The role of glutamate via the NMDA receptor in aggressive behavior after ethanol consumption in mice

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Abstract

Alcohol has been linked to two-thirds of violence in humans (Krug et al., 2002). However, only certain individuals are more likely than others to engage in aggressive behavior after drinking (Cloninger, 1987). Mice that display more than two standard deviations of aggressive behavior after ethanol drinking compared to water drinking can be considered alcohol-heightened aggressive (AHA) mice (Miczek et al., 1998) and are clinically relevant models. Serotonergic projections from the dorsal raphe nucleus (DRN) to the prefrontal cortex (PFC) are involved in modulation of aggression. Because the N-Methyl-d-aspartate receptor (NMDAR) has been implicated in ethanol-associated phenomena such as tolerance, dependence, withdrawal, craving, and relapse (Trujillo et al., 1995; Krystal et al., 2003, this study sought to examine the role of glutamate via the NMDAR in alcohol-heightened aggression in mice. Because ethanol antagonizes the NMDAR (Lovinger et al., 1989; Follesa & Ticku, 1996; Kumari & Ticku, 1998; Roberto et al., 2004; Qiang & Ticku, 2005), researchers expected an increase in aggressive behavior after alcohol consumption.

Male CFW mice were trained to self-administer 1 g/kg of 6% EtOH or the equivalent volume of water. In the first experiment, mice were systemically injected with memantine (1,3,10,17 mg/kg) immediately after drinking and then confronted with an intruder mouse in the home cage. Aggressive behavior significantly increased at the lower doses of memantine (1 and 3 mg/kg) after ethanol but not water consumption. In the second experiment, mice were microinjected with AP-5 (0.1,1 μg) in the DRN. There was a significant drug effect in AHA animals but not in ANA animals. This study suggests that glutamate specifically via the NMDAR may have a role in regulating alcohol-heightened aggression in mice; however, this role may not be limited to the NMDAR or the DRN

The Human Condition

The link between alcohol and aggression has been recognized in the scientific community for a long time; some may consider the coupling of the two even a "natural phenomenon" (Pernanen, 1991). Alcohol has been linked to an overwhelming two-thirds of violent acts in humans (Krug et al., 2002). Epidemiological and experimental data point to a correlation between alcohol and aggression in studies of domestic violence, child abuse, and rape, assault, and homicide after alcohol consumption in both victims and perpetrators (Roizen, 1997; Collins and Messerschmidt, 1993; Arseneault et al., 2000; Caetano et al., 2001; Scott et al., 1999). Significant findings in this area of research could potentially guide public health and judicial policies.

Laboratory studies in humans have found similar correlations to the epidemiological studies. One meta-analysis of 30 studies focusing on experiments that used between-subjects design, male confederates, and male social drinkers found that alcohol significantly induced aggression; however, the analysis also concluded that the results were only valid under the methodological parameters used in the study and future studies should set up procedures that would better mimic real world situations (Bushman, 1990).

Not all individuals experience a spike in aggressive behavior after the consumption of alcohol. On the contrary, alcohol drinking usually produces social, relaxing, or sedative behavior (Miczek, 1987); however, some individuals became more likely than others to engage in aggressive behavior after alcohol (Cloninger, 1987). In order to study the clinically relevant individuals with significantly higher levels of aggressive behavior after alcohol consumption, one must account for the individual differences (Miczek et al., 1997). Investigating the neural

mechanisms responsible for this individual variation would help predict which individuals are more likely to engage in aggressive behavior after alcohol consumption.

Little is known about the source of these individual differences or why some individuals are violent after alcohol consumption while others are not. Due to the difficulty of studying the alcohol-aggression link in humans (Higley, 2001), animal models that represent the human condition have been useful in studying the biological mechanisms underlying alcohol-heightened aggression.

Animal Models

Animal models have allowed researchers to manipulate and measure a range of variables related to alcohol and aggression that cannot be examined in human studies. Researchers using animal models are often able to explore neural mechanisms more in depth because of more invasive manipulations and measurements that would not be appropriate in human studies. Alcohol and aggressive behavior, in particular, have been studied in a variety of animal models from rodents to primates. The mouse (*Mus musculus*) is the most studied laboratory vertebrate (Brain et al., 1989). Studying mice offers many advantages including but not limited to: consistent aggressive behavior in laboratory settings, the availability of a large number of inbred strains, and established means of assessing aggression (Brain et al., 1989). Mouse aggression can be observed by using the laboratory resident-intruder aggression paradigm, in which a breeding male "resident" mouse defends the site of the deme by attacking an "intruder" mouse placed in his territory (Miczek and O'Donnell, 1978). Mice that display species atypical levels of aggression in these resident-intruder confrontations can be used as clinically relevant models of

excessive aggression. These models have also allowed researchers to assess the issue of individual differences in aggressive behavior after ethanol consumption (Miczek et al., 1998). These individual differences in mice mimic those observed in human populations.

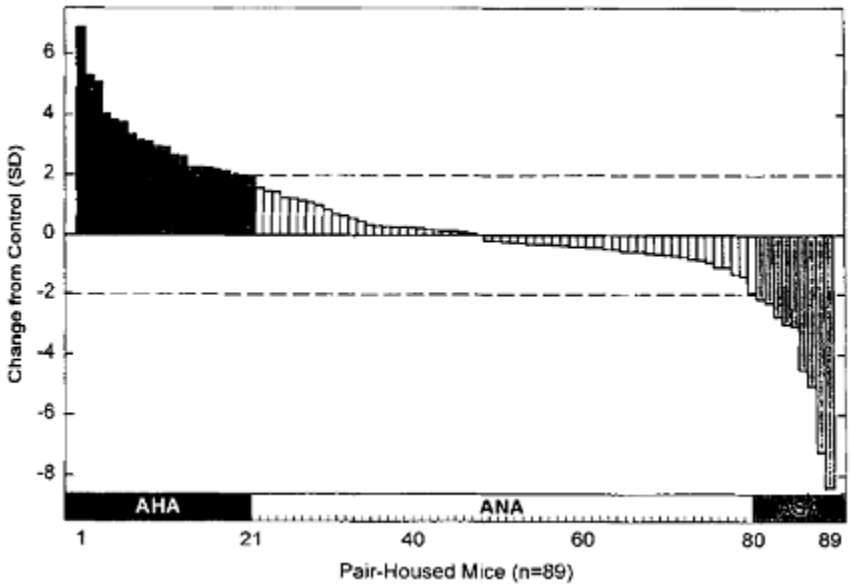

Figure 1. Alcohol-heightened aggressors are a distinct subpopulation in mice. Change in the frequency of attacks bites after consumption of 1.0 g/kg of alcohol as compared to consumption of the equivalent volume of water. From Miczek et al. 1998

Miczek and colleagues (1998) were able to identify a subgroup of mice showing significantly increased aggressive behavior after alcohol consumption (alcohol-heightened aggression, AHA) in comparison to a different subgroup that showed no change aggressive behavior after alcohol consumption (alcohol-non-heightened aggression, ANA). The increase in aggressive behavior in the AHA subgroup of mice was replicable and stable. This lends evidence to the idea that the increased level of aggression after alcohol consumption is a behaviorally stable trait with an underlying neuronal mechanism (Miczek et al., 1998).

Neurobiological Mechanisms of Aggression

Aggressive behavior occurs in all classes of vertebrates (Scott, 1958), and for this reason, it has been actively investigated for the greater part of the last century. Much of this research has focused on identifying neural structures and neurotransmitters that may mediate aggressive behavior.

One of the first major explorations that focused on neuroanatomical structures potentially responsible for mediating aggressive behavior was conducted by Flynn (1967) in which he discovered that stimulation of different brain sites, the ventral and lateral hypothalamus, led to different forms of aggressive behavior, affective defense and quiet biting attack, respectively, in cats. In rodents, different brain regions have been proposed to control aggressive behavior depending on social context. The posteroventral medial amygdala and ventromedial hypothalamus have been found to be important in defensive aggression, whereas the posterodorsal medial amygdala may play a more important role in offensive aggressive behavior (Nelson & Trainor, 2007).

Selective breeding for certain levels of aggressive behavior have led to the creation of highly aggressive and nonaggressive strains of mice—an example of "top-down" genetics (Miczek et al., 2001). Alternatively, using "bottom-up" genetics, behavioral studies conducted after the deletion or overexpression of particular genes have revealed a wide variety of potentially important genetic components of aggressive behavior (Miczek et al., 2007).

Species atypical aggression or inappropriate aggressive behavior can be seen as an inability to control impulses, a function primarily modulated by the prefrontal cortex (PFC) (Amat et al., 2005). In humans, both structural magnetic resonance imaging and functional

positron emission topography imaging studies have shown that many aggressive individuals have reduced volume and metabolism in the PFC (Nelson, 2005). Serotonin (5-HT), dopamine (DA) and gamma-aminobutyric acid (GABA) and specific receptor subtypes have all been implicated in a potential neural circuit (Miczek et al., 2002; De Almeida et al. 2005). 5-HT, in particular, has been highlighted as the prominent neurotransmitter across a variety of aggression types ranging from maternal aggression to escalated aggression (Miczek et al., 1998; Quadros et al., 2010). The majority of the serotonergic innervation to the PFC stems from the dorsal raphe nucles (DRN; Amat et al., 2005). In rats, uncontrollable stress was found to significantly increase activation of DRN 5-HT neurons as compared to a controllable stress resulting in a sensitization period for these neurons. Additionally, the medial PFC was found to inhibit activation of the DRN when a stressor was controllable (Amat et al., 2005). Animals that exhibit species atypical aggressive behavior may do so due to a defect in the neural system responsible for control and impulsivity. Neural changes induced via the DRN to medial PFC via somatodendritic autoreceptors (Adell et al., 2002) may be responsible for the behavioral phenomenon of species atypical aggression.

Serotonin

Serotonin is the most widely studied neurotransmitter in aggression research (Quadros et al., 2010). There is no definitive stance on the role of the neurotransmitter in aggressive behavior as the nature of its effects on many of the investigated neural systems is complex. Several discrepancies within both preclinical and clinical research have made the creation of an all-encompassing proposal of serotonin's actions difficult. The first human studies found reduced

levels of the serotonin metabolite 5-HIAA in cerebral spinal fluid in marine soldiers dismissed from service due to aggressive behavior (Brown et al., 1979). Although this inverse trend was seen in many other human studies, it was notably absent in a significant portion. The same inconsistency was seen in animal studies in which some species of monkeys showing the inverse correlation while not in others (Miczek et al., 2007). Pharmacological serotonin agonist and antagonist challenges as well as a tryptophan-depletion study, although plagued with the same discrepancy seen in the aforementioned human studies, pointed to hypothesis that deficiency in serotonin resulted in aggressive behavior (Miczek et al., 2007). The study of serotonergic receptors, specifically the 5-HT_1 and 5-HT_2 receptors, as well as the 5-HT transporter, SERT, have contributed more detailed information in regards to 5-HT's role in aggressive behavior.

5-HT has also been extensively studied in regards to stressor controllability. Grahn and colleagues (1999) fond that uncontrollable stress activates DRN 5-HT neurons more than controllable stress as seen by increased extracellular 5-HT in the DRN and in regions that the DRN projects to such as the mPFC. Increased secretion of 5-HT neurons in the DRN after microinjections of $GABA_B$ agonist, baclofen, has been associated with increased levels of aggression in mice (Takahashi et al., *submitted* 2010). Although the correlation between 5-HT firing levels in the DRN and aggressive behavior is not clear, investigation of the relationship between serotonergic neurons in the DRN and their projection to the mPFC, a stressor controllability modulator, may yield a less ambiguous explanation for 5-HT's role in aggressive behavior.

However, the neural mechanism for aggression cannot be attributed to the influence of one single neurotransmitter. Although 5-HT is a major player in the neural circuits of aggression,

it's interactions with monoamines, peptides, and steroids are of major significance to understanding the underlying mechanism for the behavior.

Glutamate

An early hypothesis by Mandel and colleagues proposed that aggressive behavior could be seen as a product of an imbalance between the excitatory and inhibitory neurotransmitters, glutamate and GABA, respectively (Miczek et al., 2007). Aggressive animals have lower levels of GABA and higher levels of glutamate in the whole brain, hypothalamus, amygdala, frontal cortex, and olfactory bulbs as compared to nonaggressive animals (Miczek et al., 2007); additionally, pharmacologically lowering GABA and glutamate levels decreases aggressive behavior (Miczek et al., 2007). Glutamatergic neurons are particularly important in the hypothalamic attack area, a region consisting of portions of the hypothalamus thought to be important in offensive attack behavior (Miczek et al., 2007; Hrabovsky et al., 2005). In addition to mediating the output signals responsible for behavior, glutamate is also important in processing olfactory cues that prompt or inhibit the display of aggressive behavior. This is evidenced by an increase in glutamate activity in the mPFC of sheep following novel olfactory cues that provoke aggressive behavior (Broad et al., 2002).

NMDA Receptor

The N-methyl-D-aspartic acid (NMDA) glutamate receptor is an ionotropic receptor, a ligand-gated ion channel that allows the influx of sodium and calcium and the efflux of

potassium (Kew & Kemp, 2005). The NMDA receptor (NMDAR) is voltage-dependent as a result of a magnesium ion block in the ion channel and will only be activated when both intracellular voltage is changed and glutamate and glycine bind to extracellular sites on the receptor (Kew & Kemp, 2005).

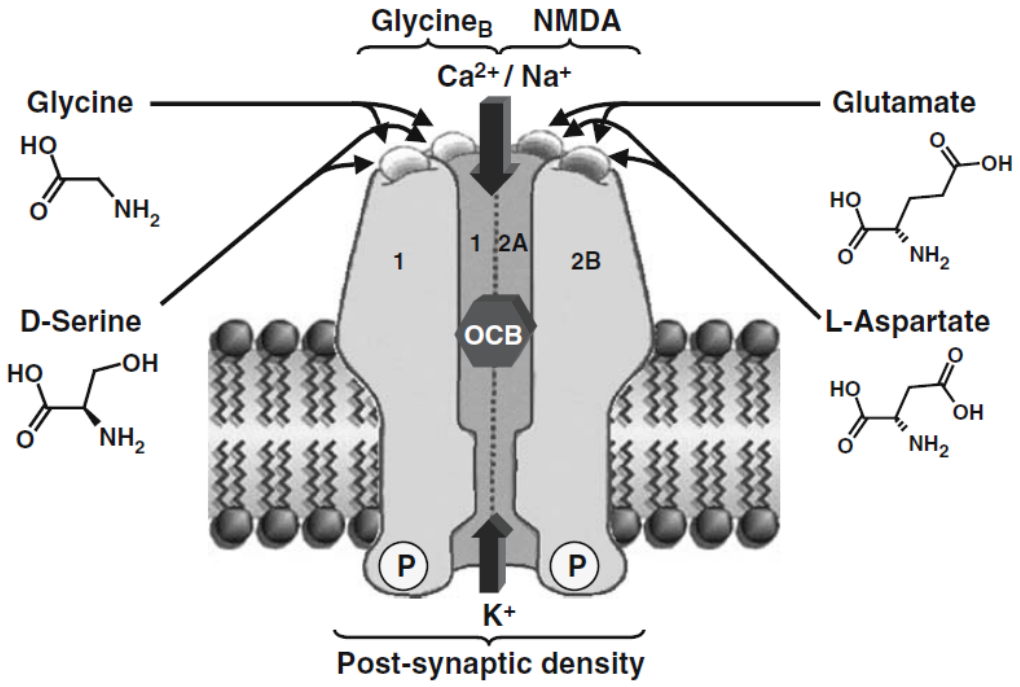

Figure 2. The NMDAR Receptor. *Figure from Millan (2005).*

The NMDAR has been especially studied in regards to its role in the phenomenon of long-term potentiation (LTP), the increase in synaptic strength after low-frequency, intense electrical stimulation (Rudy, 2008). The NMDAR is essential for the induction but not the expression of LTP. This biological mechanism is especially important in the explanation of associative memory, the strengthening of an association. The strengthening of synaptic connections may underlie the strengthening of associations (Rudy, 2008).

NMDARs in the DRN are found on both serotonergic as well as GABAergic neurons (Adell et al., 2002). When lower concentrations of NMDA (25 μM) were infused in the DRN of rats, there was a decrease of extracellular 5-HT in the DRN accompanied by an increase of extracellular 5-HT in the prefrontal cortex (Pallotta et al., 1998); however, when a higher concentration of NMDA was infused (100 μM), the opposite effect was seen—an increase in extracellular 5-HT in the DRN and a decrease of extracellular 5-HT in the prefrontal cortex (Pallotta et al., 1998). This effect was attenuated or completed blocked by the selective NMDAR antagonist 2-amino-5-phosphonopentanoic acid (AP-5) and enhanced by the application of the $GABA_A$ receptor antagonist, bicuculline (Pallotta et al., 1998; Gartside et al., 2007; Mokler et al., 2009).

Ethanol antagonizes the NMDAR primarily by inhibiting ion current (Ron & Wang, 2009). In vitro studies in rat hippocampal and cortical slices (Lovinger et al., 1989; Wright et al., 1996; Lovinger et al., 1990) have lent support to this idea; however, the exact mechanism of this antagonism is not yet certain. Because of the rapid nature of this acute inhibition, ethanol may work directly on the ion channel (Ron & Wang, 2009). In addition to its acute effects, chronic ethanol exposure has been shown to alter NMDA receptor function in rats by changing the subunit expression leading to excitability and increased receptor expression (Roberto et al., 2004; Kumari & Ticku, 1998; Follesa & Ticku, 1996; Qiang & Ticku, 2005). These changes in receptor subunit expression could be responsible for cellular changes that would result in significant behavioral changes such as tolerance.

Both preclinical and clinical data have supported an NMDAR role in chronic ethanol-associated phenomena such as tolerance, dependence, withdrawal, craving, and relapse (Trujillo et al., 1995; Krystal et al., 2003). NMDAR antagonists have been introduced in clinical settings

to alleviate alcoholism-related events from psychosis to craving (Ron & Wang, 2009). Acamprosate (Campral) is an FDA approved anticraving and relapse drug currently used in the treatment of alcoholics. Some *in vitro* rat studies have found that acamprosate acts as a partial coagonist or weak antagonist, activating NMDAR activity at low concentrations with low NMDAR levels and inhibiting NMDAR activity at high concentrations with high receptor levels (Naassila M. et al, 1998). The greatest issue with NMDAR antagonists is their nonselective effects. At clinically effective doses, many NMDAR antagonists produce undesirable side effects. However, memantine's moderate potency and clinical tolerance make it an ideal candidate for clinical treatment (Bisaga & Evans, 2004).

In regards to aggression, the role of the NMDAR is not as clear. The effects of the NMDAR antagonists tested have had different effects depending on the behavioral history of the animal. Animals naive to aggressive confrontations showed increased levels of aggressive behavior while animals repeatedly exposed to aggressive confrontations showed decreased levels of aggressive behavior after NMDAR antagonist treatment (Nelson, 2006). Additionally, most of these NMDAR antagonists do not show selective effects on aggressive behavior. In a study using the resident-intruder mice paradigm, results showed that aggressive behavior was only attenuated in doses that produced ataxia (Belozertseva & Bespalov, 1999). However, these drugs may be important when used to treat drug-related escalation in aggressive behavior. When a noncompetitive NMDA receptor (MK-801) was given to mice in the resident-intruder set-up after morphine withdrawal, doses that lowered aggression were two-to-three fold times lower than those that caused ataxia (Sukhotina & Bespalovr, 2000).

Memantine

Memantine hydrochloride is a noncompetitive NMDA receptor antagonist (Kew & Kemp, 2005). It is the only FDA-approved treatment for moderate to severe forms of Alzheimer's disease (Witt et al., 2004) and is clinically well-tolerated (Parsons et al., 1999) unlike many of its fellow NMDAR antagonists which produced a variety of central nervous system side effects such as hallucinations in clinical trials (Witt et al., 2004). Memantine at high concentrations affects a variety of other neurotransmitter uptake and receptor activity including serotonin and dopamine uptake, nicotinic acetylcholine receptors, serotonin receptors, sigma-1 receptors, and voltage-activated Na+ channels (Johnson & Kotermanski, 2006).

Because of its tolerance, memantine has been used in a variety of clinical trials investigating the role of the NMDAR in alcohol dependence or alcohol craving. In a study by Bisaga and Evans (2004), memantine administered prior to alcohol consumption in moderate (10-30 drinks per week) drinkers attenuated the craving for alcohol before administration but not after administration. A similar study using recovering alcohol-dependent subjects found that memantine reduced cue-induced cravings for alcohol (Krupitsky et al., 2007). Although subjects in the study experience alcohol-like subjective effects after memantine administration, these effects did not stimulate alcohol craving (Krupitsky et al., 2007).

Memantine's influence over aggressive behavior has recently been investigated using preclinical models of aggression. In one study, memantine was administered to see the effects of the NMDAR antagonist on aggression using the frustration and instigation models of aggression in mice (Miczek & Chu, *unpublished* 2008). In the frustration paradigm mice were trained to nose poke for sucrose reinforcement which was later unexpectedly omitted before confrontation

with an intruder mouse. In the instigation paradigm, an instigator mouse in a protective cage was placed in the cage of a resident mouse. The resident mouse was then moved to a neutral cage and confronted by an intruder. Both frustration and instigation produced increased levels of aggressive behavior in resident mice. Systemic injections of memantine (0, 3, 10, 17, and 30 mg/kg) reduced aggressive behavior at the 17 and 30 mg/kg doses (Miczek & Chu, *unpublished* 2008).

AP-5

D-(-)-2-Amino-5-phosphonopentanoic acid or D-AP-5 is a high-affinity, competitive NMDA antagonist (Kew & Kemp, 2005). AP-5 has not been widely used in aggression studies, save for investigations of conditioned defeats (Nelson, 2006). Various preclinical studies have used the competitive antagonist in ethanol preference and drinking experiments. Microinjection of AP-5 into nucleus accumbens of rats reduced ethanol self-administration in rats (Rassnick et al., 1992). Delivery of AP-5 to cerebrospinal fluid reduced ethanol preference in untrained but not trained rats supporting the hypothesis that the NMDAR may be important for acquisition of ethanol preference (Lin & Hubbard, 1995). Because of the high degree of distribution of NMDARs in the brain, AP-5 administration may produce non selective behavioral effects which would discourage the use of the compound in preclinical and clinical settings.

Objective

Many studies have examined the NMDAR's role in the issues of aggression and alcohol separately. The objective of the study, however, was to investigate glutamate's role specifically via the NMDAR in alcohol-heightened aggression in mice using systemic injections of memantine. Furthermore, the study aimed to see if the NMDARs in the DRN are critical for glutamate's influence in alcohol-heightened aggression as assessed by AP-5 microinjections. Because ethanol antagonizes the NMDAR, we would expect additional antagonism (via systemic memantine injections or microinjections of AP-5) to amplify the increase in aggressive behavior after ethanol consumption.

Materials & Methods

Animals

Subjects were adult Swiss-derived male Carworth Farms Webster mice (CFW; Charles River Laboratories, Wilmington, MA) weighing approximately 23-25 g upon arrival and 30-35 g during testing. 'Resident' mice were pair housed with female mice in clear polycarbonate cages (28x17x14 cm) with pine and cedar shavings. 'Intruders', adult Swiss-derived male CFW mice, Charles Rive Laboratories, Wilmington, MA)weighing 23-25 g upon arrival and during testing, were group housed with no more than 7 other males in larger clear polycarbonate cages (17 X 48 X 13 cm) with corn cob bedding (Shephard's Specialty Blend Alpha-dri/Cob Blend, Shephard's Specialty Papers). The vivarium was kept at a controlled humidity and temperature (35–40%, 21±1°C) on a 12-hour light/dark cycle (lights on at 7:00 AM) with free access to food (LabDiet 5001 Rodent Diet; PMI Nutrition International, Brentwood, MO, USA) and tap water. After two weeks of pair housing, residents were water restricted for 21 hours for 5 days a week. Animals were cared for according to the 'Principles of Laboratory Animal Care' (NIH publication No.85-

23, revised 1996). All procedures were approved by the Institutional Animal Care and Use Committee of Tufts University.

Apparatus & Measurements

Data was recorded using a Panasonic WV-CP280 1/3" CCD camera connected to a Panasonic video cassette recorder recorder. Frequency and duration of aggressive and non-aggressive behaviors were assessed using *The Observer* software (XT version 8.0.330, Noldus Information Technology, Wageningen, The Netherlands). Refer to Table 1 for classification of aggressive and non-aggressive behaviors recorded. Trained observers completed the analysis by recording behaviors on a custom keyboard. Inter- and intra- reliability was controlled.

Table 1. Aggressive and Non-aggressive Behaviors Recorded

Aggressive Behaviors	Non-aggressive Behaviors
• Bite: Resident contacts intruder with teeth • Pursuit: Resident quickly follows intruder • Sideways Threat: Resident positions itself at right angles to intruder's body but does not deliver bite • Tail Rattle: Resident rapidly undulates tale	• Contact: Resident intiates non-aggressive naso-nasal or ano-genital contact • Groom: Resident washes face with paws or licks flank • Rear: Resident lifts front paws off of grown, rests on hind legs • Walk: Resident displays motor behavior using all four limbs

Alcohol Self-Administration

Female and pups were removed and an aluminum panel (16.5 X 15.9 cm) with two drinking troughs, one that delivered reinforcements and one that did not, was placed in the home cage of the resident (Miczek & de Almeida, 2001). The active or reinforced drinking trough was further identified by an overhead cue light. Fluid was delivered to the trough via plastic tubing connected to a syringe pump (Med Associates, St. Albans, VT, USA). Each reinforcement was accompanied by an audible click and brief inactivation of the house light while the fluid was delivered. Resident mice were trained to perform a nosepoke behavior where every fifth poke was rewarded (Fixed Ratio 5 schedule) with 40 μL of either water or 6% ethanol (Pharmco-Aaper Ethyl Alcohol 190 Proof, 95%) delivery. A nosepoke response was registered when a photobeam above the drinking trough was broken. On the first day, residents were trained according to a Fixed Ratio 1 schedule, and on the second day, a Fixed Ratio 2 schedule with sessions lasting for 30 minutes on both days. On the third day, a 6% ethanol solution was introduced as the fluid reinforcement for animals enrolled in the memantine study. For animals under the AP-5 and varying ethanol treatments, during the first six days of training, nosepokes were only reinforced with water delivery. On the third day, animals began an FR5 schedule. On the seventh day, the 6% ethanol solution was introduced as the fluid reinforcement. From day 7 to the end of the training period, ethanol and water were alternated every other day. After day 3 of training, animals nosepoked for the delivery of 1 g/kg of ethanol or the equivalent volume of water. Drinking sessions were terminated once the animal completed the required number of nosepokes to receive 1 g/kg of ethanol, as this dose has been shown as optimal for enhancing aggressive behavior (Miczek et al. 1998). Drinking sessions were limited to 10 minutes in the next phases of the experiment where resident-intruder confrontations occurred after nosepoke sessions. The panel and pump were connected to an interface controlled by a PC running

Windows Med-PC, which simultaneously recorded the number of times each mouse poked both the active and inactive troughs, the response rate of poking, and total duration of the drinking session (MED-PC for Windows, v. 4.1; Med Associates).

Resident Home Cage Aggression Testing

Female and any pups were removed from the resident's home cage. An intruder mouse was placed into the opposite end of the resident's home cage. Resident-intruder confrontations lasted for five minutes after the first attack bite from the resident. If no bite occurred, sessions were stopped at five minutes. If the resident did not bite for two consecutive sessions, a new intruder was introduced. The same intruder-resident pair was used unless the intruder mouse attacked or the resident stopped fighting. During training, only attack bites were recorded. For the first 6-7 sessions, assessments of aggressive behavior were conducted separately from drinking in order to any influence on drinking behavior.

After aggressive behavior became stable (<20% variation in number of attack bites), resident-intruder confrontations occurred 15 minutes after the drinking sessions terminated. When fighting stabilized under these paired conditions (<20% variation in number of attack bites), aggression levels were compared after intake of water and alcohol in order to describe a mouse's aggressive behavior as alcohol-heightened (AHA) or non-alcohol heightened (ANA). This "characterization" period lasted for six sessions with alternating water and ethanol trials for a total of three trials per liquid. An AHA mouse is one that greater aggressive behavior as defined by attack bites after consumption of alcohol (2 z-scores) compared to after drinking water (Miczek et al., 1998a) during these three stabilized trials.

After characterization, aggression testing was conducted after drug treatment (Miczek & O'Donnell, 1978; Winslow & Miczek, 1983). Resident-intruder confrontations throughout the experiment occurred three times a week. Resident-intruder confrontations that followed drug administration were video recorded and later analyzed for aggressive and non-aggressive behaviors.

Experiment 1: Intraperitoneal Injections of Memantine hydrochloride

Residents (n = 20) were habituated to handling by the researcher for five days. During this habituation period, residents received an intraperitoneal injection (i.p.) of saline (1 ml/100 g, 26ga needle) once after a resident-intruder confrontation and once immediately after the termination of a drinking session. Resident-intruder confrontations after this habituation saline injection occurred 20 minutes after the injection.

On test days, residents consumed water or 1.0 g/kg alcohol and immediately were injected i.p. with 1ml/100g vehicle (dH2O) or memantine (1, 3, 10, or 17 mg/kg, i.p.). A resident-intruder confrontation followed 20 minutes after the injection of memantine or vehicle. The lower doses of memantine (1, 3 mg/kg) were administered first in order to avoid potential receptor changes after a higher dose (10, 17 mg/kg). At least 10 tests were conducted for each individual, with residents receiving a vehicle injection after two drug injections in order to ensure fighting levels remained stable. Mice who failed to show stable levels of fighting (less than 20% variance in at least 3 fighting sessions or mice who had more than two intruder changes) during testing (n = 4), complete at least 9 of the injections (n = 2), or that died (n = 2)

during the study were excluded from the final analysis. At the highest dose of memantine, one outlier was excluded.

Experiment 2: Intra-DRN Microinjections

Another group of residents (n = 12) was anesthetized with a 10 ml/kg i.p. injection of Ketamine/Xylazine combination (10:1.8 mg/mL) and underwent stereotaxic surgery (Kopf Instruments, Tujunga, CA) in order to implant a 6 mm, 26-ga guide cannula (Plastics One, Roanoke, VA) aimed at the dorsal raphe nucleus (DRN; AP, –4.2mm; ML, +1.5mm; DV, –1.9mm; 26º from bregma). A 33-ga obdurator (Plastics One) was inserted into the cannula in order to prevent substances from contaminating or blocking the cannula. The obdurator tip was coated with guanine to prevent destruction of the cannula by the female cage mate. Daily handling of the obdurator, removal, and replacement when necessary, was done in order to habituate the mice to handling of the obdurator and to prevent blockage of the cannula. Residents were housed separely from female cage mates for 3 days and allowed 2 weeks of recovery from the surgery before being returned to the testing protocol. Residents in this experiment were characterized after the surgery with new intruders.

Resident-intruder confrontation tests occurred three times per week, separated by at least 24 hours. On days when testing did not occur, residents performed the nosepoke task to maintain self-administrated drinking behavior. On test days, mice consumed water or 1.0 g/kg and immediately thereafter received a microinjection of vehicle (aCSF) or AP-5 (0.1, 1.0 nmol). Microinjections were performed by removing the obdurator and inserting a 33-ga injector (Plastics One, Roanoke, VA) that extended 2 mm beyond the guide cannula. The injector was

connected by flared 50 polyethylene tubing (CMA Microdialysis, North Chelmsford, MA) to a glass syring and pump. Fluid was infused at a rate of 0.2 µl/min. The injector was then left inside the cannula for one minute after the infusion to allow time for diffusion. Mice were free to move about during the infusion. Ten minutes after the infusion, mice were confronted by an intruder.

Tests occurred in a systematically varied sequence by varying the dose of the drug and fluid consumed. At least 6 tests were conducted for each individual. Mice who failed to fight an intruder after more than two sessions (n = 2) were excluded from the final analysis.

After the completion of testing for the given doses, mice were deeply anesthetized (ketamine/xylazine, 100:10 mg/kg) and intracardially perfused with 0.9% saline and 4% paraformaldehyde. Brains were extracted and suspended in a 4% paraformaldehyde solution. Brains were then transfered to a 10% sucrose solution and kept at XºC. Brains were then frozen and sliced on a cryostat in 50µm coronal sections, which were then stained with cresyl violet in order to visualize the placement of the cannula into the DRN. Mice with inaccurate placements served as controls.

Drugs

The 6% ethanol solution was obtained by diluting 95% ethyl alcohol (Pharmco-Paper Products Inc., Brookfield, CT) with tap water. A portion of the memantine used in this study was generously donated by Forest Laboratories (Jersey City, New Jersey, USA). Additional memantine was obtained from Sigma-Aldrich (St. Louis, MO). Memantine was freshly dissolved

in dH2O and diluted to obtain the different doses used. AP-5 (DL-2-Amino-5-phosphonovaleric acid; Sigma-Aldrich, St. Louis, MO) was dissolved in aCSF.

Statistics

For both the memantine and AP-5 experiments, a two-way analysis of variance (ANOVA) with repeated measures ($p < 0.05$) was used to analyze the behavioral effects of each drug condition after ethanol and water consumption (Sigmastat, version 3.11.0). The Holm-Sidak post-hoc test was used when appropriate.

Results

Alcohol Self-Administration

All mice ($n = 31$) were trained successfully to self-administer 1 g/kg of 6% EtOH on a FR5 schedule of delivery under both drinking acquisition protocols. Response rate per minute increased as a function of number of sessions in a linear style under Protocol 1 and a curvilinear style under Protocol 2. Ethanol self-administration acquisition increased in a linear function under both protocols. See Figure 3.

Screening for Aggressive Behavior

All mice included in analysis ($n = 19$) developed stable levels of aggressive behavior as defined by less than 20% variance in attack bites after three consecutive fighting sessions. See Figure 4.

Experiment 1: Intraperitoneal injections of memantine

No animals in the first experiment (n = 12) exhibited two or more standard deviations above species typical aggressive behavior after ethanol consumption. See Table 2.

Aggressive behaviors

Aggressive behaviors were analyzed as percent change from ethanol and water control. Memantine (1 and 3 mg/kg) significantly increased percent change of attack bites after ethanol consumption as compared to control but not after water drinking ($F(11,4) = 2.596$, $p = 0.050$; $t = 2.715$, $p < 0.01$; $t = 2.894$, $p < 0.01$, respectively). See Figure 5. Additionally, memantine at the 1 and 3 mg/kg doses increased frequency of sideways threats after ethanol consumption but not after water drinking ($F(11,4) = 3.190$, $p = 0.022$; t = 2.549, $p < 0.05$; $t = 2.924$, $p < 0.01$, respectively). See Figure 6. An aggression score was generated for each animal by combining on the number of attack bites and sideways threats delivered. There was a significant increase in the percent change from the aggression score of control at the 1 and 3 mg/kg memantine doses ($F(11,4) = 3.086$, $p = 0.026$; $t = 2.726$, $p < 0.01$; $t = 2.973$, $p < 0.01$, respectively). See Figure 7. There was no significant change in tail rattle behavior at any drug dose ($F(11,4) = 1.675$, $p = 0.173$) or drink condition ($F(11,4) = 0.000838$, $p = .977$). See Figure 8.

Nonaggressive behaviors

Nonaggressive behaviors were measured in seconds. There were no significant effects at any level of drug for all nonaggressive behaviors measured—walking ($F(11,4) = 1.293$, $p = 0.287$), grooming ($F(11,4) = 1.792$, $p = 0.148$), and rearing ($F(11,4) = 0.700$, $p = 0.596$). There were no significant effects at any drink condition for all nonaggressive behaviors measured—walking ($F(11,4) = 0.543$, $p = 0.477$), grooming ($F(11,4) = 0.000151$, $p = 0.990$), and rearing ($F(11,4) = 0.142$, $p = 0.713$). See Figures 9, 10 and 11.

Experiment 2: Microinjections of AP-5 into the DRN

Animals were considered AHA mice if they displayed two or more standard deviations more aggressive behavior as defined by number of attack bites after ethanol consumption compared to aggressive behavior after water consumption ($n = 3$). Animals that did not meet this criterion were considered to be ANA mice ($n = 4$). See Table 3 and Figure 12.

Aggressive behaviors

Aggressive behaviors were analyzed after grouping animals in either the AHA or ANA category. Aggression score was calculated by summing the number of attack bites and sideways threats delivered. ANA animals showed no significant changes in ($F(3,2) = 1.304$, $p = 0.339$), attack bites ($F(3,2) = 0.694$, $p = 0.536$), sideways threat ($F(3,2) = 1.635$, $p = 0.271$) or tail rattles ($F(3,2) = 2.384$, $p = 0.173$) under any drug treatment. See Table 4. ANA animals showed no significant changes in aggression score ($F(3,2) = 10.023$, $p = 0.051$), attack bites ($F(3,2) = 2.090$, $p = 0.244$) or tail rattles ($F(3,2) = 2.457$, $p = 0.215$) under any drink level; there was a significant

increase in sideways threats after ethanol drinking compared to water drink ($F(3,2) = 15.087$, $p = 0.030$). A significant main effect of drug on aggression score under the 1.0 µg AP-5 dose was present in AHA animals ($F(2,2) = 19.947$, $p < 0.010$) though this did not depend on the drink condition ($F(2,2) = 0.121$, $p = 0.761$). See Figure 13. There was no effect on sideways threat ($F(2,2) = 0.236$, p = 0.675) or tail rattle ($F(2,2) = 0.000490$, p = 0.984) in AHA animals depending on drinking condition or drug treatment ($F(2,2) = 3.730$, $p = 0.122$; $F(2,2) = 0.798$, $p = 0.511$, respectively). See Table 5.

Nonaggressive behaviors

Nonaggressive behaviors were measured in seconds using both AHA and ANA groups together. There was no significant effect on walking, rearing, or grooming under any of the drug treatment ($F(6,2) = 2.500$, $p = 0.115$; $F(6,2) = 2.126$, $p = 0.159$; $F(6,2) = 0.256$, $p = 0.778$, respectively) or drink conditions ($F(6,2) = 3.257$, $p = 0.116$; $F(6,2) = 0.754$, $p = 0.417$; $F(6,2) = 0.000524$, $p = 0.982$, respectively). See Figures 14, 15 and 16.

Histology

Correct cannulae placement in the DRN was verified for seven of the animals originally enrolled in the experiment. See Figures 17 and 18.

Discussion

Memantine significantly increased aggressive behavior as measured by percent change from control after ethanol but not water drinking. Since ethanol is known to antagonize the

NMDAR (Ron & Wang, 2009), it is expected that additional antagonism of the receptor via memantine administration should escalate aggressive behavior induced by ethanol. These ANA animals did not show any significant escalation after ethanol drinking compared to water. However, they did show a dose-dependent increase in aggressive behavior at doses specific to antagonism of the NMDAR (1 and 3 mg/kg, i.p.) after ethanol but not water consumption. These data support the connection between ethanol and the NMDAR. There was no significant change in walking, rearing, or self-grooming behaviors. Besides supporting the selective effects of the drug, this is important in a clinical setting since many of the NMDAR antagonists used before have had undesirable side effects such as hallucinations (Witt et al, 2004). In a clinical study, memantine administration led to the experience of alcohol-like subjective effects (Bisaga and Evans, 2004). Although the mechanism behind ethanol's NMDAR antagonism remains unclear, it may be similar to the way memantine antagonizes the receptor, by binding to the ion channel site. In this study, memantine had no effect on aggression after water drinking; however, this is in contrast to previous studies which looked at frustration and instigation. At higher, clinically irrelevant doses (17 and 30 mg/kg), memantine dose-dependently reduced aggressive behavior.

Although the link between aggression and alcohol holds much clinical significance, the mechanisms behind the correlation have not been fully investigated. Some individuals are more likely than others to engage in aggressive behavior after ethanol drinking (Cloninger, 1987). These individual differences are essential to exploring the mechanism behind ethanol and aggression. Due to the large amount of variability during the characterization period in many of the animals in the first experiment, the identification of animals of AHA animals was compromised. It is important to recognize that those individuals that show species atypical levels of aggression after alcohol consumption are clinically relevant models. Because there were no

identified AHA animals, it is difficult to assess the degree to which glutamate, specifically the NMDAR, plays a role in alcohol-heightened aggression. Nevertheless, these data support the idea that some of ethanol's effects may be mediated by glutamate specifically by the NMDAR.

In addition to the in vitro studies that have shown ethanol's antagonism of the NMDAR (Lovinger et al., 1989; Wright et al., 1996; Lovinger et al., 1990), clinical studies using NMDAR antagonists confirm a connection between ethanol and the receptor. Most clinical antagonists studies have focused on alcohol-related phenomena such as dependence or craving (Trujillo et al., 1995; Krystal et al., 2003). The ability of NMDAR antagonists to reduce alcohol cravings not only supports the ethanol-NMDAR connection but also emphasizes the clinical importance of understanding the mechanism behind the ethanol-NMDAR relationship.

The DRN has been targeted as a potential important site for alcohol-heightened aggression in mice (Hwa et al., *unpublished* 2009). In this study, glutamate's role in this phenomenon was investigated in this region. Because NMDAR receptors are found on GABAergic and serotonergic neurons in the DRN (Adell et al., 2002), activation or inactivation of these receptors in conjunction with ethanol consumption may produce behavioral effects associated with alcohol-heightened aggression. Previous studies have shown that levels of extracellular 5-HT in the DRN are inversely correlated with levels of 5-HT in the prefrontal cortex; these levels can be manipulated depending on the concentration of NMDA injected into the DRN (Pallotta et al., 1998). These effects are blocked by the application of AP-5 (Pallotta et al., 1998; Gartside et al., 2007; Mokler et al., 2009). Additionally, studies have shown an increase in extracellular serotonin in the mPFC after an uncontrollable stress as compared to a controllable stress (Amat et al., 2005). Glutamatergic modulation of serotonergic levels in the

DRN and consequently in the mPFC may underlie the response to a controllable versus an uncontrollable stressor.

Animals in the AP-5 microinjection experiment were able to be categorized into two subgroups—AHA and ANA. In AHA animals but not ANA animals, there was a significant drug effect at both drug doses; however, there was not a statistically significant interaction with alcohol. Because there were only three AHA animals, there was a high degree of variability which may have hidden any statistically significant interaction. Currently, more animals are being added to this experiment. Additionally, since there are NMDARs on serotonergic as well as GABAergic neurons in the DRN, there may have been a nullification of the effect of NMDA antagonism.

Because NMDARs are implicated in learning and memory and synaptic plasticity (Collingridge & Singer 1990; Danysz et al., 1995), antagonism of the receptor also disrupts normal synaptic transmission which can result in many undesirable behavioral effects (Parsons et al., 1999). This is more likely at higher doses of memantine and AP-5; although there were no significant changes in nonaggressive behaviors, there were visible signs of impairment at the 10 and 17 mg/kg memantine doses and the 1.0 μg AP-5 dose. The results of this study are promising because the doses at which we saw significant increases in aggressive behavior were lower, more NMDAR selective doses that did not produce visible or significant behavioral effects.

Currently, we are investigating the relationship between a fixed memantine dose and ethanol at varying ethanol doses. The ethanol doses currently being tested were used in a Miczek and colleagues study in which they saw an inverse U-shaped trend with the peak level of aggressive behavior at the 1 g/kg dose (Miczek et al., 1998). We expect from this experiment

that memantine administration in combination with ethanol should shift the aggression curve after ethanol drinking up and have no effect after water drinking.

Since differential levels of NMDA yielded different effects on serotonergic activity in the DRN (Pallotta et al., 2008), we may see a different trend for different levels of NMDAR inactivation. Besides additional AP-5 doses, it may be interesting to investigate different types of antagonism in the DRN. In a study examining the discriminating effects of ethanol, uncompetitive antagonism (MK-801) not competitive antagonism (CPP) of the NMDAR in the CA1 hippocampal area and nucleus accumbens resulted in full substitution for ethanol (Hodge & Cox, 1998).

Results from the current study are a stepping stone in investigating the role glutamate may play in alcohol-heightened aggression; it would be important to explore different receptors and their particular influence over serotonergic innervation in the DRN. Glutamate's effects on alcohol-heightened aggression may be co-mediated by AMPA/kainite receptors in the DRN. Ethanol has been shown to inhibit AMPA receptor function in mouse hippocampal and cortical neurons (Möykkynen et al., 2008). Additionally, AMPA receptors like NMDARs regulate 5-HT firing activity in the DRN (Gartside et al., 2007). Future research should consider the function that these glutamate receptors have in alcohol-heightened aggression.

Although in this experiment we targeted the DRN, it may be interesting to investigate different brain areas. One study showed that ethanol inhibited NMDAR-induced activity in the inferior colliculus and hippocampus and not the lateral septum (Simpson et al., 1993). Similarly, NMDAR subunit modification by ethanol is not present in all brain areas (Ron & Wang, 2009). There is also substantial evidence that glutamatergic inputs from the mPFC affect 5-HT neurons

in the DRN (Tao & Auerbach, 2000; Celada et al., 2001). Future studies may consider targeting the hippocampus or medial prefrontal cortex to see if outside DRN glutamate modulation plays a role in alcohol-heightened aggression.

After seeing an increase in aggressive behavior with a memantine-ethanol combination, we should expect to see a decrease in alcohol-induced aggressive behavior with an NMDA agonist and ethanol combination. However, no one neurotransmitter is responsible for alcohol-heightened aggression. Instead, the interactions between neurotransmitters are responsible for the behavioral changes associated with alcohol-induced aggression. In particular, the $GABA_A$ receptor may mediate alcohol-heightened aggression in the DRN alongside the NMDAR. Kock and colleagues (2006) postulated the NMDAR activation could indirectly facilitate GABAergic input on DRN serotonergic neurons. Addition of bicuculline, a $GABA_A$ receptor antagonist, in the DRN amplified the response of 5-HT cells to NMDA and AMPA (Gartside et al., 2007). The two receptors may be connected in terms of influencing alcohol-induced aggressive behavior.

In conclusion, the present study suggests that glutamate specifically via the NMDAR may have a role in regulating alcohol-heightened aggression in mice; however, this role may not be limited to the NMDAR or the DRN.

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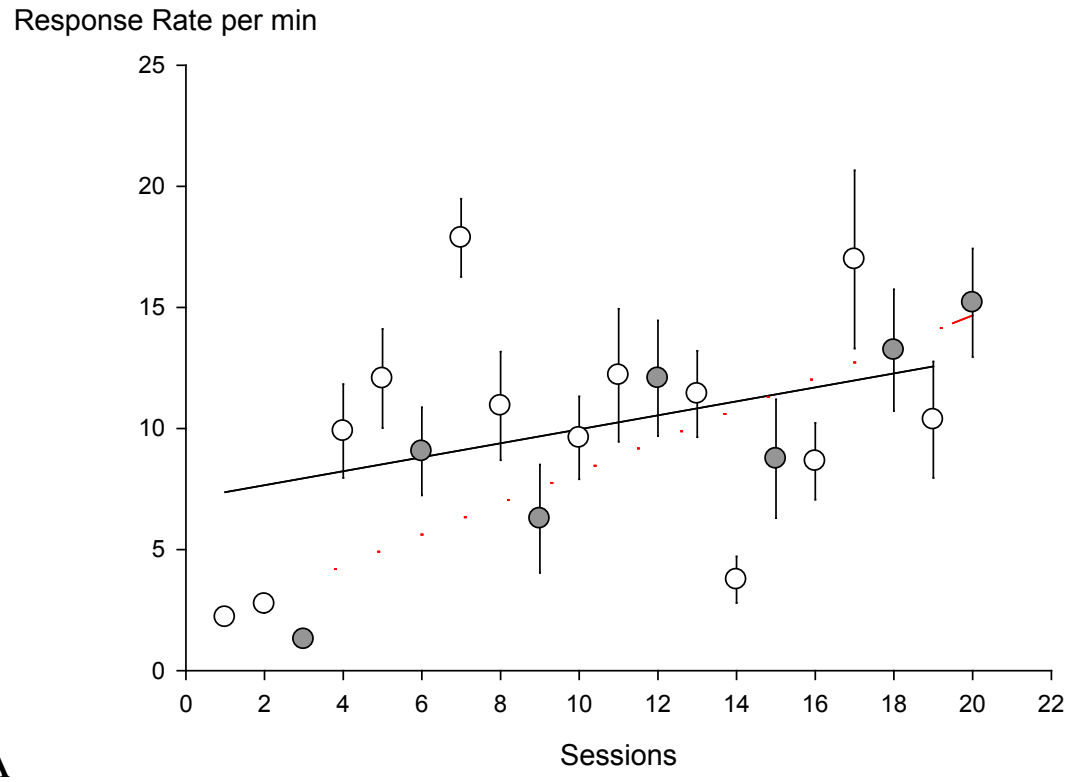
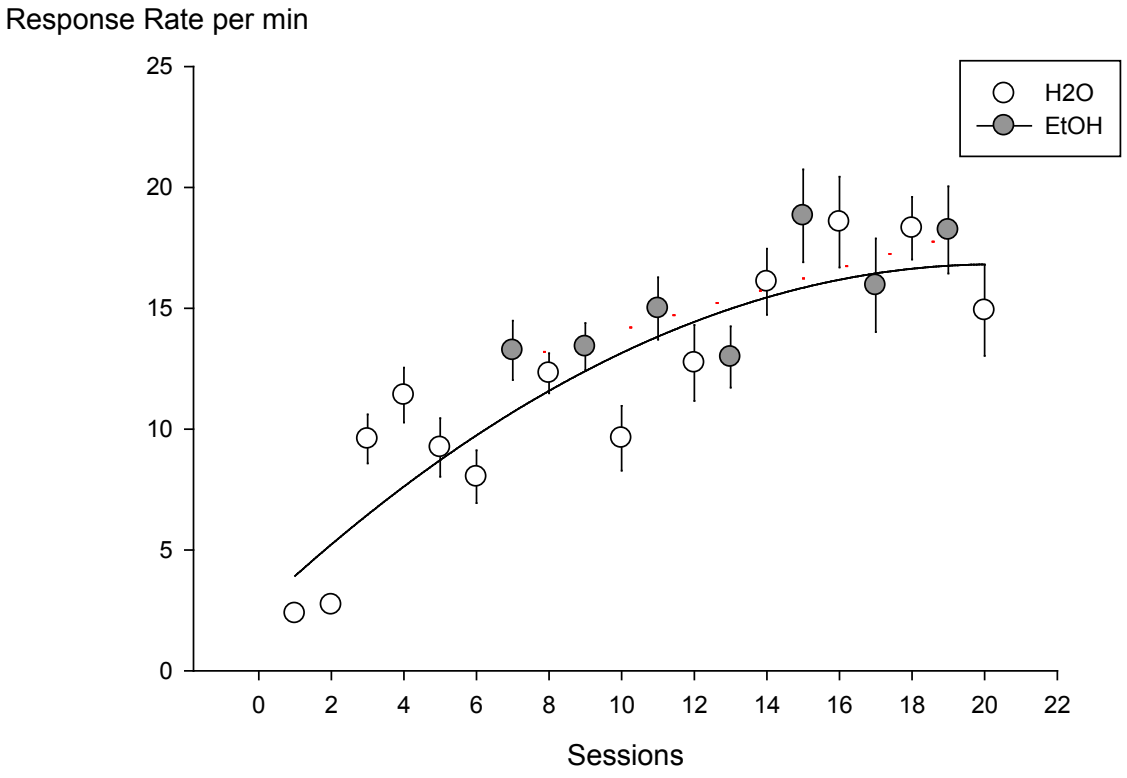
Figure 3. Response rate per minute versus session number. Nosepoke response rate per minute according to session. Clear circles represent days of water consumption. Dark grey circles represent days of ethanol consumption. **A** Protocol 1 **B** Protocol 2



A


Response Rate per min
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
20
22
Sessions
H2O
EtOH

B

Figure 4. Aggressive behavior stabilization according to session number. Animals showed stabilized levels of fighting, less than 20% variance in attack bites, after 7-8 sessions of fighting.

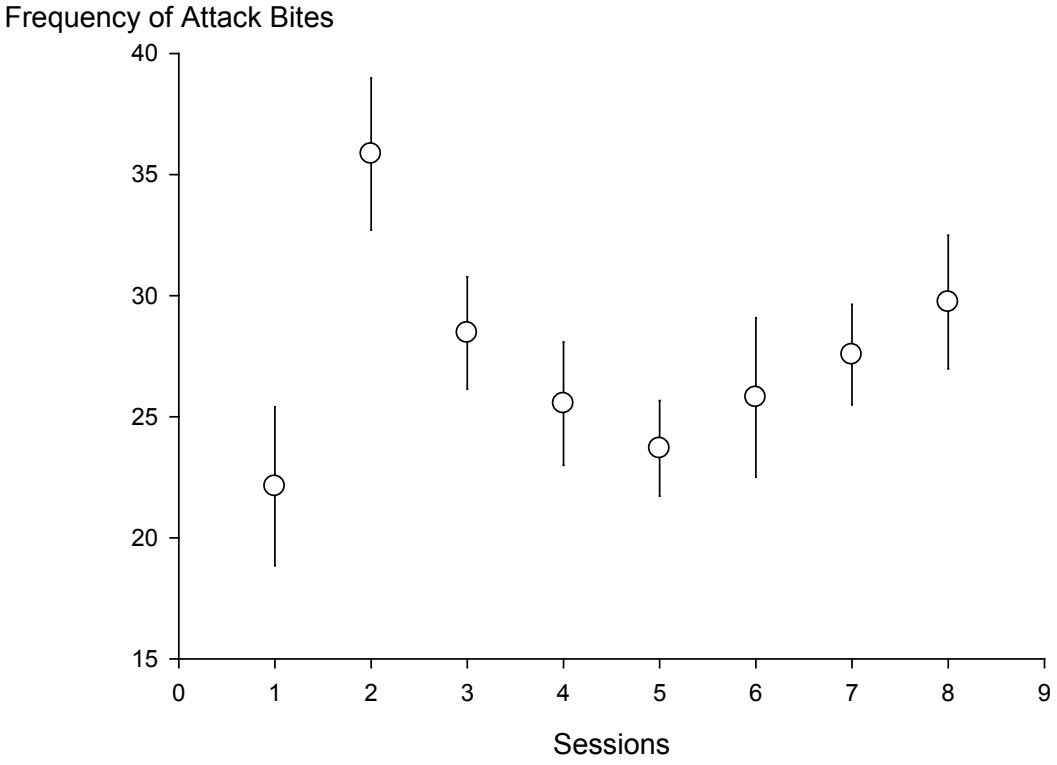


Table 2. Memantine characterization data. Water average of attack bites plus two standard deviation comparison to ethanol average of attack bites.

Animal	H2O AVG	STDEV	STDEV+2	EtOH AVG
843	12	3	18	14.66667
846	15.66667	1.154701	17.97607	23.33333
849	35.66667	1.527525	38.72172	33.33333
850	17	7.211103	31.42221	12.33333
851	28.33333	8.144528	44.62239	32.33333
853	48	22.71563	93.43127	41.66667
856	18.33333	6.658328	31.64999	15.66667
1672	17.33333	8.082904	33.49914	28
1673	11.33333	5.131601	21.59654	17.33333
1674	26.66667	6.429101	39.52487	31
1675	8.333333	4.725816	17.78496	11
1676	17.66667	3.05505	23.77677	16

Figure 5. Percent change of attack bites from control versus memantine dose. Animals showed an increase in percent change of attack bites from control in combination with memantine under ethanol but not water. Animals showed significantly higher increase at 3 mg/kg ($p < 0.05$).

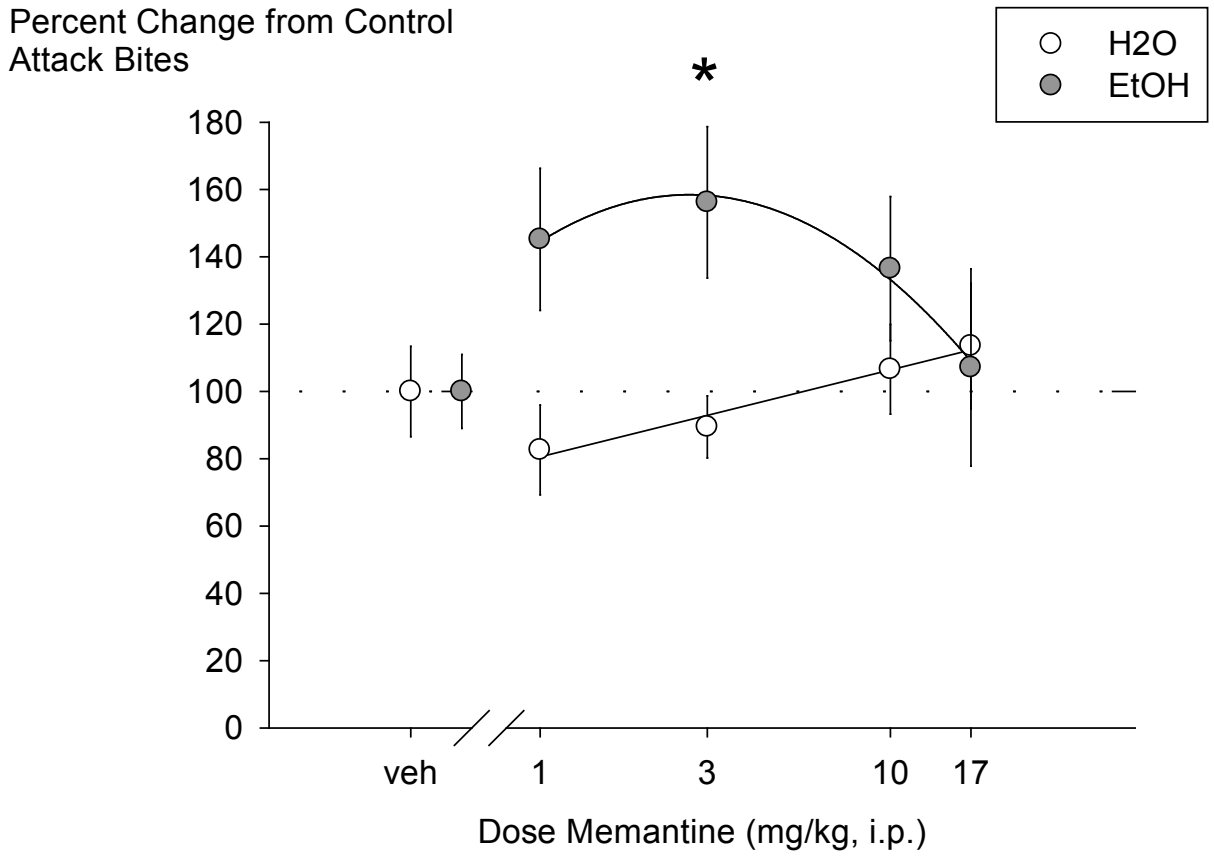


Figure 6. Percent change of sideways threats from control versus memantine dose. Animals showed an increase in percent change of sideways threats from control in combination with memantine under ethanol but not water. Animals showed significantly higher increase at 1 and 3 mg/kg ($p < 0.05$ and $p < 0.01$, respectively).

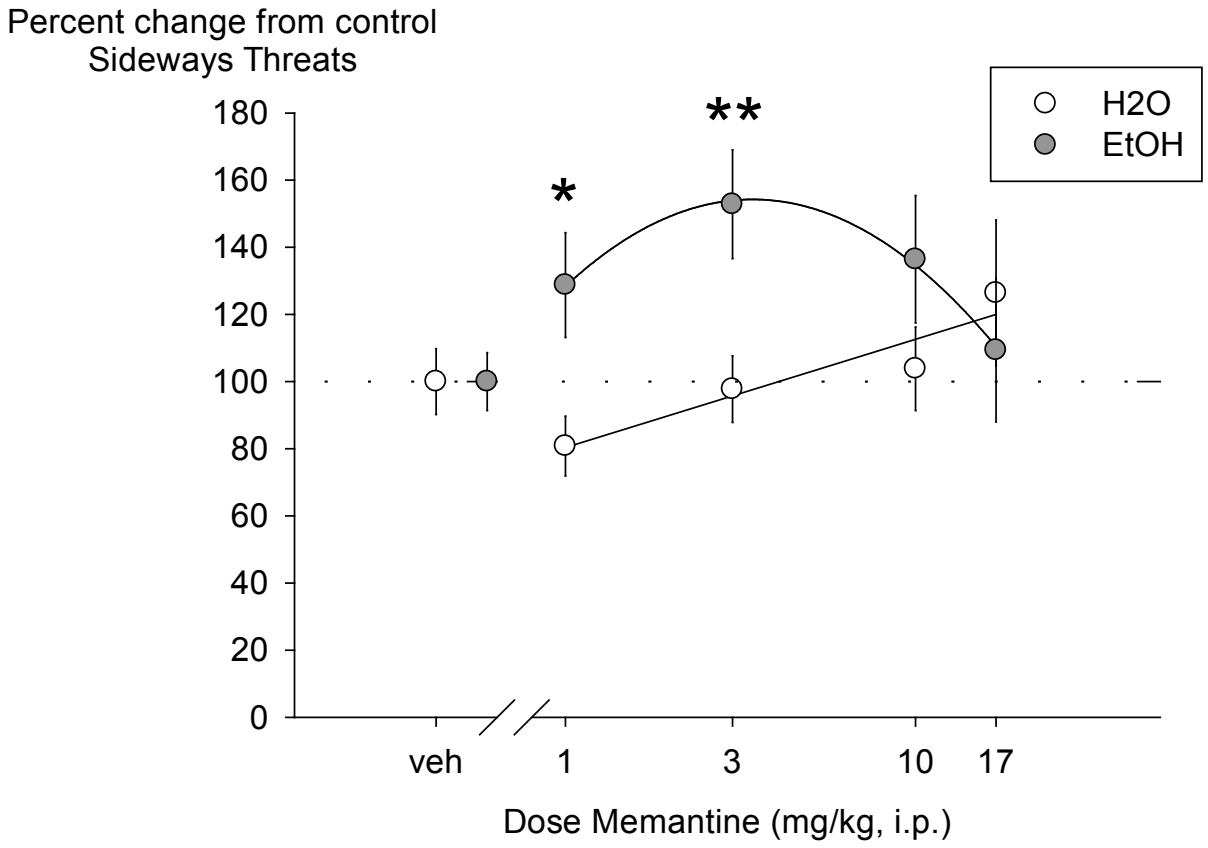


Figure 7. Percent change of aggression score from control versus memantine dose. Animals showed an increase in percent change of aggression score from control in combination with memantine under ethanol but not water. Animals showed significantly higher increase at 1 and 3 mg/kg ($p < 0.05$).

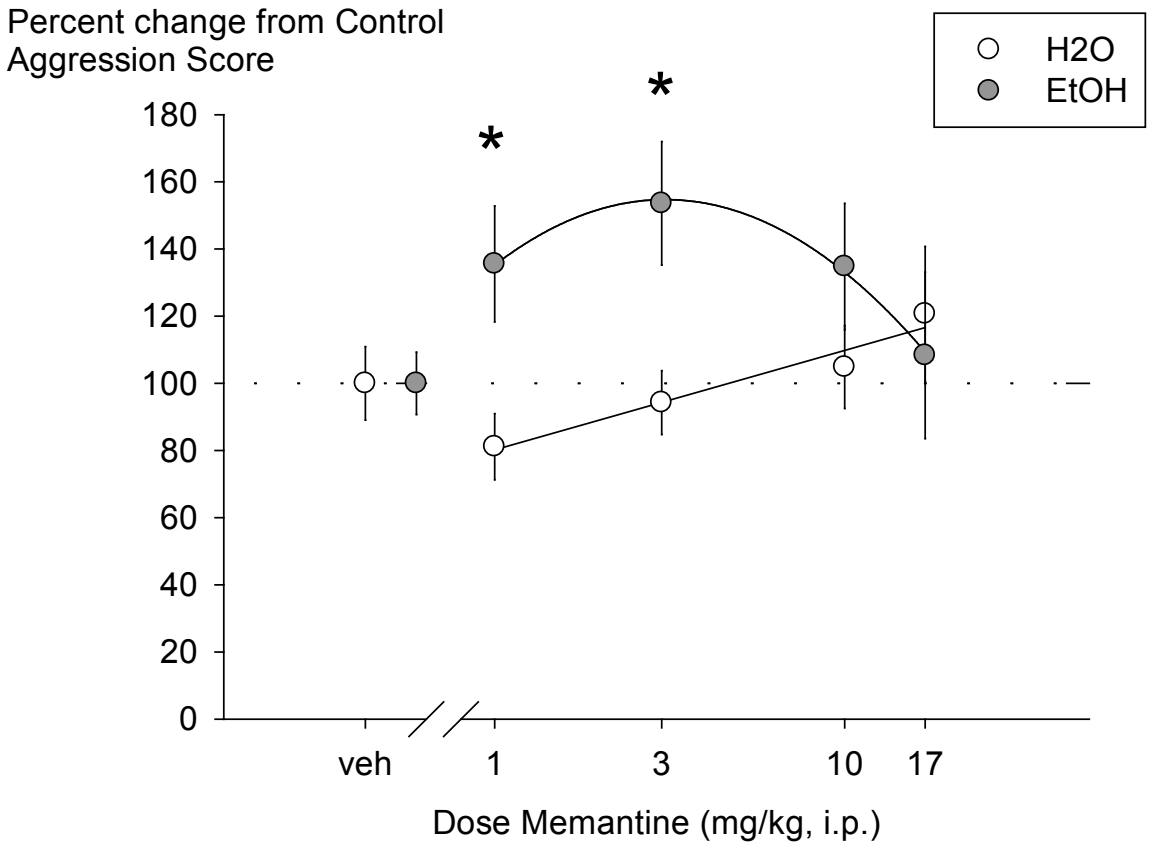


Figure 8. Percent change of tail rattles from control versus memantine dose. Animals did not show any significant change in tail rattle behavior under any drink or drug condition.

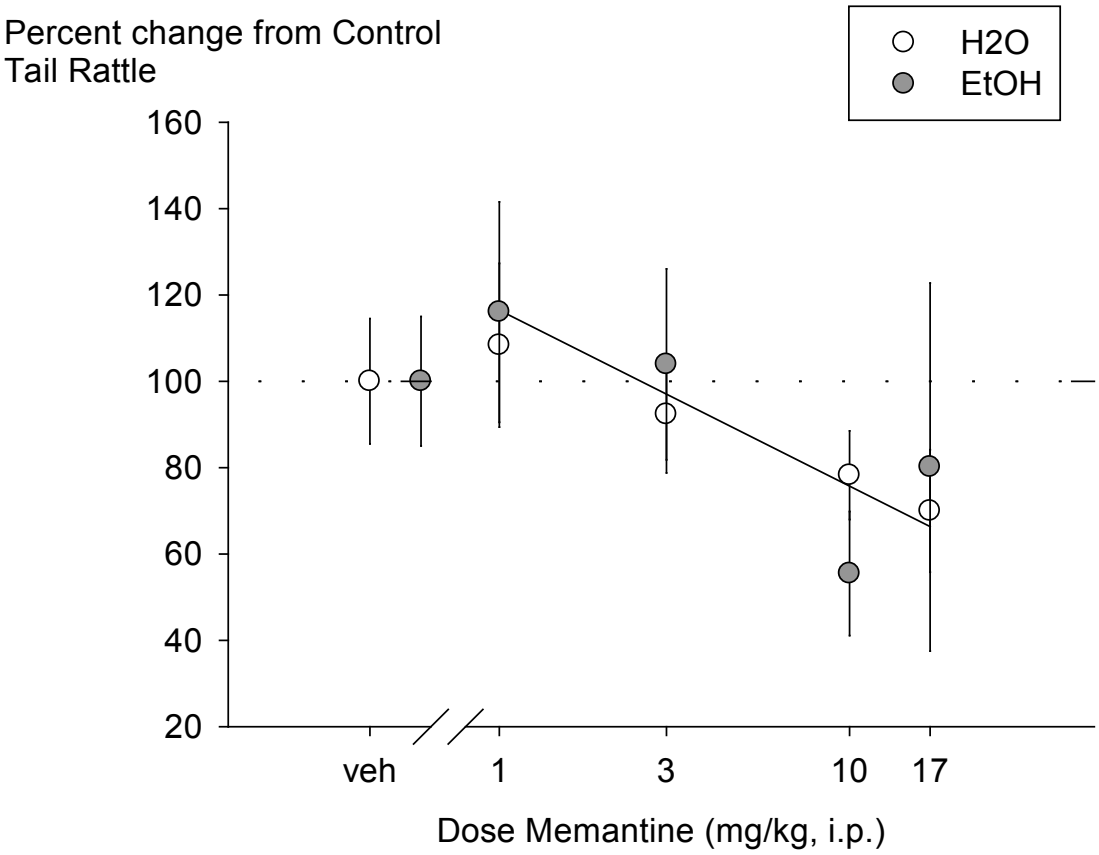


Figure 9. Rearing behavior versus memantine dose. Animals did not show any significant difference in rearing behavior under any drink or drug condition.

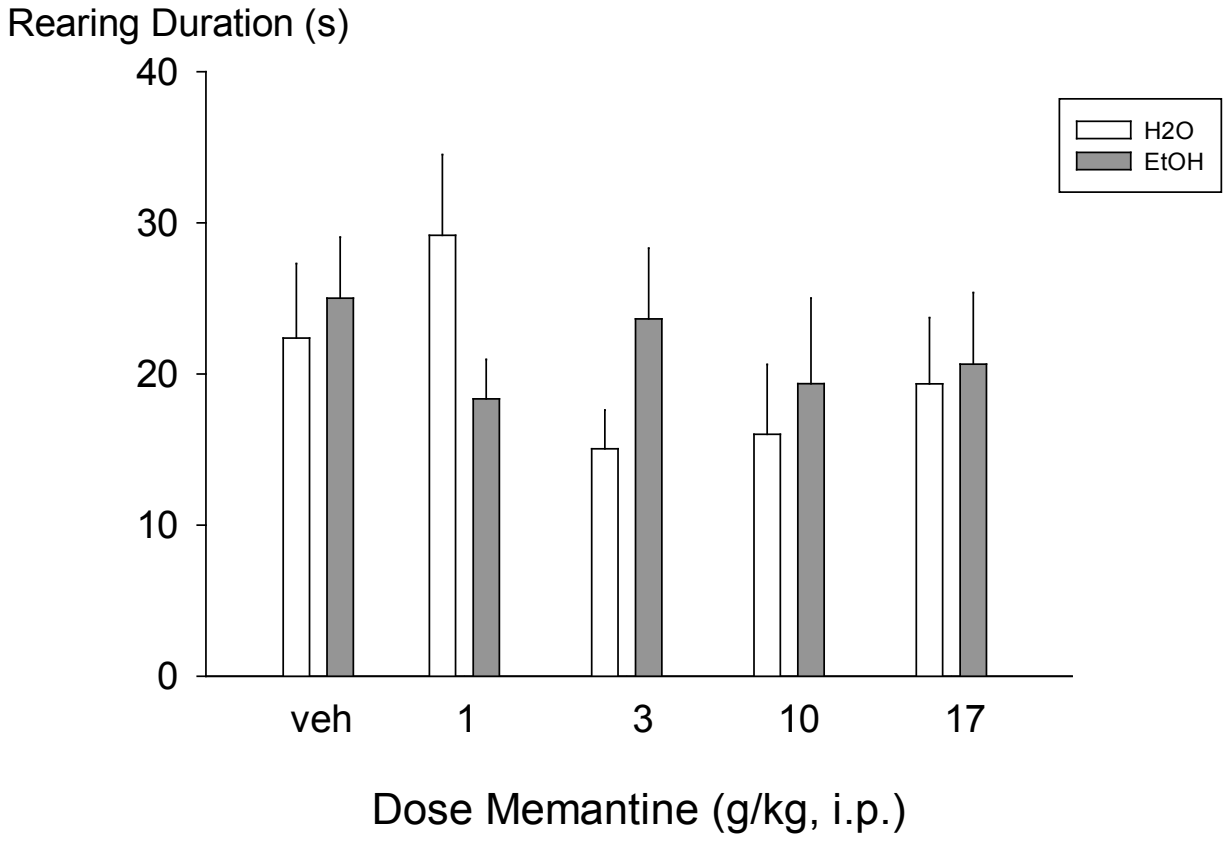


Figure 10. Walking behavior versus memantine dose. Animals did not show any significant difference in walking behavior under any drink or drug condition.

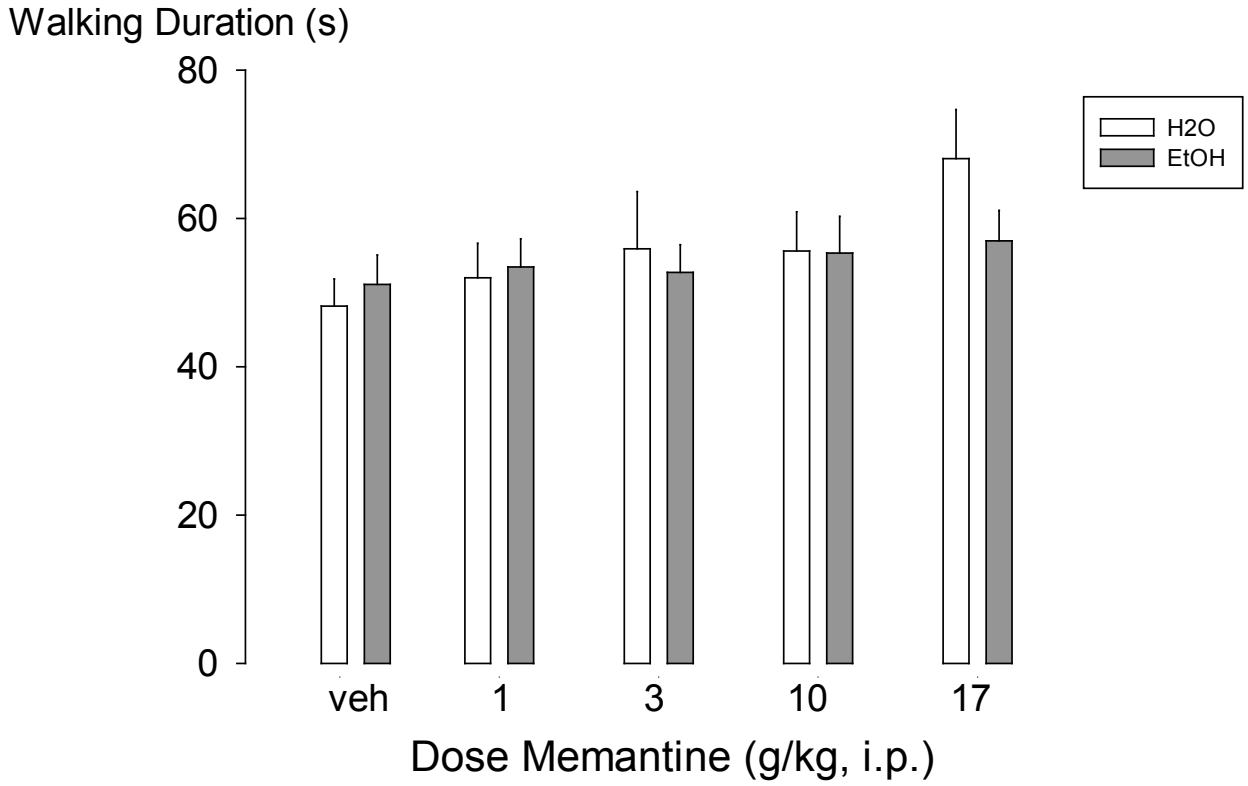


Figure 11. Self-grooming behavior versus memantine dose. Animals did not show any significant difference in self-grooming behavior under any drink or drug condition.

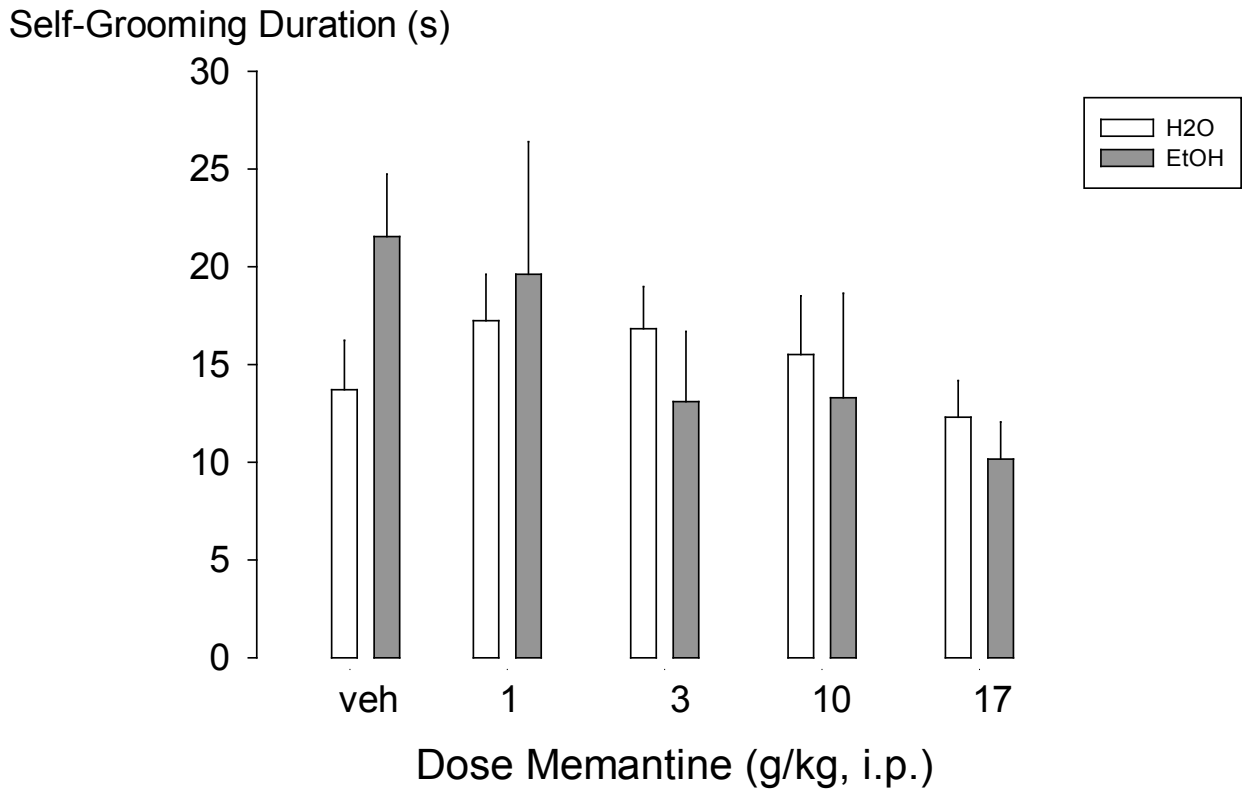


Table 3. AHA ANA AP-5

Figure 12. Frequency of attack bites according to sub-groups of alcohol-non-heightened aggressors (ANA) and alcohol-heightened aggressors (AHA).

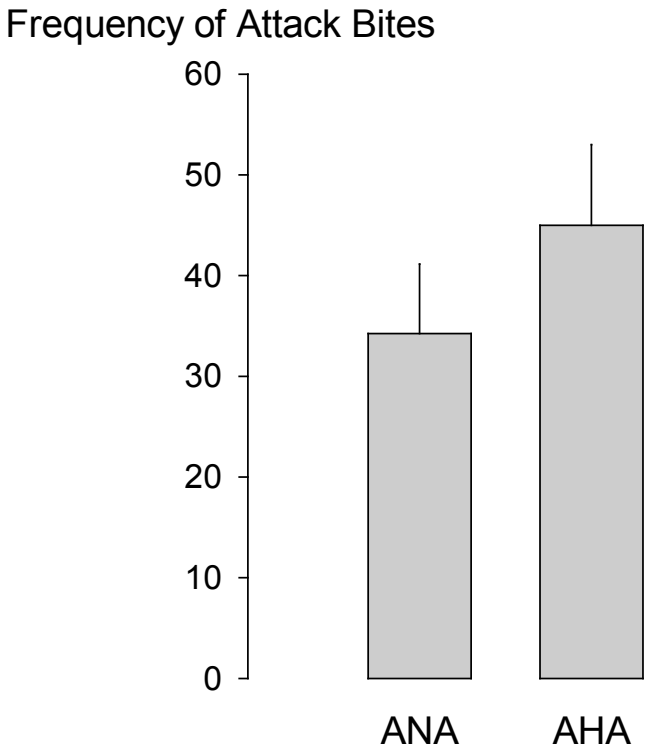


Table 4. AP-5 characterization data. Water average of attack bites plus two standard deviation comparison to ethanol average of attack bites. Italicized animals represent AHA animals. 1677-1683 are currently being tested.

Animal	AVG H2O	STDEV	STD +2	VAR/AVG	AVG EtOH
1007	*13.66667*	*2.081666*	*17.83*	*0.152317*	*23.66667*
1009	*12.33333*	*3.05505*	*18.44343*	*0.247707*	*20.33333*
1010	30.66667	11.93035	54.52737	0.389033	26.33333
1011	12.33333	5.507571	23.34847	0.44656	14.33333
1014	20	1.732051	23.4641	0.086603	14.33333
1017	*14.66667*	*3.05505*	*20.77677*	*0.208299*	*23.66667*
1018	9.666667	5.507571	20.68181	0.569749	8.666667
1677	16.33333	6.658328	29.64999	0.407653	21.33333
1678	14	2.645751	19.2915	0.188982	11
1679	*30.33333*	*5.033223*	*40.39978*	*0.16593*	*43*
1683	*4*	*1*	*6*	*0.25*	*9*

Figure 13. Aggression score for AHA animals (*n* = 3) versus AP-5 dose. Aggression score was calculated by summing total number of attack bites and sideways threats. There was a significant drug effect at the 1.0 µg drug dose ($p < 0.05$)

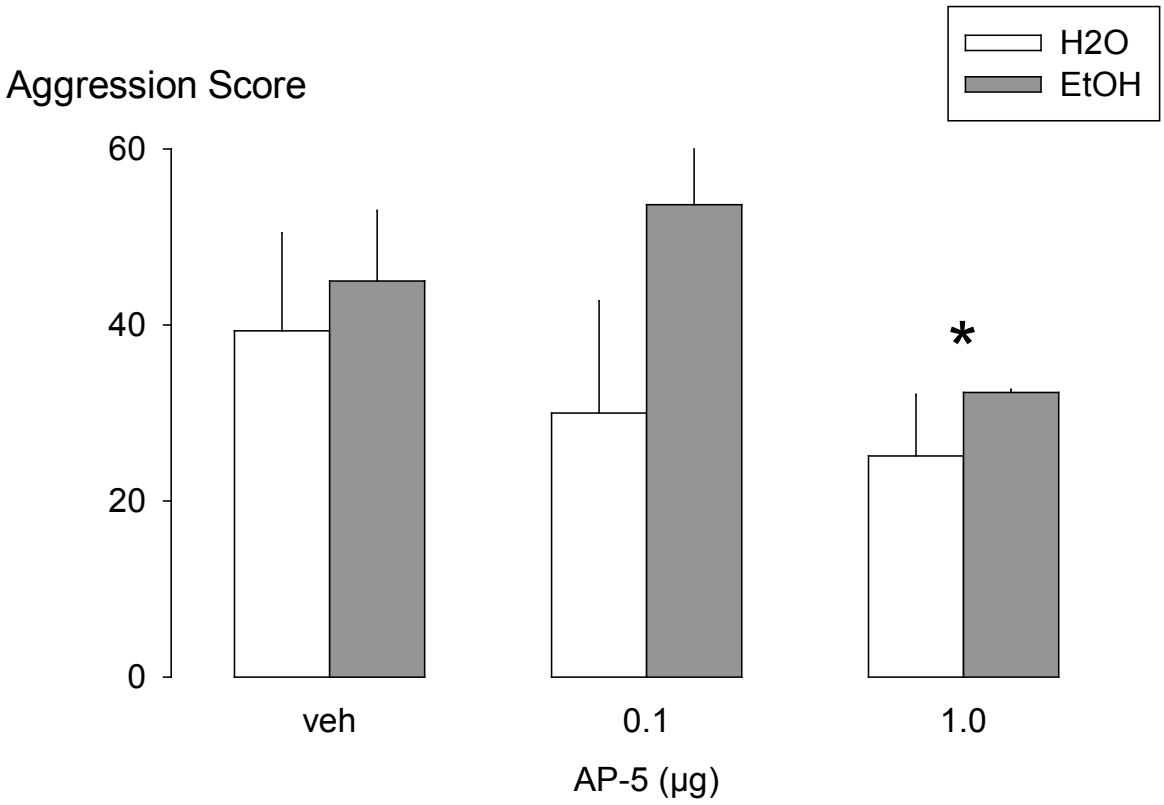


Table 5. Frequencies of attack bites, sideways threats, and tail rattles in AHA animals (*n* = 3). Numbers in parentheses represent standard error of the mean.

Behavior	Dose AP-5 (µg)	Water		Ethanol	
Attack Bites	Vehicle	16	*(5.5)*	13	*(2.5)*
	0.1	11.3	*(4.1)*	13.3	*(2.7)*
	1.0	8.9	*(1.7)*	10.7	*(2)*
Sideways Threats	Vehicle	23.3	*(6.5)*	23	*(6)*
	0.1	18.7	*(8.7)*	28	*(8.1)*
	1.0	16.2	*(5.2)*	20.7	*(1.3)*
Tail Rattles	Vehicle	8.7	*(2.4)*	11.7	*(5.7)*
	0.1	11.7	*(5.2)*	11	*(4.4)*
	1.0	8.3	*(3.4)*	6.3	*(1.8)*

Figure 14. Self-grooming behavior versus AP-5 dose. Animals did not show any significant difference in self-grooming behavior under any drink or drug condition.

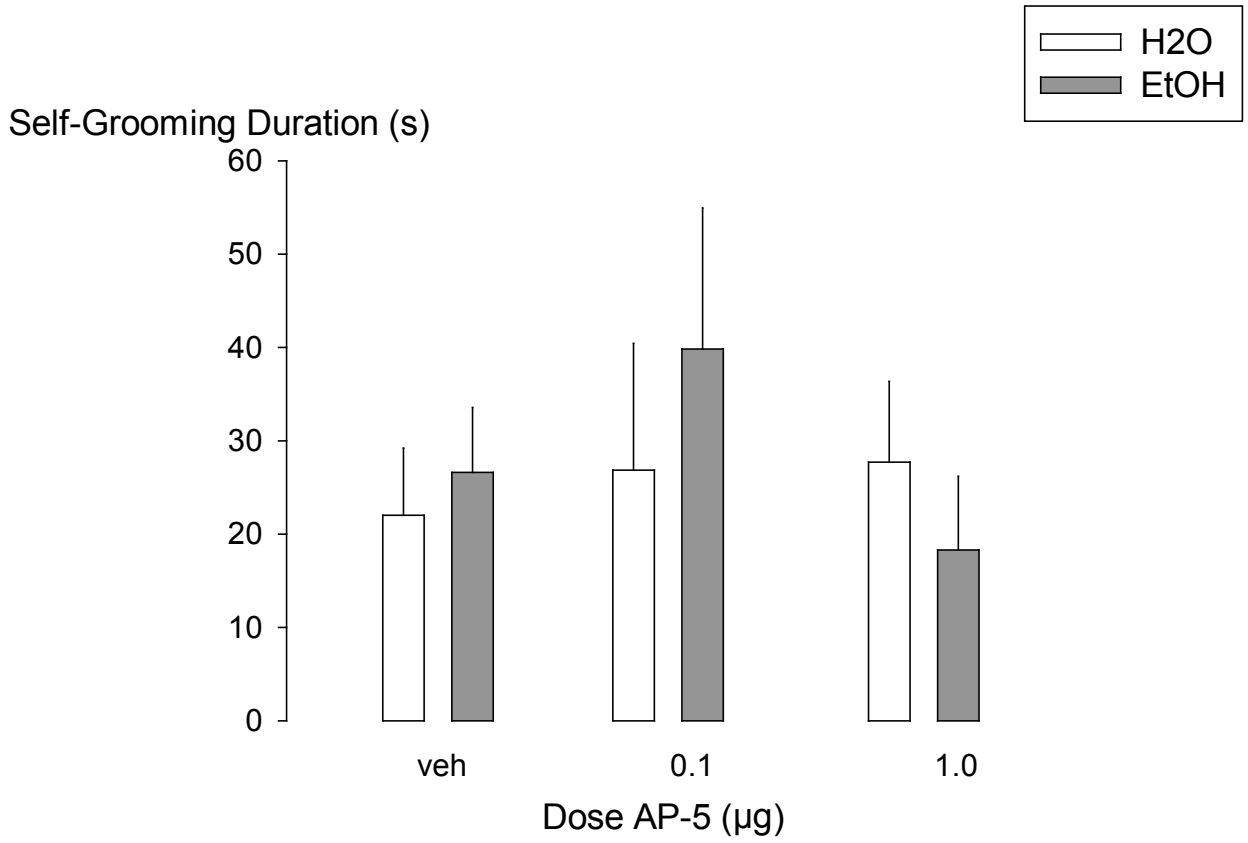


Figure 15. Walking behavior versus AP-5 dose. Animals did not show any significant difference in walking behavior under any drink or drug condition.

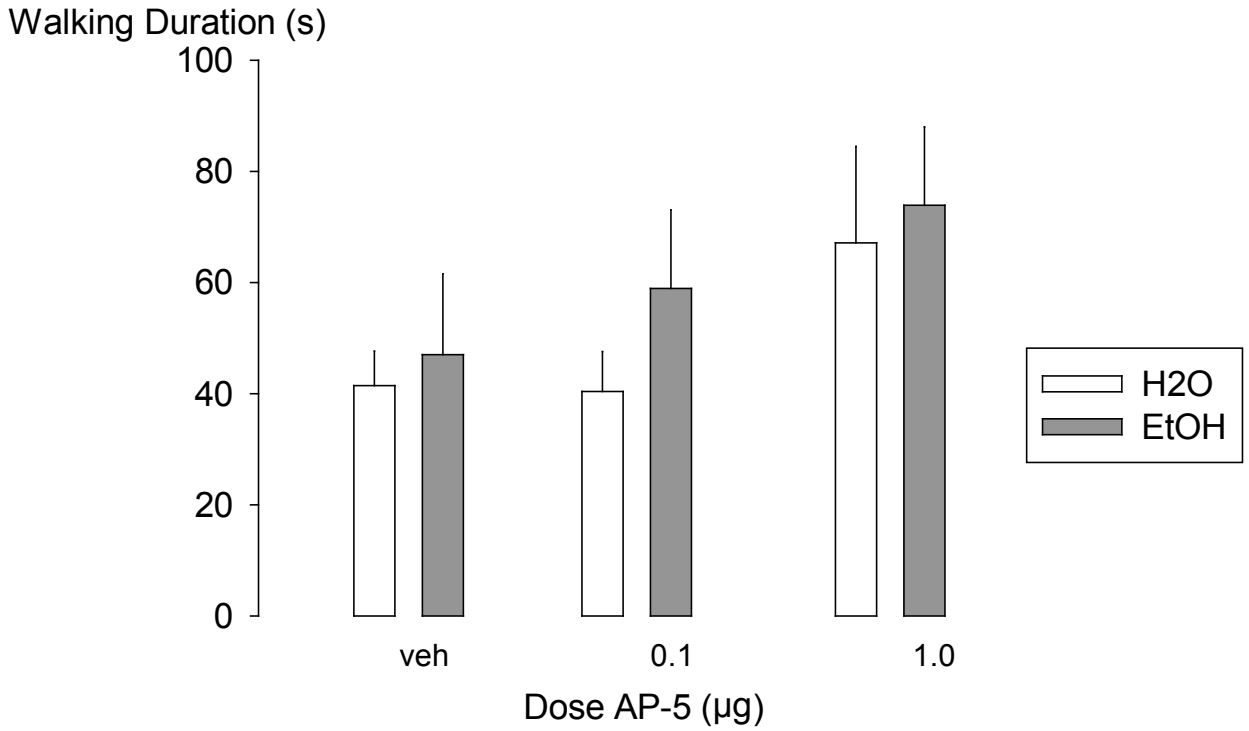


Figure 16. Rearing behavior versus AP-5 dose. Animals did not show any significant difference in rearing behavior under any drink or drug condition.

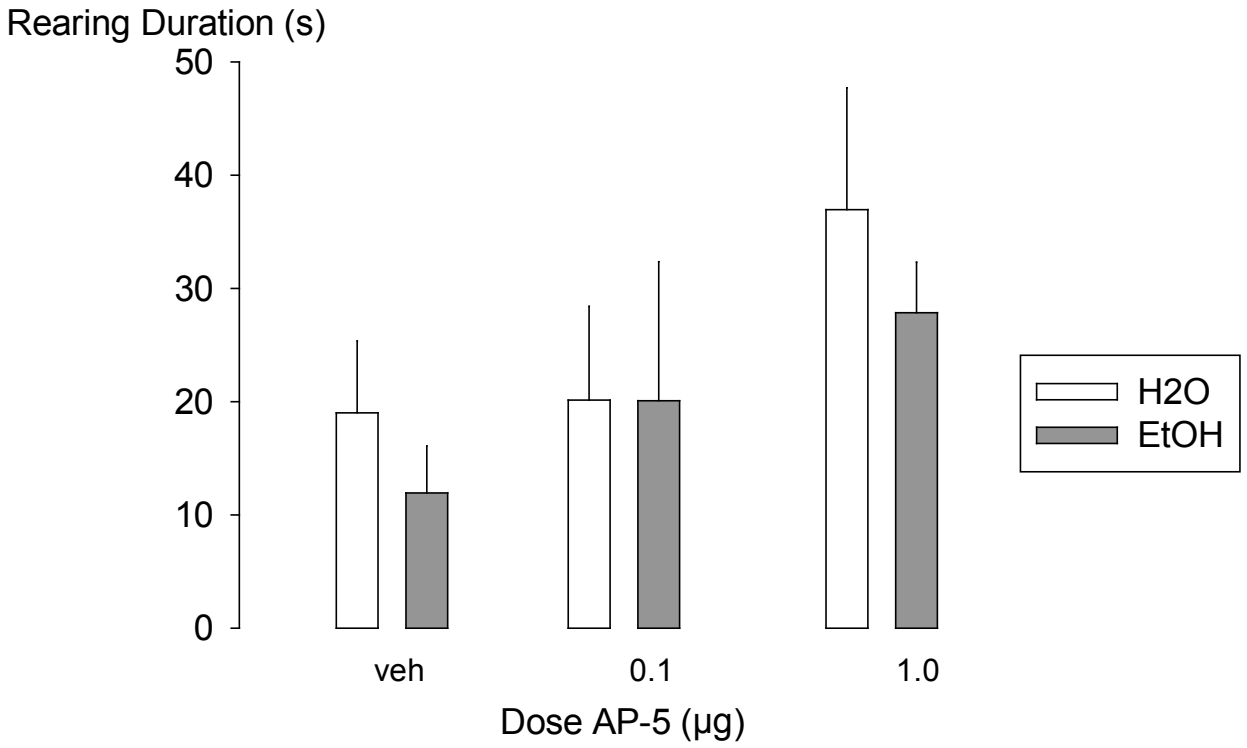


Figure 17. Ccannulae verification for AP-5 animals. Mouse atlas view of cannulae placements.

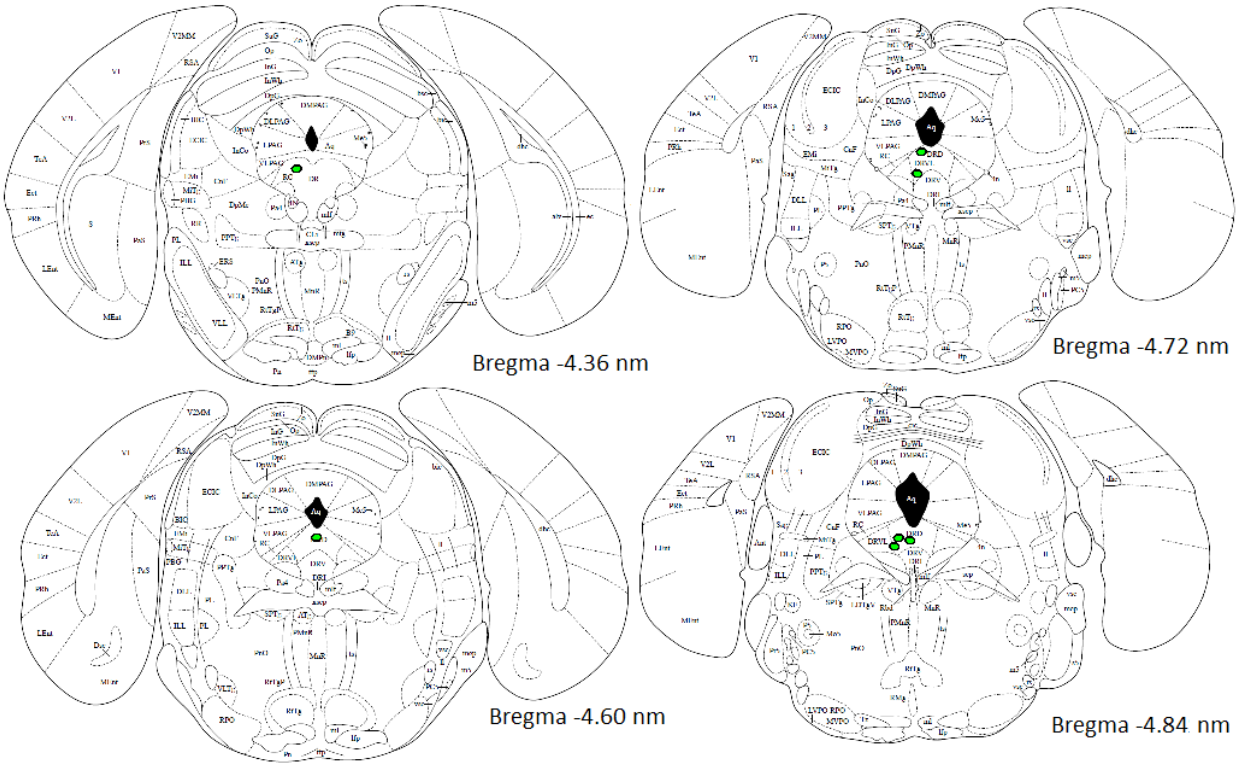


Figure 18. Example of intra-DRN cannula placement.