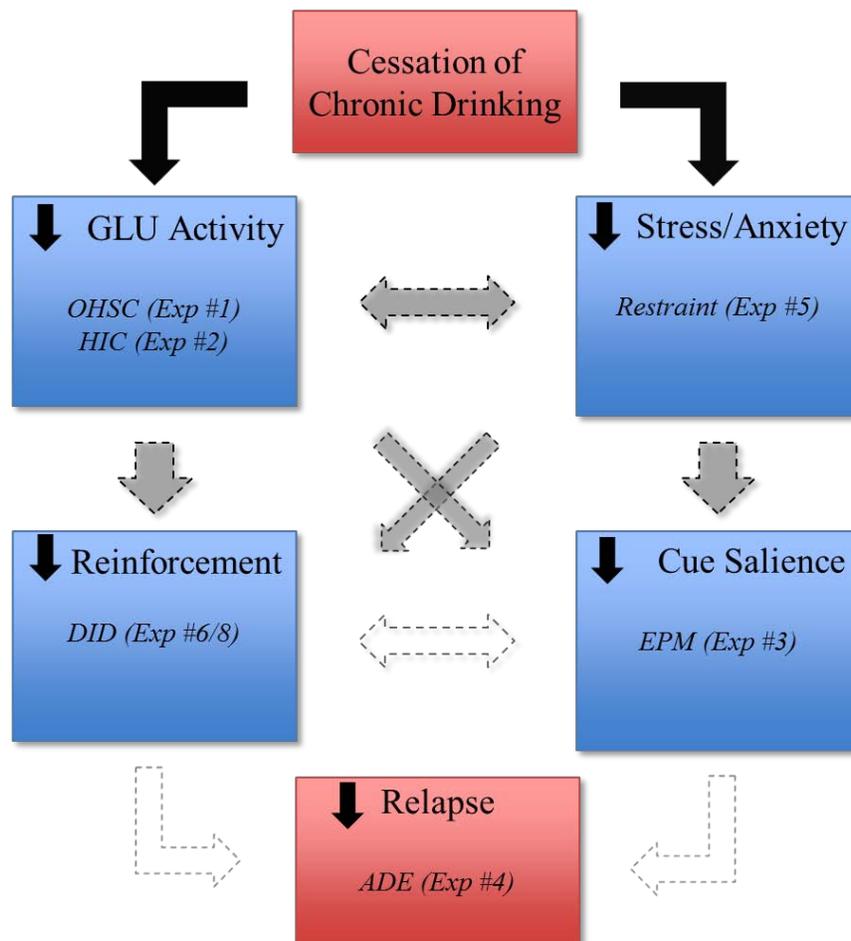
including baclofen, CRF antagonists, mGLUr antagonists, mecamylamine and lobeline (Moore & Boehm, 2009; Sajja & Rahman, 2011; Gupta et al., 2008; Hendrickson, Zhao-Shea & Tapper, 2009; Sparta et al., 2008). While somewhat surprising that NMDAr compounds have remained unexamined, our results serve the dual-purpose of advancing the development of CP and JR, as well as further implicating the role of glutamate (in general), NMDAr (in particular) and polyamine-sensitive receptor subtypes (specifically) in binge-like intake, a behavior which they are not well-recognized for.

Chapter 8. General Discussion

8.1 Summary of Results

Current evidence suggests that JR-220 and CP-101,606 (Haradahira et al., 2002; Mott et al., 1998) are both inhibitory modulators of the NMDAR, whose mechanisms of action involve interference with polyamine binding. Thus, for numerous reasons discussed above, it was hypothesized that both would perform positively in screens designed to examine withdrawal symptoms, neurotoxicity, ETOH reinforcement, relapse behavior, stress-associated drinking and ETOH-associated cue conditioning (see Figure XX, below). Furthermore, based on success in these screens, efficacy in a standardized DID model was hypothesized. Our results were consistent with these hypotheses, despite the recognitions that CP needs to be examined in additional screens (e.g., ADE) and that some findings deserve more thorough re-examination (e.g., reductions in stretched-attend posture).

Figure 42. Model of Drinking Progression With Experimental Evidence for Intervention



Our results suggested that both JR and CP possess neuroprotective potential in an *in vitro* screen using organotypic hippocampal slices withdrawn from ETOH. Consistent with this demonstration of *in vitro* neuroprotection, our behavioral HIC screen demonstrated the ability of these compounds to reduce withdrawal-associated convulsive behaviors, which are associated with underlying excitotoxic insult and index withdrawal severity. Taken together with molecular screens conducted previous to this dissertation, and with the results of behavioral data from neonatal studies which used JR and CP as neuroprotective interventions during developmental ETOH exposure (Lewis et al.,

submitted; Wellmann et al., *in press*), evidence for the neuroprotective potential of both compounds is strong.

Our EPM screen suggested JR's ability to reduce ETOH-associated cue conditioning, although these data should be interpreted carefully, given the potential hypoactive and/or anxiolytic effects of JR in this paradigm. Results from the ADE screen indicated anti-relapse efficacy. Our restraint-stress screen demonstrated JR's ability to reduce ETOH's negative reinforcing value, while our DID experiments suggested the ability to reduce its positive reinforcing value. Taken together, these results suggest JR's ability to reduce multiple motivational factors contributing to relapse. While full screening of CP was not possible, its similar mechanism of action, similar action in HIC screening, and positive performance in all DID experiments suggests that it too possesses strong potential, however more thorough screening is required. *Taken together, the performance of these compounds in our screening procedure provides strong evidence for both drugs as potential pharmacotherapeutics for alcoholism, and further implicate polyamines and NR2B subunits in multiple mechanisms of ETOH consumption and withdrawal.*

Although these two drugs are thought to have similar mechanisms of action, albeit in variable receptor populations, dissociations in their performance were particularly interesting. For instance, CP-101 appeared far more potent in the screens for neuroprotective potential (OHSC and HICs). Current evidence suggests that NR2B/2B-containing receptors represent a high-risk subpopulation that is particularly sensitive to such insults. However, at equal doses CP's performance was not distinguishable from JR's in the DID paradigm, suggesting that while NR2B/2B subpopulations appear to contribute to this type of drinking behavior, their contribution is shared. A particularly interesting, if somewhat puzzling, dissociation in the behavioral effects of these compounds involves the bidirectionality of their effects on activity. CP appears to increase open-field activity, while JR-220 appears to reduce it. This difference may be indicative of the differential contributions of NR2B-containing subpopulations to activity. It is possible that antagonism of NR2B/2B stimulates motor activity, while antagonism of

a more heterogenous receptor population depresses it. While supported by the observation that nonspecific antagonism depresses activity (Ghasemi et al., 2010) the lack of clear indication in the literature as to ifenprodil/eliprotil effects on open field activity makes this interpretation tentative, at best. An alternate interpretation would suggest that one or both compounds may be interacting with unknown and unanticipated transmitter systems, the resulting in disrupted activity. Regardless, such differences illustrate the clear need for further investigation of both compounds.

8.2 Clinical Futures

This dissertation has used our group's screening procedure as a hypothesis-testing device. Although the screens provide useful data which allows for the constant re-evaluation and alteration of our underlying hypotheses, their primary utility is the discovery and development of novel compounds for eventual clinical use. However, even perfect performance throughout the screening hierarchy cannot be taken as evidence of the compounds' ability to clear any of the numerous hurdles involved with progression from 'bench to bedside'. While not the focus of this dissertation, potential future difficulties in the advancement of JR-220 (and similar agmatine analogues) are worth consideration, and may guide future studies.

Recent estimates suggest that for every one compound receiving FDA approval, approximately 10,000 molecules are synthesized in drug discovery programs. Of these 10,000, roughly 2.5% will advance to preclinical testing and only .05% to Phase I trials. That said, it must be noted that while JR-220's influence on ETOH intake behavior is marked, little more than gross toxicity analyses have been performed; none of the numerous toxicology screens (e.g., genotoxicity, hemolysis, cardiotoxicity, mitochondrial toxicity, reactive metabolite screening, etc.) have been conducted, nor have any 'ADME' (absorption, distribution, metabolism and excretion) screens. Our application to the NIH Rapid Access to Interventional Development (RAID) program, if accepted, will begin such examination. If JR-220 survives the significant failure rate associated with such

toxicological/kinetic measures and advances to Phase I testing, its chances remain low: CNS drugs have a historically low success rate, with roughly 7.5% of Phase 1 trials producing marketable compounds. While JR-220 has several advantages which may increase these odds, including its similarity to an endogenous molecule (agmatine), and its modulatory action at the target (thought to be a self-limiting characteristic in terms of toxicity) its chances of advancement to bedside are limited. An objective acceptance of the sizeable odds against JR-220's success is useful to avoid the recognized pitfall of "championing" a promising compound which represents a significant amount of invested time and money, past the point at which evidence suggests its limited utility. However the recognition of such sizeable odds should not be taken as reluctance or reservation to continue JR-220's investigation. Unlike other areas of drug discovery, many of which have existing pharmacotherapies with acceptable effectiveness, the success of current FDA-approved medications for treating alcohol related disorders has been marginal, at best. When considered alongside the number of people affected by alcohol dependence issues, the 'big risk/big reward' assessment of medications development work in this field becomes apparent. Thus, despite probable failure, the cost-benefit analysis of continuing to invest in JR-220 and in future medications development endeavors of this nature seems clear.

In contrast with JR, CP has already surmounted many of the challenges involved in progressing to humans. CP is orally bioavailable (Taylor et al., 2006), and appears well-tolerated, with a favorable side-effect profile. However, clinical trials for several (non ETOH-related) diseases have revealed only modest effects. A clinical TBI study indicated outcome improvements and reductions in mortality rates, however effects were only marginally significant (p 's = .07 and .08, respectively; Yurkewicz et al., 2005). A clinical Parkinson's study found CP was effective at reducing levodopa-induced dyskinesia effects, but not Parkinsonism itself (Nutt et al., 2008). Interestingly, it appears to produce antidepressant effects in patients unresponsive to serotonin selective reuptake inhibitors (Skolnick et al., 2009). However, interest in the compound appears to be decreasing; pubmed searches indicated 15 publications related to CP during 2004-2005

(at the time when our group noted the compound and began examining it in FASD experiments), however only 5 five were noted from 2010 to the current date, all of which utilize it as a pharmacological tool in preclinical examinations. Such loss of interest may reflect clinical shortcomings of CP, but may also be related to the recognition that CP shares discriminative stimuli with phencyclidine; it is self-administered in non-human primates who were experienced with PCP administration (Nicholson et al., 2007), consistent with observations that CP is dose-dependently associated with dissociative and amnestic effects (Nutt et al., 2008).

The current work is focused specifically on alcoholism and alcohol-related effects, however, as the aforementioned investigations of CP indicate, NMDAR modulators are implicated as potential pharmacotherapeutics for a startlingly large number of conditions, including chronic pain, Huntington's, Parkinson's, migraine, major depression, stroke/ischemic injury, epilepsy, and others. While speculative, it is not unlikely that in the discovery and development of inhibitory modulators for alcohol dependence, compounds with utility in some, if not many, of these additional disorders may be produced. An alternate future for these compounds, mentioned only briefly in the introduction, involves neuroprotection from ETOH insult in utero, and in particular, from the prolonged withdrawal following birth. No pharmacotherapeutic intervention is currently available for FASDs, which are thought to involve similar if not identical excitotoxic mechanisms as those presented here, providing yet another potential avenue of JR-220 development.

Though not explicitly stated thus far, one potential contribution of the current work may not include either compound, but rather the demonstration of this type of project's utility. JR's transition from simple molecular screening to success in a strong preclinical model (the majority of which is contained herein) provides evidence that a small research team can accomplish this type of directed drug discovery and development. Furthermore, our inclusion of CP in various screens demonstrates the

flexibility of the program, which allows for both ‘bottom-up’ examination of novel compounds, as well as ‘top-down’ testing of known compounds.

Finally, while this dissertation represents only one of the first few steps toward the potential clinical development of these compounds, our results provide strong evidence for both drugs as potential pharmacotherapies, further implicate polyamines and NR2B subunits as critical mechanisms in ETOH consumption and withdrawal and demonstrate the utility of academic drug discovery and development programs.

Appendix A: Table Legend

Table 1. Species/Strain Use by Experiment

Table 2. JR-220 Treatments & Subjects

Experiments with JR utilized six litters (N=217), distributed across 20 treatment groups. These included Control (n=23), ETOH (n=22), Control + 20N (n=18), ETOH + 20n (n=19), Control + MK (n=18), ETOH + MK (n=20), Control + 50 µM JR (n=8), Control + 60 µM JR (n=7), Control + 75 µM JR (n=16), Control + 80 µM JR (n=4), Control + 90 µM JR (n=3) Control + 100 µM JR (n=4), Control + 125 µM JR (n=4), ETOH + 50 µM JR (n=7), ETOH + 60 µM JR (n=8), ETOH + 75 µM JR (n=18), ETOH + 80 µM JR (n=3), ETOH + 90 µM JR (n=4), ETOH + 100 µM JR (n=4) and ETOH + 125 µM JR (n=6).

Table 3. CP-101 Treatments & Subjects

Experiments with CP utilized five litters (N=177), distributed across 16 treatment groups. These included Control (n=21), ETOH (n=21), Control + 20N (n=12), ETOH + 20N (n=12), Control + MK (n=12), ETOH + MK (n=12), Control + 5 nM CP (n=6), Control + 10 nM CP (n=10), Control + 25 nM CP (n=12), Control + 50 nM CP (n=12), Control + 75 nM CP (n=4), ETOH + 5 nM CP (n=6), ETOH + 10 nM CP (n=9), ETOH + 25 nM CP (n=12), ETOH + 50 nM CP (n=12) and ETOH + 75 nM CP (n=4).

Table 4. Handling-Induced Convulsion Assessment Scale

Adapted/modified from Wilson & Little, 1998.

Table 5. Sucrose Fading Schedule

Appendix B: Figure Legend

Figure 1. Simplified Polyamine Biosynthesis (adapted from Coffino, 2001)

Figure 2. Contributions of ETOH Withdrawal to Reinforcement and Cue Salience

Figure 3. Molecular Structure of Ifenprodil and its Analogue, CP-101,606

Figure 4. Molecular Structure of Agmatine and its Analogue, JR-220

Figure 5. Treatment Distribution Within a Single Culture

Figure 6. JR-220 in CA1

PI florescence, by group mean + S.E.M, in CA1 of organotypic hippocampal slice cultures. Data collapsed across sex and converted to % control. Horizontal black line indicates 100% control fluorescence. * indicates significance ($p < .05$) relative to ETOH control group; # indicates significance relative to ETOH-naïve control group.

Figure 7. JR-220 in CA3

PI florescence, by group mean + S.E.M, in CA3 of organotypic hippocampal slice cultures. Data collapsed across sex and converted to % control. Horizontal black line indicates 100% control fluorescence. * indicates significance ($p < .05$) relative to ETOH control group; # indicates significance relative to ETOH-naïve control group.

Figure 8. JR-220 in DG

PI florescence, by group mean + S.E.M, in DG of organotypic hippocampal slice cultures. Data collapsed across sex and converted to % control. Horizontal black line indicates 100% control fluorescence. * indicates significance ($p < .05$) relative to ETOH control group; # indicates significance relative to ETOH-naïve control group.

Figure 9. CP-101,606 in CA1

PI florescence, by group mean + S.E.M, in CA1 of organotypic hippocampal slice cultures. Data collapsed across sex and converted to % control. Horizontal black line indicates 100% control fluorescence. * indicates significance ($p < .05$) relative to ETOH control group; # indicates significance relative to ETOH-naïve control group.

Figure 10. CP-101 in CA3

PI florescence, by group mean + S.E.M, in CA3 of organotypic hippocampal slice cultures. Data collapsed across sex and converted to % control. Horizontal black line indicates 100% control fluorescence. * indicates significance ($p < .05$) relative to ETOH control group; # indicates significance relative to ETOH-naïve control group.

Figure 11. CP-101 in DG

PI florescence, by group mean (+S.E.M.), in CA3 of organotypic hippocampal slice cultures. Data collapsed across sex and converted to % control. Horizontal black line indicates 100% control fluorescence.

* indicates significance ($p < .05$) relative to ETOH control group; # indicates significance relative to ETOH-naïve control group.

Figure 12. Representative Slices From Select Treatment Groups

Including Control (a), ETOH (b), 20 μ M NMDA (c), 50 μ M JR-220 (d), 25 nM CP-101 (e), and 20 μ M MK-801 (f).

Figure 13. BEC as a Function of Time and 4MP Treatment

Blood ethanol content, presented as mean (\pm S.E.M.) mg/dl, following 2.5 g/kg ETOH administration. Groups included 4MP (9 mg/kg) and control. * indicates significance ($p < .05$) between groups.

Figure 14. JR-220 HICs, Percent Control Values

Handling Induced Convulsion scores, presented as mean (\pm S.E.M.) percentage of ETOH-naïve scores. * indicates significance ($p < .05$) relative to drug-naïve (0 mg/kg) group.

Figure 15. CP-101,606 in HICs, Percent Control Values

Handling Induced Convulsion scores, presented as mean (\pm S.E.M.) percentage of ETOH-naïve scores. * indicates significance ($p < .05$) relative to drug-naïve (0 mg/kg) group.

Figure 16. Stretched-Attend Behaviors

Stretched-attend postures, presented as mean (\pm S.E.M.). Groups included ETOH-treated and ETOH-naïve animals, treatments included saline and 20 mg/kg JR-220. * indicates significance ($p < .05$) between treatment conditions.

Figure 17. Time Spent in Closed vs. Open arms

Time in open and closed arms, respectively, presented by as mean (\pm S.E.M.) seconds. Groups included ETOH-treated and ETOH-naïve animals, treatments included saline and 20 mg/kg JR-220. * indicates significance ($p < .05$) between treatment conditions.

Figure 18. Total Arm Entries

Arm entries (open and closed), presented as mean (\pm S.E.M.) entries. Groups included ETOH-treated and ETOH-naïve animals, treatments included saline and 20 mg/kg JR-220. * indicates significance ($p < .05$) between treatment conditions.

Figure 19. 20 mg/kg JR in ADE

ETOH consumption during baseline, untreated ADE period, and treated ADE period. Presented as daily mean (\pm S.E.M.) g/kg. Treatments included vehicle and 20 mg/kg JR-220. * indicates significance ($p < .05$) relative to baseline consumption.

Figure 20. Water consumption in ADE reinstatement phases (20 mg/kg)

Water consumption during baseline, untreated ADE period, and treated ADE period. Presented as daily mean (\pm S.E.M.) g/kg. Treatments included vehicle and 20 mg/kg JR-220. * indicates significance ($p < .05$) relative to baseline consumption.

Figure 21. 10 mg/kg JR-220 in ADE

ETOH consumption during baseline, untreated ADE period, and treated ADE period. Presented as daily mean (\pm S.E.M.) g/kg. Treatments included vehicle and 10 mg/kg JR-220. * indicates significance ($p < .05$) relative to baseline consumption.

Figure 22. Water Consumption during ADE reinstatement phases (10 mg/kg)

Water consumption during baseline, untreated ADE period, and treated ADE period. Presented as daily mean (\pm S.E.M.) g/kg. Treatments included vehicle and 10 mg/kg JR-220. * indicates significance ($p < .05$) relative to baseline consumption.

Figure 23. Restrained vs. Unrestrained Drinking

ETOH consumption, presented as mean (\pm S.E.M.) g/kg. Treatments included restraint-stress and control handling. * indicates significance ($p < .05$) between treatments.

Figure 24. JR-220 Treatment (Following 5-day Restraint Period)

ETOH consumption among restrained animals, presented as mean (\pm S.E.M.) g/kg. Treatments included 0, 10, 20 and 30 mg/kg JR-220. * indicates significantly ($p < .05$) increased consumption relative to all other groups. # indicates significantly ($p < .05$) reduced consumption relative to all other groups.

Figure 25. Unrestrained Animals, 0 vs. 30 mg/kg JR

ETOH consumption among unrestrained animals, presented as mean (\pm S.E.M.) g/kg. Treatments included 0 and 30 mg/kg JR-220.

Figure 26. Latin-Square Design for Screen 1 and Screen 2

Screen 1: Saline, 10 mg/kg CP, 10 mg/kg JR; Screen 2: Saline, 20 mg/kg JR, 300 mg/kg Acamprosate

Figure 27. DID ETOH Access Schedule

Figure 28. Daily DID Consumption (4hr/day) Prior to Treatment Phase

ETOH consumption during the last three weeks of untreated 4 hr consumption, presented as mean (\pm S.E.M.) g/kg ETOH consumption.

Figure 29. CP-101,606 (10 mg/kg) and JR-220 (10 mg/kg) in DID

ETOH consumption during the first DID treatment phase, presented as mean (\pm S.E.M.) g/kg ETOH consumption. Treatments included CP-101 (10 mg/kg), JR-220 (10 mg/kg), and saline. * indicates significant ($p < .05$) difference from vehicle treated drinking.

Figure 30. JR-220 (20 mg/kg) and Acamprosate (300 mg/kg) in DID

ETOH consumption during the second DID treatment phase, presented as mean (\pm S.E.M.) g/kg ETOH consumption. Treatments included JR-220 (20 mg/kg), acamprosate (300 mg/kg) and saline. * indicates significant ($p < .05$) difference from vehicle treated drinking.

Figure 31a. Open Field Quadrant Crosses (By Block) Following Saline, JR and CP

Open field activity among Swiss Webster mice, presented as presented as mean (\pm S.E.M.) quadrant crosses, per 2 min block. Groups includes JR-220 (10 and 40 mg/kg), CP-101 (1 and 20 mg/kg) and saline.

Figure 31b. Open Field Quadrant Crosses (Total) Following Saline, JR and CP

Open field activity among Swiss Webster mice, presented as presented as mean (\pm S.E.M.) total quadrant crosses. Groups includes JR-220 (10 and 40 mg/kg), CP-101 (1 and 20 mg/kg) and saline. * indicates significant ($p<.05$) difference relative to saline control.

Figure 32. Open Field Center Dwell-Time Following Saline, JR and CP Treatment

Center zone activity among Swiss Webster mice, presented as mean (\pm S.E.M.) time spent (sec). Groups includes JR-220 (10 and 40 mg/kg), CP-101 (1 and 20 mg/kg) and saline. * indicates significant ($p<.05$) difference relative to saline control.

Figure 33. Activity 30-60 min Following JR-220 Administration (Sprague Dawleys)

Open field activity among Sprague-Dawley rats, recorded 30 min following drug administration, presented as mean (\pm S.E.M.) distance (cm) in 5 min bins. Treatments included JR-220 (10 and 20 mg/kg) and saline. *indicates significant ($p<.05$) treatment effect, such that activity differed between saline and 20 mg/kg treated animals.

Figure 34. Activity 4-4.5 hr Following JR-220 Administration (Sprague Dawleys)

Open field activity among Sprague-Dawley rats, recorded 4 hr following drug administration, presented as mean (\pm S.E.M.) distance (cm) in 5 min bins. Treatments included JR-220 (10 and 20 mg/kg) and saline. *indicates significant ($p<.05$) treatment effect, such that activity differed between saline and 20 mg/kg treated animals.

Figure 35. Open Field Quadrant Crosses Following Saline or CP (C57 Mice)

Open field activity among C57BL/6J mice, presented as mean (\pm S.E.M.) quadrant crosses in 2 min bins. Treatments include 0, 5, 10 or 20 mg/kg CP-101.

Figure 36. Open Field Quadrant Crosses Following Saline or JR (C57 Mice)

Open field activity among C57BL/6J mice, presented as mean (\pm S.E.M.) quadrant crosses in 2 min bins. Treatments include 0, 10, 20 or 30 mg/kg JR-220. * indicates significance ($p<.05$) relative to saline control.

Figure 37. Open Field Quadrant Crosses (Total) Following Saline, 10, 20, or 30 mg/kg JR

Open field activity among C57BL/6 mice, presented as presented as mean (\pm S.E.M.) total quadrant crosses. Groups includes 0, 10, 20 and 30 mg/kg doses of JR-220. * indicates a significant ($p<.05$) difference relative to all other groups.

Figure 38. Latin Square Treatment Design

Weeks 1-2: 0, 5, 10, 20 mg/kg CP; Weeks 3-4: 0, 5, 10, 20 mg/kg JR

Figure 39. DID ETOH Access Schedule

Figure 40. Ethanol Consumption Following CP-101,606 Administration

ETOH consumption during the first DID treatment phase, presented as mean (+S.E.M.) ETOH consumption (mL), following injections of 0, 5, 10, and 20 mg/kg CP-101,606. * indicates significant ($p < .05$) difference from vehicle treated drinking.

Figure 41. Ethanol Consumption Following JR-220 Administration

ETOH consumption during the first DID treatment phase, presented as mean (+S.E.M.) ETOH consumption (mL), following injections of 0, 5, 10, and 20 mg/kg JR-220. * indicates significant ($p < .05$) difference from vehicle treated drinking.

Figure 42. Model of Relapse Progression with Experimental Evidence for Intervention

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- Publications
- Wellmann, K.A., Lewis, B., and Barron, S. (2010). Agmatine reduces ultrasonic vocalization deficits in female rat pups exposed neonatally to ethanol. *Neurotoxicology and Teratology* 32(2): 158-163.
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