Role of Glutamate Receptors in a Mouse Model of Alcohol-Heightened Aggression

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Abstract

Serotonin is the neurotransmitter most implicated in the neural mechanism of aggressive behavior, including the heightened aggressive behavior that occurs in some individuals after alcohol consumption. Recent data suggest that the glutamate NMDA receptor (NMDAR) may modulate the serotonergic pathway involved in alcohol-heightened aggression. The objective of the present study was to further characterize the role of the NMDAR in alcohol-heightened aggression via pharmacological manipulations at the NMDAR in a series of preclinical studies. Male Swiss-Webster mice self-administered 1.0 g/kg (w/v) ethanol and were assessed for aggressive behavior using the resident-intruder paradigm, a translational rodent model of aggression. D-Cycloserine (0-100 mg/kg), ifenprodil (0-10 mg/kg), and amantadine (0-30 mg/kg) were injected intraperitoneally immediately following ethanol self-administration to investigate the effects of manipulations at the NMDAR glycine-binding site, NR2B subunit, and channel pore, respectively. While these compounds did not significantly interact with ethanol to affect aggressive behavior, further investigations are warranted before relegating these sites as irrelevant in alcohol-heightened aggression. Specifically, in a subpopulation of mice characterized as alcohol-heightened aggressors (AHA), ethanol significantly blocked the apparent anti-aggressive effect of D-cycloserine. Furthermore, 17 mg/kg amantadine significantly increased aggressive behavior after ethanol drinking compared to water drinking, and a similar trend was seen after 0.3 mg/kg ifenprodil. Given the individual differences in aggression after alcohol consumption, it would be useful to examine these compounds in a larger population, and particularly in a population of alcohol-heightened aggressors.

Glutamate Receptors in a Mouse Model of Alcohol-Heightened Aggression

Alcohol is one of the oldest and most widely-used psychoactive substances, linked to more acts of violence than any other substance (Heinz, Beck, Meyer-Lindenberg, Sterzer, & Heinz, 2011; Roizen, 1997; Miczek et al., 1994). While alcohol-heightened aggression thus represents a substantial social and public health problem, alcohol only shows pro-aggressive effects in a small subset of the general population, suggesting that there are individual differences that complicate the story. In fact, the precise neural mechanism alcohol-heightened aggression remains to be elucidated. Given the significance of alcohol-heightened aggression, it would be useful to identify such a neural mechanism in order to direct therapeutic interventions. Considering the ethical complications associated with studying aggression in humans, preclinical investigations use translational rodent models such as the resident-intruder confrontation protocol. The results of previous preclinical studies in this field implicate various brain regions and neurotransmitters in the neural circuitry of alcohol-heightened aggression, including serotonin, dopamine, GABA, and glutamate. The present study seeks to clarify the role of glutamate receptors in aggression after acute alcohol consumption in a mouse model of aggression.

Aggression

Aggressive behavior in humans is perhaps best defined as overt violent behavior intended to do harm to another individual (Moyer, 1971). Species-typical aggression evolved as an adaptive behavior—such as when defending a territory or competing for a mate—across many animal species (Scott, 1956). However, when aggression is expressed excessively or in inappropriate contexts, it is considered pathological and maladaptive (Nelson & Trainor, 2007).

Such aggression can have serious consequences; in fact, The World Health Organization considers violence to be one of the leading causes of death for 15-44 year olds (Krug et al., 2002). Clearly, escalated aggressive behavior in humans constitutes a major public health hazard.

From a clinical viewpoint, aggressive behavior is generally categorized into two subtypes: (1) emotionally charged impulsive and reactive aggression and (2) calculating, instrumental, and predatory aggression (Vitiello & Stoff, 1997). Feline studies of aggression were some of the earliest and most notable to translate these human forms of aggressive behaviors into an animal model (Siegel, Bandler, Jr., & Flynn, 1972). Both forms can be maladaptive and in fact are each characteristics of several psychiatric disorders including but not limited to post-traumatic stress disorder (PTSD; French, 1989), mood disorders (Michaelis et al., 2004), dementia (Cummings et al., 1994; Brodaty et al., 2003), epilepsy (Marsh & Krauss, 2000; Rodin, 1973), borderline personality disorder (New et al., 1997; Frankenburg & Zanarini, 2002), antisocial personality disorders (Blair, 2001; Moeller & Dougherty, 2001) and schizophrenia (Fava, 1997; Nolan et al., 2005). Additionally, impulsive violence is the main feature of intermittent explosive disorder, a disorder characterized by repeated, inappropriate episodes of aggression (Heinz et al., 2011; Coccaro, Lee, & Kavoussi, 2010; American Psychiatric Association, 1994).

Aggression is particularly escalated in certain conditions. In humans, forms of escalated aggression include alcohol-, social threat-, and frustration-heightened aggression (Dollard, Doob, Miller, Mowrer, & Sears, 1939; Berkowitz, 1989), all of which are examples of the impulsive and reactive subtype of aggressive behavior. There are various translational animal models that are used to study these species-atypical forms of aggression. Animals may be trained to self-administer ethanol via operant-conditioned responses in order to study alcohol-heightened

aggression (Miczek, Weerts, Tornatzky, DeBold, & Vatne, 1992). Social provocation may also result in escalated aggressive behavior and can be studied in rodents by a social instigation paradigm (Fish, Faccidomo, & Miczek, 1999). A third form of escalated aggression, frustration-heightened aggression, is modeled in animals as schedule-heightened aggression, in which omission of an expected reward results in escalated aggressive behavior (de Almeida & Miczek, 2002). The focus of the present study is the escalated aggressive behavior that may occur following alcohol consumption.

Alcohol

Alcohol (ethanol, EtOH) is one of the oldest and most widely used psychoactive substances. Ethanol intoxication, defined as a blood ethanol concentration (BEC) of 0.5 mg/mL in humans, affects motor, sensory, and cognitive functions (Goldstein, 1983). Chronic excessive usage may result in dependence in some individuals (Cloninger, 1987). Behaviorally, alcohol acts as a stimulant at low doses and as a sedative-hypnotic at high doses (Miczek et al., 2004). Because intoxication affects nearly all behaviors, it can have dangerous and life-threatening consequences (Goldstein, 1983). However, it remains an extremely popular social phenomenon. Alcohol is often used as a social lubricant, increasing sociability and gregariousness in social situations. Alternatively, inebriation may also have a depressive effect in some individuals, and an aggressive effect in others (Miczek, Fish, de Almeida, Faccidomo, & DeBold, 2004). It is evident that there are individual differences in the behavioral effects of alcohol, which are likely mediated via a complex neurobiological mechanism.

Alcohol affects a variety of receptors in the central nervous system (CNS), particularly the glutamate NMDA receptor and the GABA-A receptor (Lovinger, White, & Weight, 1989;

Woodward, 2000; Lovinger, 1996; Hoffman et al., 1990). While it is generally accepted that ethanol is an antagonist at the NMDA receptor (Lovinger et al., 1989), its precise binding site is still in question; researchers have proposed, among other sites, the glycine-binding site (Lovinger, 1996) as well as a polyamine site on the NR2B subunit (Boyce-Rustay & Holmes, 2005). Ethanol has been demonstrated to antagonize NMDARs on serotonergic and GABAergic neurons more potently than those on dopaminergic neurons; incidentally, these NMDARs have a higher proportion of the NR2B subunit (Fink & Gothert, 1996). Acute alcohol consumption impairs executive cognitive function in the prefrontal cortex (PFC), and promotes dopaminergic transmission in the ventral striatum (Heinz et al., 2011). These neurotransmitters and brain regions are also implicated in aggressive behavior and therefore likely contribute to the heightened aggressive behavior seen in some individuals after alcohol drinking.

Alcohol-Heightened Aggression

Alcohol consumption has been linked to approximately two-thirds of violent acts of humans aggression such as rape, assault, domestic violence, and murder (Arseneault, Moffitt, Caspi, Taylor, & Silva, 2000; Cunradi, Caetano, Clark, & Schafer, 1999; Krug et al., 2002; Miczek, Barros, Sakoda, & Weerts, 1998). For example, emergency room patients admitted for violence-related injuries were more likely to have a high blood alcohol concentration, to have been drinking before the incident, and to report more frequent heavy drinking (Cherpitel, 1997). However, not all alcohol consumption is related to aggressive behavior; rather, there appears to be only a small percentage of individuals who show heightened aggression following alcohol consumption (Miczek et al., 1998; Higley, 2001). A small subpopulation of alcohol-heightened

aggressors (AHA) has been identified in a translation mouse model of aggression (Miczek et al., 1998; Figure 1).

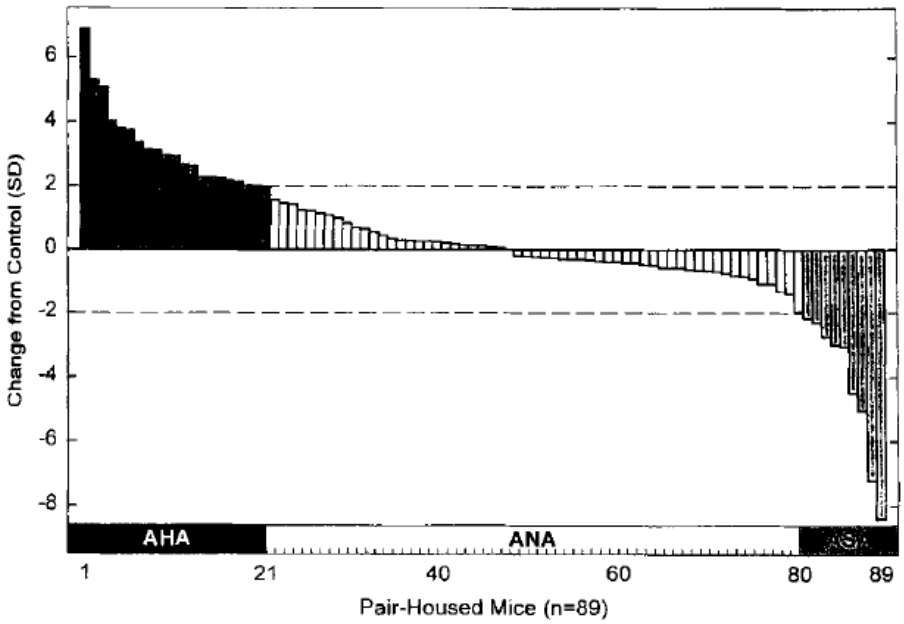

Figure 1. Characterization of mice as alcohol-heightened aggressors (AHA) and alcohol non-heightened aggressors (ANA). From Miczek et al., 1998.

Alcohol has a biphasic effect on aggression, increasing aggressive behavior at low doses but decreasing it at higher doses (Miczek et al., 1993). Additionally, the same dose of alcohol may increase aggressive behavior in some individuals while decreasing it or having no effect in others (Krug et al., 2002); individual differences are also seen in rodent and non-human primate populations (Miczek, Weerts & DeBold, 1993; Higley, 2001). Altogether, the mechanism of how alcohol affects aggressive behavior appears to be complex merits further study. However, due to ethical restraints, it is difficult to empirically study aggressive behavior in humans (Miczek, 2001; Higley, 2001). Previous studies in humans, such as Lang (1975), have used paradigms that employ the use of confederates to whom a participant may choose to administer a noxious stimulus (Lang, Goeckner, Adesso & Marlatt, 1975). Though the results from such studies give insight to alcohol-heightened aggression, the use of such protocols may cause lasting

psychological damage to participants. Furthermore, it is difficult to study the neurobiology of aggressive behavior without the use of invasive techniques and appropriate controls. Thus, while data relating to alcohol-escalated aggression in humans can provide some useful correlational information, it is necessary to collect empirical data in order to fully understand, and potentially treat, pathological aggressive behavior. To collect experimental data, investigators must instead rely on preclinical models of aggression in animal subjects. A widely-used rodent model of aggressive behavior is the resident-intruder paradigm (Miczek & O'Donnell, 1978a), in which a 'resident' male, housed in isolation or with a female, attacks a group-housed 'intruder' within the resident's home cage. This protocol can also be modified to evaluate the effects of social instigation on baseline levels of aggression (Fish et al., 1999). This paradigm is used in many investigations of aggression, and is utilized in the present study to examine the neurobiology of alcohol-heightened aggression.

Neurobiology of Aggression

Both offense and defensive aggressive behaviors are regulated by various pathways in the CNS, with key brain areas including the medial amygdala, anterior hypothalamus, ventromedial hypothalamus, lateral septum, bed nucleus of the stria terminalis (BNST), the periaqueductal grey (PAG), the medial prefrontal cortex (mPFC), and the orbitofrontal cortex (Miczek, Faccidomo, Fish & DeBold, 2007; Nelson & Trainor, 2007; Siegel, Roeling, Gregg & Kruk, 1999; Grafman et al., 1996; Carobrez, Teixeira & Graeff, 2001). In rodents, olfactory cues are received by the olfactory bulb, processed in the medial amygdala, and sent to the lateral septum, BNST, and the anterior hypothalamus (Delville, De Vries, & Ferris, 2000; DaVanzo, Sydow, & Garris, 1983). Neurons project from these brain areas to the PAG, a brain region involved in

species-specific defensive and aggressive behaviors (Carobrez et al., 2001). Lesions to these areas reduce aggression between male rats (Kruk, 1991). Conversely, expression of the immediate early gene product c-FOS increases in these brain areas during species-typical aggressive encounters in hamsters (Delville et al., 2000; Kollack-Walker & Newman, 1995) and mice (Davis & Marler, 2004; Hasen & Gammie, 2005, Lin et al., 2011). A similar pathway exists in primates, though the relevant sensory cues tend to be visual or vocal rather than olfactory (Nelson & Trainor, 2007). Electrical excitation in the anterior hypothalamus increases aggression in both male rats (Kruk et al., 1984) and rhesus monkeys (Robinson, 1967).

The neural circuitry of aggression is specific to the type of aggressive behavior (Miczek et al., 2007). Excitation of the PAG, resulting from stimulation of the ventromedial hypothalamus, will cause defensive rage behavior (which corresponds to the imupulsive aggression subtype), whereas excitation of the lateral hypothalamic attack area will yield predatory offensive behavior (which corresponds to the calculating, predatory aggression subtype) (Siegel et al., 1999; Miczek et al., 2007). In cats, brain sites associated with quiet biting predatory attack include the perifornical lateral hypothalamus, PAG, and the ventral tegmental area (Siegel et al., 1972). Conversely, studies in cats have linked defensive rage to activity in the ventromedial hypothalamus which projects to the dorsal PAG (Siegel et al., 1999).

The medial prefrontal cortex (mPFC), which projects to the amygdala, is often thought of as the origin of the circuit that mediates aggressive behavior. Specifically, the PFC, which regulates higher cortical function and social behavior, is thought to impede agonistic behavior by providing inhibitory projections to the hypothalamus and amygdala (Davidson, Putnam & Larson, 2000; de Bruin, Van Oyen & Van de Poll, 1983). Impairment in the PFC should thus eliminate this inhibition of aggressive behavior—in other words, “remove the brakes”—in

response to social and environmental provocation (Grafman et al., 1996). In support of this hypothesis, male rats with lesions in the orbitofrontal cortex (OFC), a subregion of the PFC involved in the interpretation of social cues and the execution of the appropriate behavioral response (Nelson & Trainor, 2007), show an increase in aggression (de Bruin et al., 1983). Similarly, lesions in the OFC increase aggressive behavior in dominant, but not subordinate, male rhesus monkeys (Machado & Bachevalier, 2006), although this appears to be a temporary effect (Butter & Snyder, 1972). In humans, damage to the frontal cortex has been correlated with higher aggressive behavior (Grafman et al., 1996; Anderson, Bechara, Damasio, Tranel & Damasio, 1999); similarly, there is correlational evidence that violent psychiatric patients show lower baseline metabolic activity in the prefrontal cortex compared to nonpatients (Scarpa & Raine, 1997; Volkow et al., 1995). Therefore, the prefrontal cortex is implicated in aggressive behavior across species.

Acute alcohol affects the circuitry of aggressive behavior by impairing executive function in the PFC, thereby reducing behavioral control and promoting impulsive responding, as well as by stimulating dopamingeric transmission in the ventral striatum (de Almeida, Ferrari, Parmigiani, & Miczek, 2005). Thus, the prefrontal cortex is a site of interest in alcohol-heightened aggression as well.

The PFC projects to the amygdala via serotonergic neurons. Consequentially, the amygdala is another major site of interest in the neural pathway of aggression. Here, a balance between glutamatergic excitation and GABA-ergic inhibition may mediate species-typical aggressive behavior, with escalated aggression resulting from an imbalance, or “limbic dyscontrol” (Miczek et al., 2007). Indeed, increased activation in the amygdala has been demonstrated in patients with intermittent explosive disorder who are shown angry faces. In this

group and in a control group, amygdala activation was correlated with higher scores on the Lifetime History of Aggression scale (Coccaro, McCloskey, Fitzgerald & Phan, 2007).

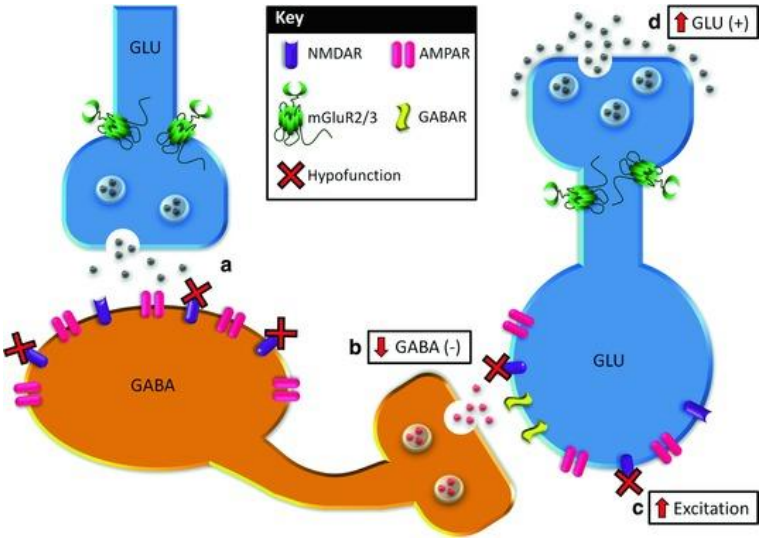

Figure 2. Inhibition and excitation via glutamatergic neurons and NMDA receptors on GABAergic neurons. From Sheffler & Jeffrey, 2010.

The neural circuitry of aggression is dependent on the activity of neurotransmitters within and between the summarized brain regions. While the majority of aggression research has focused on serotonin, other neurotransmitters such as dopamine, GABA, and glutamate also affect this pathway (Miczek, Fish, DeBold & de Almeida, 2002).

Serotonin and Aggression

Serotonin (5-HT), more than any other neurotransmitter, has been implicated in the neurobiology of impulsive aggressive behavior (Faccidomo, Quadros, Takahashi, Fish, & Miczek, 2012; Takahashi, Quadros, de Almeida, & Miczek, 2012). The prevailing theory suggests that low levels of serotonergic tone are correlated with increased impulsive aggression, as determined by measuring levels of serotonin precursors and metabolites (Faccidomo, Bannai

& Miczek, 2008; Fish et al., 1999). The role of serotonin in aggression depends on CNS localization, with the serotonin receptors in the PFC being highlighted for the execution of and recovery after an aggressive episode (Van Erp & Miczek, 2000; Miczek & Fish, 2006; Halasz, Toth, Kallo, Liposits, & Haller, 2006), as well as on the receptor family: 5-HT_1, 5-HT_2, or 5-HT_3. Within these classes, serotonin receptor types are further subdivided. The 5-HT_1 class is the one most often implicated in aggressive behavior; and above all, the 5-HT_{1A} and 5-HT_{1B} receptor subtypes (Fish et al., 1999; Takahashi et al., 2012).

Most CNS serotonin comes from the raphé nuclei; particularly, from the dorsal raphé nucleus (DRN) (Takahashi, Kwa, DeBold & Miczek, 2010). It is hypothesized that dysfunction in serotonergic projections from the DRN to the PFC is involved in aggressive behavior and impulsivity. Agonists of the 5-HT_{1A} and 5-HT_{1B} receptors have been demonstrated to dose-dependently attenuate species-typical aggression in rodent models (Faccidomo et al., 2008; Grafman et al., 1996; Spinella, 2004). Administration of a 5-HT_{1A} selective antagonist prior to administration of a 5-HT_{1A} agonist can eliminate the anti-aggressive effects of the agonist (Miczek, Hussain & Faccidomo, 1998). Additionally, mutant mice that lack the 5-HT_{1B} receptor displayed enhanced aggressive behavior compared to wild-type mice in the resident-intruder test (Saudou et al., 1994).

Serotonin is also implicated in escalated aggression following alcohol consumption. Specifically, presynaptic 5-HT_{1B} receptors in the medial PFC have been demonstrated to mediate alcohol-heightened aggression (Fish et al., 1999; Faccidomo et al., 2008). Furthermore, genes for the 5-HT_1 and 5-HT_2 receptors are expressed less in the PFC of rodents who show alcohol-heightened aggression when compared to mice who do not show escalated aggression (Miczek et al., 2007). While the role of serotonin in alcohol-heightened aggression is thus well established,

it is interesting to consider not only release from serotonergic neurons via serotonin autoreceptors, but also via glutamatergic and GABA-ergic input to serotonergic neurons in brain regions of interest (Miczek et al., 2007).

Glutamate

Glutamate is the major excitatory neurotransmitter in the brain, affecting nearly all cognitive function and behavior, with an especially crucial role in learning and memory (Bliss & Collingridge, 1993). Glutamate is the primary ligand at both ionotropic and metabotropic receptors. There are three major classes of ionotropic glutamate receptors: the NMDA receptor, the AMPA receptor, and the kainate receptor.

As previously mentioned, an imbalance between glutamatergic excitation and GABA-ergic inhibition in the limbic system has been proposed to escalate aggressive behavior (Miczek et al., 2007). Glutamate receptors influence serotonergic neurons in some brain regions. Specifically, glutamate has been demonstrated to control serotonergic tone in the DRN; infusion of glutamate agonists in the DRN yields an increase in extracellular serotonin (Tao & Auerbach, 2000). Regulation of serotonin by glutamate receptors may explain a role for glutamate in behaviors mediated by serotonin, such as aggression.

NMDA Receptor

The NMDA receptor (NMDAR; Figure 2) is an ionotropic glutamate channel permeable to Na^{2+} and Ca^{2+} and blocked by Mg^{2+} in a voltage-dependent manner. Binding of glutamate and the receptor co-agonist, glycine, causes the channel to open and allows the conduction of ions across the membrane, which stimulates neurotransmitter release into the CNS by neuronal

depolarization. Clinically, NMDAR has been implicated in treatment for many psychological disorders including alcoholism and Post-Traumatic Stress Disorder, as well as neurological disorders such as Alzheimer's Disease (AD) and epilepsy (Krystal et al., 1998; Kew & Kemp, 2005; Tsai & Coyle, 1998).

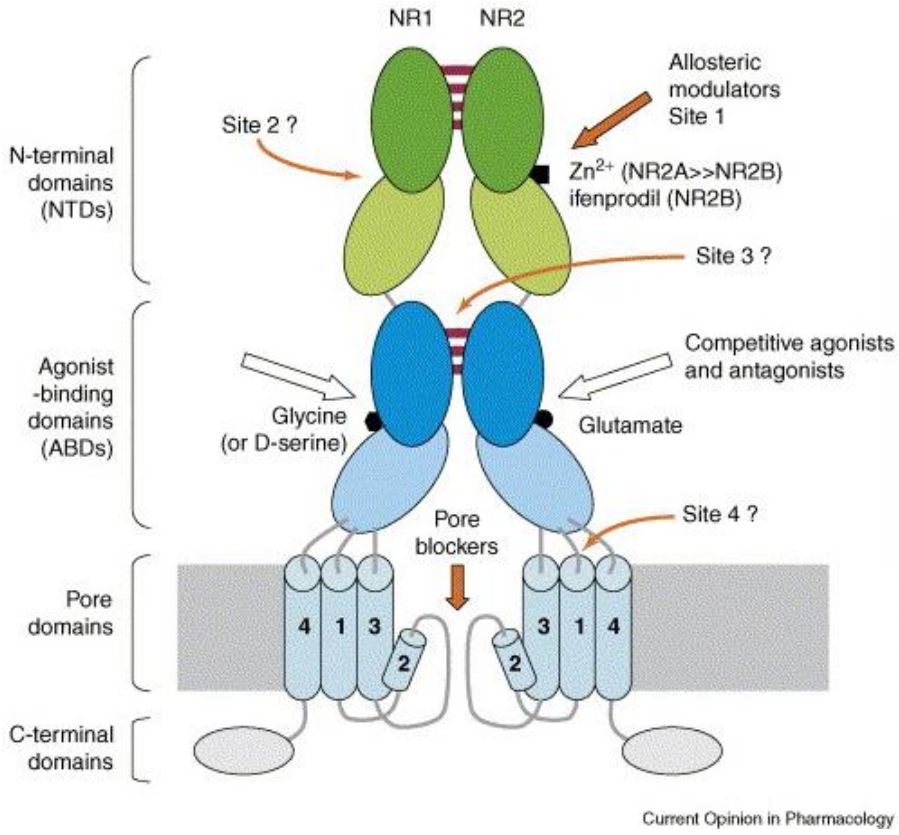

Figure 3. NMDAR composition and ligands.

Seven NMDAR subunits have been identified: NR1, NR2A-D, and NR3A-B. Glutamate binds to the NR2 subunit, while glycine binds to the NR1 subunit. A functional NMDAR is a tetramer that requires a dimer composed of NR1 subunits and a dimer of NR2A subunits (NR1/NR2A-D), but may also be composed of an NR2 subunit and an NR3 subunit (NR1/NR1A-D/NR3A) (Kew & Kemp, 2005; Hansen, Brauner-Osborne & Egebjerg, 2008; Paoletti & Neyton, 2007). The NMDAR subunits show differential localization; the NR1 and NR2A subunits are fairly ubiquitous throughout the CNS, whereas the NR2B subunit is

concentrated in the forebrain and the NR2C and NR2D subunits are principally found in the cerebellum and midbrain regions (Kemp & McKernan, 2002; Kew & Kemp, 2005; Chenard & Menniti, 1999; Mosley et al., 2010a; Traynelis et al., 2010; Parsons, Danysz & Quack, 1998; Danysz & Parsons, 1998). The NR2 subunit has been demonstrated to mediate agonist/antagonist sensitivity of the receptor, with the NR2A subunit being most sensitive to antagonism while the NR2B-D subunits being most sensitive to agonism (Woodward, 1999).

Various pharmacological manipulations result from the NMDAR composition. Agonists may act at the glutamate binding site, such as the ligand for which the receptor is named, *N*-methyl-D-aspartate (NMDA). However, agonism of the glutamate binding site generally results in excitotoxicity when applied systemically (Leeson & Iversen, 1994). It is therefore more common to systemically agonize the glycine binding site, which does not typically result in excitotoxicity (Lanthorn, 1993). Common agonists of the glycine binding site include D-serine, D-cycloserine, and (+)HA-966 (Dunn et al., 1992). Competitive antagonists, compounds that bind at the same site at the endogenous ligand glutamate, include AP-V and AP-7 (Kew & Kemp, 2005). Uncompetitive antagonists, which bind within the open channel pore and therefore can only bind to receptors at which glutamate and glycine are already bound, typically fall into two categories: PCP-like ligands and memantine-like ligands. PCP and PCP-like ligands, including ketamine and dizocilpine (MK-801) block the channel pore and tend to have undesirable side effects such as dissociative anesthesia and hallucinations (Chen & Lipton, 2006; Porter & Greenamyre, 1995). Memantine and memantine-like ligands, such as neramexane, are open-channel blockers with a fast on/off rate; as a result, they permit normal glutamatergic transmission and therefore have fewer undesirable side effects (Johnson & Kotermanski, 2006; Gilling, Jatzke, Hechenberger & Parsons, 2009).

Noncompetitive antagonists, which bind at a different site than glutamate but do not require that the co-agonists already be bound, may act at various sites on the NMDAR. Ligands have been developed to selectively antagonize different NMDAR subtypes, with NR2B antagonists such as ifenprodil and eliprodil being the most prominent and selective. Currently the most specific and selective ligand that acts primarily at the NR2A receptor is zinc (Nozaki et al., 2011) although new compounds are currently being synthesized. Derivatives of quinazolin-4-one act as selective noncompetitive antagonists at receptors containing the NR2C or NR2D subunit (Mosley et al., 2010b).

Acute ethanol acts as a functional noncompetitive antagonist at the NMDAR (Lovinger et al., 1989; Woodward, 2000; Lovinger, 1996; Hoffman et al., 1990); chronic ethanol causes upregulation of the NMDAR to compensate for the effects of acute ethanol (Hoffman et al., 1990; Allgaier, 2002). Although there is some uncertainty about its exact site of action on the NMDAR, proposed locations include a site on the NR2B subunit (Yaka, Tang, Camarini, Janak & Ron, 2003; Wang et al., 2007; Boyce-Rustay & Holmes, 2005; Badanich et al., 2011), the glycine binding site (Ferreira & Morato, 1997; Lovinger, 1996), or a phenylalanine residue in the TM3 domain of the NR1 subunit (Allgaier, 2002). Electrophysiological data show that at the NMDAR, the effects of alcohol may be dependent on glycine concentration (Rabe & Tabakoff, 1990). Evidence for action on the NR2B subunit is supported by evidence that demonstrates a greater inhibitory effect of acute ethanol on NR1/NR2A and NR1/NR2B NMDARs compared to NR1/NR2C and NR1/NR2D NMDARs (Allgaier, 2002; Masood, Wu, Brauneis & Weight, 1994; Mirshahi & Woodward, 1995). Furthermore, studies using pharmacological blockade of the NR2B subunit as well as knockout mice suggest that ethanol inhibits NMDA receptors containing the NR2B subunit only (Wills et al., 2012; Kash, Matthews, & Winder, 2008).

NMDARs in the DRN are found on serotonergic neurons (Adell, Celada, Abellan, & Artigas, 2002), and thus may modulate aggressive behavior related to low levels of extracellular serotonin. Therefore, manipulations at the NMDA receptor may affect aggressive behavior.

D-Cycloserine

D-Cycloserine is a high-affinity partial co-agonist at the glycine binding site of the NMDA receptor, located on the NR1 subunit (Hood, Compton, & Monahan, 1989; Nunnink, Davenport, Ortega, & Houpt, 2007). In the absence of glycine, D-Cycloserine appears to act as an agonist; however, as the glycine concentration increases, D-Cycloserine becomes an antagonist by competing for the receptor site and reducing the maximal level of glycine stimulation (Hood et al., 1989; Emmett et al., 1991). It has been proposed that cyclic homologues of glycine display mixed agonist/antagonist properties, and that the ratio of agonist to antagonist properties decreases as the size of the ring increases (Watson & Lanthorn, 1990). D-Cycloserine has been demonstrated to act as an agonist at doses below 20 mg/kg in mice (Danysz & Parsons, 1998), and increases in antagonistic activity as the dose increases (Lanthorn, 1994). Some reports show that the different effects of D-cycloserine may be due to varying affinities for the different NMDA subtypes; D-cycloserine has been shown to be more effective than glycine at receptors containing the NR2C subtype, but less effective than glycine at receptors containing the NR2A or NR2B subtypes. Therefore, in low doses D-cycloserine may act as an agonist at receptors containing the NR2C subtype, but in high doses will act as an antagonist at receptors containing the NR2A/B subtypes (O'Conner et al., 1996; Sheinin et al., 2001).

While most NMDA agonists result in excitotoxicity, the partial agonist character of D-cycloserine grants it a safe clinical profile (Lanthorn, 1994). For many years, it has been

clinically used as an antibiotic for tuberculosis; more recently, however, D-Cycloserine has undergone Phase III clinical trials for its therapeutic potential in relieving social inhibition in autism spectrum disorders (Posey et al., 2004), negative symptoms of schizophrenia (Goff et al., 1999). Clinically it has also been investigated for its use in enhancing implicit memory in Alzheimer's patients (Schwartz, Hashtroudi, Herting, Schwartz & Deutsch, 1996) and in associative learning to reduce phobias (Ressler et al., 2004). Preclinically, D-cycloserine has been studied for its effects on anxiety (Anthony & Nevins, 1993; Fujiwara, Takahashi & Hirai, 2004), sociability (McAllister, 1994; Deutsch, Burket, Jacome, Cannon & Herndon, 2011; Jacome, Burket, Herndon & Deutsch, 2011), cognitive function (Tin-Tin, Shiho & Shinji, 2010), learning (Nunnink et al., 2007), locomotor activity (Carlsson, Engberg & Carolsson, 1994) and sedation (Irifune, Shimizu & Nomoto, 1992).

Various preclinical studies indicate that D-cycloserine might interact with ethanol at the NMDA glycine site (Rabe & Tabakoff, 1990). D-Cycloserine has been demonstrated to block anxiolytic properties of ethanol in rats (Ferreira & Morato, 1997) as well as potentiate the antiseizure efficacy of ethanol in mice (Deutsch et al., 1990). Because D-cycloserine has been demonstrated to reduce aggressive behavior in the resident-intruder test (McAllister, 1994), it is thus interesting to consider whether D-cycloserine may affect alcohol-heightened aggression.

Ifenprodil

Ifenprodil is the prototypic noncompetitive antagonist specific for the polyamine recognition site of the NR2B subunit of the NMDA receptor (Carter et al., 1988; Williams, 1993). The compound also acts as an antagonist at both serotonin 5-HT_3 receptors (McCool &

Lovinger, 1995) as well as α_1 adrenoceptors (Malinowska, Napiorkowska-Pawlak, Pawlak, Buczko & Gothert, 1999).

Clinically, ifenprodil was first used as a cerebral anti-ischemic agent (Gotti et al., 1988). As ifenprodil and other NR2B-selective antagonists generally have a better clinical safety profile than nonselective NMDA antagonists (Kemp & McKernan, 2002; Tzschentke, 2001), these compounds are good therapeutic candidates for glutamate-related pathologies. When administered systemically in mice, ifenprodil was shown to have anxiolytic properties, and to block the anxiolytic properties of dizolcipine (Fraser, Cooke, Fisher, Thompson & Stone, 1996). Additionally, ifenprodil was demonstrated to have antidepressant activity in a mouse model of depression (Ghasemi, 2010) and to suppress the rewarding effects of morphine (Suzuki, 1999).

Preclinically, ifenprodil has been noted to interact with behavioral effects of ethanol. In vivo, ifenprodil was shown to dose-dependently increase or decrease ethanol-induced hypnosis in Swiss mice (Malinowska et al., 1999), to attenuate ethanol's amnesic effects in Swiss mice (Napiorkowska-Pawlak, Malinowska, Pawlak, Buczko & Gothert, 2000). Various in vitro studies also suggest an interaction between ifenprodil and ethanol (Allgaier, 2002; Fink & Götherd, 1996; Lovinger, 1995). Specifically, electrophysiological data demonstrate that ethanol may act specifically on an ifenprodil-specific NMDA receptor subtype (Fink & Gothert, 1996; Yang et al., 1999). Studies using knockout mice that lack the gene for the NR2B subunit also imply a role of the NR2B subunit in regulating both low-dose stimulant effects as well as the depressant effects of ethanol (Badanich et al., 2011). Studies using other NR2B antagonists, such as the compound Ro 25-6981, also appear to affect the sedative actions of ethanol in mice (Boyce-Rustay & Holmes, 2005). As a result of these studies, the NR2B subunit appears to be involved in the sensitivity of NMDA receptors to ethanol.

Amantadine

Amantadine, like memantine, acts as a low-affinity uncompetitive antagonist at the NMDA receptor, binding to the open channel pore. Like memantine, amantadine binds to a site on the NMDAR where it can remain trapped after the channel closes (Blanpied, Boeckman, Aizenman & Johnson, 1997). Historically, amantadine has been studied for its effectiveness in increasing synaptically available dopamine, particularly in the striatum (Starr & Starr, 1995). Research has shown that amantadine is most likely not an agonist at dopamine receptors (Starr & Starr, 1995), but rather may act presynaptically to increase dopamine synthesis and/or release, and block dopamine reuptake (Starr & Starr, 1995; Reis et al., 2006). The actions of amantadine at the NMDA receptor were more recently discovered. In addition to blocking the channel pore, amantadine also acts noncompetitively at the σ_1 binding site located outside the NMDA channel, a site at which memantine does not appear to bind (Kornhuber, Schoppmeyer & Riederer, 1993).

Amantadine has, for several decades, been used clinically for the treatment of influenza A virus as well as neurological conditions such as Parkinson's disease, dementia, multiple sclerosis, chronic pain, and cocaine withdrawal (Kaefer et al., 2010; Svensson & Stromberg, 1970). Preclinically, amantadine has been studied for possible effects on OCD-like behavior (Nobuaki et al., 2008), locomotor activity (Starr & Starr, 1995), stereotypies (Gumulka, Dinnendahl, Peters & Schonhofer, 1976), and alcohol drinking (Escher, Call, Blaha & Mittleman, 2006). Recent data (Newman et al., 2012, *in press*) have demonstrated that memantine increases alcohol-heightened aggression; however, amantadine has yet to be examined for its effects on aggressive behavior.

Objective

The purpose of the present study was to determine how modulation of the NMDAR affects alcohol-heightened aggression. This was done using by pharmacological manipulations that affect three distinct sites on the NMDAR that have been hypothesized to interact with ethanol antagonism: the glycine-binding site, the NR2B subunit, and the receptor channel itself. The glycine-site agonist D-cycloserine, the NR2B-selective antagonist ifenprodil, and the open-channel blocker amantadine were administered systemically following alcohol self-administration. The resident-intruder test was then used to determine whether alcohol interacted with each compound to affect aggression. Three hypotheses were tested. First, because D-cycloserine acts as a partial agonist, it was hypothesized that it would abolish escalated aggression at doses that stimulate the receptor but would potentiate alcohol-heightened aggression at higher doses. Second, it was hypothesized that ifenprodil, as a noncompetitive antagonist, would potentiate alcohol-heightened aggression. Third, it was hypothesized that amantadine, as a precursor of memantine, a compound previously shown to potentiate alcohol-heightened aggression, would also potentiate alcohol-heightened aggression. While these compounds appear to interact with alcohol, the results of these studies highlight future directions in the delineating the role of the NMDAR in the neural circuitry of alcohol-heightened aggression.

Methods

Animals

Adult male and female Swiss-derived Cartworth Farms Webster (CFW) mice were purchased from Charles River Laboratories (Wilmington, MA, USA), weighing 21-25g on

arrival. Resident males were housed in breeding pairs in clear polycarbonate cages (28 × 17 × 14 cm) with pine shavings as bedding material and stainless-steel wire lids. Rodent chow (Purina Lab Chow,) and tap water were freely available. Male "intruder" CFW mice were housed in groups of 10 per cage (48 × 26 × 14 cm) with corncob bedding and stainless-steel wire lids. Animals were kept on a reversed 12 h light/dark cycle (lights off at 07:00), in a vivarium maintained at 21±1°C. All testing was conducted during the dark cycle. Mice were cared for according to the NIH guidelines for the Care and Use of laboratory animals and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Tufts University.

Drugs

Synthetically derived D-cycloserine [(*R*)-4-Amino-3-isoxazolidone], ifenprodil (+)-tartrate [α-(4-Hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol (+)-tartrate salt], amantadine hydrochloride [1-Aminoadamantane hydrochloride], and S(-)-raclopride (+)-tartrate [3,5-Dichloro-N-(1-ethylpyrrolidin-2-ylmethyl)-2-hydroxy-6-methoxybenzamide (+)-tartrate salt] were purchased from Sigma-Aldrich (Saint Louis, MO). D-Cyloserine, amantadine and raclopride were dissolved in 0.9% saline solution. Ifenprodil was dissolved in distilled water. Final doses (D-cycloserine: 0, 10, 17, 100 mg/kg; ifenprodil: 0, 0.3, 1.0, 10 mg/kg; amantadine: 0, 10, 17, 30 mg/kg; and raclopride: 0, 0.5 mg/kg) were administered intraperitoneally (i.p.) in a volume of 10 ml/kg.

A 6% (w/v) ethanol solution was prepared by diluting 95% ethyl alcohol (Pharmco-Paper Products Inc., Brookfield, CT) with tap water and was self-administered (1.0 g/kg).

Alcohol Self-Administration.

Pair-housed residents were water-restricted Monday through Friday for 21 h daily. During self-administration and drug testing, female and pups were removed from the home cage. Resident males were conditioned to nose-poke for a liquid reward on an FR5 schedule (fixed ratio 5, in which every fifth correct response rewarded). Figure 4 illustrates the full schedule of operant conditioning task acquisition.

Figure 4. Schedule of ethanol self-administration task acquisition.

An aluminum panel (16.5 × 15.9 cm) was inserted into the home cage (Miczek and de Almeida, 2001). The panel contained cue lights over two troughs and a central house light. Animals nose-poked, half left-side and half right-side, for a 50 µl water or 6% (w/v) ethanol reward (resulting in a dose of 1.0 g/kg), delivered by a syringe pump connected to the trough by plastic tubing and operated by MED-PC software (Med Associates). Operant responses were registered when animals interrupted the horizontal photobeam in the correct trough; the house light was turned off during reward administration to facilitate associative learning. Animals were trained in this task for three weeks before beginning experimental testing. Water and ethanol sessions alternated daily once animals had acquired the operant conditioning task.

Resident-Intruder Paradigm

After acquiring the operant conditioning task, resident animals were evaluated for aggressive behavior. The female and pups were removed from the home cage, and a specific intruder mouse was introduced. During resident-intruder confrontations, the wire lid containing food pellets was removed from the home cage and replaced with a clear plastic lid. Attack bite frequency was recorded for 5 minutes following the first bite(Miczek & O'Donnell, 1978b). If no attack bites occurred, the intruder mouse was removed from the home cage after 5 minutes. A specific intruder mouse was assigned to each resident and was replaced if the intruder attacked the resident or if the resident's attack bite frequency was unusually low. If the intruder attacked the resident, the session was terminated and the intruder was immediately removed. Residents whose intruders were replaced more than five times due to unusually low attack bite frequencies were not included in the data. After becoming stable (<20% variability), residents were habituated to an i.p. injection of vehicle administered 20 minutes before the introduction of the intruder. If aggressive behavior remained stable after 6-8 tests, alcohol-heightened aggression experimental sessions began. To maintain high, stable levels of aggression, resident-intruder confrontations only occurred every 48 hours.

Characterization

After the rate of attacks stabilized (<20%), resident mice were characterized as alcohol-heightened aggressors (AHA) or alcohol non-heightened aggressors (ANA) (Miczek et al., 1998). Resident mice self-administered on alternating days either water or 1.0 g/kg of 6% (w/v) ethanol. Resident-intruder confrontations occurred 10 minutes later. Each subject had two sessions with water and two with ethanol. AHA mice were defined as resident mice with attack bite frequencies after ethanol self-administration of at least 2 standard deviations above their

attack bite frequencies after water self-administration (Miczek et al., 1998). The remaining mice were characterized as ANA mice.

Alcohol-Heightened Aggression

Resident mice self-administered either water or 1.0 g/kg ethanol immediately before being injected intraperitoneally with either 10 ml/kg drug (D-cycloserine, ifenprodil, or amantadine) or the corresponding vehicle (0.9% saline, dH_2O, and0.9% saline, respectively), in doses that were selected based on previous reports (McAllister, 1994; Malinowska et al., 1999; Escher et al., 2006) and on the basis of preliminary results. Doses were administered according to a Latin Square design. Resident-intruder confrontations occurred 20 minutes after the injection. Latency to first attack bite and frequency of attack bites within five minutes after the first bite were measured. Resident-intruder confrontations were conducted every other day for a maximum of three sessions per week.

Video Analysis

Aggressive confrontations between a resident and an intruder were recorded using a JVC Everio digital camera and the video files were uploaded to a computer. Files were analyzed at a later time by a trained observer (intra-observer reliability $r>.95$) for the frequency and duration of eight salient aggressive and non-aggressive behaviors (Table 1) shown by the resident mouse. Video files were analyzed using The Observer XT Software (Noldus v. 9.0: Wageningen, The Netherlands).

Table 1

Aggressive and Non-aggressive Behaviors Analyzed

Aggressive Behaviors		Non-aggressive Behaviors	
Bite	Resident contacts intruder with teeth	Walk	Resident displays motor activity with all four limbs
Sideways Threat	Resident angles himself at a right angle to intruder's body	Rear	Resident raises himself on hind legs either in open air or against the wall of the cage
Tail Rattle	Resident rapidly undulates tail	Self-Groom	Resident washes face with paws or licks flank
Pursuit	Resident chases intruder	Contact	Resident initiates naso-nasal or ano-genital contact but does not bite

Statistics

Repeated-measures two-way analyses of variance (ANOVAs) were performed to examine the effect of the drug treatment after ethanol and water consumption on the frequency, duration, and/or percent change from baseline for behavioral measures ($\alpha = .05$) (SigmaStat 11). The Holm-Sidak post-hoc test was used when appropriate.

Results

Aggression Screening

Animals were screened for aggressive behavior via the resident-intruder test prior to beginning the characterization phase and drug experiments. Animals achieved stable levels of aggressive behavior (<20% variability; Winslow & Miczek, 1983) in order to continue in the experiments (Figure 5). Only animals in experiments 1 and 2 (n=18) underwent 6-7 screening sessions; animals in experiment 3 that achieved stable aggressive behavior in 2-3 screening sessions proceeded in the experiment.

Experiment 1: D-Cycloserine

Aggressive Behavior

Ethanol did not affect attack bite frequency, the percent change of attack bites, or the frequency of sideways threats for the group as a whole (see Figures 6, 7 and 8). There was a significant main effect of D-cycloserine dose on attack bite frequency [F(3,9)=5.655, p=0.004], and on the frequency of sideways threats [F(3,09)=6.959, p=0.001]. D-cycloserine also had a significant main effect on the percent change of attack bites from vehicle administration [F(3,9)= 3.326, p=0.034]. Animals characterized as AHA mice were also analyzed as a separate subgroup.

Non-aggressive Behavior

D-cycloserine significantly increased the duration of walking compared to vehicle administration ([F(3,9)= 6.552, p=0.002]; see Figure 9]. There was a significant main effect of ethanol administration on rearing behavior ([F(1,9)= 7.776, p=0.021]; see Figure 10].

Aggressive Behavior in AHA Mice

Three animals were characterized as AHA (2 SD > X_{H2O}). There was a significant interaction between ethanol and D-cycloserine dose on attack bite frequency ([F(3,6)=6.336, p=0.027]; see Figure 11). Post-hoc analysis revealed a selective increase in attack bite frequency at the 10 mg/kg dose and a selective reduction at the 100 mg/kg dose following water self-administration.

Experiment 2: Ifenprodil

Aggressive Behavior

There was a significant main effect of ethanol [F(1,9)= 8.626, p=0.017] and ifenprodil dose ([F(3,9)= 5.652, p=0.004]; see Figure 12) on attack bite frequency. Post-hoc analysis revealed a selective reduction in attack bite frequency at the 10 mg/kg dose following ethanol self-administration. Additionally, there was a trend towards a higher attack bite frequency at the

0.3 mg/kg dose after ethanol but not water consumption, though this was not statistically significant [t=1.994, p=0.054]. Ifenprodil also had a significant main effect on sideways threat frequency ([F(3,9)= 6.983, p=0.001]; see Figure 13); post-hoc analysis revealed a reduction in the frequency of sideways threats at the 10 mg/kg dose after both water and ethanol self-administration. Animals did not undergo characterization in this experiment and were not divided into AHA and ANA mice.

Non-aggressive Behavior

Ifenprodil had a significant main effect on walking duration ([F(3,9)= 10.483, p<0.001]; see Figure 14) and on rearing behavior ([F(3,9)= 3.090, p=0.044]; see Figure 15). Post-hoc analysis revealed a reduction in walking duration at the 10 mg/kg dose following both water and ethanol self-administration.

Experiment 3a: Amantadine

Aggressive Behavior

Ethanol significantly increased the attack bite frequency independent of amantadine dose for the group as a whole [F(1,10)=8.982, p=0.013]; see Figure 16). There was a significant main effect of amantadine on both attack bite frequency and percent change in attack bites from baseline ([F(3,10)= 11.071, p<0.001; F(3,10)= 8.757, p<0.001, respectively]; see Figure 17). Post-hoc analysis revealed a selective increase in attack bite frequency after ethanol self-administration at the 17 mg/kg dose. Amantadine also had a significant main effect on sideways threat frequency ([F(3,10)=6.725, p=0.001]; see Figure 18). Post-hoc analysis revealed a selective increase in sideways threat frequency after ethanol self-administration at the 17 mg/kg

amantadine dose. Animals did not undergo characterization in this experiment and were not divided into AHA and ANA mice.

Non-aggressive Behavior

Amantadine had a significant main effect on walking duration ([F(3,10)=4.124, p=0.015]; see Figure 19. There was a significant main effect of ethanol on the duration of rearing ([F(1,10)=9.279, p=0.012]; see Figure 20). Amantadine also had a significant main effect on rearing duration [F(3,10)=2.911, p=0.051]; post-hoc analysis revealed a selective decrease in rearing duration after ethanol self-administration at the 17 and 30 mg/kg doses.

Experiment 3b: Raclopride Challenge

Aggressive Behavior

Pretreatment with 0.5 mg/kg raclopride significantly decreased the frequency of attack bites when administered prior to ethanol self-administration and 17 mg/kg amantadine ([F(1,5)= 14.444, p=0.013]; see Figure 21).

Non-Aggressive Behavior

Raclopride pretreatment significantly increased the duration of walking ([F(1,5)=12.312, p=0.017]; see Figure 22).

Discussion

Manipulations at the glycine-binding site and NR2B subunit did not significantly affect alcohol-heightened aggression on average, while manipulation at the channel pore via an uncompetitive antagonist potentiated alcohol-heightened aggression. This is consistent with the potentiating effect of memantine, an uncompetitive antagonist at the NMDAR, on alcohol-heightened aggression (Newman et al., *in preparation*). While all drugs showed a decrease in

aggressive behavior at the highest administered dose, this effect of ifenprodil and amantadine is likely due to a sedative effect, as implied by decreases in walking and rearing behaviors.

The effect of the uncompetitive antagonist amantadine might be due to antagonism of NMDARs on serotonergic neurons in the dorsal raphé nucleus, which project to the medial prefrontal cortex. Decreased serotonergic tone has already been proposed to lead to an increase in aggressive behavior, including after alcohol drinking, while increased tone will attenuate aggression (Faccidomo et al., 2008; Spinella, 2004; Miczek, Hussain & Faccidomo, 1998).

D-Cycloserine, a ligand at the glycine-binding site on the NMDAR, showed a decrease in aggressive behavior at the 100 mg/kg dose. This decrease does not appear to be due to sedative effects, and is consistent with previous literature (McAllister, 2004). There was no effect of alcohol on this anti-aggressive effect; however, when considering only the mice characterized as AHA, there does appear to be an ethanol effect, with alcohol consumption at the 100 mg/kg dose appearing to restore attack bite frequency to a level similar to that following vehicle administration. If D-cycloserine was analyzed purely as an agonist, this result would be consistent with the hypothesis that increased NMDA activation on serotonergic neurons in the DRN increases serotonergic tone in the mPFC and thus decreases aggressive behavior. Then, ethanol would appear to be antagonizing this effect in AHA mice. However, at 100 mg/kg, D-cycloserine is reported to act as an antagonist, not as an agonist (Danysz & Parsons, 1998; Lanthorn, 1994).

An explanation potentially lies in the differential intrinsic activity of D-cycloserine at the NMDAR subtypes; compared to glycine, D-cycloserine has an intrinsic activity of 130% at NR2C-containing NMDARs, but an intrinsic activity of 38% and 56% at NR2A- and NR2B-containing NMDARs, respectively (O'Conner et al., 1996). However, the NR2C subunit is

localized in the cerebellum, which is not hypothesized to be a part of the neural circuitry of aggressive behavior. Interestingly, there is recent literature that reports that memantine is weakly selective for NMDA receptors containing the NR2C subunit (Kotermanski & Johnson, 2009; Rogawski & Wenk, 2003), although historically memantine has been considered to be nonselective (Bresink et al., 1996). However, there is a rather large body of literature that depicts the NR2B subunit as responsible for the intoxicating effects of ethanol (Malinowska et al., 1999; Boyce-Rustay & Holmes, 2005) and for behaviors such as conditioned defeat and fear conditioning (Day, Cooper, Markham & Huhman, 2011) that could be part of a social behavior network. In fact, the specificity of ethanol for the NR2B subunit has been reported in the bed nucleus of the stria terminalis (BNST; Kash et al., 2008; Wills et al., 2012), a brain site well-established as part of the neural pathway mediating aggression (Shaikh, Brutus, Siegel, & Siegel, 1986). Furthermore, while not significant, administration of the NR2B antagonist ifenprodil seemed to increase aggressive behavior after alcohol drinking compared to water drinking. More animals should be added to the experiment to better determine if there is an ifenprodil effect.

There are several directions in which future studies could attempt to illuminate the role of the NMDAR subunit in alcohol-heightened aggression. Most importantly, more animals should be added to these experiments. The addition of AHA mice in particular would be useful. Second, infusion of compounds into specific brain regions would be informative. Given the focus on the BNST in NMDAR antagonism by ethanol, this would be one particular site of interest. Another would be the dorsal raphé, as NMDAR-containing serotonergic neurons that regulate aggressive behavior primarily originate here. Third, the utilization of knockout mice that do not express a particular NR2 subunit might be useful, although the elimination of one subunit might result in upregulation of the other subunits. It would be interesting to see whether escalated aggressive

behavior after ethanol consumption occurs in mice that do not express the NR2B subunit. A third direction would be to continue systemically administration of compounds that are selective for the other NR2 subunits. Lastly, it would be interesting to determine if administration of compounds prior to ethanol self-administration yields the same results as when administered immediately following self-administration.

One of the most important factors to consider is the high on/off rate of amantadine and memantine, which distinguishes them from other channel blockers of the NMDA receptor. Because of these unusual kinetics, amantadine and memantine only block pathological NMDA activation that results from abnormally high glutamatergic tone, but permits= normal levels of NMDA activation (Porter & Greenamyre, 1995). This type of blockage is unusual and may account for the potentiation of alcohol-heightened aggression, by amantadine, memantine, and the memantine-like compound neramexane, but not seen in ketamine (Newman et al, 2012, *in press*). If in fact it is the low affinity and rapid association/dissociation rate of memantine that is responsible for its effects on alcohol-heightened aggression, then a similar effect was predicted to occur after administration of the precursor amantadine. While there was no significant interaction of ethanol and amantadine, there is significantly heightened aggression at the 17 mg/kg dose only after ethanol consumption.

One AHA mouse was identified on the basis of preliminary data and showed a significant escalation of aggression at the 17 mg/kg dose after ethanol self-administration. While this mouse did not undergo the full characterization phase and therefore cannot be identified as AHA with confidence, the data is suggestive of an ethanol-amantadine interaction. As with cycloserine, it will be helpful to study systemic administration of amantadine in a larger population of AHA mice.

Because initial trials of amantadine administrations indicate potentiated alcohol-heightened aggression at the 17 mg/kg dose, a dopamine antagonist experiment was conducted to determine if the effects of amantadine were due to its actions at NMDA receptors or at dopamine D2 receptors. However, the results prove to be inconclusive. While raclopride challenge significantly reduced the frequency of attack bites, it appeared to increase the frequency of sideways threats; additionally, an increase in walking duration but a decrease in rearing behavior do not make it clear whether there is a sedative effect. It is also possible that there was an effect of the injection prior to drinking as well as due to aging that confound these data. When comparing data collected from the animals who received ethanol and 17 mg/kg amantadine to data from the same animals when they received a saline injection prior to ethanol and 17 mg/kg amantadine, there appears to be a difference in most behaviors, even though the drug treatment was effectively the same. Therefore, it is still unclear whether the pro-aggressive effects of amantadine after ethanol administration result from its agonist effects at D2 receptors. Dopamine has been previously implicated in the neurobiology of aggressive behavior (Miczek et al., 2002), and raclopride has been previously demonstrated to decrease aggressive behavior (Aguilar, Minarro, Perez-Iranzo, & Simon, 1994), although this may be a consequence of sedative effects (Rodriguez-Arias, Pinazo, Minarro, & Stinus, 1999). Therefore, it is still important to clarify the role of amantadine as a D2 agonist versus as an NMDAR antagonist in alcohol-heightened aggression. Ideally, the raclopride challenge could be continued in a larger population of AHA-only mice within a shorter experiment. Furthermore, the effects of amantadine or memantine infusions into the dorsal raphé could be compared to infusions in the ventral tegmental area (VTA), the brain region from which dopaminergic projections to the prefrontal cortex originate

and are activated by ethanol (Gessa, Muntoni, Collu, Vargi, & Mereu, 1985). A microinjection or microdialysis study might provide clearer data than would be obtained from systemic injections.

There are several limitations to the current experiments. Primarily, the time course of the experiments varied, with various advantages and disadvantages. This was in part because drug dosages were partially determined on the basis of preliminary results, and in part due to differences in the screening and characterization phases. Animals in the first experiment went through both a full screening and characterization phase. As a result, AHA mice could be identified with confidence. However, animals showed a reduction in baseline aggressive levels over the time course of the experiment that is most likely attributable to aging and experimental factors. To avoid this, the screening phase was shortened and the characterization phase was essentially eliminated in the second and third experiments. The advantage was that animals maintained high and stable aggression levels longer throughout the experiment time course; however, this presents a difficulty in identifying AHA mice. Before settling on any conclusions, these experiments should be replicated with animals undergoing the full screening and characterization phases; because the drug dosage will be known, the overall experimental time course should be sufficiently short to avoid diminishing aggressive behavior.

In summary, although pharmacological manipulations at the glycine-binding site and NR2B subunit of the NMDA receptor did not affect alcohol-heightened aggression, there are several future investigations that can be directed based on the results of these experiments. Ideally, these compounds should be studied in a large population of AHA mice. However, because such a small proportion of animals tend to be characterized as AHA, this is a difficult task. Microinjection studies of these compounds into the DRN and the BNST would also be illuminating, and potentially more feasible. A microinjection study of a full agonist of the

NMDAR such as D-serine should also be conducted to gain a better understanding of how modulation of the glycine-binding site does or does not affect aggressive behavior. The potentiating effect of amantadine on alcohol-heightened aggression prompts further study into the role of glutamate versus dopamine in alcohol-heightened aggression, perhaps via microinjection of amantadine into the DRN and VTA.

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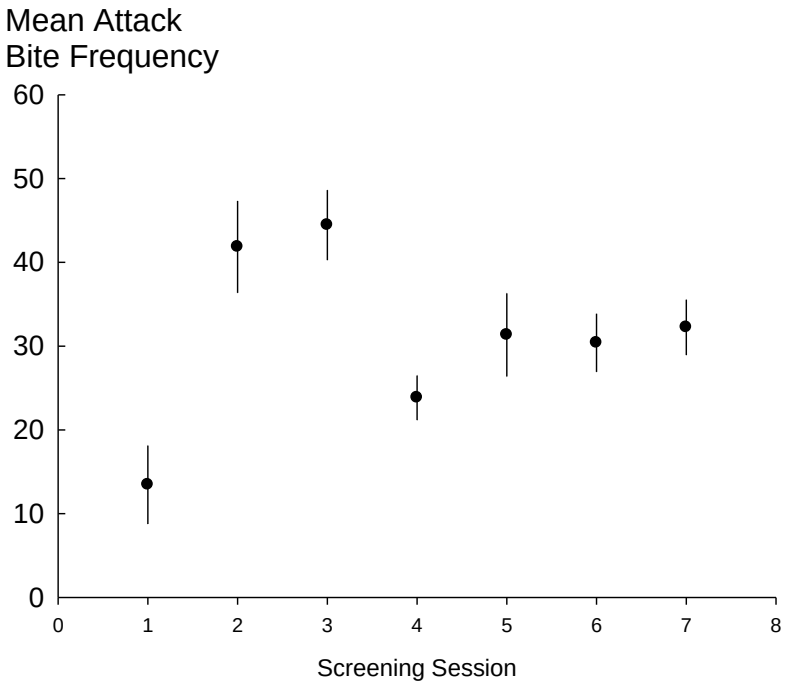

Figure 5. Stabilization of average attack bite frequency during successive home cage screens (n=18). Frequency of attack bites generally stabilized after 5-7 screens.

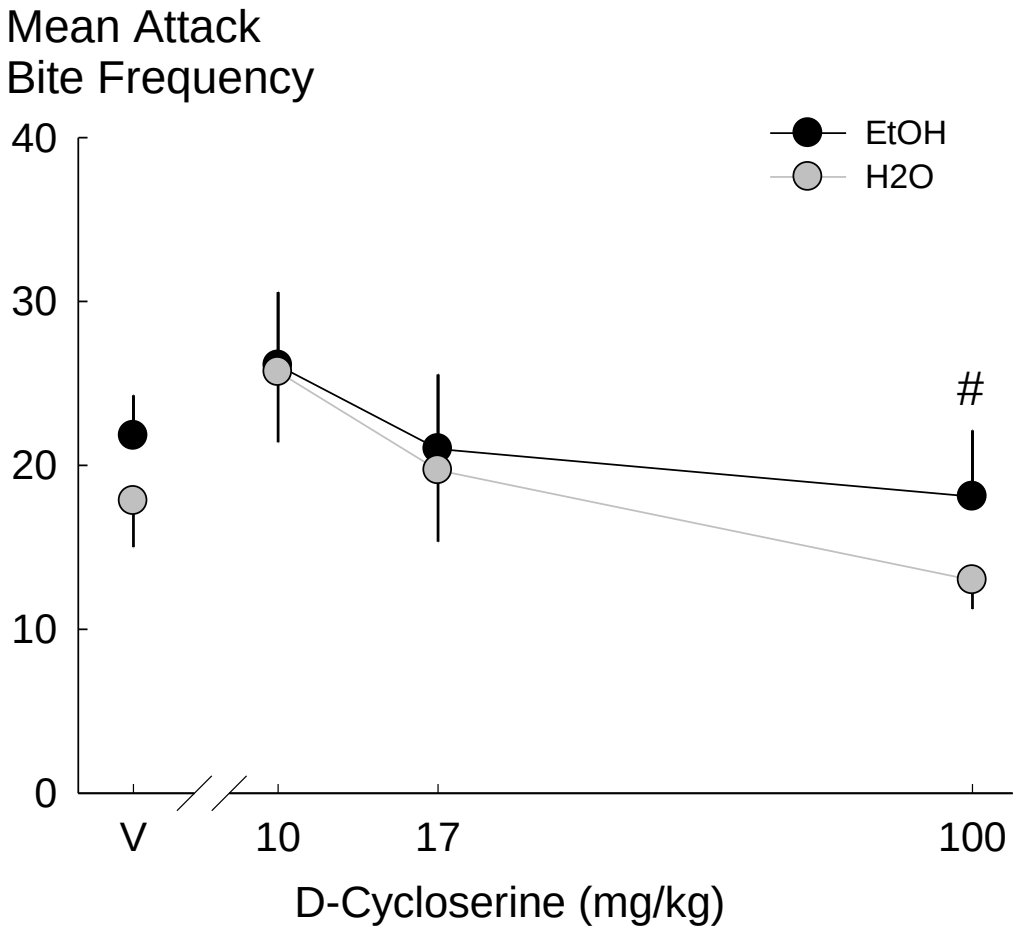

Figure 6. Mean attack bite frequency after ethanol or water self-administration and D-cycloserine administration (0-100 mg/kg, i.p., n=10). Frequency of attack bites was significantly lowered by the 100 mg/kg dose independent of self-administration condition. # $p < 0.05$ significance from water administration.

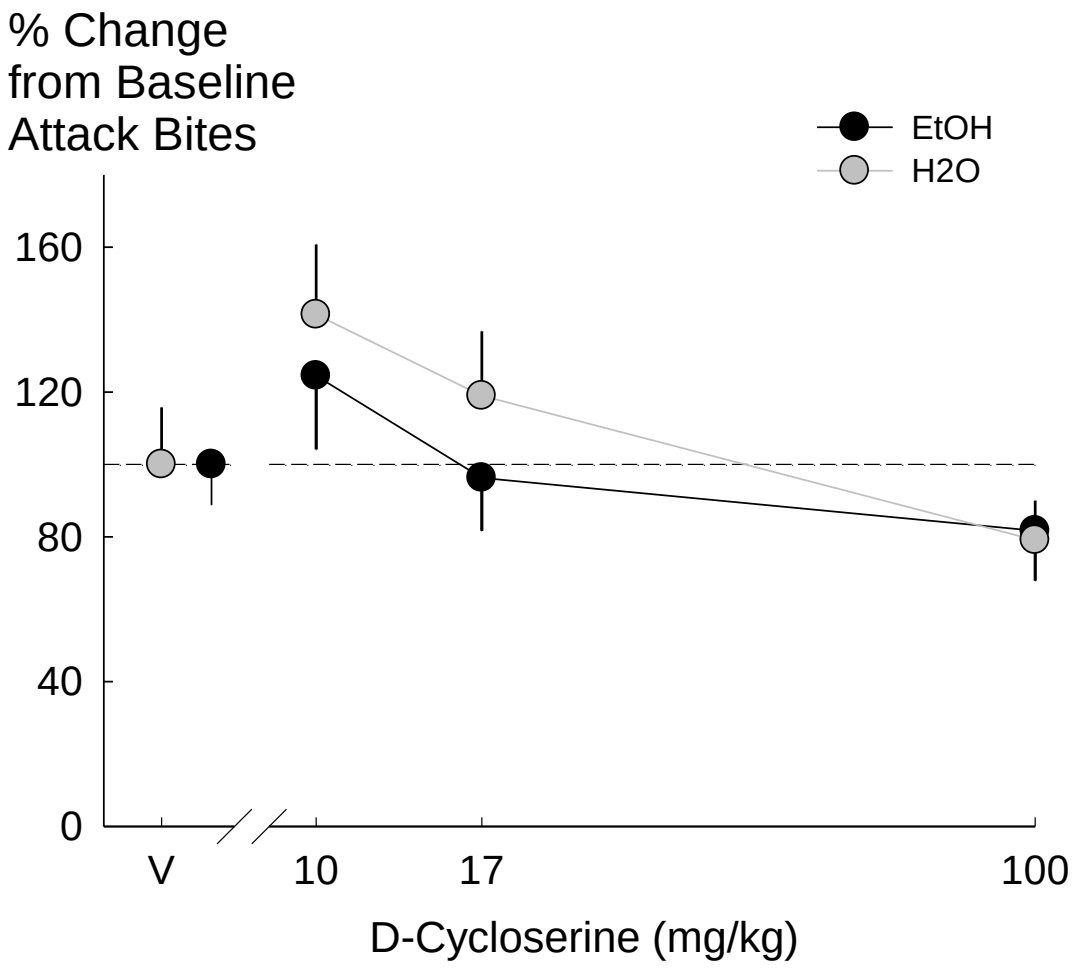

Figure 7. Percent change after 10-100 mg/kg (i.p.) D-cycloserine administration in attack bite frequency from vehicle condition (n=10). The percent change from baseline was significantly lowered by D-cycloserine independent of self-administration condition.

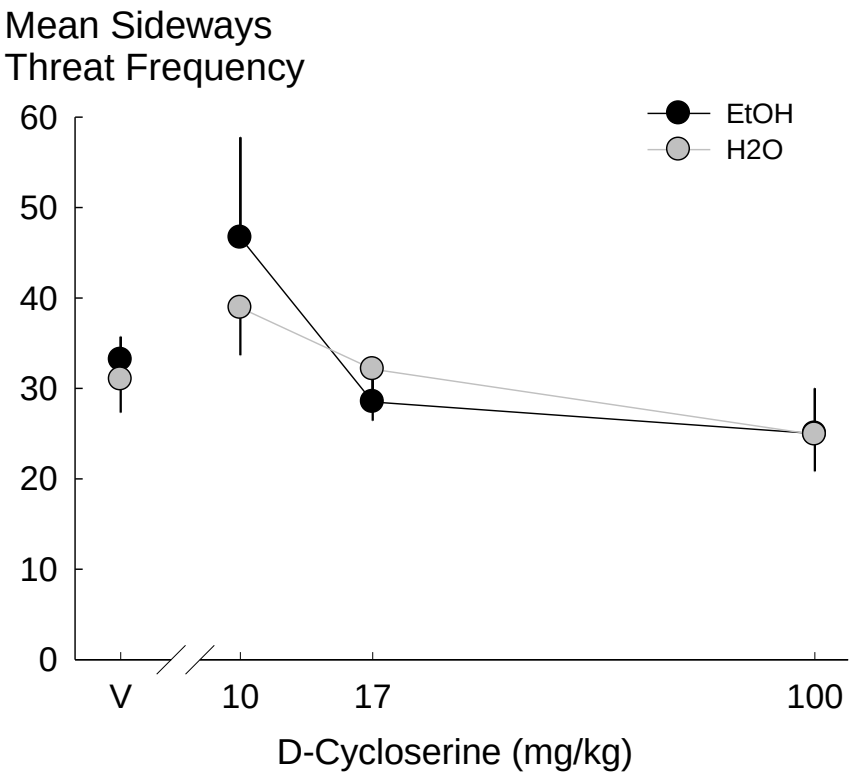

Figure 8. Frequency of sideways threats after ethanol or water self-administration and D-cycloserine administration. D-cycloserine had a significant main effect on the frequency of sideways threats.

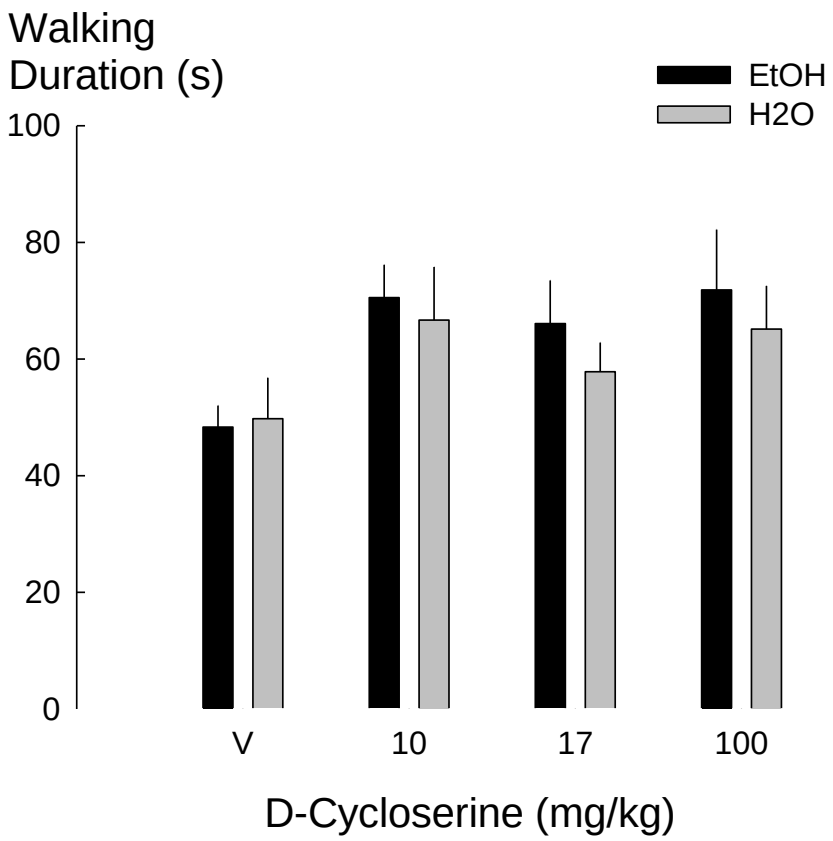

Figure 9. Walking duration after ethanol self-administration and systemic D-cycloserine. D-cycloserine significantly increased the duration of walking compared to vehicle administration.

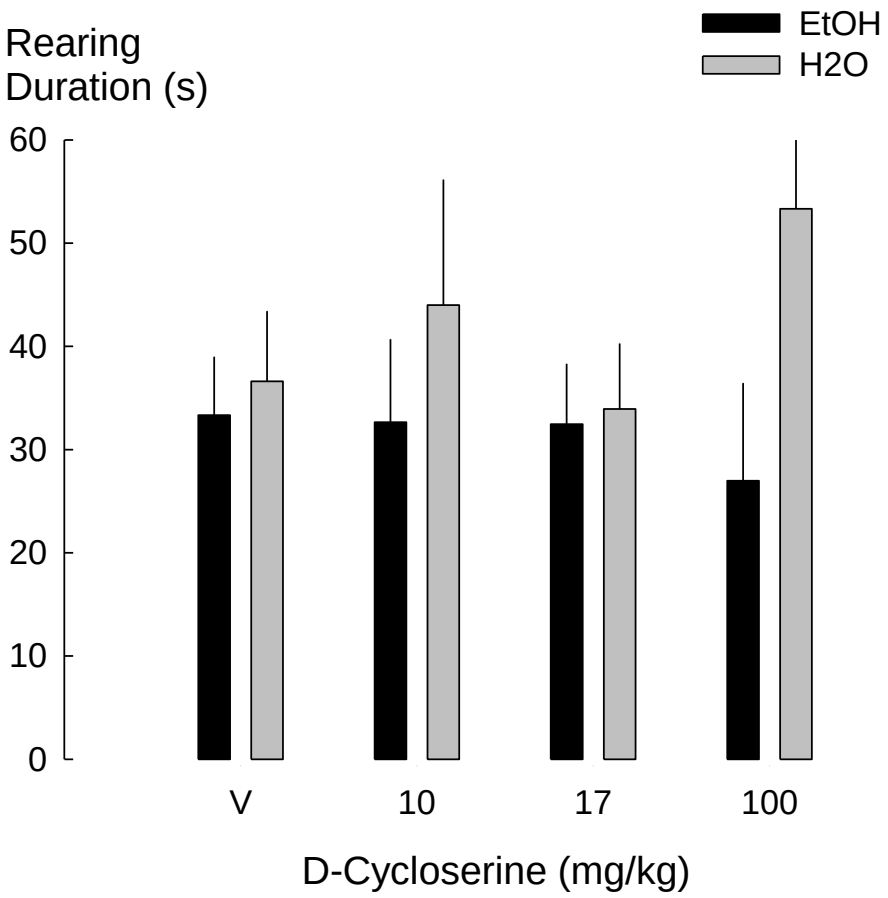

Figure 10. Rearing duration after ethanol self-administration and systemic D-cycloserine. Ethanol significantly reduced the duration of rearing compared to water self-administration.

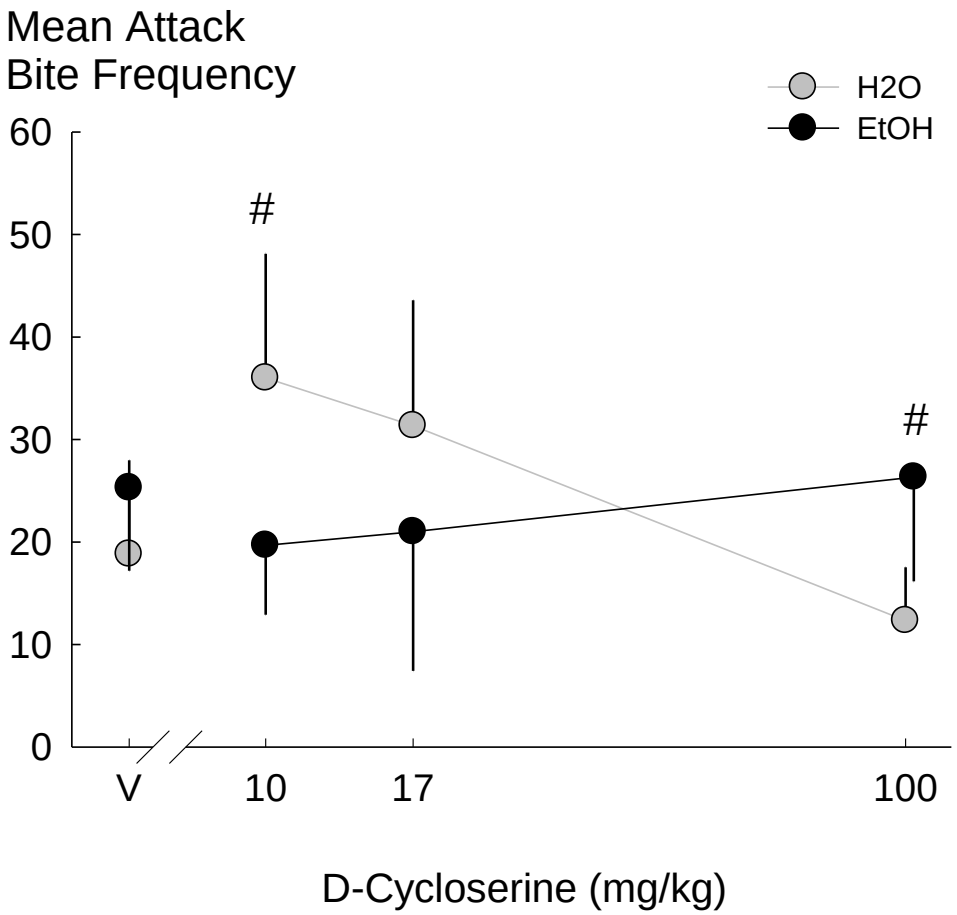

Figure 11. Attack bite frequency after ethanol self-administration and systemic d-cycloserine injection in alcohol-heightened aggressors (AHA, n=3). # $p < 0.05$ significance from water self-administration condition.

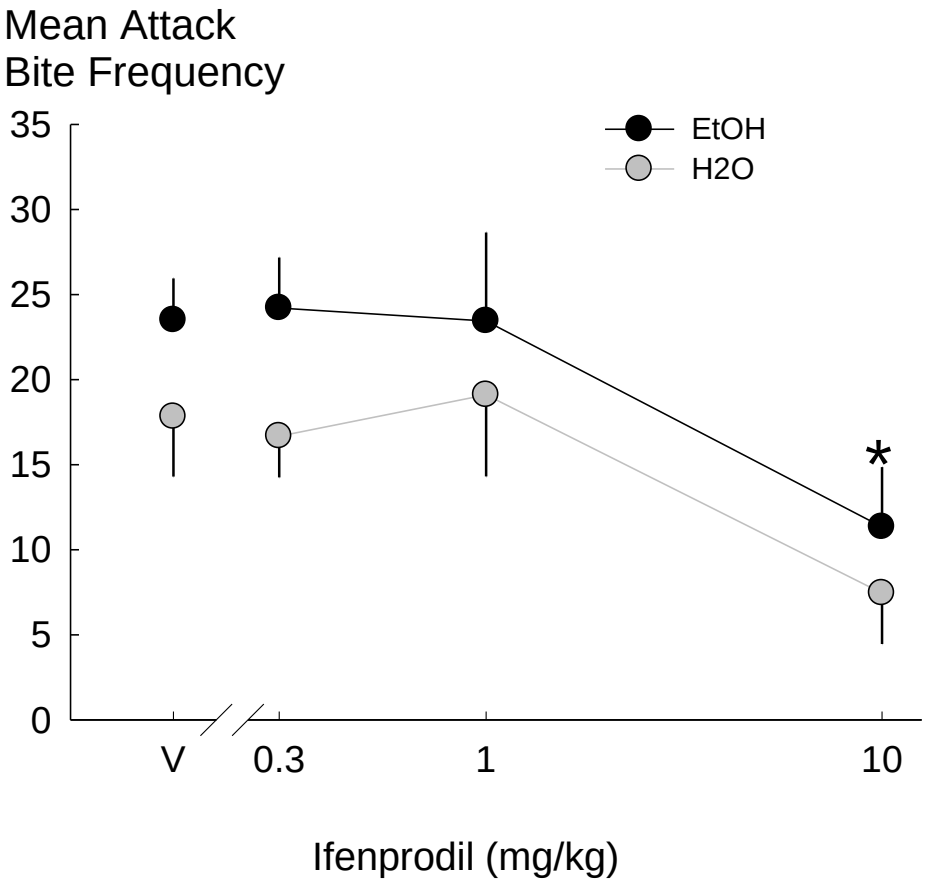

Figure 12. Attack bite frequency after ethanol self-administration and systemic ifenprodil injection (n=10). Ethanol self-administration significantly increased the frequency of attack bites compared to water self-administration. Attack bite frequency at the 10 mg/kg dose of ifenprodil was significantly reduced from vehicle after ethanol self-administration. * $p < 0.05$ significance from vehicle administration.

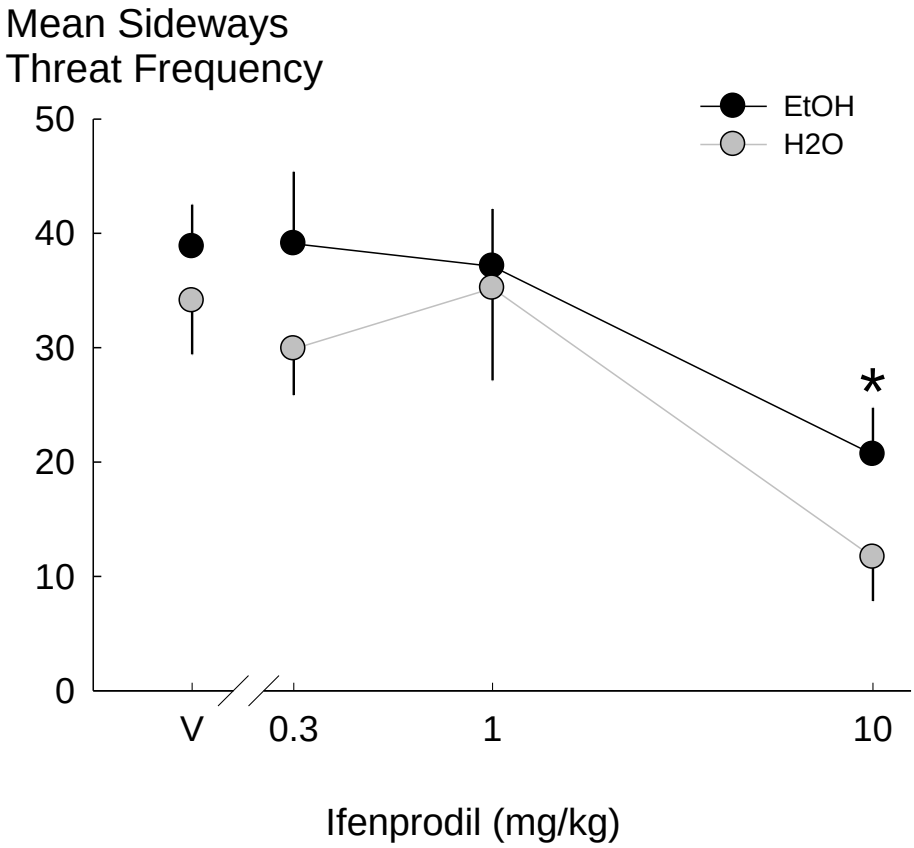

Figure 13. Average sideways threat frequency following ethanol self-administration and systemic ifenprodil. Frequency of sideways threats was significantly reduced by the 10 mg/kg dose after both ethanol and water consumption. * $p < 0.05$ significance from vehicle administration.

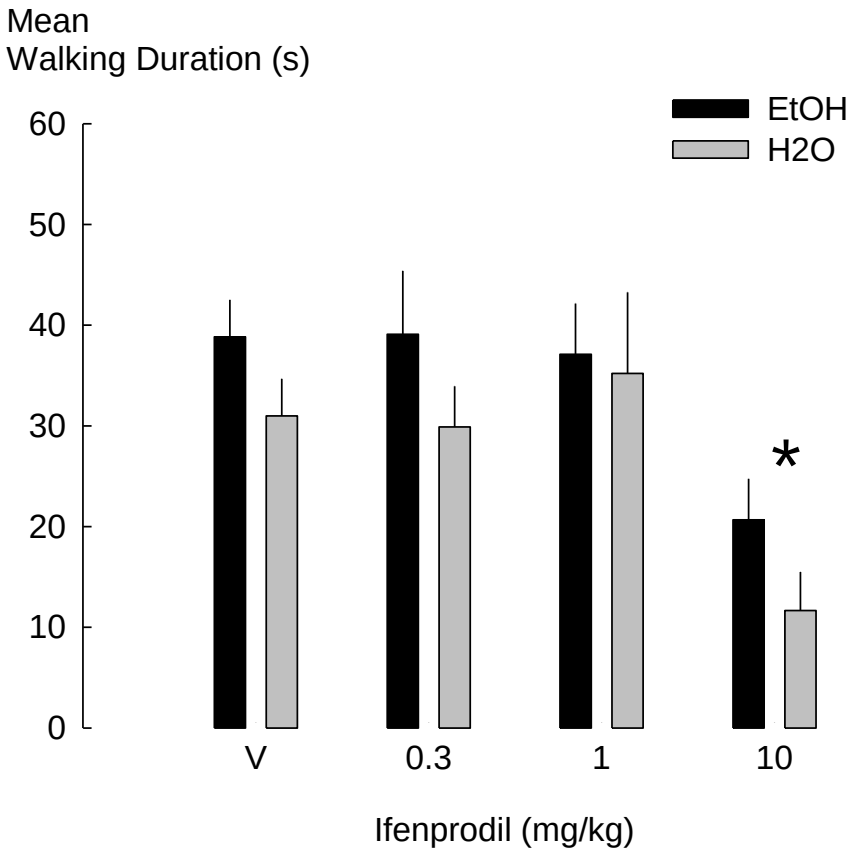

Figure 14. Mean walking duration after ethanol self-administration and systemic ifenprodil administration. Walking duration was significantly decreased by the 10 mg/kg dose after both ethanol and water consumption. * $p < 0.05$ significance from vehicle administration.

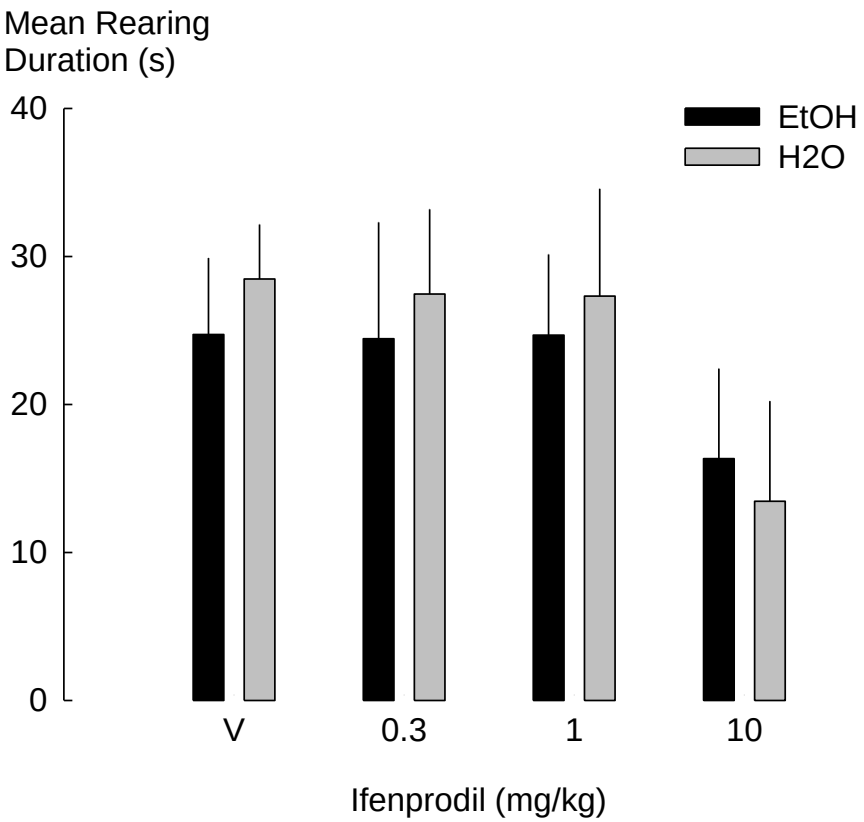

Figure 15. Average rearing duration following ethanol self-administration and systemic ifenprodil. Ifenprodil had a significant main effect on rearing duration.

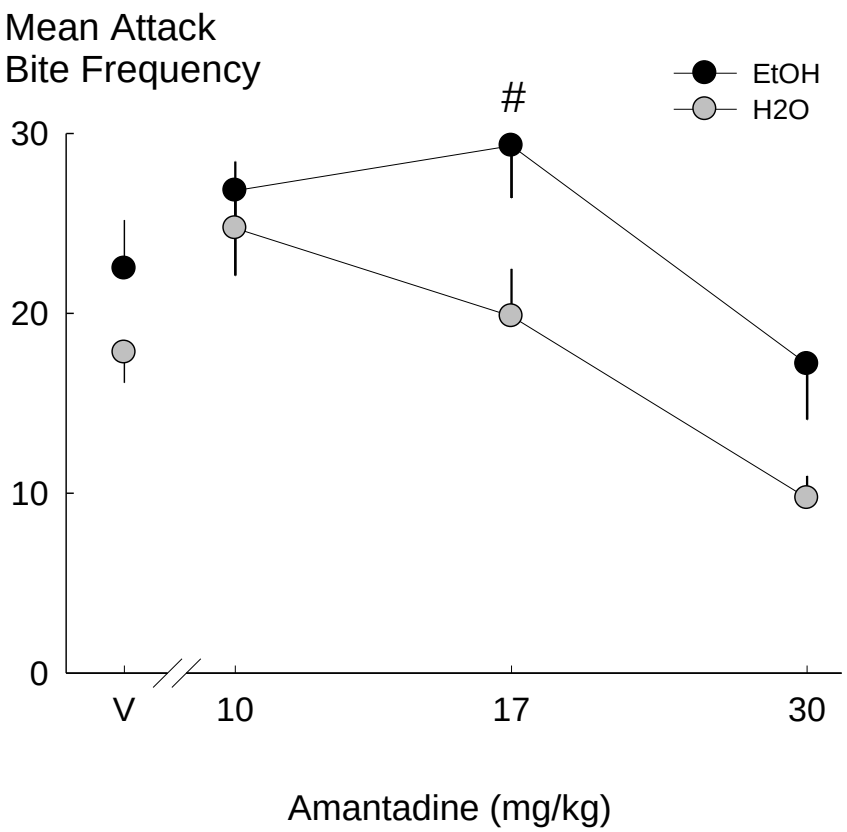

Figure 16. Mean attack bite frequency following ethanol self-administration and systemic amantadine. 17 mg/kg amantadine significantly increased the frequency of attack bites in the ethanol self-administration condition. # $p < 0.05$ significance from water administration.

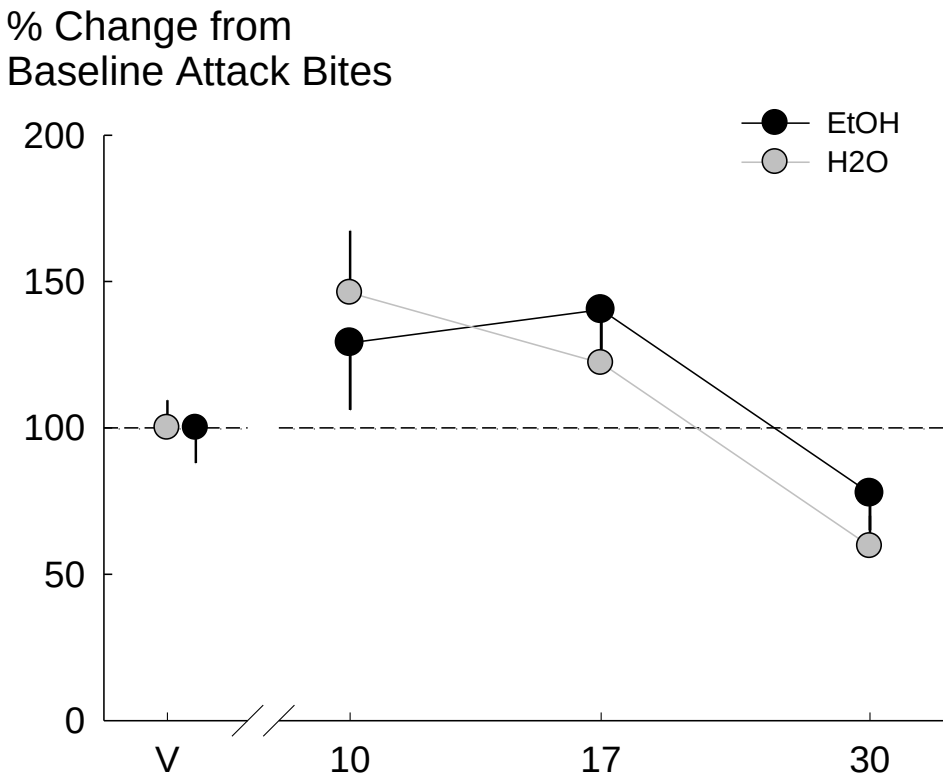

Figure 17. Percent change in attack bite frequency following systemic amantadine compared to vehicle administration. Amantadine had a significant main effect on the percent change in attack bites from baseline.

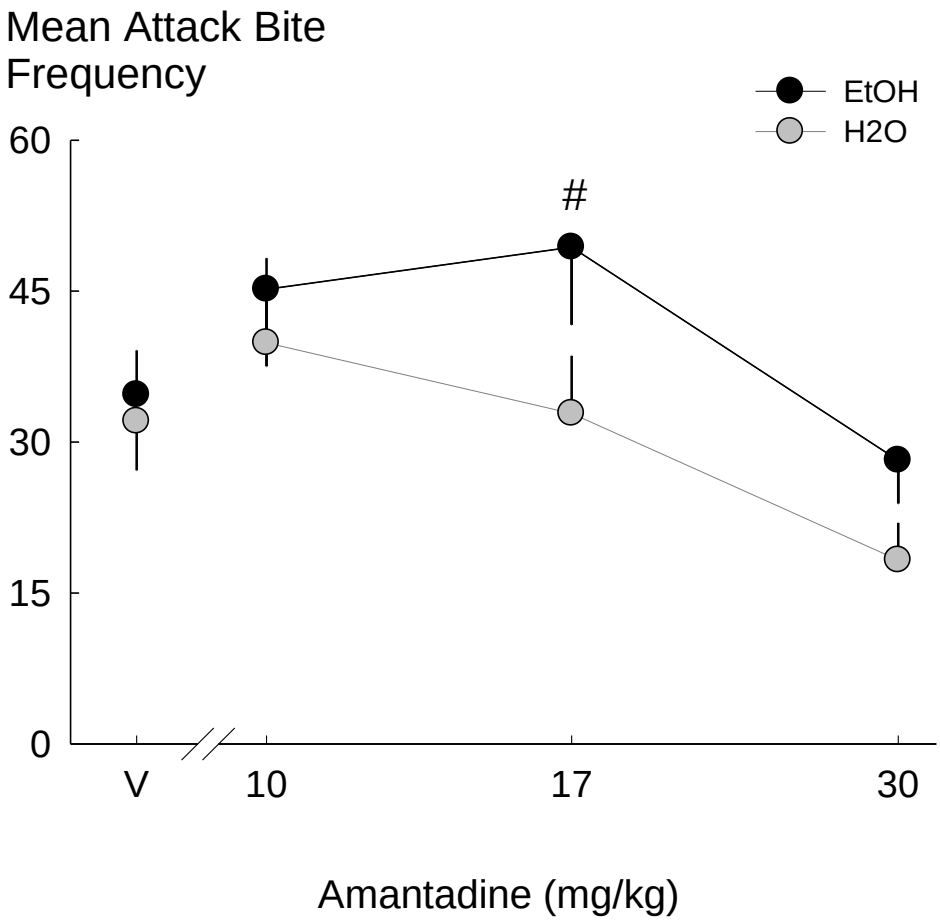

Figure 18. Average sideways threat frequency following ethanol self-administration and systemic amantadine. Frequency of sideways threats was significantly increased by the 17 mg/kg dose after ethanol consumption. # $p < 0.05$ significance from water administration.

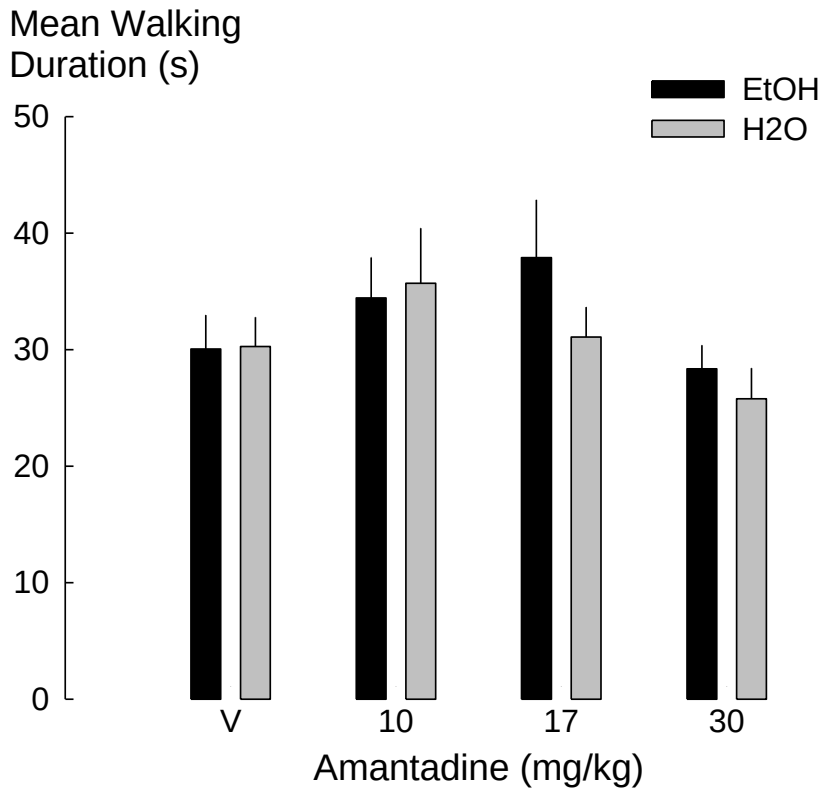

Figure 19. Mean walking duration after ethanol self-administration and systemic amantadine administration. Amantadine significantly affected walking duration. * $p < 0.05$ significance from vehicle administration.

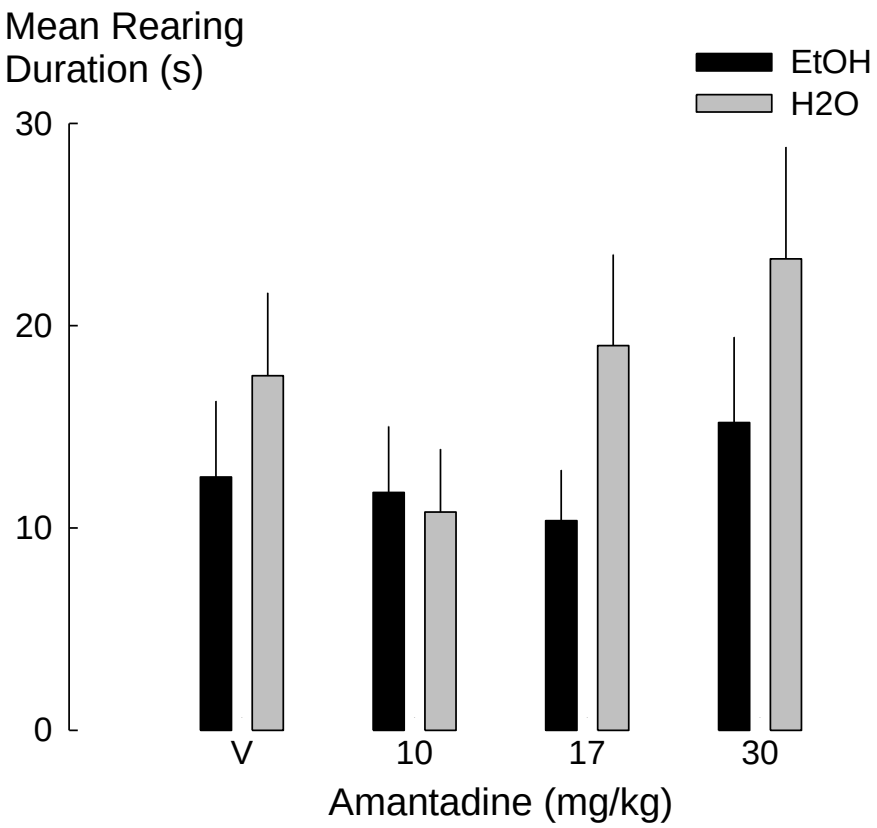

Figure 20. Average rearing duration following ethanol self-administration and systemic amantadine administration. Ethanol significantly reduced rearing duration compared to water self-administration, particularly at the 17 mg/kg dose of amantadine. # $p < 0.05$ significance from water administration.

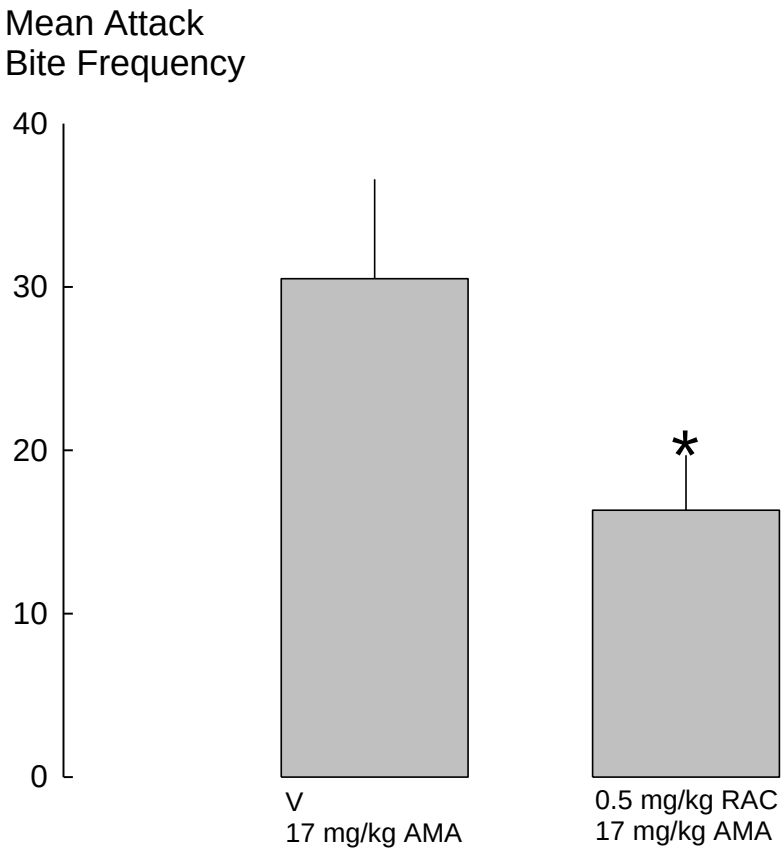

Figure 21. Mean attack bite frequency after raclopride challenge preceding ethanol self-administration and 17 mg/kg systemic amantadine (n=6). Pretreatment with raclopride attenuates the heightened aggression that occurs after ethanol self-administration and 17 mg/kg amantadine (see Figures 16 and 18). *p <0.05 from vehicle administration.

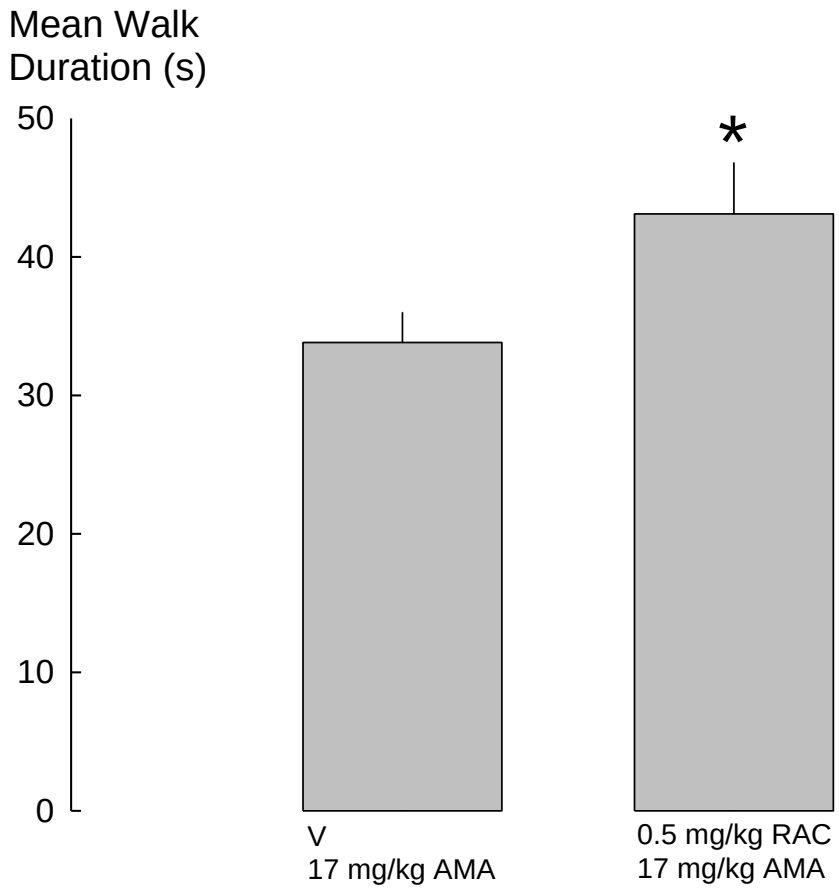

Figure 22. Mean walking duration after raclopride challenge preceding ethanol self-administration and 17 mg/kg systemic amantadine (n=6). Raclopride pretreatment increased the duration of walking. *p <0.05 from vehicle administration.