The Effect of Ketamine on Motor Coordination and Thermal Nociception in Ethanol-Withdrawn Mice

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THE EFFECTS OF KETAMINE ON MOTOR COORDINATION AND THERMAL
NOCICEPTION IN ETHANOL-WITHDRAWN MICE

by

Jameson M. Ford

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of the Requirements for a Degree with Honors
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Abstract

This experiment aimed to establish a model for long and/or short-term ethanol-withdrawal induced motor deficits and thermal sensitivity in mice. Additionally, the effects of ketamine on these phenomena were evaluated. Human studies of alcoholics have documented, along with behavioral depression, deficiencies in gait and balance, as well as an increase in pain sensitivity, immediately following abstinence, and in some cases, lasting many years. Ketamine, an NMDA antagonist, has been shown alleviate behavioral depression in both animal and human models, but its physiological mechanisms are still being evaluated. Ethanol dependence was induced using chronic intermittent alcohol vapor exposure. Low-dose ketamine was injected immediately following the 8-day ethanol vapor protocol. Animals were then tested for motor coordination via an accelerating rotarod (ARR), and for thermal nociception via the radiant heat tail-flick test. Tests were run at 6 hours, 24 hours, then each day for two weeks post-withdrawal. Data show a significant, late-emerging effect of ethanol on ARR scores, establishing a novel model for long-term ethanol withdrawal-induced motor coordination deficiencies in mice. Additionally, no effects of ketamine were observed. Finally, No effects of either ethanol or ketamine were seen in the tail-flick assay.
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Introduction

Ethanol Withdrawal Syndrome: An Overview

Alcoholism, otherwise known as alcohol dependence, is a disorder affecting millions of people every day in the US and beyond. The National Institute of Alcohol Abuse and Alcoholism (NIAAA) estimates 17 million Americans have an alcohol use disorder, including alcoholism and other harmful drinking, and sights alcohol use as the leading cause of death and disability globally in persons ages 15-49 (NIAAA, 2014). Unfortunately, complications related to alcoholism do not cease when a person gives up drinking and becomes abstinent from alcohol. Most are aware of the symptoms that can arise during the acute stages of ethanol withdrawal, known as alcohol withdrawal syndrome, which include tremors, agitation, autonomic hyperactivity, delirium, seizures, gait and balance deficiencies, and increased pain sensitivity (hyperalgesia) (Soto et al., 1985; Jochum et al., 2010; Heilig et al., 2010; Fein et al., 2013; WebMD, 2014). Such symptoms usually resolve within a few days of abstinence. In the weeks to follow, anxiety, depression, sleep and circadian rhythm disruption, and continuing motor deficiencies often occur (Soto et al., 1985; Soto et al., 1989; Heilig et al., 2010; Fein et al., 2013). In some cases, protracted withdrawal symptoms can be observed for months, or even years, after a person stops consuming alcohol, including long-term depression and anxiety, sleep disturbances, stress-induced craving and relapse, and persistent gait and balance disruption (Soto et al., 1985; Soto et al., 1989; Martinotti, 2008; Heilig et al., 2010; Fein et al., 2013). These symptoms contribute to a condition known as post-acute-withdrawal syndrome, or PAWS (Addictions and Recovery, 2014). The incidence of
post-acute-withdrawal syndrome is a subject of contention, as an unknown number of individuals may experience this condition without seeking medical attention. Based on various studies conducted in the later half of the twentieth century, PAWS can last anywhere from a few months up to 10 years or more, though in all cases symptoms tended to become less severe over time (Soto et al., 1985).

Causes of PAWS can be attributed both to chemical and psychocognitive mechanisms (Rimondini et al., 2008). The acute behavioral effects of alcohol consumption result in part from the activation of GABA<sub>A</sub> receptors in a few key areas of the brain, including the amygdala (related to anxiety relief), hippocampus (cognitive effects), cerebellum (balance deficits), and preoptic area (sleep effects) (Sanna et al., 2003; Clapp et al., 2008). Repeated long-term exposure to alcohol, as seen in alcoholics, produces a tolerance to the behavioral effects due to decreased responsiveness in GABA<sub>A</sub> receptors as a result down regulation of the genetic transcription of specific GABA<sub>A</sub> subunits. During withdrawal, the central nervous system enters a state of hyperexcitability as a result of these adaptive changes in the balance of excitatory and inhibitory neurotransmission, which has been implicated in relation to the symptoms of alcohol withdrawal. Thus, the pharmacological management of acute withdrawal involves the use of drugs that counteract this hyperexcitability, such as benzodiazepines, and more recently, acamprosate (Clapp et al., 2008). The time frame for which GABA<sub>A</sub> receptor function returns to normal, baseline levels post-withdrawal is widely variable between patients, and may be related to why some individuals develop PAWS and others do not (Sanna et al., 2003).
The upregulation of glutamate release, in many of the same key brain areas as we see GABA affected, is a second important factor in alcohol dependence and long-term withdrawal, and thus should be considered in relation to PAWS. Acute exposure to ethanol has been found to inhibit glutamatergic transmission in the hippocampus, cerebellum, cerebral cortex, and amygdala, contributing to the anxiolitic, sedative, and intoxicifying effects of alcohol (Clapp et al., 2008). In the presence of chronic alcohol exposure, adaptive neural mechanisms compensate for the glutamatergic depression, leading to an eventual increase in glutamate release. Here we see interplay between GABA and glutamate systems, in that heightened activity in glutamate interneurons further inhibit action from GABA neurons (Clapp et al., 2008). Upon removal of alcohol following chronic exposure, the brain enters a hyperglutamatergic state, contributing to symptoms of withdrawal including anxiety and agitation, and thus posing risk for relapse (Clapp et al., 2008).

Other studies postulate that up-regulation of corticotrophin releasing hormone (CRH), both within the hypothalamic-pituitary-adrenal (HPA) axis and the amygdala, may play a role in the development of PAWS, as heightened CRH levels are observed in these systems during both acute and protracted withdrawal (Clapp et al., 2008; Heilig et al., 2010). Whether this chemical imbalance is the cause or a byproduct is a point of contention, as the stress, anxiety, and depression associated with PAWS may bring about the rise in CRH. Additionally, the role of mesolimbic dopamine reward pathways has been implicated in underlying the symptomatology of both alcohol withdrawal syndrome and PAWS through motivational learning and reward models (Heilig et al., 2010).
Motor Deficits Following Ethanol Withdrawal

In human studies, disturbances in gait and balance are exceptionally well documented in abstinent alcoholics. In fact, impairments in these areas are considered to be among the most consistent and noticeable results of chronic alcoholism (Smith et al., 2011). Research has shown that the initial disturbances in motor coordination experienced by abstinent alcoholics does improve with time, but the length of time associated with recovery is still a matter of debate (Smith et al., 2011; Fein et al., 2012). Studies suggest that little improvement in gait and balance deficits occurs over the first year after withdrawal; return to normal balance begins during year 2 of abstinence and progresses slowly for a period of up 10 years. Causes of motor impairment following alcoholism are generally attributed to cerebral damage caused by frequent exposure to high concentrations of ethanol. Notable damage has been observed in the cerebellum, hippocampus, and corpus callosum following alcoholism, all areas related to movement and coordination (Fein et al., 2012).

Animal research in this area is far more limited. Studies have established the accelerating rotarod (AAR) as effective tool for testing motor coordination in mice (Philibin et al., 2011). While the AAR has been used extensively to measure intoxication in animals, its use to test persistent motor deficits following alcohol withdrawal has only been demonstrated in a few studies. An AAR consists of a shaft upon which a mouse is placed and required to balance. The shaft then rotates at progressively increasing speeds, requiring a higher degree of coordination to prevent falling. The height of the rod is enough to induce a desire not to fall, but not enough to cause injury when the fall occurs (Figure 1). Philibin et al. (2011) treated mice with chronic intermittent ethanol vapor
exposure to induce alcohol dependence, and found significant deficits in ARR scores compared to air exposed mice. However, this study only tested CIE withdrawing mice at 8-hours post-withdrawal. An additional experiment conducted by Philibin et al. (2012) exposed animals to constant ethanol vapor, and performed ARR testing for 1 week following. The results of this study found ethanol effects to last between 1-4 days. No work has previously been conducted to establish the long-term (i.e. longer than 1 week) effects of CIE exposure on motor coordination in mice.

Pain Sensitivity Following Ethanol Withdrawal

There is a somewhat more prevalent literature on pain sensitivity in abstinent alcoholic animals, but the research on this area in humans is sparse. However, studies have indeed found that humans undergoing withdrawal from alcohol dependence experience an increase in heat pain sensitivity (thermal nociception), though the length of
time that this effect persists was not concluded (Jochum et al., 2009). This is consistent with the animal research showing a decreased latency in radiant heat tail-flick tests in withdrawing alcohol-dependent rats (Gatch et al., 1999; Gatch et al., 2006). Radiant heat tail-flick tests function by placing the tail of an animal on a heated surface that steadily increases in temperature; the amount of heat an animal can withstand before moving its tail provides a measure of its pain sensitivity (Figure 2). Therefore, a shorter amount of time before a tail-flick occurs in abstinent alcoholic mice suggests an increase in pain sensitivity. Gatch et al., 1999, administered alcohol via liquid diet for a period of 10 days, and tested thermal nociception over the next 36 hours, finding significant hyperalgesia for only the first 12 hours. However, similarly to the animal studies of balance and gait impairment, these tests did not look at long-term effects. Furthermore, no studies to date have evaluated thermal nociception in animals following chronic intermittent ethanol exposure.

http://ja.brc.riken.jp/

Figure 2: Radiant Heat Tail-flick Test

Prior Work

The chronobiology lab of Dr. Alan M. Rosenwasser has conducted extensive research on models of alcoholism in animals. While many past studies have focused on
circadian rhythm disruptions revolving around alcohol consumption and withdrawal, lately the lab has begun explorations into alcohol withdrawal syndrome, post-acute withdrawal syndrome, and investigating various pharmaceuticals to treat these conditions. Logan et al. (2010, 2011) employed a chronic intermittent ethanol (CIE) exposure protocol previously developed by Becker et al. (1997) to induce alcohol dependence in mice, as an analog for human alcoholism. This protocol involved the use of ethanol vapor chambers, with alternating periods of ethanol exposure and plain air. Following treatment, Logan observed a significant reduction in wheel running activity in ethanol-exposed mice, lasting approximately 1 week before returning to baseline levels. These two papers found that B6 mice show a 1-week deficit but C3H mice show an effect that lasts at least 30 days. Wheel running is a pleasure seeking activity for caged mice and a reduction in wheel running would suggest a reduction in pleasure seeking behaviors, known as anhedonia (Brene et al., 2007). Anhedonia is a commonly cited symptom of human depression, such as that seen in withdrawing abstinent alcoholics, and thus would suggest the mice exhibiting this symptom are experiencing a state analogous to human depression.

Following the success of the Logan et al. (2010, 2011) studies, the Rosenwasser lab began research on how antidepressant pharmaceuticals would impact wheel-running activity in CIE withdrawing animals. Desipramine, a tricyclic antidepressant prescribed in humans, was the first drug to be evaluated. Unfortunately, the results of this study were disappointing, in that chronic desipramine completely failed to reverse withdrawal-induced hypolocomotion (unpublished data). The present study evaluated the effects of a different drug, ketamine, on CIE withdrawing animals. As described below, ketamine is
currently under intense experimental investigation as a possible rapid-acting antidepressant drug. A study conducted in parallel by Edwards et al., 2014, examined the effects of ketamine on wheel running activity, while this study diverged in a direction not previously taken by the Rosenwasser lab, looking at motor coordination and nociception following CIE exposure.

Potential Therapeutic Effects of Ketamine

Ketamine, an NMDA receptor antagonist, has been used as an intramuscular and intravenous anesthetic in both human and veterinary medicine since the 1970s (Salvadore & Singh, 2013). Beginning in the early 2000s with a study by Berman et al., 2000, the possible role of ketamine in the treatment of major depression began to be explored, with a number of other studies being conducted from that time to present day. Research has shown that a single, sub-anesthetic dose of ketamine can produce rapid antidepressant effects in treatment-resistant depressed patients within 2 hours, lasting for about a week (Burman et al., 2000). While the safety and mechanisms by which ketamine produces these dramatic antidepressant effects are not yet completely understood, researchers remain hopeful for the future use of ketamine to treat major depression.

At anesthetic doses, ketamine blocks the function of NMDA glutamate receptors (Salvadore & Singh, 2013). However, at low, sub-anesthetic doses, we see a very different phenomenon occur, in that ketamine dramatically increases glutamate release for an acute period (around 2 hours) (Salvadore & Singh, 2013). One hypothesis suggests that low-dose ketamine may block NMDA receptors on GABAergic interneurons, thus disinhibiting glutamatergic pyramidal cells and increasing glutamate release in the prefrontal cortex (Duman et al., 2012). It should also be noted that the response of AMPA
receptors to the upsurge of synaptic glutamate release is imperative to the antidepressant effects of ketamine, as AMPA antagonists administered before ketamine injections completely abolish these effects (Salvadore & Singh, 2013). The role of synaptic potentiation has also been implicated in ketamine’s antidepressant properties, as acute increased translation of brain-derived neurotrophic factor (BDNF), an essential component in forming neural connections, has been observed in the hippocampus shortly following ketamine administration (Salvadore & Singh, 2013). This may be a crucial factor in ketamine’s mechanism of action as decreased synaptic connectivity in various regions of the brain, including the hippocampus, can be observed in patients with major depression, likely due to heightened levels of CRH (Zhou, 2013).
The Present Study

The aims of this study are:

1. To establish a long-term animal model of motor impairment following CIE withdrawal.
2. To establish an animal model for heightened pain sensitivity following CIE withdrawal.
3. To evaluate the effects of ketamine on motor coordination and pain sensitivity in CIE withdrawing mice, in order to hypothesize how these symptoms may be influenced if ketamine were used to treat PAWS-related depression in humans.

Materials and Methods

Subjects and Apparatus

Upon arrival in the laboratory, 6 to 8 week old male C3H mice (Jackson Laboratories, Bar Harbor, ME), were weighed and housed in groups of 5 in cages without running wheels (Figure 3). Food (Prolab RMH 3000; LabDiet, St. Louis, MO) placed on the bottom of the cage, and tap water through long drinking tubes, were freely available throughout the process.
Animals underwent chronic intermittent ethanol exposure (CIE) while group-housed in standard mouse cages (Figure 4). CIE exposure protocol was based on the work of Logan et al., University of Maine. CIE animals (n=20) were administered a priming injection containing 1.6 g/kg ethanol and 68.1 mg/kg pyrazole HCl to stabilize blood ethanol concentration and inhibit ethanol metabolism. Pyrazole was dissolved in 20% ethanol solution and injected subcutaneously in a volume of 10mg/kg of body weight, while control animals (n=20) were given a comparable dose of pyrazole in 0.9% saline solution at the same volume. All animals were weighed prior to and following the 8-day CIE cycle to ensure appropriate injection volumes, and to monitor possible CIE-induced changes in body weight. Air and ethanol vapor were delivered to the exposure chambers at a rate of 10 to 12 l/min, allowing for satisfactory airflow to meet the animals’ breathing requirements. Ethanol was vaporized using a pressurized pump to push air through a porous diffusing stone submerged in a 1.0-l bottle filled with 95% ethanol. To ensure ethanol vapor concentrations were within an appropriate range (10 to 12 mg/l) and stable across treatment days, 5.0-ml air samples were extracted from the
ethanol chambers using a 60-ml syringe and mixed with 55.0 ml of ambient air. The diluted sample was injected into a breathalyzer (Lifeloc FC-10; Wheat Ridge, CO) and the resultant readings were compared to a standardized calibration curve of known ethanol concentration to determine chamber ethanol concentration. Lights were kept on an LD 12/12 cycle, with 16 hours of vapor exposure followed by 8 hours of plain air daily. Ethanol vapor was initiated 2 hours before the dark phase of the cycle, with pyrazol HCL and saline injections occurring shortly before vapor exposure each day; vapor was terminated 2 hours into the light portion of the cycle. This allowed the maximum vapor exposure to occur during the peak activity periods of the animals. Vapor chamber treatments lasted 8 days. The entire experiment was conducted twice due to space limitations, with 20 animals in each round, for a total of 40 animals with 10 in each treatment condition.

Figure 4: Ethanol Vapor Chambers

Measurement of Blood Ethanol Content

BECs were measured in experimental animals at the termination of the 8-day CIE exposure. Briefly, each mouse was removed from the cage and gently placed in a plastic restraining tube, and a small (approximately 10 ll) blood sample was collected from the
tip of the tail, then centrifuged for 2 minutes to separate plasma from serum. BECs were determined from 5 ll plasma samples using an AM-1 alcohol analyzer (Analox Instruments, Lunenburg, MA).

**Ketamine Injections**

Immediately following removal from the vapor chambers at the end of the 8-day CIE protocol, test animals (n=20, 10 from ethanol exposed treatment and 10 from air exposed controls) were administered a single, sub-anesthetic injection of ketamine (10mg/kg). Following injections, animals were returned to their cages for 6 hours before testing began.

**Accelerating Rotarod Test**

An Accelerating Rotarod was used to test balance and motor coordination in CIE-withdrawn animals. ARR protocol was based on the work of Philbin et al. (2012). 3 animals at a time were placed on the rod in individual compartments, at a height in which falling would not cause physical harm, but still stimulate falling avoidance behavior. Once all animals were in position, the motor was turned on and accelerated from 0rpm at a constant rate of 20rpm/min. Sensors recorded the time at which each animal fell from the rod. Once all had fallen, the motor was reset animals were given 1 minute before repeating the test. Each animal was subjected to 3 tests in a row per testing period. ARR Testing was conducted at 6hr post-withdrawal, daily for 1 week, then weekly for 1 month.
Tail-flick Assay

A Tail-Flick Analgesia Meter (Columbus Instruments, Columbus, OH, USA) was used to test post-withdrawal thermal nociception in the animals. The tail-flick assay procedure used was based on the work of Gatch et al. (1999). Mice were placed in clear plastic restrainers, with their tails extending through a notch in the device, and tested individually. Tails were laid across an adapter block with movement sensors, and the a light beam set at a nominal intensity level of 10 was projected on a point approximately half way down the tail. A 0-60 second timer began as soon as the light beam shutter was opened, and stopped when tail movement was registered by the sensors. Each animal was given 1 minute before repeating the measure, and were tested twice per testing period. Tail-flick assays were conducted at 6hr post-withdrawal, then daily for 1 week.

Ethical Considerations

This experiment was approved by the University of Maine Institutional Care and Animal Use Committee.

Data Analysis

Both accelerating rotarod and tail-flick test scores were first analyzed using a three-factor analysis of variance (ANOVA) with “ethanol vs. air” and “ketamine vs. saline” as between-groups factors and time-blocks as a repeated-measures factor. This approach allowed us to detect possible main effects of ethanol exposure, ketamine treatment, and time-blocks, as well as possible ethanol-by-ketamine-by-time interactions. This analysis was followed by two-factor (ethanol, ketamine) ANOVA for specific time blocks and finally by standard t-tests for pairwise comparisons.
Results

**Accelerating Rotarod**

Mean accelerating rotarod scores fell between 25-50 seconds for the initial 6 hour post-withdrawal test session, and then appeared to gradually increase over the next month (Figure 1), probably indicative of a practice effect in this task. Not surprisingly, then, the overall ANOVA detected a significant main effect of time-blocks ($F(10,340) = 23.59, p < 0.001$). In addition, there was a significant main effect of ethanol, in that ethanol-exposed animals showed reduced time on the rotarod across time-blocks relative to air-exposed animals ($F(1,340) = 7.51, p = 0.01$). There was no main effect of ketamine, nor any interactions detected. Follow-up analyses revealed significant effects of ethanol at 3, 4, 6 and 7 days post-withdrawal, but no ketamine effect at any time-point. Together, these results suggest a late-emerging effect of ethanol withdrawal on motor coordination that emerges a couple of days following withdrawal and lasts about a week.
Figure 5. Mean (± standard error of the mean) ARR scores in seconds at time points between 6 hours and 4 weeks post-withdrawal across all treatment groups. Asterisks (*) denote a significant difference ($P < .05$) between control (Air) and treatment groups on ARR performance.

**Tail-Flick Assay**

While the ethanol exposed group appeared to show reduced tail flick latency not present in the ethanol-ketamine exposed group at 6 hours post-withdrawal, this effect was not significant. Scores did not show any differences between 6 hours and 5 days (Figure 2). Day 6 revealed a significant ($P < .05$) difference between the ethanol exposed group and all other groups, suggesting a potential late emerging effect of ethanol corrected by
ketamine. Tail flick latency did not appear to change within groups between testing periods.

![Tail-Flick Graph](image)

Figure 6. Mean (± standard error of the mean) tail-flick scores in seconds at time points between 6 hours and 1 week post-withdrawal across all treatment groups. Asterisks (*) denote a significant difference ($P < .05$) between control (Air) and treatment groups on tail-flick performance.

**Discussion**

**Motor Coordination**

These results suggest late-emerging motor deficits in CIE exposed mice beginning at day 4 of withdrawal and ending after 1 week post-withdrawal. This effect differs from that found previously by Philibin et al., 2012, which showed effects of ethanol at 8 hours.
post-withdrawal but did not continue testing at later time points. It should be noted that in the first round of the present study, impairment was present both in the first 24 hours of testing, then again appeared at a late emerging time point, while in the second round, the immediate impairment was not apparent. This would suggest that CIE vapor exposure protocol produces long-lasting motor deficits in animals, similar to those seen in human abstinent alcoholics. The establishment of this animal model for motor deficits following alcohol withdrawal opens the door for future research on treatment for this salient condition. Mechanisms for this phenomenon may revolved around ethanol-induced damage to motor centers in the brain, and/or hippocampal GABA_A receptor subunit abnormalities, as seen in human alcoholics, though direct neurobiological investigation would be needed to verify this hypothesis.

In the first round of this study, ketamine appeared to produce and/or exacerbate motor coordination deficits for the first 24 hours after administration, yet when combine with the results of the second study, this effect was no longer apparent. Thus, we may conclude that while ketamine does not reverse motor impairment, it does not worsen it. This is applicable to the use of ketamine to treat PAWS-related depression, as motor deficits would be an unpleasant side effect.

**Pain Sensitivity**

Contrary to previous research, the present study failed to find an effect of ethanol withdrawal on thermal nociception in mice. The first trial of this study saw a significant difference between ethanol-exposed animals and air exposed animals at the 6 hour time point that was reversed by ketamine, but when combined with data from the second round, this effect was no longer significant. There is no conclusive explanation for the
appearance of a significant difference between ethanol exposed and air exposed animals on day 6 post withdrawal; further research is needed to investigate this finding. Again, there was no significant effect of ketamine on pain sensitivity. While this does not imply possible treatment options for PAWS patients experiencing analgesia, we do not see reason to believe ketamine treatment to adversely affect this symptom.

**Future Directions**

This study has produced promising implications for the future of research on treatment for alcoholism, yet there are still questions to be answered and further work to be done. Firstly, to conclude more certainly that ketamine does not have effects on the processes in question, a dose-response curve for ketamine in mice should be generated. While the dose used was sub-anesthetic and thus theoretically comparable to the doses used in humans, the potential for differing responses in mice is likely. Second, as results obtained between the two trials of the study were varied, more trials should be conducted. The statistical analysis assumes that both trials were identical when in reality uncontrolled factors may have been at play, for example the time of year, atmosphere, mice (assumed to be clones, but in reality phenotypic variations exist), and individuals conducting testing. More trials would increase the number of subjects and thus the power of the study, minimizing the effects of random variables. Finally, recent studies have suggested that multiple doses of ketamine may provide longer lasting symptom relief from major depression (Murrough et al., 2013). Thus, examining the effects of repeated doses on motor coordination and pain sensitivity in CIE withdrawing mice may be beneficial to understanding the side effects of such treatments.
References


Author’s Biography

Jameson M. Ford was born in Kansas City, Missouri on April 10, 1992. He spent the majority of his childhood growing up in Falmouth, Maine, where he graduated from Falmouth High School in 2010. Jameson then attended the University of Maine, majoring in psychology with minors in neuroscience and dance. Aside from his academic career, Jameson participated in theatre and dance at UMaine, serving as the President of Alpha Psi Omega National Theatre Honors Society and as the President of the UMaine Student Dance Division. Jameson graduated from the University of Maine in 2014, with a BA in Psychology and earning High Honors from the Honors College.