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Ethanol and Retrograde Amnesia: Can Rats Have Blackouts and Does Caffeine Help?

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Ethanol and Retrograde Amnesia: Can Rats Have Blackouts and Does Caffeine Help?

by

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Dedication

This dissertation is dedicated to my father Dr. John Joseph Spinetta who has served as the single most inspiring individual in my life: as a philosopher, as a scholar, as a researcher, as an excellent professor, as a father, and as an incredible man.

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Ethanol and Retrograde Amnesia: Can Rats Have Blackouts and Does Caffeine Help?

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The work in this dissertation aims to describe a simple new test for odor-recognition memory in rats that can be readily performed and results in an easily observable and lasting form of memory. This test has allowed for the demonstration of ethanol-induced retrograde memory impairments in rats when ethanol is administered during both the consolidation and reconsolidation phases of memory encoding. The observation that a high-dose of ethanol can cause retrograde memory impairments when administered immediately or within hours after learning has taken place is an original finding that may have implications for understanding human blackouts. Furthermore, the finding that ethanol can disrupt the reconsolidation of a previously consolidated memory has not been previously established. It is also demonstrated that caffeine can prevent ethanol's memory impairing effects, a result that contributes a new piece of evidence for caffeine's effects on the learning and memory process. This effect has been further

investigated mechanistically and attributed to caffeine's dual role as a phosphodiesterase type 5 inhibitor and adenosine A_{2A} antagonist. Neither of these mechanisms alone appear to be sufficient enough to prevent the retrograde memory impairments seen with ethanol. It is hoped that this test and our findings will prove useful for future investigations into the effects of ethanol on learning and memory and the human phenomenon of alcohol-induced blackouts.

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GENERAL INTRODUCTION

> "However, what must be brought to the fore, following Aristotle, is the reference to the authority of the "thing" remembered in relation to its present evocation. The cognitive dimension of memory, its character of knowing, lies in this reference. It is by virtue of this feature that memory can be held to be trustworthy or not and that properly cognitive deficiencies are to be accounted for, without our rushing to construe them according to a pathological model, under the heading of this or that form of amnesia."
>
> Paul Ricouer

Amnesia comes from the Greek word Anamnesis: *mnesis* meaning memory or to remember and *ana* meaning returning to. This word was developed by Plato as a response to the mystery of not recalling events that had previously occurred. Aristotle's explanation of this phenomenon could, with our modern understanding of the brain, be referred to simply as failed recollection or the act of forgetting. The quote by Ricouer is important because it emphasizes two vital points: 1) that the ability to accurately remember events is a fundamental component of human cognition and 2) the importance of dissociating *normal forgetting* from the *loss of memory* for events that have already happened. The work presented in this dissertation will focus on the latter and will attempt to provide evidence, in an animal model, for an *active loss of memory* for events as a result of post-learning acute ethanol exposure, a phenomenon that is referred to as retrograde amnesia.

The foundations of modern scientific investigation into retrograde amnesia can be found in the work of a clinician named Ribot. His work with patients suffering from retrograde amnesia following traumatic brain injury led to his formulation of the "loi de regression", or the law of regression, which states that events experienced immediately before brain trauma has occurred are the most likely to be forgotten (Ribot 1881). This led to scientific investigations into the theory of consolidation, which in Latin literally

means, “to make firm.” Consolidation or *Konsoliderung* a term first proposed by Muller and Pilzecker (1900) is the transfer of memory from a short-term labile state, into a more stable long-term state. Pioneering work by McGaugh (1966), based on countless clinical observations of amnesia for newly acquired memory after cerebral injury in human patients, led to the development of an animal model of retrograde amnesia. Classic studies into retrograde amnesia employed the inhibitory avoidance task, a highly emotionally charged test, in which an animal learns to pair an otherwise neutral dark environment with an aversive component. When electroconvulsive shock (ECS) is delivered minutes to hours after learning has occurred (Schneider & Sherman 1968; McGaugh 1970) memory is impaired when assessed at 24 to 48-hours post learning (Martinez, Jensen & McGaugh 1981; Maki 1985). These studies demonstrated that consolidation of a learned avoidance response could be disrupted through the administration of ECS. These mice responded in the post-training test as if they had received no shock. Their ability to consolidate the memory of the dark chamber being paired with shock was impaired, showing that indeed the process of consolidation was labile and could be disrupted.

Ethanol, one of the most widely consumed drugs in the world, has a variety of effects on memory depending on when it is given in relation to learning. A review of acute alcohol intoxication by Fleming (1935) revealed “the almost infinite diversity of symptoms that may ensue from the action of this single toxic agent”. Acute exposure to ethanol in high doses, typically during episodes of binge drinking, can cause blackouts, defined as periods of amnesia during which a subject participates in mundane or even emotionally salient events that they later cannot remember (Goodwin 1995, Hartzler &

Fromme 2003, Haltzer and Fromme 2003a; Wixted 2005). The blackout phenomenon is not exclusive to alcoholics, but can frequently occur in non-alcoholics, as demonstrated in populations of college students (White 2003). In humans it has been shown that the consumption of alcohol can impair the acquisition of new information in learning tasks, resulting in impaired recall in later tests of memory (Parker & Birnbaum 1976; Birnbaum 1978; Bruce et al., 1999; Moulton et al., 2005). Nelson et al., 1986 suggested that this impairment may be due at least in part to ethanol's effects on retrieval from long-term memory.

Similarly in animals, acute ethanol administered prior to learning can impair performance in follow up tests of memory (MacInnes & Uphouse 1973; Melia 1996, Matthews 1999; Acheson, Ross & Swartzwelder 2001; Weitemier 2003; Gonenc et al., 2005). Chronic ethanol ingestion likewise impairs memory acquisition (Freund 1970; Brioni, McGaugh & Izquierdo 1989; Kogan, Frankland & Silva 2000; Mikolajczak et al.,, 2001; Garcia-Moreno et al., 2002; Carpenter-Hyland, Woodward & Chandler 2004). However, it is unclear whether the impairment reflects ethanol's influence on, for example, encoding, storage, retrieval, and/or factors that might more tangentially contribute to encoding efficiency such as sensory, motor, emotional, motivational, or attentional mechanisms (Ryabinin et al., 2002).

One approach to this problem, used in the present series of studies, is to administer ethanol immediately after the learning experience, well after short-term memory is unambiguously established, so that there is no intoxication during learning. Non-memory related factors are ruled out because the animal is not intoxicated during learning or when tests of memory are later conducted. In humans (Parker 1980; Mueler,

Lisman & Spear 1983; Mann, Young & Vogel-Sprout 1984; Lamberty, Beckwith & Petros 1990; Tyson & Schirmuly 1994; Hewitt, Holder & Laird 1996; Bruce & Pihl 1997) and animals (Alkana 1979; Parker et al., 1981; Colburn, Sharek, Zimmermann 1986; Babini, Jones & Alkana 1991; Prediger & Takahashi 2003; Prediger et al., 2004; Manrique 2005), consumption of ethanol after learning has been shown to enhance recall in tests of memory conducted the next day or have no effect (De Carvalho, Vendite & Izquierdo 1978). However these tests may not have been sensitive enough to detect the memory loss produced by ethanol and the doses of ethanol used may not have been high enough to produce memory deficits.

In order to address these issues, we have developed a simple and sensitive olfactory memory test, described in chapter one, that takes advantage of rats' natural preference for novelty (Carr 1980; Viola et al., 2000; Mumby 2005) and allows us to examine the effects of acute ethanol exposure on memory after learning has occurred. There has been no systematic examination of the possible retrograde influence of high doses of alcohol on relatively neutral memories (e.g., a very common memory for minimally salient events and information that do not elicit an extreme emotional reaction). We hypothesized that the administration of a high dose and not a low dose of ethanol after the learning phase of a task would produce impairments in a follow-up test of memory conducted the next day. During the learning phase of our task, rats initially show robust exploration of a novel odor in comparison to familiar odors and subsequently habituate rapidly to the novel odor (N1). After habituation, we exposed a group of rats to pentylenetetrazol, a known amnestic agent (Grossman 1967, Baratti 1987), and twenty-four hours later tested the animals' preference for this recently-novel

odor N1 in the presence of a brand new novel odor (N2). By comparing exploration of N1 and N2 by PTZ and control rats, we were able to establish a behavioral baseline for overnight memory loss, which was reflected as no difference in time spent exploring N1 and novel N2 (as seen in PTZ treated rats) and intact overnight memory retention, in which the N2 was preferred over the N1 (as evidenced in control animals). In chapter two, we found that rats given a high dose of ethanol also demonstrated a loss of overnight memory for N1; i.e., a retrograde memory impairment for that odor.

Having demonstrated that a high dose of ethanol, given immediately after exposure to a novel odor, leads to retrograde memory impairment 24 hr later in a novel odor-recognition test, we became interested in whether this memory impairment could be prevented with the administration of caffeine, a drug that is widely available and often contained in beverages consumed before, during or after alcohol. Caffeine has been reported to enhance memory in inhibitory avoidance tasks (Angelucci et al., 1999), spatial learning (Prediger et al., 2005) and odor memory tasks specifically (McLean et al., 2005). In chapter three, we tested whether caffeine, delivered before or one hour after the administration of ethanol, might reverse or possibly exaggerate the memory impairment seen with ethanol alone. We found that caffeine, delivered prior to or one-hour after learning, followed by a high dose of ethanol, prevented the amnestic effects seen with ethanol alone. Caffeine serves as both a phosphodiesterase inhibitor and an adenosine antagonist (Howell 1997), although multiple mechanisms could be responsible for the observed reversal of ethanol's amnestic effects. We therefore conducted a series of experiments to investigate if adenosine antagonism alone, phosphodiesterase inhibition alone, or in combination potentially might underlie caffeine's prevention of ethanol

induced retrograde memory impairments. Our findings indicate that neither an adenosine A_{2A} antagonist nor a phosphodiesterase-5 inhibitor alone, even at high doses, reversed or prevented retrograde amnesia when delivered one hour after ethanol (unlike caffeine, which did prevent the amnesia). However, a combination of these two drugs at the previously ineffective doses was highly effective at preventing ethanol's amnestic effects. Doubling the dose of the phosphodiesterase inhibitor did not prevent ethanol's retrograde amnestic effects.

In the fourth chapter we attempt to explore potential sites of action in the brain that might be involved in learning during our odor recognition task and the process by which consolidation of the odor recognition memory occurs in those structures. Although we have demonstrated that ethanol can disrupt memory for previously learned odors when delivered after learning has occurred, it is unclear both where in the brain and by which of its multiple mechanisms of action ethanol exerts its amnestic effects. Ethanol at high doses is known to interfere with glutamatergic action at NMDA, AMPA and Kainate receptor subtypes while it also enhances GABAergic synaptic transmission (Nevo & Hamon 1995; Schummers 2001). For new learning to undergo consolidation, (i.e. the transfer from a labile to stable state), protein synthesis must occur (Schafe 2000, Kandel 2001) and there is growing evidence that activation of NMDA receptors is a crucial step in this process (Miserndino 1990, Rodrigues 2001, Riedel 2003). Furthermore, it has been demonstrated that acute ethanol exposure can inhibit critical steps in protein synthesis, possibly through its antagonism of NMDA receptors (Chandler 2005).

Based on this evidence, we have attempted to produce the retrograde memory impairments seen with ethanol by disrupting the consolidation process more specifically

through the use of protein synthesis inhibitors, an intervention well established in the literature (Flexner 1965; Flexner, Flexner & Stellar 1965; Grollman 1967; Grollman 1967a; Dunn 1971). Ethanol's effects are widespread throughout the brain, and it is still unclear in which specific brain structures ethanol works to exert its amnestic effects. As odors represent highly salient cues for rodents, ethanol's impairment of retention of an odor memory could be due to its deleterious effects on protein synthesis in the amygdala. Inactivation of the amygdala has been shown to attenuate memory for emotionally motivated tasks (Salinas 1993) and lesions of the medial amygdala specifically have been demonstrated to disrupt performance on tasks of odor memory (Petrulis 1999). Thus we predicted that administering a protein synthesis inhibitor directly into the medial amygdala after habituation to an unfamiliar odor in our task should impair memory for that odor in a follow up test. Results indicate that compared to controls, protein synthesis inhibition in the medial amygdala disrupts overnight memory for N1, as indicated by an equal percent time spent exploring N1 and N2 in the follow-up test.

In rats (Misanin et al., 1968; Mactutus et al., 1979; Przybyslawski et al., 1997; Sara 2000; Nader, Schafe & ledoux 2000; Anagnostaras Schallert & Robinson, 2002; Debiec et al., 2002; Artinan et al., 2007) and humans (Rubin et al., 1968), once a memory is long established and resistant to amnestic agents, presentation of a cue (memory reactivation) may render some memories again vulnerable to disruption for a short time. Misanin was instrumental in discovering that not only could memory be disrupted in the initial consolidation phase, but also when that consolidated memory was reactivated by a learned cue. Using a classical conditioning paradigm, it was discovered that rats re-exposed to a cue associated with an aversive stimulus, showed no memory of that learned

association when the cue was followed by ECS. This effect was re-introduced by Przybyslawski, Sara, and Nader and termed *reconsolidation*. The theory of reconsolidation is based on the finding that once a memory becomes consolidated and stabilized through protein synthesis, it must undergo a second process of restabilization, a process that has been argued to be important for updating memory and may involve de-novo protein synthesis, once reactivated by a learned cue. It is during this time, when the memory is unstable, that interventions such as protein synthesis inhibition or seizures can cause memory impairments for the previously consolidated memory (Nader, Schafe & Ledoux 2000a). Interestingly, protein synthesis inhibition is a mechanism that is shared by ECS (Duncan 1971; Cotman et al., 1971). Using the odor recognition model, we have demonstrated that reconsolidation deficits are produced by disrupting protein synthesis after cued reactivation of the original memory with a 1-trial reminder of N1. We further investigated this effect based on ethanol's ability to disrupt key components of protein synthesis through its inhibition of NMDA receptors, finding that a high-dose of ethanol, administered after a 1-trial reminder of N1, was sufficient to disrupt the reconsolidation of odor recognition memory.

In sum, the present series of experiments describes a simple test for assessing memory in rats, developed to investigate retrograde amnestic effects of ethanol and their prevention by caffeine or related agents. The finding that ethanol can disrupt both consolidation and reconsolidation might have substantial ramifications for people who abuse alcohol and for those who suffer from unwanted memories.

Chapter 1: Developing methods to test amnesia in rodents

CHAPTER OVERVIEW

This dissertation introduces an odor-recognition test that can be used to directly assess the effects of pharmacological interventions on long-term 24-hour memory in rats. Recognition memory is the ability to distinguish between stimuli that have been previously encountered and stimuli that are novel (Brennan & Keverne 1997; Steckler, 1998). The two primary recognition memory animal models are the social odor recognition test, developed by Thor and Holloway (1987) and the object recognition test, developed by Ennaceur and Delacour (1988). These tests take advantage of a rodent's natural preference for novelty (Griffin & Taylor 1995; Arletti et al., 1997; Gheusi, Goodall & Dantzer 1997; Burman & Mendl 2002; Giaqnnaris, Cleland & linster 2002; Myskiw et al., 2008), exemplified by an initially robust exploration of unfamiliar cues, followed by habituation of exploratory behavior after repeated exposures to the same cues (Wirth, 1998). Specifically, our test uses odors, likely carried primarily in the urinary proteins (Hurst et al., 2001) obtained from novel odor donors (i.e. from the cages of other rats) as the novel cue. Since olfaction represents a highly salient (White, 2004) and ethologically relevant (Prediger, 2004) sensory modality used to guide many behaviors in rats, they readily explore and habituate to these novel odors without the presence of reward, punishment or other experimenter imposed motivational devices that are frequently used in learning and memory tests to initiate and sustain exploration and guide the learning process.

In this chapter, we present data, based on modifications of the social odor and object recognition memory tests, of 24-hour memory for a previously habituated novel odor and disruption of that memory with the use of the amnestic agent pentylenetetrazol (PTZ). PTZ was one of the first amnestic agents that was widely used to demonstrate retrograde amnesia in rodents (Essman 1968; Bookin & Pfeiffer 1977), in various memory tasks including inhibitory avoidance (Iuvone et al., 1977), passive avoidance (Putney and McCoy 1976), and taste aversion learning (Shaw & Webster 1979). PTZ is a convulsant that works as a GABA antagonist to produce seizures (Blake et al., 2004) which is the mechanism by which it causes memory impairments, although it is suggested that PTZ's interaction with the norepinephrine system could contribute to observed memory impairments (Palfai, Kurtz & Gutman 1974). PTZ was used in the 1940's to treat depression and other psychiatric disorders and has been used for decades in rat models of kindling (Pereira & Vasconcelos 1996) which produces an animal model of epilepsy (pentylenetetrazol-induced status epilepticus). However, PTZ is also administered acutely, *after* learning, to produce retrograde memory impairments (Palfai & Kurtz 1973; Millner & Palfai 1975; Baratti, Deerausquin, Faiman 1990).

ODOR RECOGNITION TEST METHODOLOGY

Subjects. Male Sprague-Dawley and Long-Evans rats (weighing 200-400g) obtained from either an on-site animal colony, or from Harlan or Charles River Laboratories, were used. Animals were housed three per cage in clear Plexiglas cages with wood shavings, maintained under a 12:12 h light:dark cycle, and given access to

food and water *ad libitum*. One week after arriving in the laboratory animal colony, rats were handled and familiarized to the researchers. All animal care and experimental procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee.

Pre-habituation procedures. Animals were removed from group-housing cages, weighed, and re-housed singly in identical cages with sawdust bedding and removable wire tops. Once singly housed, animals remained in these test cages for the duration of the experiment. During the initial 24-hour familiarization period, four 2.5cm round wooden beads with a small hole through the center (www.craftworks.com) were introduced into the test cages in order to acquire the odor of the animal and to serve as familiar odors for subsequent use in the experiment. Housing the animals in the test cages with the beads for 24 hr allowed for familiarization to both the testing environment and the presence of the beads.

Several beads were also introduced into the cages of three previously selected odor-donor groups (housed three rats per cage), whose cages had not been changed for one week to allow for a build-up of animal-specific novel odors. Wood beads incubated in these odor-donor cages provided equally-salient novel odors for the upcoming task. The hole in the center of the bead enhanced exploration, and because they were round and large, little or no gnawing took place during incubation or testing. The cages designated to provide donor odor beads were counterbalanced, so that any one odor served as either a recently-novel odor (N1) or a brand new novel odor (N2) during memory assessment for different experimental rats.

Habituation to the novel odor (N1). During the habituation phase of the task, after 24 hr of familiarization to the presence of four beads in the testing environment, the four now-familiar beads were removed for one hour. After this one hour-period, a novel-odor wood bead (N1), taken from an odor-donor cage, and three familiar beads that had been taken from their own cages one hour previously were introduced into the cage. They were exposed to these four beads for three 1-minute trials with 1-minute inter-trial intervals during which the beads were removed from the testing enclosure. This procedure allows for habituation to N1 and ensures lasting memory for it, while minimizing or preventing olfactory adaptation.

For each 1-minute trial, the three familiar-odor beads and the N1 bead were placed in the middle of the testing cage, and the rats were allowed one minute to actively explore the beads. The first approach to a bead made during this period initiated the timing of the 1-minute trial. Exploration time for each of the four beads was recorded. The spatial arrangement of the beads in the middle of the cage was randomly altered between trials. Statistical consultants recommended, based on information theory, that to maximize sensitivity of the test, one novel (N1) and at least three familiar odor beads should be used during habituation trials rather than N1 only, and that during memory retention assessment (below) four beads should be used (N1, N2, and two familiar) rather than N1 and N2 only. Thus, non-memory would yield 25% per bead investigation time rather than 50%, so fewer animals can be used to detect memory retention optimally.

Odor recognition memory assessment. 24 hours after the novel-odor habituation phase, the odor recognition test was conducted. For this phase of the task, rats were presented with the recently-novel odor N1 (which it had thoroughly explored on the previous day) in the presence of one unfamiliar novel odor bead (N2) taken from a different odor-donor cage and two familiar (own-cage) odors, following the same procedure outlined for the habituation phase. For a graphical representation of the experimental procedure for the odor recognition test, see Fig. A2.1. To dismiss scent marking as a confound, the N1 bead was discarded after habituation and replaced by another N1 bead taken from the same odor-donor cage for the recognition memory phase. Videos demonstrating rat behavior during the habituation and odor recognition test phases can be viewed on our website at http://www.schallertlab.org.

STATISTICS

Analysis of N1 salience and habituation. For the habituation phase of the task, rats explored a novel odor in the presence of three familiar odors, over three 1-minute trials. The focus of our analysis of this phase was 1) to establish habituation to the novel odor, expressed behaviorally as a reduction in time spent exploring it over each subsequent trial, and 2) to verify that all rats demonstrated a novel odor preference, expressed as a substantially longer amount of time spent exploring the novel odor over the familiar odors. T-tests were run using SPSS to assess novel odor salience and habituation, with odor type (novel vs. familiar) and trial number as within-subjects variables. Significantly more exploration of N1 than of familiar odors on the first habituation trial was regarded

as evidence for novel odor salience. A significant reduction in N1 exploration time between the first and last (third) habituation trials was regarded as reflecting adequate habituation to N1 (i.e., learning).

Analysis of memory for recently novel odor N1. On the final day of the task (odor recognition test), rats explored the recently-novel odor (N1) in the presence of an unfamiliar novel odor (N2) and two familiar odors. The focus was to assess 24-hour memory for the recently novel odor. Memory for the recently-novel odor was indicated by significantly more time spent exploring the unfamiliar novel odor (N2) than the recently novel odor (N1) on the first trial of the test phase, as determined by t-tests run in SPSS. Cohen's *d* was also calculated as a measure of effect size for the difference in percent time spent exploring N1 versus N2.

PHARMACOLOGICAL TREATMENTS

Pentylenetetrazol and Saline Controls.

To demonstrate that retrograde memory impairment could be evaluated using our social odor recognition test, rats were treated with the established seizure-producing drug and amnestic agent, PTZ (Sigma; dissolved in saline and administered at 25 mg/kg, i.p.) immediately following the last habituation trial with N1. PTZ has been used previously to cause retrograde amnesia (Baratti 1987). This dose produces a brief, mild seizure within minutes of the injection (Hernandez & Schallert 1998). PTZ-treated rats were pooled (n=26) from separate experiments carried out either alone or in tandem with experiments

examining ethanol's effects. A small group of matched controls (n=7) were administered equivalent volumes (1 ml/kg, i.p.) of saline vehicle. Assessment of memory for N1 was carried out 24 h after PTZ was administered, as outlined above and in Fig. A2.1 and A2.3.

RESULTS

Experiment 1: Pentylenetetrazol and Saline Controls

Habituation. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation as seen in Table 1.1a. Initially, of course, N1 was much more salient than the familiar odors. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads, as seen in Table 1.1b. These animals persisted in exploring N1 more than the familiar odors across habituation trials; however, as seen in Fig. 1.1, there was a dramatic reduction in the mean difference between the time spent exploring N1 and the average time spent exploring the 3 familiar odors on each subsequent trial.

Recognition Test. The seizure/amnesia inducing drug PTZ appeared, as expected, to cause retrograde memory impairment. Recognition memory test data for the saline- versus PTZ-treated rats are shown in Fig 1.2 and Table 1.2a and 1.2b. Data are expressed as mean percent of total exploration time, calculated by dividing the mean time spent

exploring each individual odor by the total time spent exploring all odor beads during the first one-minute trial. Absolute mean exploration times are summarized separately in Table 4. Control rats spent significantly more time exploring the new novel odor (N2) than the recently-novel odor (N1) whereas PTZ treated rats did not show a difference in exploration of N1 versus N2 indicating an impairment of overnight memory for N1.

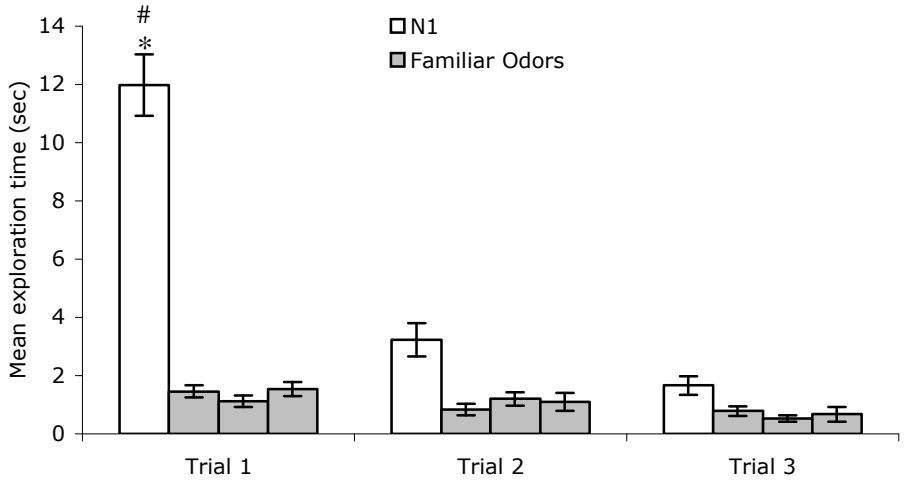


Figure 1.1 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to PTZ immediately following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
PTZ	11.9±1.0	1.66±.32	11.5	<.0001

Table 1.1a Habituation to N1 (PTZ)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
PTZ	11.9±1.0	1.45±.20	9.8	<.0001

Table 1.1b Novel-odor preference in the habituation phase (PTZ)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

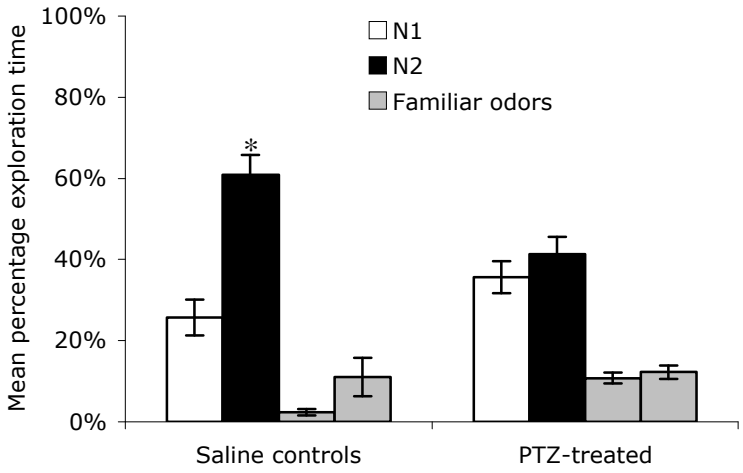


Figure 1.2 PTZ disrupts odor memory

Recognition of the recently-novel odor on the next day, reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor, is seen only in the saline control rats. PTZ rats explore both odors equally, indicating an impairment of overnight memory. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.01$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
PTZ	26	0.35±.03	0.41±.04	0.10±.01	0.12±.01	0.99	.325	0.27
Control (saline)	7	0.25±.03	0.60±.04	0.02±.007	0.11±.047	5.29	<.0001	2.82

Table 1.2a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (PTZ)

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
PTZ	3.04±.55	3.46±.69	1.0±.18	0.78±.12
Control (saline)	3.59±.54	9.41±1.5	0.3±.09	1.6±.67

Table 1.2b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (PTZ)

DISCUSSION

The primary goal of this chapter was to validate the odor recognition test as a legitimate tool to assess long-lasting recognition memory. This was accomplished first by demonstrating a novel-odor preference in all rats, the quintessential component in forming a memory for N1. As seen in all of our groups of rats during habituation, N1 was explored preferentially over the familiar home cage odors. This signified that the rats had no olfactory deficits and were readily capable of distinguishing novelty from familiarity. Secondly, it was observed that all rats significantly reduced their exploration of N1 over the course of three trials, indicating habituation to the novel odor, or "learning" of that odor. 24 hours later, in the follow up recognition test, control rats demonstrated long-term overnight memory, as evidenced by their preference for N2 in the presence of the previously encountered and habituated N1. Taken together, these results indicate that the test serves as a valid test of long-lasting odor recognition memory.

In the first experiment a behavioral baseline for amnesia in our task was established with the acute exposure of the rats to PTZ, administered immediately after habituation to N1. This caused a loss of overnight memory for N1 in the follow up test the next day. This observation allowed for a definition of retrograde memory impairment as the lack of preference for N2 over N1 when the two odors were presented simultaneously. These results are consistent with data showing retrograde impairments of recognition memory for objects and odors (Ennaceur & Aggleton 1997; Mumby & Glenn 2000; Mumby et al., 2002; Gaskin et al., 2003; Mumby et al., 2005).

Chapter 2: Overnight Memory Disruption With Ethanol

CHAPTER OVERVIEW

Acute ethanol exposure before or during learning can cause memory impairments in follow up tests of memory. This effect has been demonstrated in both humans and animals and can result from acute episodes of binge drinking or the administration of high doses of ethanol respectively. Ethanol has multiple mechanisms of action that could contribute to memory impairments including inhibition of GABA and NMDA receptors (Browning & Hoffer 1992; Nevo & Hamon 1995; Valenzuela 1997; Faingold, N'Gouemo & Riaz 1998; Little 1999; Dodd et al 2000; Schummers 2001; Allgaier 2002; Arizzi et al., 2003; Costa, Ferreira & Valenzuela 2003; Suvarna et al., 2005) long-term potentiation (LTP) (Givens & McMahon 1995) and exerts these effects in multiple areas throughout the brain (Pyapali et al., 1999; Givens, Williams & Gill 2000). At issue is whether these observations are due to the effects of ethanol on memory specifically or rather result from a combination of other factors, including ethanol's effects on the ability to process information and engage in learning tasks (Ryabinin 2002). The present chapter will provide behavioral evidence, in an animal model, of ethanol-induced retrograde memory impairment when a high dose of ethanol is administered after learning has occurred and thus rules out non-specific attention processing deficits as contributing factors to the observed memory impairments.

PHARMACOLOGICAL TREATMENTS

Lower dose ethanol. Immediately following the last N1 habituation trial, animals (n=13) were given i.p. injections of 20% (w/v) ethanol at a dose of 1.0 g/kg ethanol, with matched controls (n=7) receiving equivalent volumes of saline i.p. The next day overnight memory for N1 was assessed. As outlined in Fig. A2.1 and A2.4.

Higher dose ethanol. Immediately following the last habituation trial, Long-Evans rats (n=25) were given i.p. injections of 20% (w/v) ethanol at a dose of 3.0 g/kg ethanol, with matched controls (n=11) receiving equivalent volumes of saline. Sprague-Dawley rats were treated identically in a second experiment to determine whether another strain would show retrograde memory impairment with a high dose of ethanol (ethanol group n = 13; saline group n = 13). In both strains assessment of memory for N1 was carried out 24 h after ethanol or saline was administered, as outlined in Fig. A2.1 and A2.5.

48 hour hangover control. Rats were given i.p. injections of either 20% (w/v) ethanol at a dose of 3.0 g/kg (n = 6) or saline (n=6) immediately, after habituation. 48 hr after this, all rats were tested for recognition memory. In this procedure the longer time-span between ethanol administration and the memory test was sufficient for memory consolidation to occur, yet hangover symptoms would presumably be absent during the recognition test since it is performed 48 hr after administration of high-dose ethanol. As outlined in Fig. A2.7.

RESULTS

Experiment 1: Lower-dose ethanol

Habituation test data are shown in Fig. 2.1 and Tables 2.1a and 2.1b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition memory test. Recognition memory 24 hr after habituation to N1 was not detectably affected by administration of the lower dose of ethanol after learning. Data for the saline- versus ethanol 1.0 g/kg-treated rats are shown in Fig. 2.2 and Tables 2.2a and 2.2b. Both control and ethanol-treated rats spent significantly more time exploring N2 than N1, indicating retained memory for N1 in both groups.

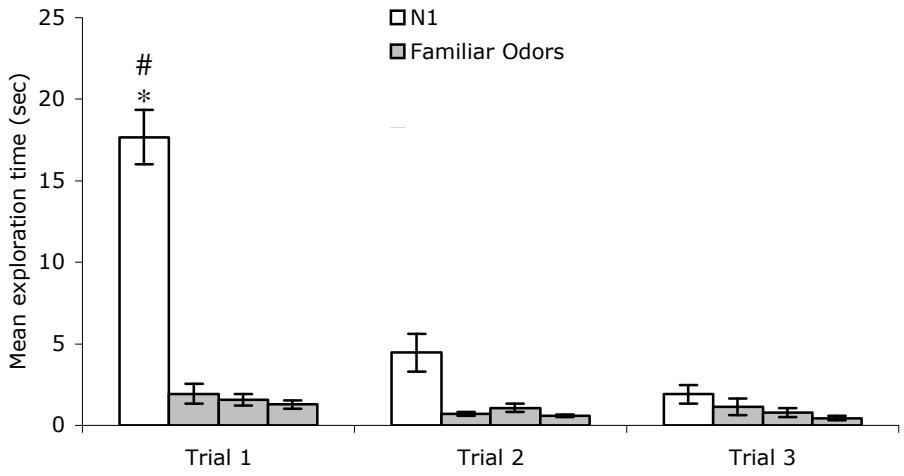


Figure 2.1 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to ETOH 1.0 g/kg immediately following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
ETOH 1.0	17.67±1.6	1.9±.57	9.569	<.0001

Table 2.1a Habituation to N1 (ETOH 1.0)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
ETOH 1.0	17.67±1.6	1.9±.61	8.8	<.0001

Table 2.1b Novel-odor preference in the habituation phase (ETOH 1.0)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

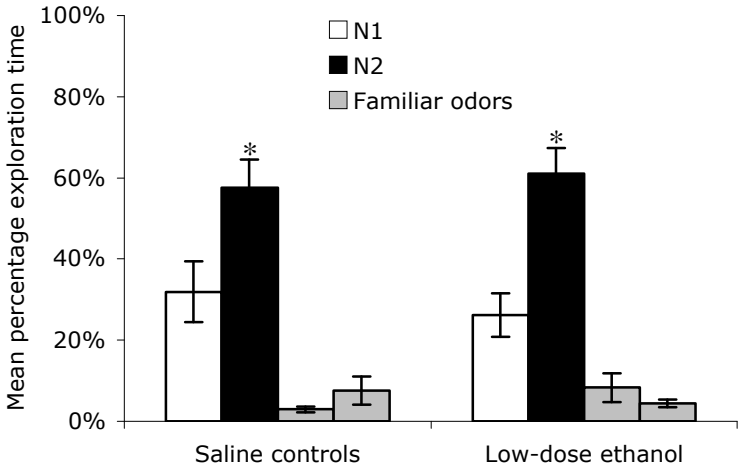


Figure 2.2 Lower-dose ethanol does not impair odor memory

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor in both groups. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.05$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Low-dose ethanol	13	0.26±.05	0.61±.06	0.08±.03	0.04±.009	4.23	<.0001	1.65
Control (saline)	7	0.31±.07	0.57±.06	0.02±.007	0.07±.03	2.51	.028	1.34

Table 2.2a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (ETOH 1.0)

Group	Odors			
	N1	N2	Familiar	Familiar
Low-dose ethanol	3.04±.55	3.46±.69	1.0±.18	0.78±.12
Control (saline)	3.59±.54	9.41±1.5	0.3±.09	1.6±.67

Table 2.2b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (ETOH 1.0)

Experiment 2: Higher-dose ethanol Sprague-Dawley

Habituation test data are shown in Fig. 2.3 and Tables 2.3a and 2.3b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition memory test. The higher dose of ethanol led to what might be considered severe retrograde memory impairment. Recognition memory was undetectable 24 hr after ethanol, which had been delivered immediately after habituation to N1. Data for the saline vs. ethanol 3.0 g/kg-treated Sprague Dawley rats are shown in Fig. 2.4, and Tables 2.4a and 2.4b. Control rats explored N2 significantly more than N1 whereas rats receiving 3.0 g/kg ethanol did not.

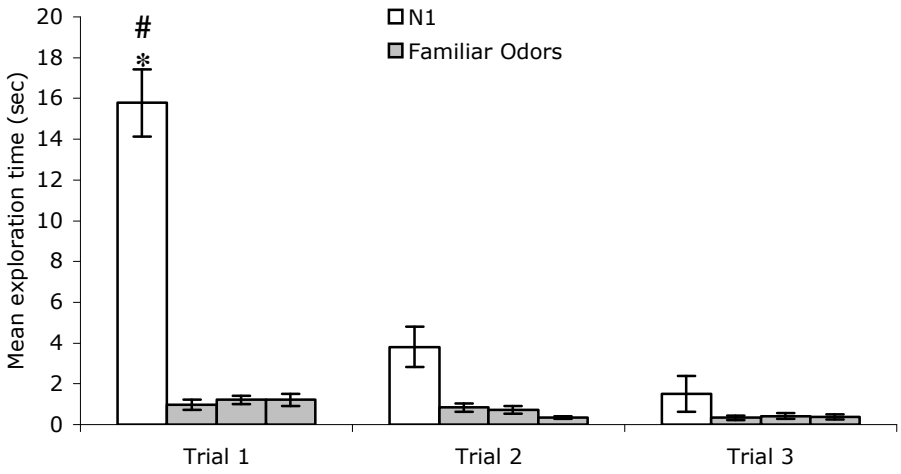


Figure 2.3 Novel odor preference and habituation to a novel odor across three trials in drug naïve Sprague-Dawley rats that will be exposed to ETOH 3.0 g/kg immediately following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
ETOH 3.0 Sprague-Dawley	15.78±1.6	1.5±.86	8.136	<.0001

Table 2.3a Habituation to N1 (ETOH 3.0 Sprague-Dawley)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
ETOH 3.0 Sprague-Dawley	15.78±1.6	0.9±.25	8.868	<.0001

Table 2.3b Novel-odor preference in the habituation phase (ETOH 3.0 Sprague-Dawley)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

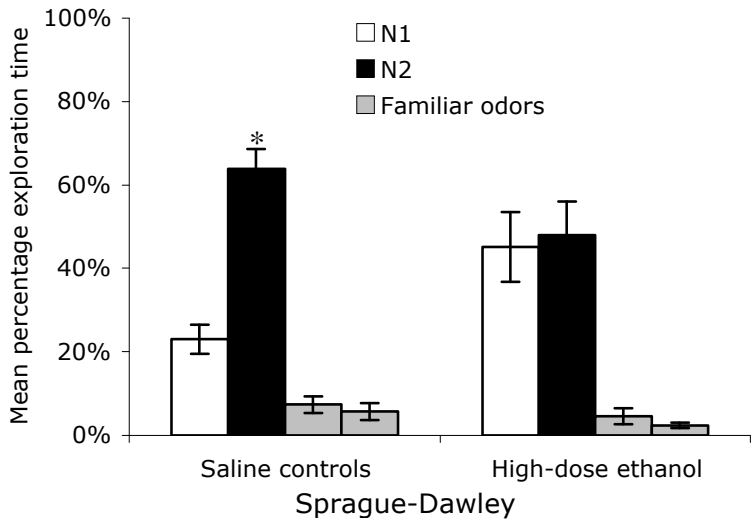


Figure 2.4 Higher-dose ethanol disrupts odor memory in Sprague-Dawley rats

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor only in the saline control rats. Ethanol-treated rats explored both odors equally, indicating an impairment of overnight memory. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.01$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
High-dose ethanol (Sprague-Dawley)	13	0.45±.08	0.47±.08	0.04±.01	0.02±.006	0.24	.812	0.09
Control (saline; Sprague-Dawley)	13	0.23±.03	0.63±.04	0.07±.02	0.05±.02	6.97	<.0001	2.7

Table 2.4a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (ETOH 3.0 Sprague-Dawley)

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
High-dose ethanol (Sprague-Dawley)	5.57±1.3	5.87±1.4	0.37±.13	0.29±.08
Control (saline; Sprague-Dawley)	2.22±.61	6.65±1.6	0.42±.08	0.29±.08

Table 2.4b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (ETOH 3.0 Sprague-Dawley)

Experiment 3: Higher-dose ethanol Long-Evans

Habituation test data are shown in Fig. 2.5 and Tables 2.5a and 2.5b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition memory test. The higher dose of ethanol led to what might be considered severe retrograde memory impairment. Recognition memory was undetectable 24 hr after ethanol, which had been delivered immediately after habituation to N1. Data for the saline vs. ethanol 3.0 g/kg-treated Long-Evans rats are shown in Fig. 2.6, and Tables 2.6a and 2.6b. Control rats explored N2 significantly more than N1 whereas rats receiving 3.0 g/kg ethanol did not.

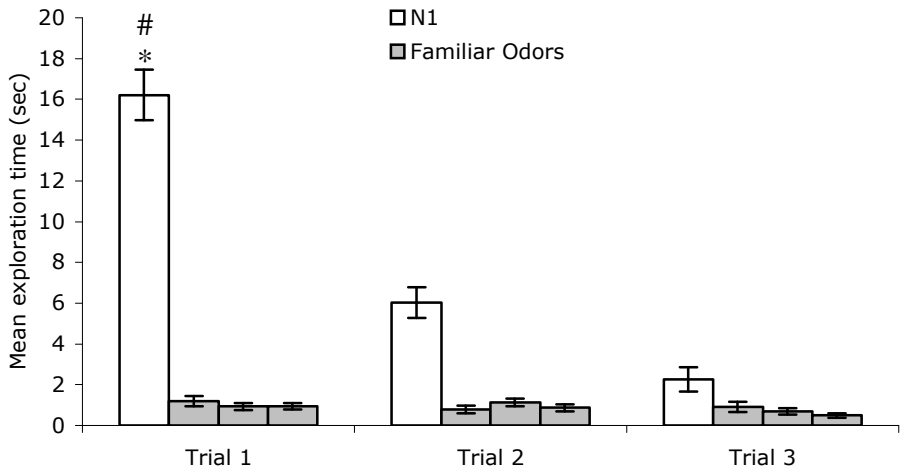


Figure 2.5 Novel odor preference and habituation to a novel odor across three trials in drug naïve Long-Evans rats that will be exposed to ETOH 3.0 g/kg immediately following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
ETOH 3.0 Long-Evans	16.2±1.2	2.2±.60	11.32	<.0001

Table 2.5a Habituation to N1(ETOH 3.0 Long-Evans)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
ETOH 3.0 Long-Evans	16.2±1.2	1.2±.25	11.82	<.0001

Table 2.5b Novel-odor preference in the habituation phase (ETOH 3.0 Long-Evans)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

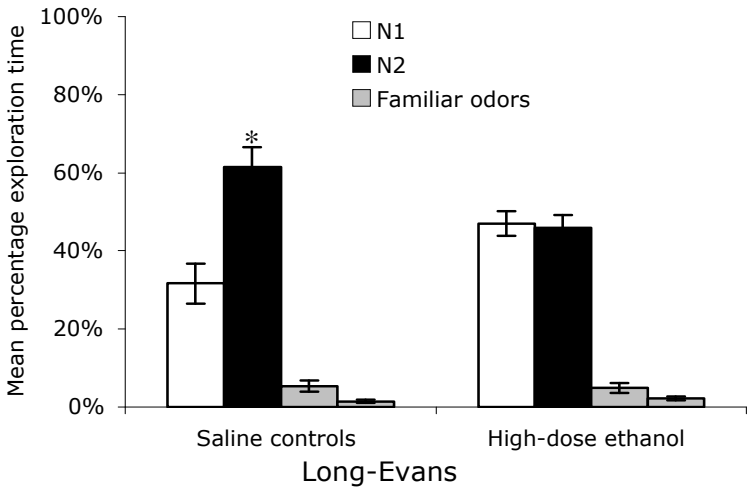


Figure 2.6 Higher-dose ethanol disrupts odor memory in Long-Evans rats

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor only in the saline control rats. Ethanol-treated rats explored both odors equally, indicating an impairment of overnight memory. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.01$. Data are means ± SEM

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
High-dose ethanol (Long-Evans)	24	0.47±.03	0.45±.03	0.04±.01	0.02±.004	0.24	.809	0.07
Control (saline; Long-Evans)	11	0.31±.05	0.61±.05	0.05±.01	0.01±.004	4.18	<.0001	1.78

Table 2.6a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (ETOH 3.0 Long-Evans)

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
High-dose ethanol (Long-Evans)	8.09±.88	9.81±1.8	0.72±.14	0.39±.09
Control (saline; Long-Evans)	5.19±1.2	9.82±1.6	0.85±.33	0.20±.06

Table 2.6b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (ETOH 3.0 Long-Evans)

Experiment 5: Hangover control

Habituation. Habituation test data are shown in Fig. 2.7 and Tables 2.7a and 2.7b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition memory test. When habituation was followed 24 hr later (rather than immediately) by high-dose ethanol, and recognition was assessed 24 hr after that, both control and ethanol-treated rats spent significantly more time exploring the novel odor N2 than N1 indicating retained memory for N1 as seen in Fig. 2.8 and Tables 2.8a and 2.8b. This shows not only that memory for N1 was detectable 48 hr after habituation, but also that the residual effects of ethanol administered 24 hr earlier (i.e., "hangover") did not contribute to the disruption of performance in the recognition memory task when ethanol was delivered immediately after habituation in the high-dose ethanol experiments.

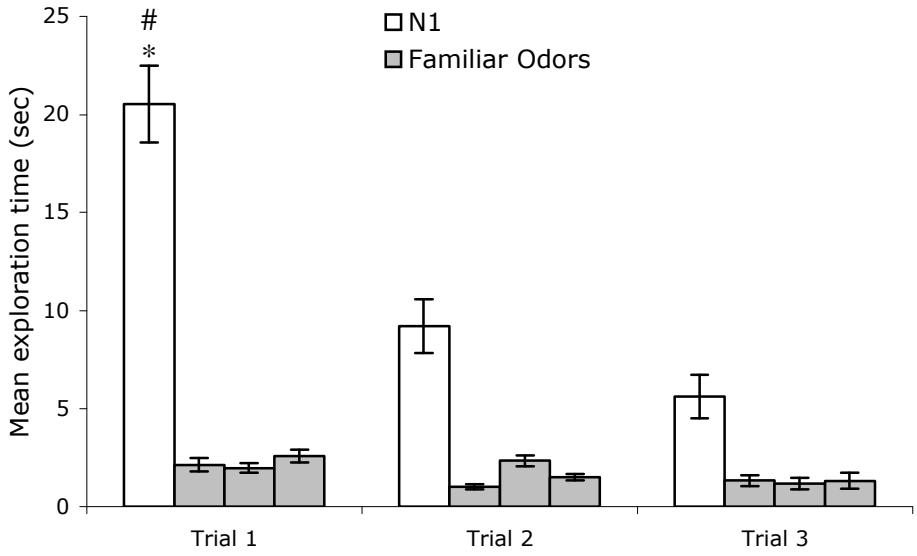


Figure 2.7 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to ETOH 3.0 g/kg immediately following the third habituation trial and tested 48 hours later

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
ETOH 3.0 Hangover control	20.5±1.9	5.6±1.0	8.02	<.0001

Table 2.7a Habituation to N1 (Hangover control)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
ETOH 3.0 Hangover control	20.5±1.9	2.11±.34	9.255	<.0001

Table 2.7b Novel-odor preference in the habituation phase (Hangover control)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

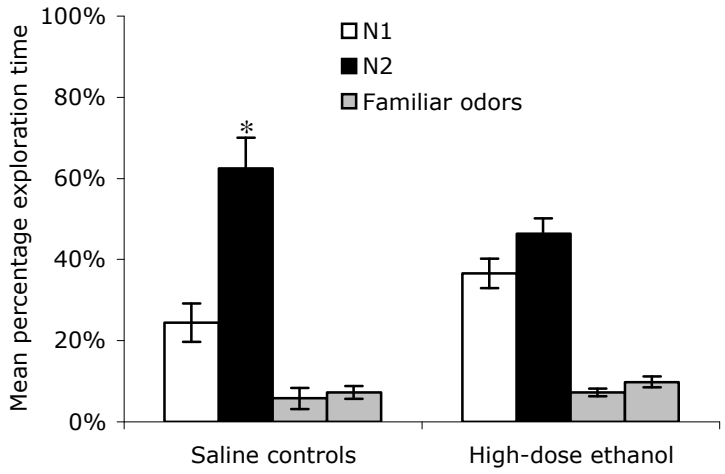


Figure 2.8 Higher-dose ethanol delivered immediately after habituation disrupts odor memory tested 48-hours later

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor only in the vehicle control rats. Rats that received ethanol injections explore both odors equally, indicating a loss of overnight memory.

Group	*n*	Odors	t	P	Effect

		N1	N2	Familiar	Familiar	value	value	Size
Ethanol (Hangover)	35	0.36±.03	0.46±.037	0.1±.01	0.1±.01	1.9	.06	0.45
Saline controls	8	0.24±.04	0.62±.07	0.06±.02	0.07±.01	4.24	.001	2.12

Table 2.8a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (Hangover control)

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
Ethanol (Hangover)	6.54±1.15	8.8±1.56	0.85±0.10	1.22±0.1
Saline control	3.9±0.68	15.0±4.8	0.76±0.31	1.22±0.31

Table 2.8b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (Hangover control)

DISCUSSION

Chapter 2 demonstrates that rats given a high dose and not a low dose of ethanol demonstrate the same retrograde memory impairment that is observed with the administration of PTZ in the follow up test of memory. Rats given a high, but not a low, dose of ethanol delivered immediately after repeated exposure to a N1 showed a loss of memory 24 hr later, in that they explored both N1 and a new odor N2 for an equivalent amount of time, whereas Control animals overwhelmingly explored N2 more than N1. These impairments cannot be attributed to the rat's inability to perform the task as ethanol was administered after learning had occurred. Secondly, as control and low-dose ethanol animals demonstrate long-term 24-hour overnight memory for the previously encountered odor, the memory impairments cannot be attributed to the inability of the animals to form a lasting memory for the odor. Third, the argument cannot be made that the observed loss of overnight memory for the recently-novel odor (i.e.: the even split in time between the two odors) is the result of reinforcement of the recently-novel odor by ethanol as opposed to a retrograde memory impairment for that odor, as rats given a low, but still substantial dose of ethanol (as in Manrique, 2005) show no such effect. Finally, as the odors were removed for one minute in between each of the three habituation trials, it cannot be argued that sensory adaptation to the novel odor (Best et al., 2005), which could potentially result in the observed impairments, occurred as opposed to true habituation.

The argument could be made that the observed retrograde memory impairments are not due to ethanol's effect on memory itself, but rather result from a "hangover" effect. Potentially, the administration of such a high dose of ethanol the previous day would produce an aversive state in the rat, which would in turn affect the rat's ability to explore the odors in the follow up test of memory and cause the observed impairment. However, a close inspection of the total mean exploration times of control and reveals no substantial differences between groups. Had this dose of ethanol truly caused a hangover effect, thus impacting odor exploration ability, this effect would have revealed itself in the total time spent exploring the odors.

It will be important to extend these studies by varying the delay of ethanol, varying the dose of ethanol, varying the level of experience with ethanol (most human consumption is not in alcohol-naïve individuals) and testing whether self-administered and experimenter administered lower doses of alcohol might lead to retrograde memory impairment if retention interval is delayed (in humans, simple information that is retained at 24 hrs is often lost after several days). It is important also to establish whether ethanol might can disrupt memory for flashbulb type memories such as that found in fear conditioning or spatial learning in a water maze, which is stressful. Human studies indicate that fragmentary blackouts are the most common memory impairment with ethanol. That is, major events occurring under the influence (and, based on our data, events just prior to alcohol consumption) may be recalled if they are extraordinarily salient, but modestly or minimally salient memories are lost, especially with some amount of time passed.

Chapter 3: Prevention of ethanol's amnestic effects with caffeine and related drugs

CHAPTER OVERVIEW

The third objective was to investigate whether the retrograde memory impairments observed with ethanol could be reversed or prevented with the administration of caffeine. Caffeine is often consumed before, during or shortly after ethanol and without ethanol has been demonstrated to improve cognition and mental performance on tasks of learning and memory in humans (Battig et al., 1984; Erikson et al., 1985; Lieberman et al., 1987; Jarvis 1993; David & Warburton 1995). Caffeine at moderate doses has also been shown to facilitate memory acquisition and retention in animals on various learning tasks (Izquierdo et al., 1979; Furusawa 1991; Buffalo et al., 1993; Molinengo, Scordo & Pastorello 1994; Molinengo et al., 1995; Martin & Garfield 2006) including recognition memory (Costa 2008). However, at high doses, caffeine can disrupt memory acquisition (Corodimas, Pruitt & Steig 2000). There is a paucity of literature on the effects of caffeine and ethanol combinations on memory however, with studies focusing primarily on caffeine's influence on the depressor effects of alcohol (Ferreira et al., 2004; Ferriera et al., 2004a; Ferriera et al., 2006).

Caffeine is both a phosphodiesterase inhibitor and an adenosine antagonist (Nehlig, Daval & Debry 1992; Howell et al., 1997; Fredholm et al., 1999). As an adenosine antagonist, caffeine has equal affinity for A_1 and A_{2A} subtypes of adenosine receptors (Prediger 2005a) with the behavioral activating effects of caffeine associated with antagonism of A_{2A} receptors (Svenningsson et al., 1997, Sveningsson et al., 1997a). It has been shown that antagonists specific to the A_{2A} subtype (ZM241385) (Yang et al.,

2007), at doses of 1.0mg/kg and not antagonists specific to the A_1 subtype of adenosine can improve memory in social odor recognition tasks (Prediger 2005b). Similarly, it has been demonstrated that antagonists specific to the A1 subtype can affect memory in tasks with a strong emotional component, such as inhibitory avoidance (Normile & Barraco 1991; Normile, et al., 1994; Zarrindast & Shafagi 1994). Furthermore, as a phosphodiesterase inhibitor, caffeine is non-selective for multiple subtypes of phosphodiesterase. Inhibition of two of these subtypes, PDE4 and PDE5 lead to increases in intracellular cAMP and cGMP respectively. Zaprinast, a potent inhibitor of the PDE5 subtype has been shown to improve memory consolidation in an object recognition task and only at a dose of 10 mg/kg (Prickaerts et al., 1997; Prickaerts et al., 2004; Blokland et al., 2006). Based on this literature, we used each of these drugs, Zaprinast and ZM241385 separately and in unison, in an attempt to prevent ethanol-induced retrograde memory impairments.

PHARMACOLOGICAL TREATMENTS

Caffeine post-ethanol. Rats (n=9) were given injections of 3.0 g/kg ethanol, with matched saline-injected controls (n=9), immediately following the last N1 habituation trial. Then, after a one-hour delay, these rats (all 18) received i.p. injections of 5 mg/kg caffeine dissolved in saline. They were then left alone until odor recognition testing the following day. As outlined in Fig. A2.8.

Caffeine pre-ethanol. Twenty minutes *before* the first novel-odor habituation trial, rats were given intraperitoneal injections of 5 mg/kg caffeine (MP Biomedicals, dissolved at 5 mg/ml in saline). Then, following the last N1 habituation trial, subgroups of these caffeine-treated animals were given either 20% (w/v) ethanol at a dose of 3.0 g/kg i.p. (n=23) or equivalent volumes of saline (n=20). Assessment of memory for N1 was carried out 24 h after ethanol was administered. As outlined in Fig. A2.9.

PDE5 inhibitor post-ethanol. Rats (n=10) were given injections of 3.0 g/kg ethanol, with matched saline-injected controls (n=4), immediately following the last N1 habituation trial. Then, after a one-hour delay, all of these rats received i.p. injections of the PDE5 inhibitor zaprinast (Tocris Bioscience, dissolved in 100% DMSO; 10 mg/0.1 ml/kg). They were then left alone until odor recognition testing the following day. In a follow-up test, 6 additional rats received a 2X dose of Zaprinast (20 mg/kg) one hour after habituation to N1 and ethanol and were tested the next day for recognition memory. As outlined in Fig. A2.10.

A_{2A} *antagonist post-ethanol.* Rats (n=11) were given injections of 3.0 g/kg ethanol, with matched saline-injected controls (n=6), immediately following the last N1 habituation trial. Then, after a one-hour delay, these rats (all 17) received i.p. injections of 1 mg/0.1 ml/kg ZM241385 (Tocris Bioscience, dissolved in 100% DMSO). They were then left alone until odor recognition testing the following day. As outlined in Fig. A2.11.

Combination PDE5 inhibitor and A_{2A} *antagonist post-ethanol.* Immediately following the last trial of habituation to N1, rats (n=8) were given injections of 3.0 g/kg ethanol, with matched saline-injected controls (n=7), Then, after a one-hour delay, these rats (all 15) received i.p. injections of both 10 mg/kg Zaprinast and 1 mg/kg ZM241385. They were then left alone until odor recognition testing the following day. As outlined in Fig. A2.12.

RESULTS

Experiment 1: Caffeine post-ethanol

Habituation test data are shown in Fig. 3.1 and Tables 3.1a and 3.1b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial

3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition memory test. Caffeine delivered one hour after exposure to N1 prevented retrograde recognition memory disruption by the higher dose of ethanol (Fig. 3.2 and Tables 3.2a and 3.2b). Both control and ethanol 3.0 g/kg treated rats spent significantly more percent time exploring the novel odor than the recently-novel odor.

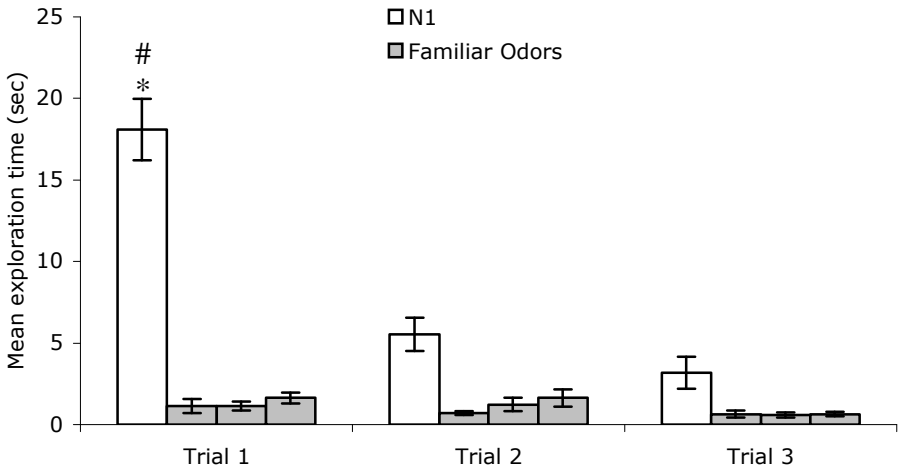


Figure 3.1 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to ETOH 3.0 g/kg and caffeine 5 mg/kg immediately following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
Caffeine Post Ethanol	18.1±1.9	3.2±.97	8.027	<.0001

Table 3.1a Habituation to N1 (Caffeine post ethanol)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
Caffeine Post Ethanol	18.1±1.9	1.1±.42	8.715	<.0001

Table 3.1b Novel-odor preference in the habituation phase (caffeine post ethanol)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

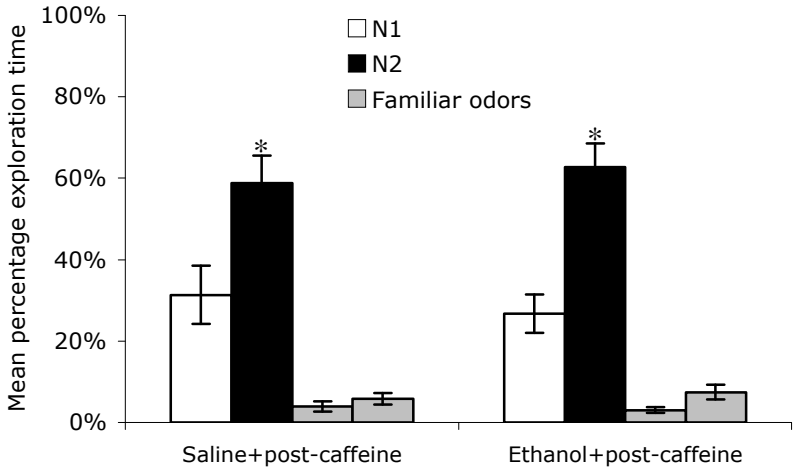


Figure 3.2 Caffeine administered after a post-learning high-dose of ethanol also, as pre-learning caffeine, prevents memory disruption

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor in both groups. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.05$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Ethanol + caffeine one hour after	9	0.26±.04	0.62±.05	0.03±.007	0.07±.01	4.87	<.0001	2.29
Control (saline + caffeine)	9	0.31±.07	0.58±.06	0.03±.01	0.05±.01	2.78	.015	1.39

Table 3.2a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (Caffeine post ethanol)

Group	Odors			
	N1	N2	Familiar	Familiar
Ethanol + caffeine one hour after	4.67±1.5	11.4±2.3	1.15±.34	0.47±.13
Control (saline + caffeine)	4.94±1.3	9.18±2.5	0.61±.21	0.5±.17

Table 3.2b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (Caffeine post ethanol)

Experiment 2: Caffeine pre-ethanol

Habituation test data are shown in Fig. 3.3 and Tables 3.3a and 3.3b. Caffeine administered prior to habituation trials did not affect habituation to N1 or absolute bead exploration times. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition Memory Test. Caffeine delivered 20 min before the first exposure to N1 prevented retrograde recognition memory disruption by a subsequent high dose of ethanol. Recognition of N1 appeared to be intact the next day despite administration of 3.0 g/kg ethanol following habituation. The behavior of rats that received caffeine 20 minutes prior to habituation is shown in Fig. 3.4 and Tables 3.4a and 3.4b. Both control- and ethanol 3.0 g/kg-treated rats pre-exposed to caffeine spent significantly more time exploring N2 than N1.

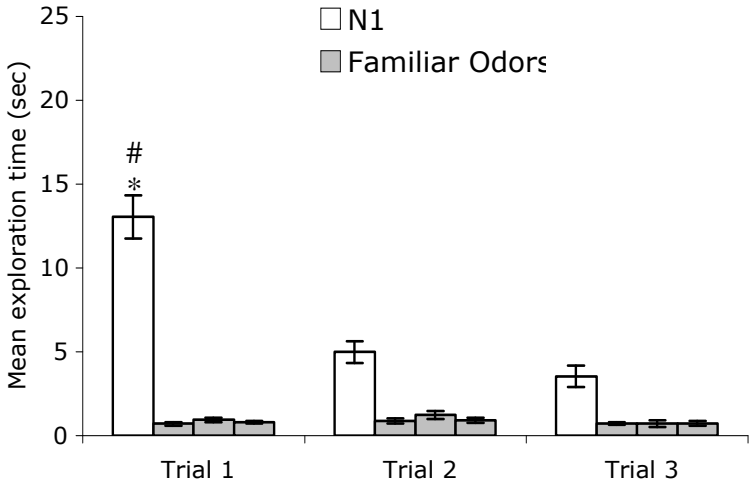


Figure 3.3 Novel odor preference and habituation to a novel odor across three trials is maintained in rats pretreated with caffeine

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
Pre-Caffeine	13.3±1.3	3.6±.66	7.4	<.0001

Table 3.3a Habituation to N1 (Caffeine pre ethanol)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
Pre-Caffeine	13.3±1.3	.72±.1	9.5	<.0001

Table 3.3b Novel-odor preference in the habituation phase (Caffeine pre ethanol)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

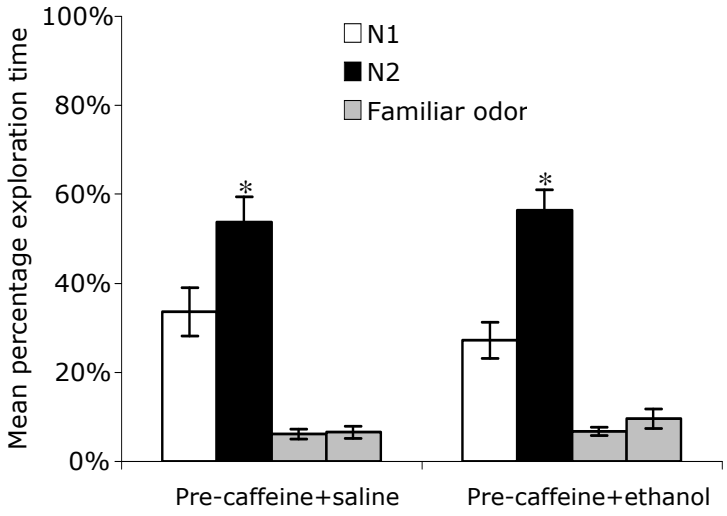


Figure 3.4 Caffeine administered before learning prevents the disruption of odor memory by a very high-dose of ethanol administered post-learning

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor in both groups. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.05$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Caffeine before habituation + ethanol	23	0.27±.04	0.56±.04	0.06±.009	0.09±.02	4.75	<.0001	1.4
Control (caffeine + saline)	20	0.33±.05	0.53±.05	0.06±.01	0.06±.01	2.55	.015	0.8

Table 3.4a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (Caffeine pre ethanol)

Group	Odors			
	N1	N2	Familiar	Familiar
Caffeine before habituation + ethanol	2.75±.52	5.76±.87	0.68±.09	0.56±.07
Control (caffeine + saline)	4.7±.81	8.8±1.5	0.85±.16	0.79±.14

Table 3.4b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (Caffeine pre ethanol)

Experiment 3: PDE5 inhibitor post-ethanol

Habituation test data are shown in Fig. 3.5 and Tables 3.5a and 3.5b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition Memory Test. Neither dose of the PDE5 inhibitor, delivered one hour after exposure to N1, prevented ethanol-induced retrograde recognition memory disruption (Fig. 3.6, Tables 3.6a and 3.6b). There were no differences between the ethanol 3.0 g/kg- treated rats that received 10 or 20 mg/kg zaprinast; therefore, these groups were combined for analysis. Ethanol treated rats showed no difference in percent time exploring N1 versus N2, whereas control rats spent significantly more time exploring N2 than N1.

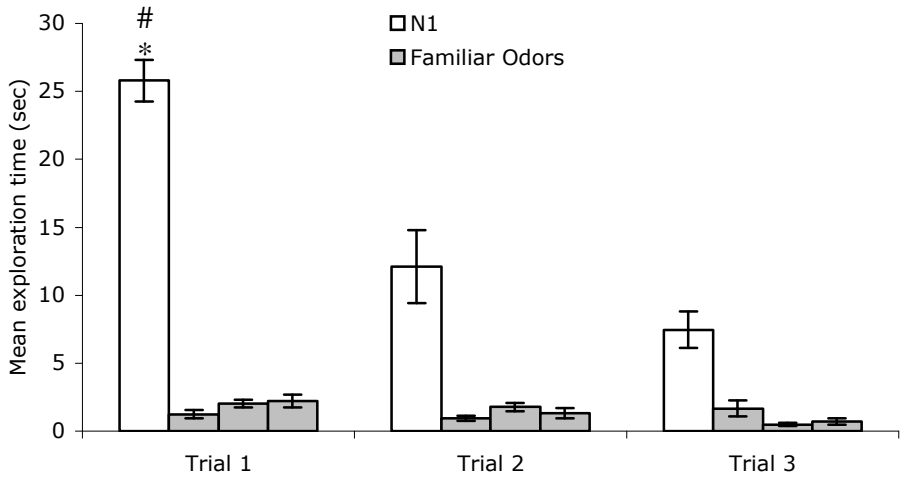


Figure 3.5 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to ETOH 3.0 g/kg and a PDE5 inhibitor immediately following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
PDE5 Inhibitor	25.7±1.5	7.4±1.3	9.878	<.0001

Table 3.5a Habituation to N1 (PDE5)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
PDE5 Inhibitor	25.7±1.5	1.24±.29	15.927	<.0001

Table 3.5b Novel-odor preference in the habituation phase (PDE5)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

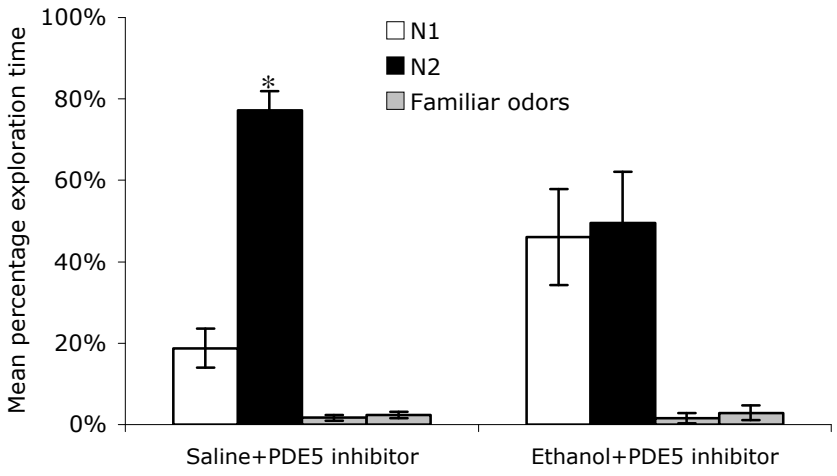


Figure 3.6 A PDE5 inhibitor administered one hour after a post-learning high-dose of ethanol does not prevent memory disruption

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor in saline controls. Ethanol-treated rats explore both odors equally, indicating an impairment of overnight memory. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.05$. Data are means ± SEM

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Ethanol + PDE5 inhibitor	10	0.46±.11	0.49±.12	0.01±.01	0.02±.01	0.41	.686	0.18
Control (saline + PDE5 inhibitor)	4	0.18±.04	0.77±.04	0.01±.006	0.02±.008	8.76	<.0001	6.19

Table 3.6a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (PDE5)

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
Ethanol + PDE5 inhibitor	8.66±1.6	10.2±1.9	0.25±.09	0.52±.21
Control (saline + PDE5 inhibitor)	3.95±2.5	12.1±4	0.18±.08	0.5±.25

Table 3.6b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (PDE5)

Experiment 4: A_{2A} antagonist post-ethanol

Habituation test data are shown in Fig. 3.7 and Tables 3.7a and 3.7b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition Memory Test. The A_{2A} antagonist, ZM241385, by itself did not negate ethanol-induced retrograde memory impairment. Fig. 3.8 and Tables 3.8a and 3.8b show that rats receiving this drug one hour after habituation to N1 and subsequent exposure to a high dose of ethanol did not preferentially explore either N1 or N2 when tested for recognition memory 24 hr later, in contrast to control rats which significantly preferred N2.

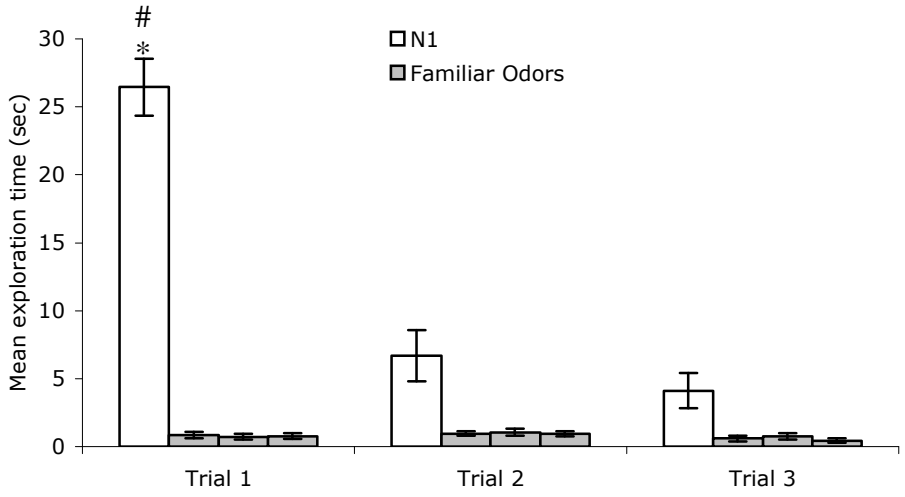


Figure 3.7 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to ETOH 3.0 g/kg and an A_{2A} antagonist immediately following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
A_{2A} Antagonist	26.4±2.1	4.1±1.3	9.569	<.0001

Table 3.7a Habituation to N1 (A_{2A})

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
A_{2A} Antagonist	26.4±2.1	0.86±.23	12.225	<.0001

Table 3.7b Novel-odor preference in the habituation phase (A_{2A})

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

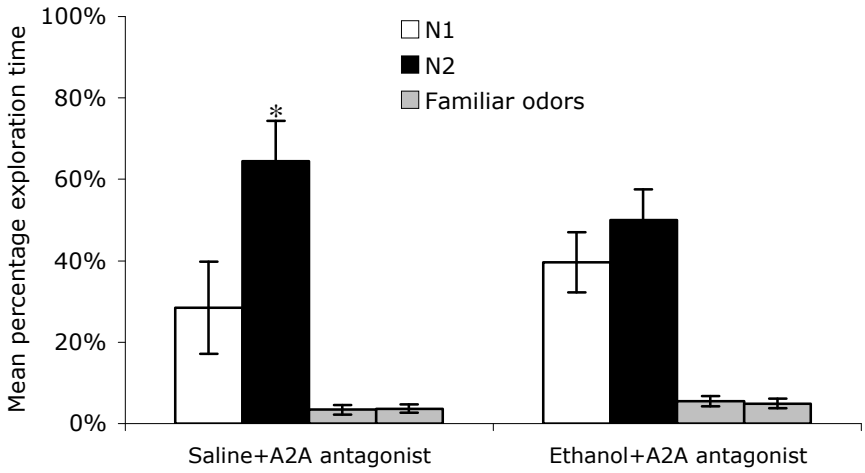


Figure 3.8 An Adenosine A_{2A} antagonist administered after a post-learning high-dose of ethanol does not prevent memory disruption

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor in saline controls. Ethanol-treated rats explore both odors equally, indicating an impairment of overnight memory. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.05$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Ethanol + A_{2A} antagonist	11	0.39±.07	0.49±.07	0.05±.01	0.04±.01	0.98	.338	0.41
Control (saline + A_{2A} antagonist)	6	0.28±.11	0.64±.09	0.03±.01	0.03±.01	2.40	.037	1.38

Table 3.8a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (A_{2A})

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
Ethanol + A_{2A} antagonist	7.24±2.5	7.38±1.9	0.64±.25	0.49±.10
Control (saline + A_{2A} antagonist)	5.5±2.3	9.4±3.1	0.35±.04	0.38±.05

Table 3.8b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (A_{2A})

Experiment 5: Combination PDE5 inhibitor and A_{2A} antagonist post-ethanol

Habituation test data are shown in Fig. 3.9 and Tables 3.9a and 3.9b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition Memory Test. A combination of the PDE5 inhibitor (10 mg/kg Zaprinast) and the adenosine A_{2A} antagonist (1 mg/kg ZM241385) administered one hour following the higher dose of ethanol prevented ethanol-induced retrograde memory impairment (Fig. 3.10 and Tables 3.10a and 3.10b). Both control and ethanol 3.0 g/kg treated rats spent significantly more time exploring N2 than N1, indicating retained overnight memory for N1. It should be noted that animals in the 3g/kg ethanol groups indeed lost the righting reflex and appeared behaviorally to be asleep. When injected with caffeine or the combination of the PDE5 inhibitor and A_{2A} antagonist, the animals did not regain the righting reflex and showed no overt sign of rescue from "sleep"

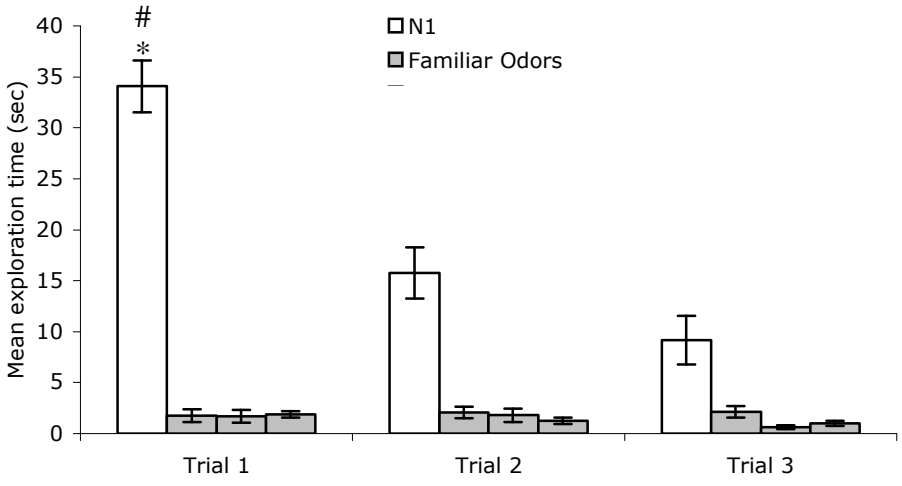


Figure 3.9 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to ETOH 3.0 g/kg and a combination PDE5 inhibitor/ A_{2A} antagonist immediately following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. **#** Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; ***** Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
A_{2A} /PDE5 Combination	34.0±2.5	9.2±2.4	9.024	<.0001

Table 3.9a Habituation to N1 (PDE5/ A_{2A})

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
A_{2A} /PDE5 Combination	34.0±2.5	1.76±.64	12.340	<.0001

Table 3.9b Novel-odor preference in the habituation phase (PDE5/ A_{2A})

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

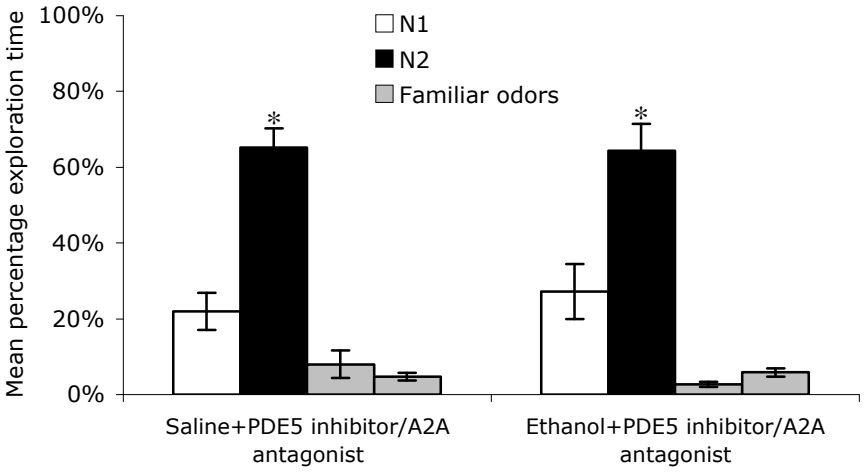


Figure 3.10 The combination of a PDE5 inhibitor and an adenosine A_{2A} antagonist administered after a post-learning high-dose of ethanol prevents disruption of odor memory

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor in both groups. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.05$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Ethanol + PDE5 inhibitor + A_{2A} antagonist	8	0.27±.07	0.64±.07	0.02±.006	0.05±.01	3.63	.003	1.81
Control (saline + both drugs)	7	0.21±.04	0.65±.04	0.08±.03	0.04±.009	6.19	<.0001	3.31

Table 3.10a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (PDE5/ A_{2A})

Group	Odors			
	N1	N2	Familiar	Familiar
Ethanol + PDE5 inhibitor + A_{2A} antagonist	3.675±.97	9.85±2.5	0.35±.08	0.8±.17
Control (saline + both drugs)	4.74±1.1	15.6±3.7	1.6±.68	1.04±.21

Table 3.10b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (PDE5/ A_{2A})

DISCUSSION

The goal of this chapter was to assess the effects of caffeine and related drugs on retrograde memory impairments observed with a high dose of ethanol. A high dose of ethanol, administered after learning, causes retrograde memory impairment 24-hours later during the recognition test. This memory impairment could be prevented with the administration of caffeine, a drug often contained in beverages consumed before, during or after alcohol. Caffeine somehow prevented the ethanol-induced retrograde amnesia, not only when delivered just prior to learning and exposure to ethanol, but remarkably also even when delivered one hour after ethanol, ruling out caffeine effects on brain levels of ethanol and indicating that the effects of ethanol on memory consolidation require disruption of neural events that are not short-term.

It was also found that neither an adenosine A_{2A} antagonist nor a phosphodiesterase-5 inhibitor alone prevented retrograde amnesia when delivered one hour after ethanol (unlike caffeine, which did prevent the amnesia). However, a combination of these two drugs was highly effective at the previously ineffective doses, which were selected on the basis of the dose of caffeine. Thus, it appears that mimicking two of caffeine's key mechanisms of action simultaneously with a PDE5 inhibitor and an A_{2A} antagonist is at least sufficient, if not necessarily required, for prevention of retrograde amnesia by ethanol.

Chapter 4: Consolidation and Reconsolidation disruption through protein synthesis inhibition and ethanol.

CHAPTER OVERVIEW

In chapter 3, it was demonstrated that an acute high dose of ethanol can cause retrograde memory impairments when delivered after learning has occurred. Ethanol might exert these deleterious effects by disrupting memory consolidation through inhibition of receptors vital to protein synthesis. In this final series of experiments, we attempted to disrupt the consolidation process more directly through the use of protein synthesis inhibition. We also attempted to shed light on the phenomenon of reconsolidation. It is well documented in fear conditioning paradigms that anisomycin, a protein synthesis inhibitor, delivered after learning and memory reactivation can disrupt consolidation and reconsolidation respectively and cause retrograde memory impairments in follow-up tests of memory (Nader 2003; Artinian et al., 2007, Wang et al., 2005; Przybylaski & Sara 1997, Przybyslawski, Roullet & Sara 1999; Miller & Matzel 2000).

The process of reconsolidation has been proposed to require four main components: 1) After the consolidation of a memory, the presentation of the learned cue reactivates the consolidated memory, returning it to a labile state that renders it vulnerable to disruption. That reactivated memory must go through a second protein-synthesis dependent consolidation process, re-consolidation, the disruption of which, through the use of a protein-synthesis inhibitor, disrupts the learning that has taken place. The absence of the CS reminder cue leaves the consolidated memory untouched, thus protein-synthesis independent, as a protein-synthesis inhibitor has no effect on a memory that has not been reactivated. 2) The process of protein-synthesis dependent re-

consolidation has a temporal component that returns the labile reactivated memory to a more stable protein-synthesis independent state after enough time has passed. 3) The length of time that a memory consolidation has to take place has no effect on the ability of that memory to be disrupted if it is reactivated. Once reactivated via the CS reminder cue, protein synthesis must take place in order for the memory to be reconsolidated. Thus, it is labile and vulnerable to disruption during this period of reactivation. 4) Reactivated short-term memory is subserved by a different function than reactivated long term memory. As a result, while short-term memory can remain robust over a short period of time, the long-term re-consolidation of that memory is subserved by different intracellular processes and therefore can be disrupted. Having identified the medial amygdala as our target for protein synthesis inhibition, we delivered anisomycin into the medial amygdala after habituation to a novel odor or reactivation of that learned cue, (i.e. a one-trial re-introduction of the habituated odor). Findings suggest that inhibition of protein synthesis during either consolidation or reconsolidation impairs memory the following day in a follow-up odor recognition test.

Surgery and histology

Prior to surgeries, animals are tamed by frequent handling to minimize stress associated with injections and behavioral measurements. On the day of surgery, rats are deeply anesthetized with a mixture of ketamine 100 mg/kg and xylezine 20 mg/kg. When fully anesthetized, as verified by lack of tail pinch and corneal responses, the animals are placed in a stereotaxic apparatus and the scalp is shaved and swabbed with betadine. A midline incision is made to expose bregma and the skull is leveled along the dorsal-

ventral plane. Two small burr holes are made and rats are bilaterally implanted with 22-gauge stainless steel cannulas into the medial amygdala. Coordinates for the medial amygdala, derived from Paxinos and Watson (1998) are 2.3mm posterior to bregma, 3.4mm lateral to the midline and 8.0mm ventral from the skull surface. No supportive care is typically required during this type of surgery, but body temperature, breathing and the plane of anesthetization are continuously monitored. Placement on a heating pad and supplemental injections of the anesthetic are performed as needed. Rats are given at least 7 days to recover from surgery before they are used in the odor-recognition task. Following the experiment, rats are transcardially perfused with a 4% formaldehyde solution and their brains removed. These brains are sectioned at 50microns thickness and stained with cresyl violet in order to verify bilateral cannula implantation into the medial amygdala. Rats without bilateral cannulae in their medial amygdalae are excluded from subsequent statistical analysis.

RECONSOLIDATION METHODOLOGY

For the reconsolidation experiments, rats habituate to the novel odor and 24 hours later are presented with the habituated novel-odor for 1-minute as a "reminder" cue in order to reactivate the consolidated memory. Immediately following this reactivation trial, anisomycin is infused into the medial amygdala. 24 hours later, the odor-recognition test is performed. For a graphical representation of the reconsolidation method, please see Fig. A2.2.

PHARMACOLOGICAL TREATMENTS

Intra-Medial Amygdala Anisomycin Infusions (Consolidation group)

Immediately following habituation in the consolidation procedure outlined in Fig. A2.1 and followed in all of the previous experiments, 62.5ug of anisomycin (Sigma) dissolved in 0.5ul of ACSF is infused via infusion pump into each medial amygdala at 0.25ul per min for two minutes in (n=9) rats with (n=10) vehicle controls. Injectors are left in place for one minute following infusion to allow for diffusion from the tip. Anisomycin is dissolved in HCl, diluted with ACSF and adjusted to a pH of 7.4 using NaOH. This dose of anisomycin was chosen based on previous studies showing that it was the minimum dose needed to effectively inhibit protein synthesis and thus consolidation (Ben-Mamou 2006). As outlined in Fig. A2.1 and A2.13.

Intra-Medial Amygdala Anisomycin Infusions (Reconsolidation group)

24-hours after habituation, a 1-minute, 1-trial reminder of N1 (reactivation trial) was run. Immediately following this reactivation trial in the reconsolidation procedure, 62.5ug of anisomycin (Sigma) dissolved in 0.5ul of ACSF is infused via infusion pump into each medial amygdala at 0.25ul per min for two minutes in (n=8) rats with (n=5) vehicle controls. Injectors are left in place for one minute following infusion to allow for diffusion from the tip. Anisomycin is dissolved in HCl, diluted with ACSF and adjusted to a pH of 7.4 using NaOH. As outlined in Fig. A2.2 and A2.14.

Ethanol Reconsolidation

24-hours after habituation, a 1-minute, 1-trial reminder of N1 (reactivation trial) was run. Immediately following this reactivation trial in the reconsolidation procedure, Long-Evans rats (n=9) were given i.p. injections of 20% (w/v) ethanol at a dose of 3.0 g/kg ethanol, with matched controls (n=6) receiving equivalent volumes of saline. The recognition memory test was performed 24 hours following ethanol injections. As outlined in Fig. A2.2 and A2.15.

Non-reactivated Control

Rats were given i.p. injections of either 20% (w/v) ethanol at a dose of 3.0 g/kg (n = 6) or saline (n=6) at 24 hours, rather than immediately, after habituation. 24 hr after this, all rats were tested for recognition memory. In this procedure the longer time-span between learning and ethanol administration was sufficient for memory consolidation to occur, yet hangover symptoms would presumably still be present during the recognition test since it is still performed 24 hr after administration of high-dose ethanol, as in the other experiments and outlined in Fig. A2.6.

Ethanol Reconsolidation hangover control

24-hours after habituation, a 1-minute, 1-trial reminder of N1 (reactivation trial) was run. Immediately following this reactivation trial in the reconsolidation procedure, Long-Evans rats (n=8) were given i.p. injections of 20% (w/v) ethanol at a dose of 3.0 g/kg ethanol, with matched controls (n=7) receiving equivalent volumes of saline. 48

hours following ethanol injections the recognition memory test was performed, as outlined in Fig. A2.16. A small group of rats were run concurrently and are identified as 72-hour controls. These rats received an equivalent volume of saline immediately following habituation and were tested 72 hours later to ensure that memory was intact as outlined in Fig. A 2.17.

RESULTS

Experiment 1: Intra-Medial Amygdala Anisomycin Infusions (Consolidation group)

Habituation test data are shown in Fig. 4.1 and Tables 4.1a and 4.1b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the

first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads. This suggests that neither surgery, nor implanted cannulae had any demonstrable effect on the ability to habituate to a novel odor or novel odor preference.

Recognition Memory Test. Recognition memory test data for the ACSF and Anisomycin treated rats are shown in Fig. 4.2 and Tables 4.2a and 4.2b. Anisomcin treated rats showed no difference in percent time exploring N1 versus N2, suggesting an impairment of consolidation for the memory of N1. Control rats spent significantly more time exploring N2 than N1.

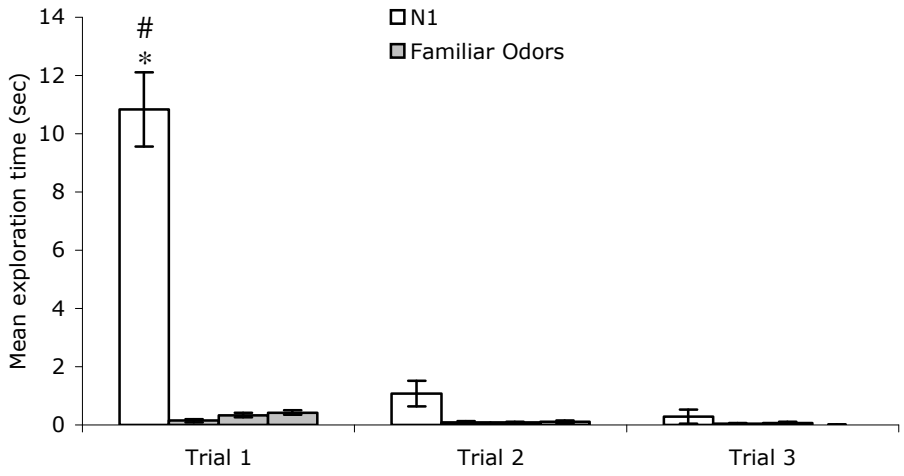


Figure 4.1 Novel odor preference and habituation to a novel odor across three trials in drug naïve cannulated rats that will be receive Anisomycin infusions immediately following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. **#** Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; ***** Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
Anisomycin Consolidation	10.8±1.3	0.28±.24	8.212	<.0001

Table 4.1a Habituation to N1 (Anisomycin consolidation)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
Anisomycin Consolidation	10.8±1.3	0.14±.06	8.393	<.0001

Table 4.1b Novel-odor preference in the habituation phase (Anisomycin consolidation)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

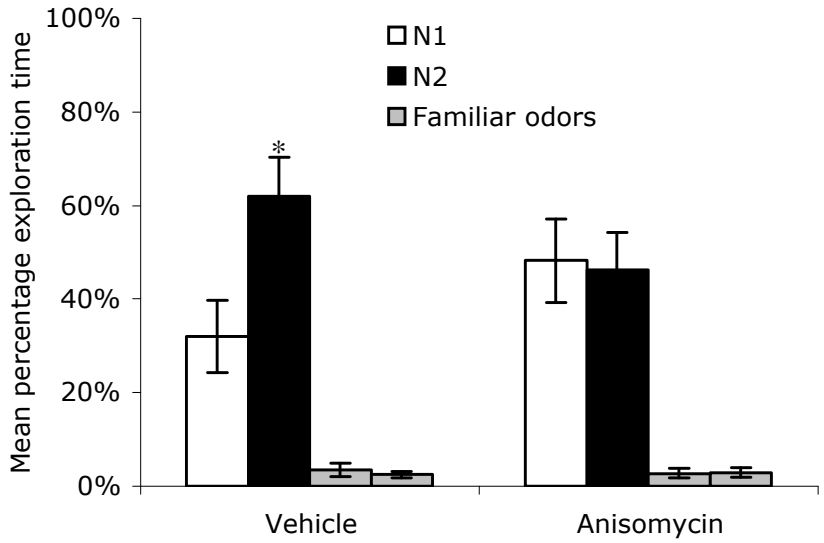


Figure 4.2 Anisomycin disrupts odor recognition memory

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor only in the vehicle control rats. Rats that received Anisomycin infusions explore both odors equally, indicating a loss of overnight memory. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.05$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Anisomycin consolidation	9	0.48±.08	0.46±.08	0.03±.01	0.03±.01	0.17	.868	0.079
Vehicle control	10	0.32±.07	0.62±.08	0.03±.01	0.02±.01	2.65	.016	1.18

Table 4.2a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (Anisomycin consolidation)

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
Anisomycin consolidation	5.527±1.18	5.75±1.33	0.29±0.09	0.29±0.09
Vehicle Control	7.74±2.57	3.68±1.4	0.18±0.05	0.39±0.15

Table 4.2b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (Anisomycin consolidation)

Experiment 2: Intra-Medial Amygdala Anisomycin Infusions (reconsolidation group)

Habituation test data are shown in Fig. 4.3 and Tables 4.3a and 4.3b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition Memory Test, 24 Hours Following Reactivation. Recognition memory test data for the ACSF and Anisomycin treated rats are shown in Fig. 4.4 and Tables 4.4a and 4.4b. Anisomcin treated rats showed no difference in percent time exploring N1 versus N2, suggesting an impairment of reconsolidation for the memory of N1. Control rats spent significantly more time exploring N2 than N1.

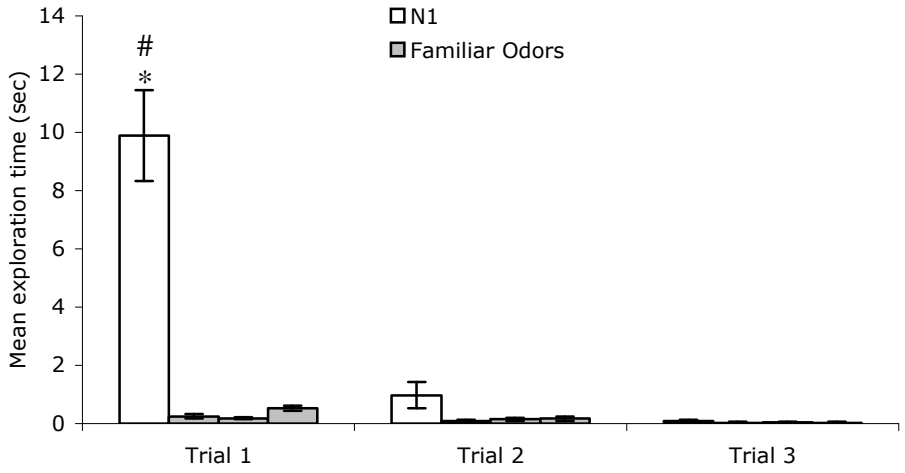


Figure 4.3 Novel odor preference and habituation to a novel odor across three trials in drug naïve cannulated rats that will receive Anisomycin infusions 24-hours following the third habituation trial, after a 1-trial reminder of N1

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. # Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; * Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
Anisomycin Reconsolidation	9.8±1.6	0.08±.05	6.512	<.0001

Table 4.3a Habituation to N1 (Anisomycin reconsolidation)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
Anisomycin Reconsolidation	9.8±1.6	0.25±.07	6.369	<.0001

Table 4.3b Novel-odor preference in the habituation phase (Anisomycin reconsolidation)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

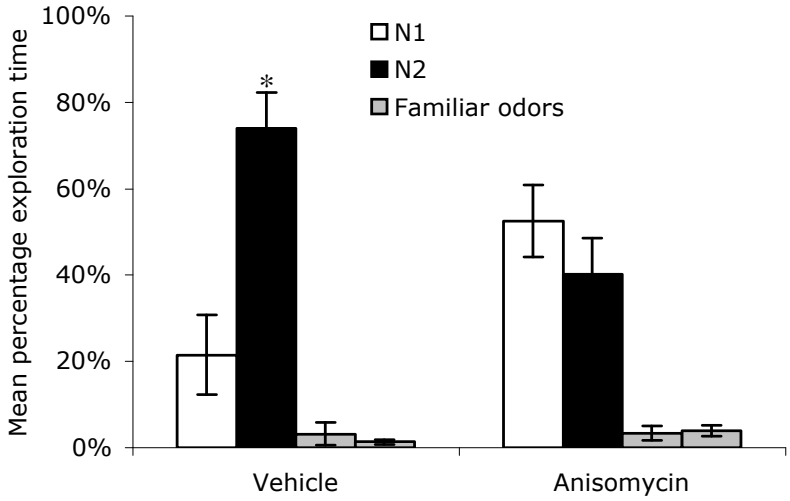


Figure 4.4 Anisomycin disrupts reconsolidation of odor recognition memory

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor only in the vehicle control rats. Rats that received Anisomycin infusions explore both odors equally, indicating a loss of overnight memory. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.05$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Anisomycin reconsolidation	8	0.52±.08	0.40±.08	0.03±.01	0.03±.01	0.983	.342	0.49
Vehicle control	5	0.21±.09	0.73±.08	0.03±.02	0.01±.01	3.84	.005	2.42

Table 4.4a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (Anisomycin reconsolidation)

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
Anisomycin reconsolidation	5.335±1.93	3.46±1.04	0.15±0.05	0.275±0.11
Vehicle Control	1.6±0.81	5.25±2.34	0.12±0.05	0.44±0.39

Table 4.4b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (Anisomycin reconsolidation)

Experiment 3: Ethanol Reconsolidation

Habituation test data are shown in Fig. 4.5 and Tables 4.5a and 4.5b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition Memory Test, 24 Hours Following Reactivation. Recognition memory test data for the ethanol and control rats are shown in Fig. 4.6 and Tables 4.6a and 4.6b. Ethanol treated rats showed no difference in percent time exploring N1 versus N2, suggesting an impairment of reconsolidation for the memory of N1. Control rats spent significantly more time exploring N2 than N1.

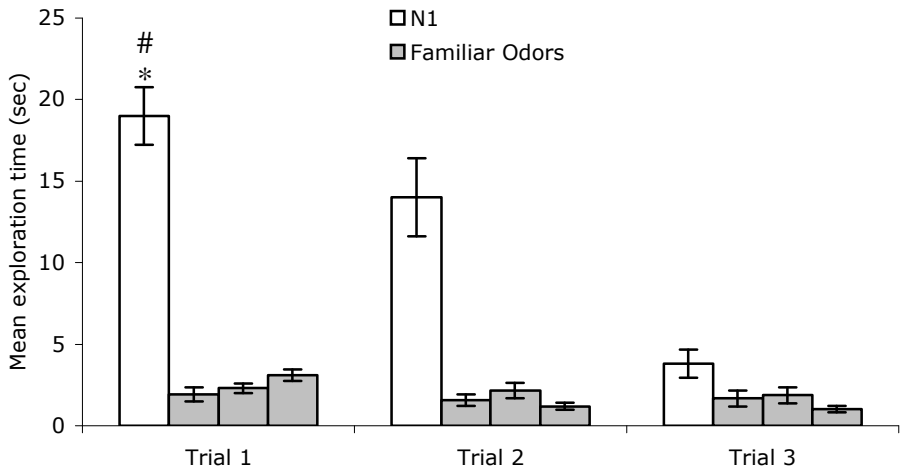


Figure 4.5 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to ETOH 3.0 g/kg 24-hours following the third habituation trial, after a 1-trial reminder of N1

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. **#** Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; ***** Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
Ethanol Reconsolidation	19.0±1.76	3.8±.87	8.303	<.0001

Table 4.5a Habituation to N1 (ETOH reconsolidation)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
Ethanol Reconsolidation	19.0±1.76	1.9±.43	9.424	<.0001

Table 4.5b Novel-odor preference in the habituation phase (ETOH reconsolidation)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

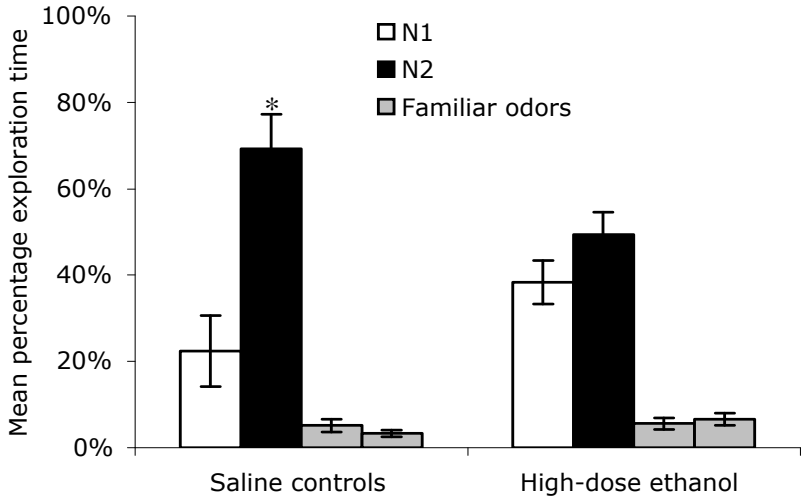


Figure 4.6 Ethanol disrupts reconsolidation of odor recognition memory

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor only in the saline control rats. Rats that received ETOH 3.0 g/kg explore both odors equally, indicating a loss of overnight memory. * Indicates significantly more time spent exploring the novel odor than the recently novel odor with $p<.05$. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Ethanol reconsolidation	9	0.38±.05	0.49±.05	0.05±.01	0.06±.01	1.524	.147	0.72
Saline controls	6	0.22±.08	0.69±.08	0.05±.01	0.03±.01	4.085	.002	2.358

Table 4.6a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (ETOH reconsolidation)

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
Ethanol reconsolidation	5.15±1.05	7.07±1.64	0.7±0.17	0.77±0.17
Saline control	4.26±1.05	15.78±3.53	0.92±0.25	0.65±0.16

Table 4.6b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (ETOH reconsolidation)

Experiment 4: Non-Reactivated Control

Habituation test data are shown in Fig. 4.7 and Tables 4.7a and 4.7b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition memory test. When habituation was followed 24 hr later (rather than immediately) by high-dose ethanol, and recognition was assessed 24 hr after that, both control and ethanol-treated rats spent significantly more time exploring the novel odor N2 than N1 indicating retained memory for N1 as seen in Fig. 4.8 and Tables 4.8a and 4.8b. This shows not only that memory for N1 was detectable 48 hr after habituation, but also that without a 1-trial reminder of N1, the memory for that odor does not get reactivated and thus is not vulnerable to ethanol.

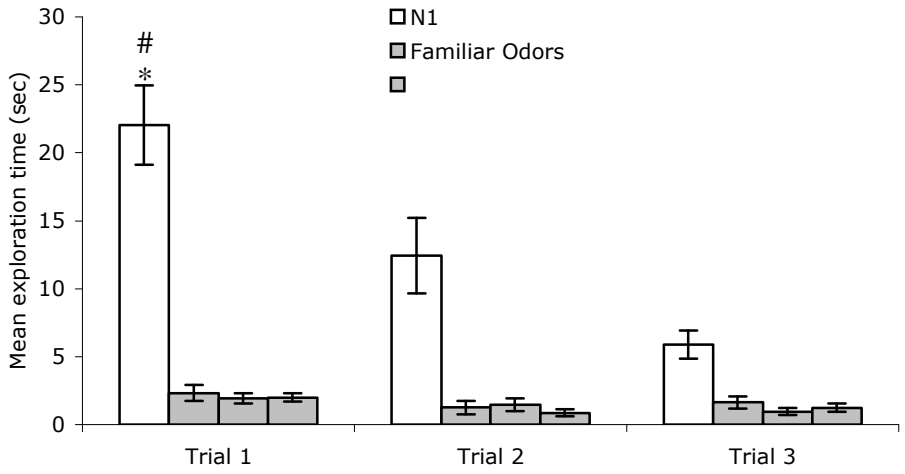


Figure 4.7 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to ETOH 3.0 g/kg 24-hours following the third habituation trial

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. **#** Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; ***** Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
ETOH 3.0 Non-Reactivated	22.0±2.9	5.9±1.0	4.907	<.0001

Table 4.7a Habituation to N1 (Non-Reactivated control)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
ETOH 3.0 Non-Reactivated	22.0±2.9	2.3±.59	6.616	<.0001

Table 4.7b Novel-odor preference in the habituation phase (Non-Reactivated control)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

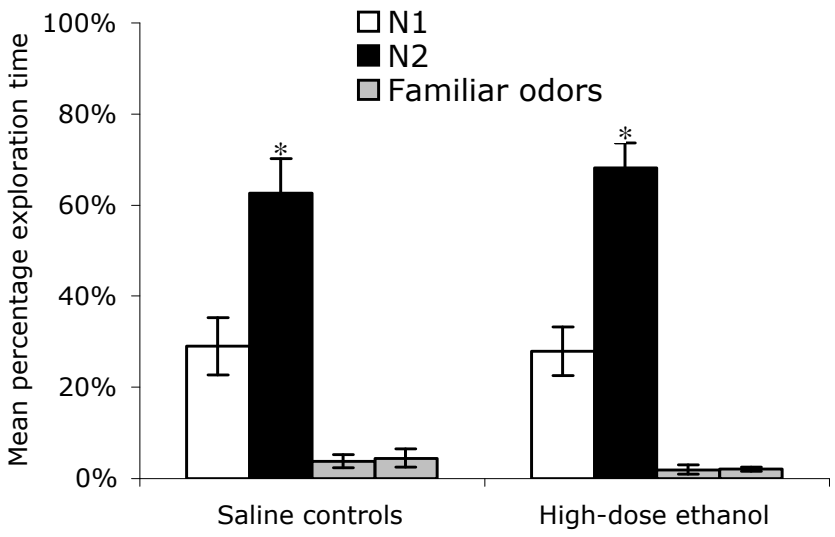


Figure 4.8. Higher-dose ethanol delivered 24-hours after habituation does not disrupt odor memory

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor only in the vehicle control rats. Rats that received ethanol injections explore N2 preferientially over N1, indicating no loss of overnight memory.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Ethanol Non-Reactivated	6	0.27±.05	0.68±.05	0.01±.01	0.02±.01	5.23	.0001	3.02
Saline controls	6	0.29±.06	0.62±.07	0.03±.01	0.04±.01	3.43	.006	1.98

Table 4.8a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (Non-Reactivated control)

Group	Odors			
	N1	N2	Familiar	Familiar
Ethanol Non-Reactivated	7.13±1.8	17.65±3.32	0.33±0.16	0.47±0.1
Saline control	6.4±1.87	13.68±3.35	0.72±0.36	0.87±0.47

Table 4.8b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (Non-Reactivated control)

Experiment 5: Ethanol Reconsolidation hangover control

Habituation test data are shown in Fig. 4.9 and Tables 4.9a and 4.9b. Rats showed a significant reduction in the amount of time spent exploring N1 between trial 1 and trial 3, suggesting marked habituation. A comparison of individual odor exploration within the first trial revealed that rats spent significantly more time exploring N1 than the familiar odor beads.

Recognition Memory Test, 48 Hours Following Reactivation. Recognition memory test data for the ethanol and control rats are shown in Fig. 4.10 and Tables 4.10a and 4.10b. Ethanol and saline treated rats showed no difference in percent time exploring N1 versus N2, suggesting an impairment of memory for N1 at the 72 hour timepoint. 72 hour control rats also showed no preference for N1 over N2, suggesting that 72 hours may be the upper-limit duration of recognition memory in the odor task.

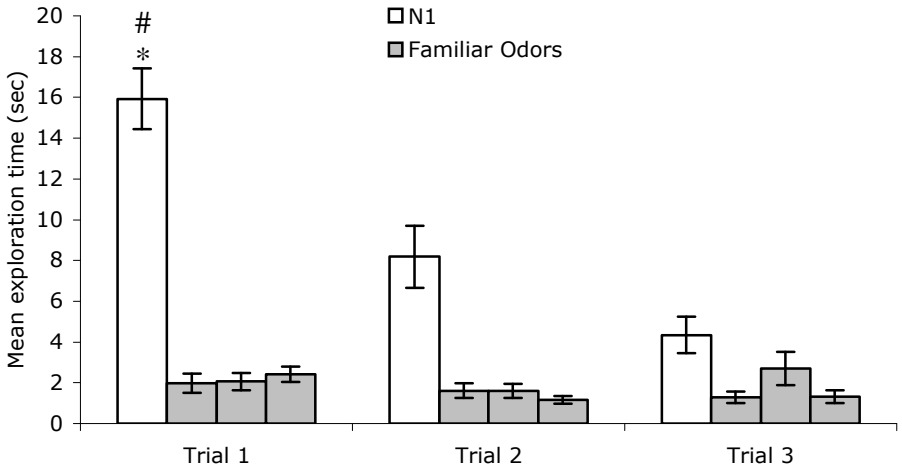


Figure 4.10 Novel odor preference and habituation to a novel odor across three trials in drug naïve rats that will be exposed to ETOH 3.0 g/kg or saline immediately following the third habituation trial, after a 1-trial reminder of N1 and tested 48-hrs later

Rats initially show intense interest in a novel odor upon its first presentation but habituate to it by the third presentation. **#** Indicates significantly more time exploring the novel odor than familiar odors within the first trial with $p<.01$; ***** Indicates a significant reduction in time spent exploring the novel odor between trial 1 and trial 3 with $p<.01$. Data are means ± SEM.

Group	Odors		t value	P value
	N1 Trial 1	N1 Trial 3		
Ethanol reconsolidation hangover control	15.9±1.49	4.34±.89	8.013	<.0001

Table 4.10a Habituation to N1 (Ethanol reconsolidation hangover control)

Data reflect mean exploration time (in seconds) of N1 on the trial indicated, ± SEM.

Group	Odors		t value	P value
	N1	Familiar		
Ethanol reconsolidation hangover control	15.9±1.49	1.98±.47	8.902	<.0001

Table 4.10b Novel-odor preference in the habituation phase (Ethanol reconsolidation hangover control)

Data reflect mean exploration time (in seconds) of the odor indicated during the first habituation trial, ± SEM.

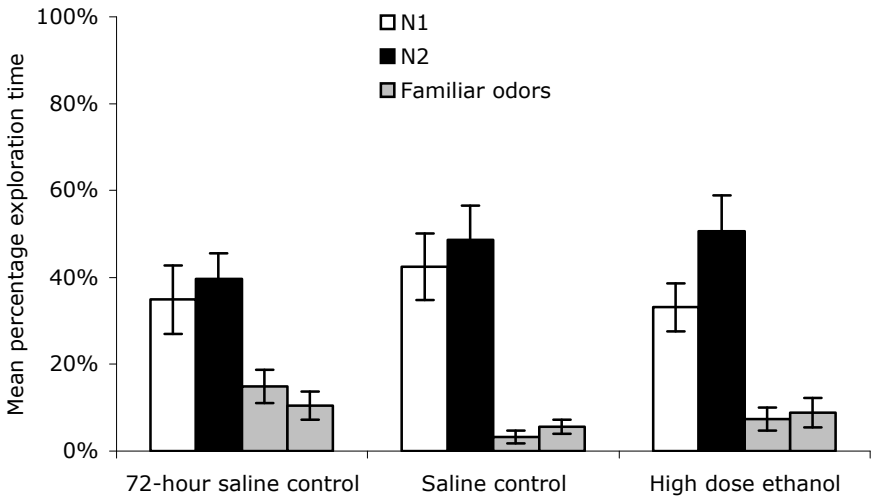


Figure 4.10 72-hours is the upper-limit for detection of odor memory in the recognition test

Recognition of the recently-novel odor on the next day is reflected in reduced exploration of the recently-novel odor compared to a brand-new novel odor only in the saline control rats. Rats that received ETOH 3.0 g/kg or saline explore both odors equally, indicating a loss of memory. Similarly, rats administered saline immediately following habituation and tested 72-hours later show no detectable memory for N1. It is possible that the delay allowed for a renewal of salience for N1. Data are means ± SEM.

Group	*n*	Odors				t value	P value	Effect Size
		N1	N2	Familiar	Familiar			
Ethanol reconsolidation hangover control	8	0.33±.05	0.50±.08	0.07±.02	0.08±.03	1.771	.101	0.885
Saline controls	7	0.42±.07	0.48±.07	0.03±.01	0.05±.01	0.574	.577	0.306
72-hr controls	5	0.34±.07	0.39±.05	0.14±.03	0.10±.03	0.485	.640	0.307

Table 4.10a Mean proportion exploration time (± SEM) in the odor recognition test, with statistics (Ethanol reconsolidation hangover control)

Shaded rows indicate groups where the comparison between N1 and N2 was not statistically significant, indicating a loss of overnight memory for N1.

Group	Odors			
	N1	N2	Familiar	Familiar
Ethanol reconsolidation	4.03±0.83	8.6±2.82	0.7±0.22	1±0.23
Saline control	6.8±2.3	7.7±2.3	0.48±0.15	0.68±0.16
72-hr controls	3.1±0.6	3.7±0.7	1.4±0.4	0.94±0.27

Table 4.10b Mean absolute exploration times (seconds, ± SEM) in the odor recognition test (Ethanol reconsolidation hangover control)

DISCUSSION

In chapter 4, it was demonstrated that odor recognition memory can be impaired by disrupting functionality of the medial amygdala through protein synthesis inhibition. However, one cannot conclude that the medial amygdala is the only structure involved in memory for our task. It has been suggested that the hippocampus and surrounding structures such as the entorhinal cortex (Brown & Aggleton 2001; Kurt, Bunsey & Riccio 2003; Fortin, Wright & Eichenbaum 2004; Mayeaux & Johnston 2004; Petrulis, Alvarez & Eichenbaum 2005) and the orbitofrontal cortex (Ramus & Eichenbaum 2000) are at least partially responsible for memory in recognition tasks. Dudai (2004) suggests that at the systems level, information may first be encoded by the hippocampus (Kapur 1999; Jarrard 2001; Murray & Bussey 2001; Sutherland et al., 2001; Wincour, McDonald & Moscovitch 2001) and subsequently passed to other systems such as the amygdala and frontal cortex. The particular task that is employed to investigate the roles of these various structures is inextricably linked to the results obtained. The version of the social odor recognition task employed by Eichenbaum (2004, 2005) requires the rat to remember an odor associated with a particular place and context, both of which might add a hippocampal component to an odor recognition task. Regardless, it is possible that all of these structures are involved in odor recognition at various points during the process of consolidation and further investigation is warranted.

The finding that that an established memory can be disrupted by delivery of a high dose of ethanol after cued reactivation of the original memory, but only if the

memory is cued, is a new finding that has not been demonstrated elsewhere. However, this result as well as our demonstration of reconsolidation deficits with anisomycin corresponds with similar results from the large body of reconsolidation literature. Essential to a demonstration of reconsolidation deficits are the effects of the proposed amnestic treatment, in this case ethanol, on a non-reactivated memory. Our non-reactivated control group demonstrates this effect. These rats are given 24-hours to consolidate the memory for N1, then they are administered a high dose of ethanol and tested 24-hours later. When the ethanol is administered, the rats do not receive a "reminder cue" (one-minute trial with N1) before ethanol intoxication, and as a result express memory for N1 in the recognition test by exploring N2 preferentially. This is in contrast to the ethanol group that were given the N1 reminder before ethanol and subsequently showed memory impairment for N1, suggesting a reconsolidation deficit.

GENERAL DISCUSSION

Binge consumption of alcohol can cause memory impairments, but surprisingly little is known about these impairments even though they are common, potentially dangerous, socially and economically costly, and linked to alcohol abuse (Sweeney 1989; Anthenelli et al., 1994; Jennison & Johnson, 1994; Buelow & Koeppel, 1995; Buelow & Harbin, 1996; Hartzler & Fromme 2003; Silvers et al., 2003; Matthews & Silvers 2004). To our knowledge there are no studies in the literature indicating that these memory impairments might include retrograde mechanisms or that they might be preventable or their occurrence limited.

In the present series of experiments, it was found that a very high dose of ethanol, given immediately after exposure to a novel odor, led to retrograde memory impairment in a memory recall test conducted 24 hr later. This memory impairment could be prevented with the administration of caffeine, a drug often contained in beverages consumed before, during or after alcohol. Caffeine somehow prevented the ethanol-induced retrograde amnesia, not only when delivered just prior to learning and exposure to ethanol, but also even when delivered one hour after ethanol. Multiple mechanisms could be responsible for the observed reversal of ethanol's amnestic effects, including an undetectable reduction in the hypnotic effects of ethanol through adenosine A_{2A} receptor blockade (Yacoubi et al., 2003).

Neither an adenosine A_{2A} antagonist nor a phosphodiesterase-5 inhibitor alone prevented retrograde amnesia when delivered one hour after ethanol (unlike caffeine, which did prevent the amnesia). However, a combination of these two drugs was highly effective at the previously ineffective doses, which were selected to on the basis of the

dose of caffeine. Thus, it appears that mimicking two of caffeine's key mechanisms of action simultaneously with a PDE5 inhibitor and an A_{2A} antagonist is at least sufficient, if not necessarily required, for prevention of retrograde amnesia by ethanol. It must be noted however, that a double dose of the adenosine A_{2A} antagonist was not administered to the rats after habituation. It is suggested by (Rebola et al., 2008) that adenosine A_{2A} receptors are critical for long term potentiation at NMDA synapses. Adenosine A_{2A} receptors are found throughout the olfactory bulb in high densities (Kaeling-Lang, Lauterburg & Burgunder 1999; Dluzen et al., 2000; Cunhan 2001; Ribiero, Sebastiao & Mendoca 2003) and (Dorhman & Diamond 1997; Schummers, Bentz & Browning 1997) suggest some of ethanol's deleterious effects may be mediated by action at adenosine receptors. In fact, (Arolfo et al., 2004) demonstrated that ethanol self-administration is attenuated by blockade of A_{2A} receptor sites with the antagonist DMPX. As our task uses odor as the primary stimulus, it might be possible that a 2X dose of ZM241385 could prevent ethanol induced retrograde memory impairments through negating ethanol's action at the adenosine receptor sites found in the olfactory bulb.

While our experimental design requires the administration of ethanol immediately after habituation to address memory impairments, this is not an accurate model of how a binge drinking episode takes place in humans. On the contrary, a binge episode will include a large volume of alcohol consumed over a period of hours, not delivered all at once as in our ethanol groups. A more accurate parallel to human consumption would be to deliver a lower-dose (1.0 g/kg) every 20 minutes for one hour, to achieve the same dose of 3.0 over a slower period of time. It remains possible that caffeine and related agents would not have negated memory impairments had the ethanol been administered

during learning. However, to rule out nonspecific factors such as attention and sensorimotor function impairments that might influence how well, or even whether, a memory is laid down, the present study was designed to ensure that memory for N1 was established in a completely sober state. Binge-alcohol induced blackouts in people may be primarily anterograde; however, the present study raises the possibility that at least some memory impairment could be retrograde, reflecting a degradation of memories laid down just prior to exposure to very high levels of alcohol. It is also possible that emotionally charged, extremely salient memories would be more resistant to disruption by ethanol.

Although we have gathered data that make it quite reasonable to refer to this deficit as "retrograde amnesia", we cannot assume that the memory was totally "lost". It is unknown whether the ethanol-induced retrograde memory impairment was a consolidation deficit, a retrieval (memory accessibility) deficit, or both. The greatest controversy surrounding research into retrograde amnesia is whether retrograde memory impairments observed in animal models are due to a retrieval failure or a failure to consolidate (Miller & Matzel 2006). A deficit in consolidation represents a disruption of the *storage* of information evidenced by impaired performance on tests of memory while a retrieval deficit represents successful storage of information with an *inability to access* that information at the time of testing (for review see Dudai & Morris 2000; Miller & Matzel 2006; Miller & Sweatt 2006; Squire 2006; Sara & Hars 2006; Nader & Wang 2006; Nader 2006; Riccio 2006). Those who take the retrieval view, point to the ability of a "reminder" cue, given at some point after learning and amnestic treatment, to allow for memory to be expressed (Springer & Miller 1972; DeVietti & Bucy 1975). However,

when this memory is retrieved, it is not restored to full capacity, on the contrary, it is returned in a diminished state, which serves as an argument for a deficit in storage (Squire 2006). Regardless, these data suggest that pharmacological manipulations might begin to shed new light on potential mechanisms of suppression and reversal of memory access.

It is unlikely that the effect of the higher dose of ethanol was related to reinforcement associated with its pairing with N1 because the lower dose of ethanol (which is still a substantial dose) did not enhance the salience of N1, in that 24 hr later N1 was explored significantly less than N2 (indicating intact memory and no increase in interest in N1 relative to controls. Furthermore, during the odor recognition task, the presentation of N1 serves as a reminder cue in the presence of N2. Regardless, rats treated with PTZ and a high-dose of ethanol show no preference for N2, suggesting that the presence of a "reminder" cue is not enough to restore the memory for N1. Finally, the 48-hour hangover control provides evidence that 48-hours post ethanol, the memory for N1 is not present. It cannot be argued that at this time-point ethanol has not been fully metabolized. If these rats were to express memory for N1 at 48 hours and not 24 hours, then a strong argument could be made for ethanol affecting the ability of the rats to retrieve memory for N1, as it does not, it is likely that this 48-hour hangover control provides evidence for a deficit in storage of memory for N1 and not retrieval.

It is also unlikely that the effect of the high dose of ethanol was due to lingering ("hangover") effects that might influence performance, for several reasons. First, the total mean exploration times (combining N1 and N2) of the control and 3.0 g/kg ethanol treated Long-Evans rats were not very different between groups: control (11.94 sec) and

3.0 g/kg ethanol (14.67 sec). Rats exposed to the higher dose of ethanol 24 hr earlier did not reduce exploration. Secondly, the 3.0g/kg dose of ethanol would be metabolized by the time the recognition test was performed 24-hours after habituation. Third, a separate group of rats administered 3.0 g/kg ethanol 24 hr after habituation, well past the time when memory consolidation should have been established and therefore should be resistant to disruption, showed apparently normal memory for the recently-novel odor the following day, when the "hangover" effects would be assumed to be taking place. That is, on the day following the higher dose of ethanol, these rats had no problem distinguishing between N1 and N2, showing the greatest preference for N2 (the most novel of the two odors), while maintaining a preference for N1 relative to familiar home cage odors. Also, rats treated with caffeine or the combination of the PDE5 inhibitor and the A2A antagonist one hour after exposure to the higher dose of ethanol behaved similarly to control animals, including no impairment in memory retrieval. Because these rats showed normal memory and no ill-effects of the ethanol 23 hr later, together these data suggest that the retrograde memory impairment observed with the higher dose of ethanol in the absence of caffeine or the combination of the PDE5 inhibitor and A2A antagonist was not likely due to veisalgia ("hangover" effects on some aspect of performance). However, it must be noted that all of the ethanol was delivered acutely via i.p. injection. This delivery method, as well as repeated intermittent doses of ethanol have been demonstrated to cause stress in rats (Zhang et al., 2007). Based on our experimental design this outcome is unavoidable as voluntary-self administration of ethanol does not provide the control necessary to directly assess the effects of an acute high dose of

ethanol retrogradely on memory. However, the acute injection of the low dose of ethanol perhaps would have produced a comparable level of stress.

Ethanol also has many different mechanisms of action. For example, ethanol at high doses is known to interfere with glutamatergic action at NMDA, AMPA and kainate receptors while it also enhances GABAergic synaptic transmission with a surprising degree of specificity in memory related areas of the brain such as the hippocampus (White et al., 2000). It has also been suggested that both acute and chronic ethanol exposure increases extracellular levels of adenosine (Dorhman & Diamond 1997). Furthermore, it has been argued that for new learning to undergo consolidation (i.e., the transfer from a labile to stable state), protein synthesis may be critically involved (Flexner & Stellar, 1965; Schafe & LeDoux 2000; Kandel 2001) and there is growing evidence that activation of NMDA receptors is a crucial step in this process (Miserendino et al., 1990; Rodrigues et al., 2001; Riedel et al., 2003). It has been demonstrated that acute ethanol exposure can inhibit critical steps in at least some protein synthesis pathways, possibly through its antagonism of NMDA receptors (Chandler & Sutton 2005).

Ethanol's effects are widespread throughout the brain, and it will be difficult to learn which specific brain structures and mechanisms of action are necessary or sufficient to cause retrograde amnesia. However, Matthews & Silvers (2004) reviewed their own work and that of others and argue that ethanol's effects on memory may have remarkably specific action in the hippocampus, particularly by enhancing GABAergic potency at $GABA_A$ receptors and by interfering with glutamate at NMDA receptors, to affect spatial memory. Memory-impairing effects of ethanol may influence GABAergic activity by

increasing levels of allopregnanolone in the hippocampus. Indeed, finasteride (which reduces ethanol-induced allopregnanolone levels by almost 50%), when combined with ethanol, blocked ethanol-induced inhibition of hippocampal pyramidal neurons and spatial memory deficits. These investigators go on to suggest that ethanol's potentiation of $GABA_A$ receptor activity in the hippocampus may reduce hippocampal levels of acetycholine. Anticholinergic drugs, like injury to the hippocampus directly or indirectly via traumatic brain injury or stroke, are known to interfere with hippocampal dependent memory, particularly when the learning procedure requires memory for strategy switching for optimal spatial performance (e.g., Lindner & Schallert, 1988; Day & Schallert, 1996; Choi et al., 2006).

The recent use of topiramate to prevent alcohol relapse in abstinent individuals (in addition to its use to treat acute withdrawal effects) might be of significance because this drug, like ethanol, is a kainate glutamate antagonist and to a lesser extent an AMPA antagonist, and it may reduce glutamate release via inhibition of glutamine synthetase activity and blocking of sodium channels (see Krupitsky et al., 2007 for review). Topiramate might interfere with memory when taken during learning (Martins de Lima et al., 2007) and could conceivably cause retrograde memory impairment effects when administered just after new learning (during consolidation). This would have considerable implications for its use in people, and the memory evaluating techniques presented in this paper may be useful in assessing this possibility.

Memantine, an NMDA antagonist used to slow the progression of Alzheimer's disease, does not appear to interfere with acquisition of new spatial or other learning, but at doses that are high enough to reduce neural degeneration it may disrupt overnight

memory (Creeley et al., 2006). Whether it can induce retrograde memory impairment, either alone or in combination with very low doses of ethanol, is a question that has never been addressed. The NMDA antagonist MK-801 has been shown to cause retrograde amnesia (e.g., Packard & Teather, 1997; but see Nilsson et al., 2007), whereas the non-NMDA glutamate receptor antagonist NBQX does not appear to affect recognition memory (Pitsikas et al., 2002).

Attempting to shed light on the mechanisms underlying consolidation of memory and the disruption of memory through ECS, Kandel (2001) began work that would eventually lead to the modern theory of LTP and the necessary mechanism for LTP; protein synthesis. In order to understand mechanisms for consolidation, Kandel utilized a simple organism (Aplysia Californica) and classical conditioning. The nervous system of the Aplysia contains a defined number of neurons that are in fact labeled and numbered universally. As a result, it was possible to condition the animal and observe the animal's learning of the conditioned behavior, while at the same time, record activity from a distinct number of neurons involved in that learning. Aplysia have a moderate gill withdrawal reflex that occurs when the mantle shelf or the siphon of the gill is touched. In a classical conditioning paradigm, the touching of either mantle or siphon (CS) is paired with a tail shock (US), eliciting an enhanced gill withdrawal reflex. Once this conditioning has taken place, the CS alone will produce the enhanced gill withdrawal. At the cellular level, if the number of pairings is increased, the sensitization becomes long-term. This occurs as a result of changes to the structure of the neuron itself, and is referred to as LTP (long-term potentiation of a pathway, resulting in a subsequent potentiation of the learned response. The processes of consolidation and re-consolidation

themselves are forms of long-lasting LTP, as they serve to encode experience from short-term to longer-lasting forms (Schafe, Nader & LeDoux 2001; Dudai 2002)

Amongst reconsolidation theorists, the general consensus is that the necessary step in eliciting LTP through consolidation/re-consolidation is protein synthesis (Judge 1982; Litvin 2000; Nader 2000; Taubenfeld 2001; Debiec, Ledoux & Nader 2002; Milekic & Alberini 2002) although there is much debate about this requirement (for the most recent reviews, see: Gold 2008; Radulovic & Tronson 2008; Rudy 2008; Routtenberg; Alberini 2008; Klann & Sweatt 2008; Abraham & Williams 2008; Miyahita et al., 2008; Won & Silva 2008; Hernandez & Abel 2008; Lu, Christian & Lu 2008; Helmstetter, Parsons & Gafford 2008; Rodriguez-Ortiz 2008). Protein synthesis is a process that involves the stimulation of a G-protein coupled receptor and the initiation of intracellular mechanisms that culminate in the production of new proteins, growth of new synapses and long-term changes in the structure of the synapse. This takes place in three phases: 1) in the short term, such as with 1 trial learning, these intracellular mechanisms lead to an increase in neurotransmitter firing, due to increases in intracellular levels of CA2+, 2) in the intermediate, across a few more trials, the intracellular protein cascade begins a loop that eventually feeds back on itself, perpetually elevating levels of CA2+ and causing elevated neurotransmitter release, 3) in the long-term, many trials over a number of days, the changes in the structure and function of the neuron itself are initiated. This long-term step in protein synthesis is what is referred to as long-term potentiation. As consolidation is a process involving long-term LTP, it too should be disrupted if protein synthesis (Grollman 1967; Grollman 1967a) is disrupted (Flexner, Flexner & Stellar 1965; Dunn 1971).

There are many investigators whose research has driven them to the conclusion that reconsolidation is the process by which a memory is updated. Reconsolidation disruption is classically demonstrated in fear conditioning paradigms, in which a rodent learns to pair a cue such as a tone, with an aversive stimulus, such as a footshock. When this cue is presented enough with the footshock, the animals freeze to the tone alone as it is a signal for upcoming footshock (Fendt & Faneslow 1999). The theory of reconsolidation posits that when an animal learns a new rule for a previously learned rule-set, a single memory trace is updated with new information, thus suppressing or perhaps even "erasing" the old information contained in that memory trace (Judge and Quartermanin 1982; Schafe et al., 2001; Duvarci & Nader 2004; Nader, Hardt, Wong 2005). Behaviorally, this is evidenced by less freezing to the tone in follow-up tests of memory, only after that tone has been played to reactivate the memory trace and followed by protein synthesis inhibition. However, there are also those who believe that this explanation of memory disruption is far too simple for such a dynamic process as memory (Miller & Matzel 2000). In opposition to reconsolidation theory is the theory of extinction, also demonstrated in fear conditioning paradigms (Milad & Quirk 2002; Fisher et al., 2004; Ouyang & Thomas 2005). Extinction theorists posit that when an animal learns a new behavior, this learning is consolidated and a new memory trace is formed. If this new behavior is reinforced enough, then the old memory trace will become suppressed, but will still actively exist in the brain. Evidence for this theory comes from that fact that often when rats are presented with a learned cue, such as in a fear conditioning paradigm, long after the extinction of the memory association with an aversive component, they will nonetheless spontaneously

recover that old information and behave fearfully by freezing (Power, Berlau & McGaugh 2006).

There is a wide degree of variability within the literature in regards to utilizing the most advantageous behavioral assays and drug administration doses to assess reconsolidation disruption or extinction (Myers & Davis 2003) although Inda, Delgado-Garcia & Carrion (2005) demonstrate that protein synthesis may underlie both reconsolidation and extinction. It is suggested that research into consolidation and reconsolidation, which has been growing exponentially over the years, be tempered by the lessons learned from the long history of memory research: "the need to carefully attend to the learning/performance distinction, to rely equally on synthetic as well as reductionistic, and to avoid the seduction of simplicity" by (Cahill & McGaugh 2001). Regardless of these differences, there is an underlying concept that unifies the studies in consolidation and reconsolidation of memory: the concept that memory is a dynamic process, that requires any organism to constantly reorganize its behavior as a result of ongoing experiences in their environment, as in Przybyslawski & Sara (1997). In other words, if memory were a stable and unchanging entity, then how would it be possible to update behavior in a way that was advantageous to a given situation? For an animal living in a changing environment, this is especially important in terms of emotionally charged experiences that require the utilization of many different pieces of learned information; food, shelter, predators, copulation etc. On the other hand, some memories are undesirable and not advantageous to human beings: traumatic experiences that lead to depression, obsession (Rubin, Fried & Franks 1968), PTSD (as in Przybyslawski & Sara 1999) and drug seeking behavior that is brought on by contextual cues and conditioned

stimuli, as in Sara (2000). Perhaps a lesson can be taken from Gold (2006) who proposes the use of the term "memory modulation and re-modulation" as opposed to consolidation and reconsolidation as most researchers would agree that memories are constantly being modulated and updated in some form via multiple mechanisms.

In summary, we have developed a test for odor-recognition memory that can be carried out in the home cage, that rats can readily perform, and that results in an easily observable and lasting form of memory that avoids some of the problems associated with standard memory assessment models. Conspecific social odor memory was confirmed to be established in a sober state but was disrupted by subsequent heavy ethanol intoxication. That is, ethanol appeared to cause impairment either of the capacity to adequately store a modestly-salient memory or the ability to retrieve that memory, whereas caffeine and related agents appeared to prevent the memory impairment. It is hoped that these data will be useful for understanding how binge ethanol consumption can cause memory impairments in humans and how caffeine or other agents might ameliorate these impairments. Furthermore, it is expected that the original finding that ethanol can disrupt both consolidation and reconsolidation will have substantial ramifications for people who abuse alcohol and for those whose trauma causes suffering from unwanted memories.

Appendices

Appendix 1: Abbreviations Index

A_{2A}	Adenosine A_{2A} subtype
cGMP	cyclic guanosine monophosphate
ECS	electroconvulsive shock
ETOH	ethanol
F	Familiar odor
GABA	gamma-amino butyric acid
N1	Novel odor 1
N2	Novel odor 2
NMDA	N-methyl-D-aspartic acid
PDE5	Phosphodiesterase type 5
PTZ	pentylenetetrazol

Appendix 2: Methodological Figures

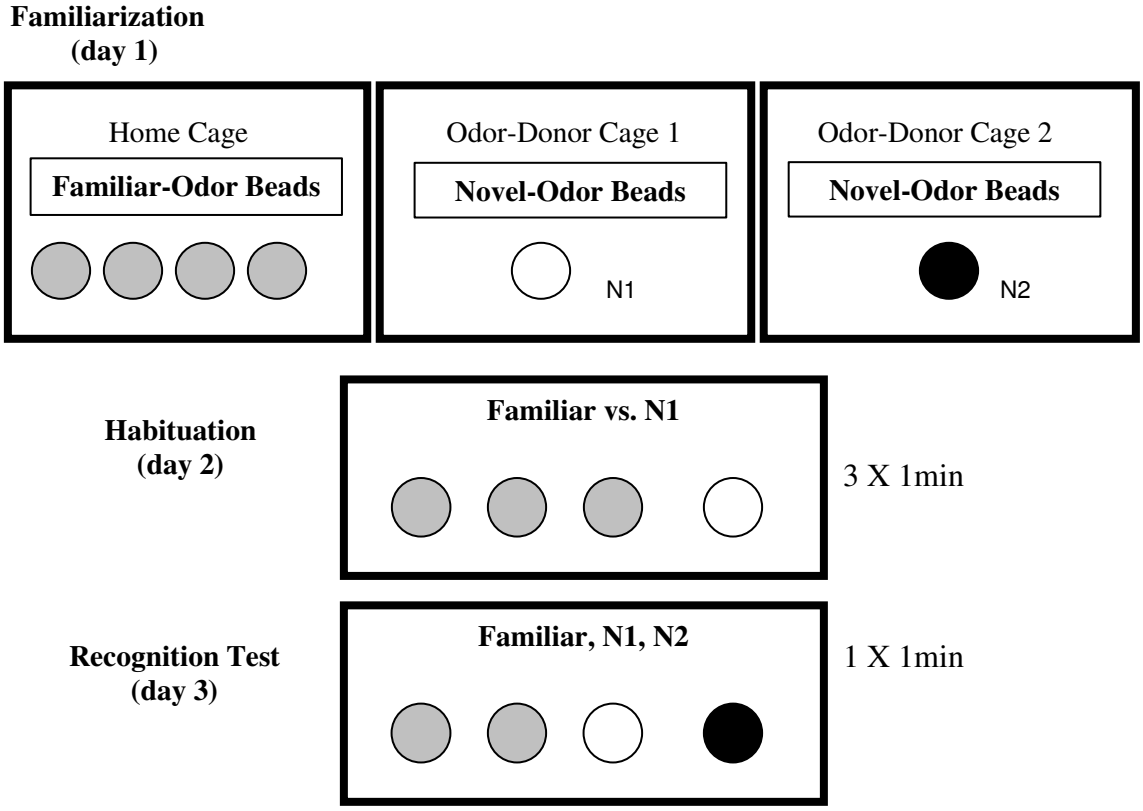


Figure A2.1 Graphical representation of the experimental procedure for the odor recognition task

For the habituation phase, three 1-minute trials with 1-minute inter-trial intervals were used for each animal. For the recognition test phase, one 1-minute trial with a 1-minute inter-trial interval was used.

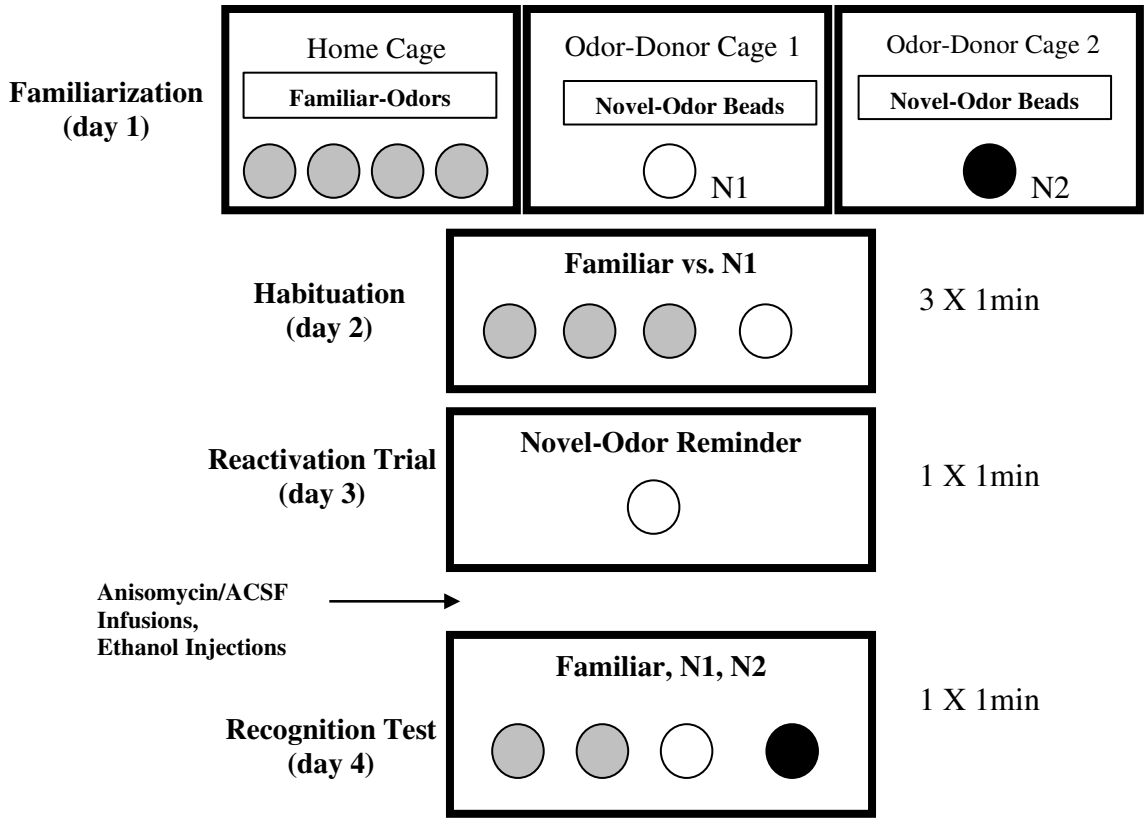


Figure A2.2 Graphical representation of the experimental procedure for the reconsolidation experiments

For the habituation phase, three 1-minute trials with 1-minute inter-trial intervals were used for each animal. The reactivation trial and recognition test phases each consisted of one 1-minute trial with a 1-minute inter-trial interval.

Figure A2.3 PTZ Procedure

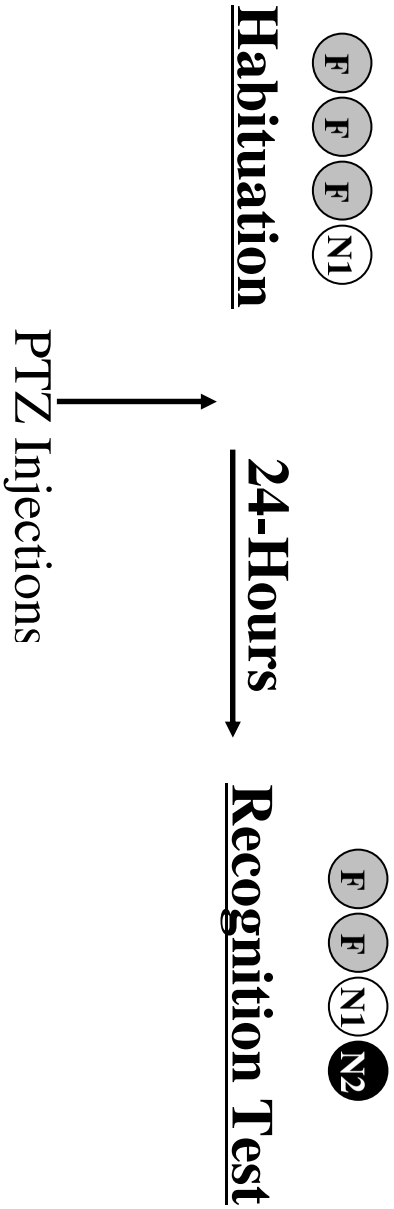


Figure A2.4 Low-dose ethanol procedure

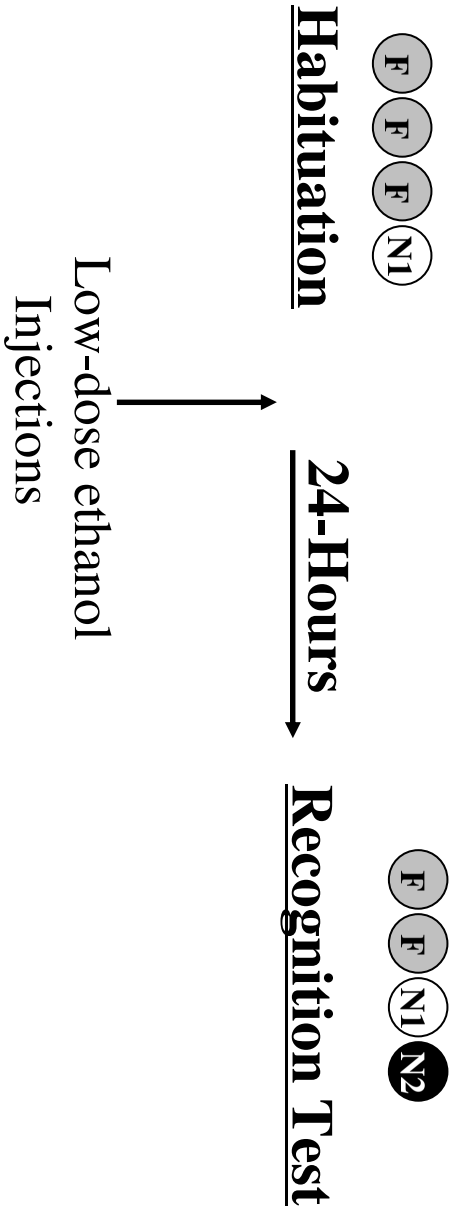


Figure A2.5 High-dose ethanol procedure

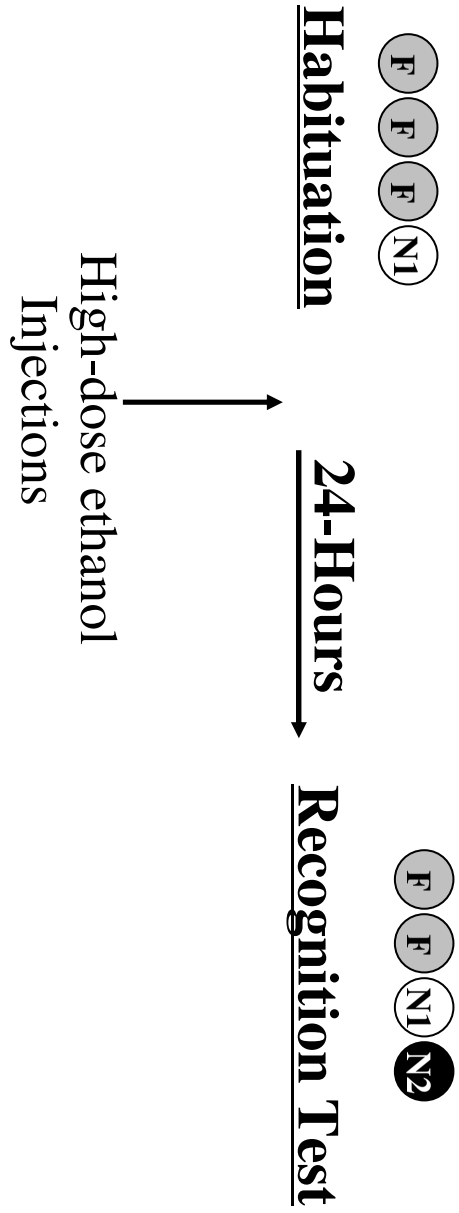


Figure A2.6 Hangover control procedure

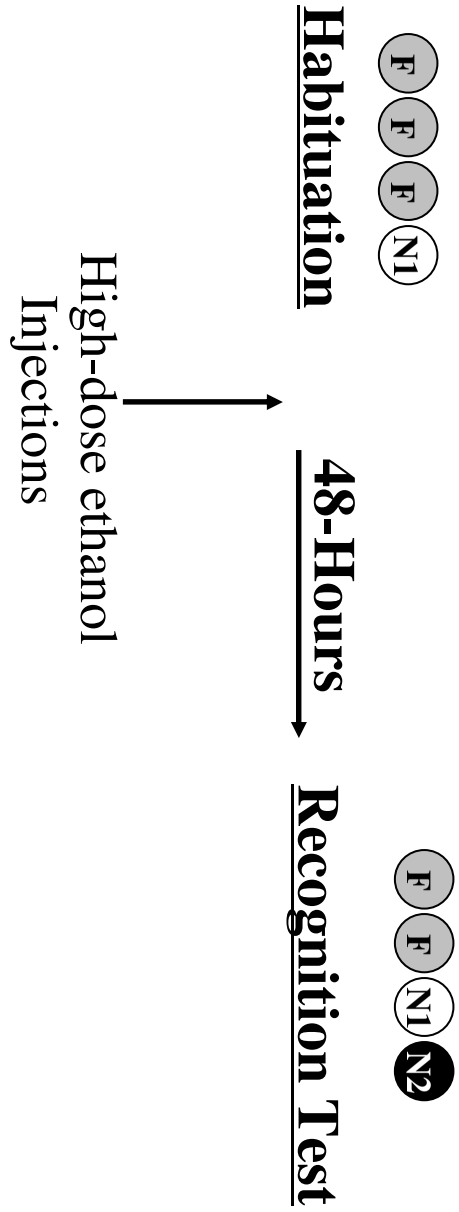


Figure A2.7 Caffeine pre- ethanol procedure

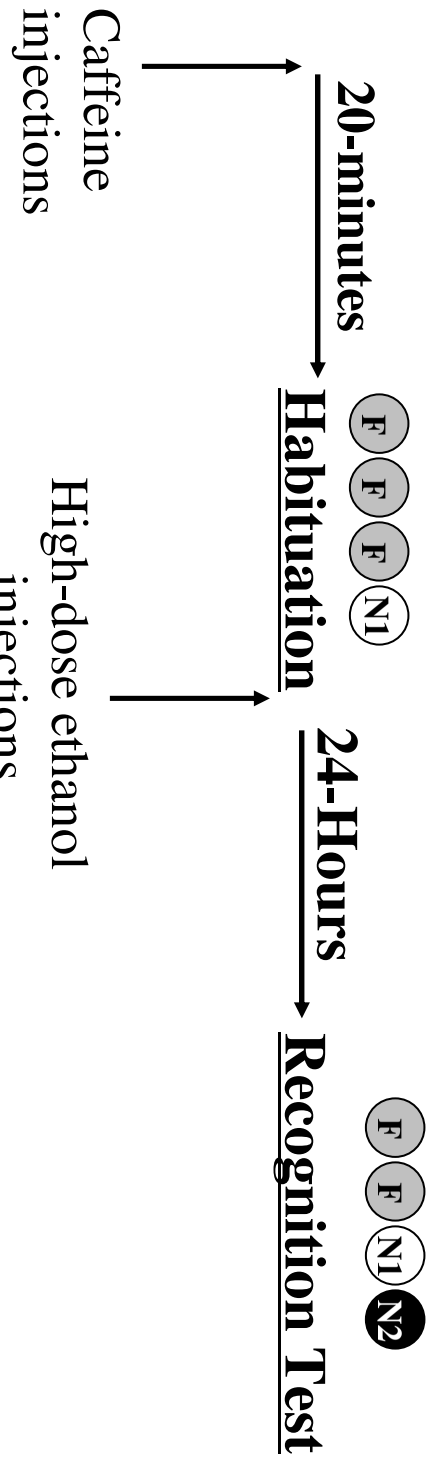


Figure A2.8 Caffeine post-ethanol procedure

Figure A2.9 PDE5 inhibitor procedure

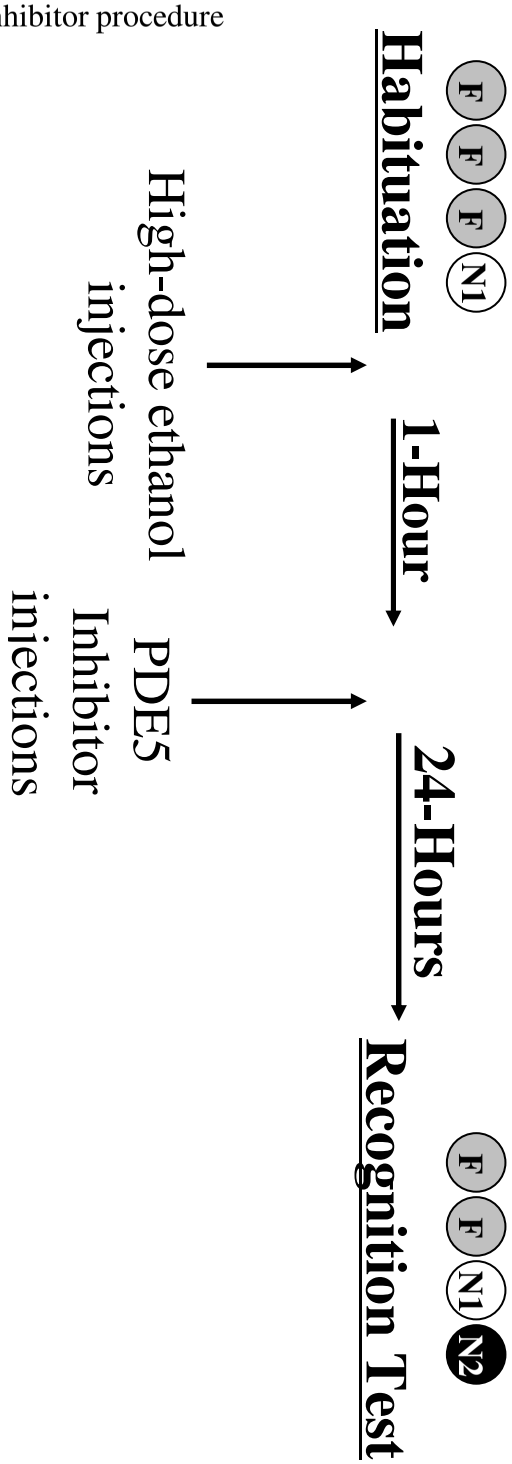


Figure A2.10 A_{2A} antagonist procedure

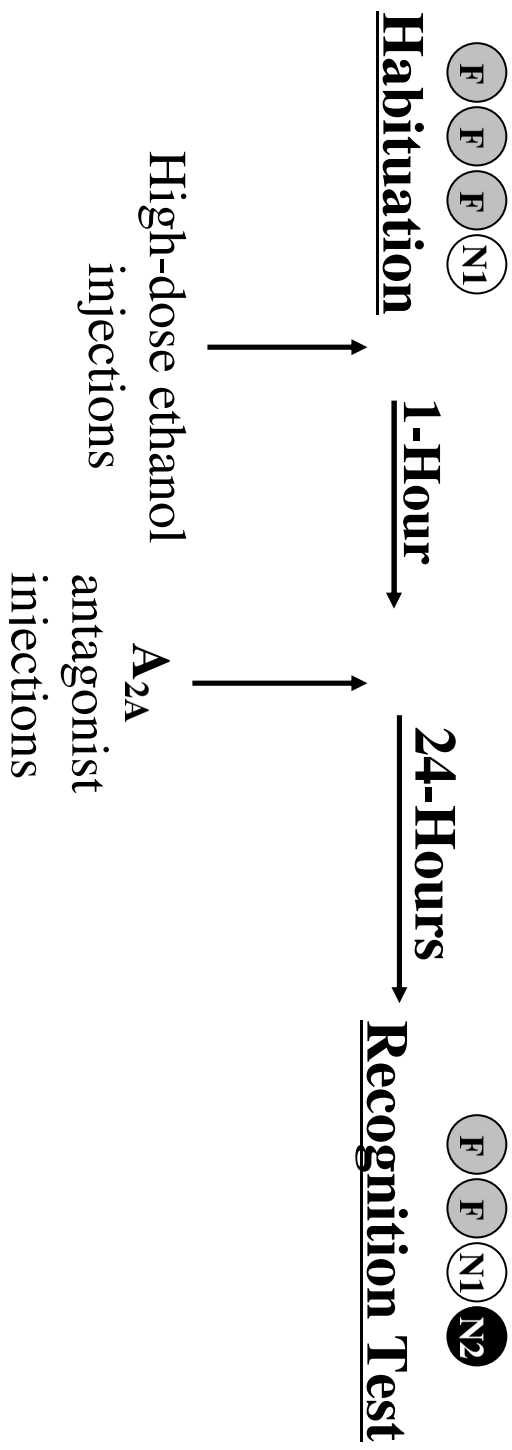


Figure A2.11 Combination PDE5/ A_{2A} procedure

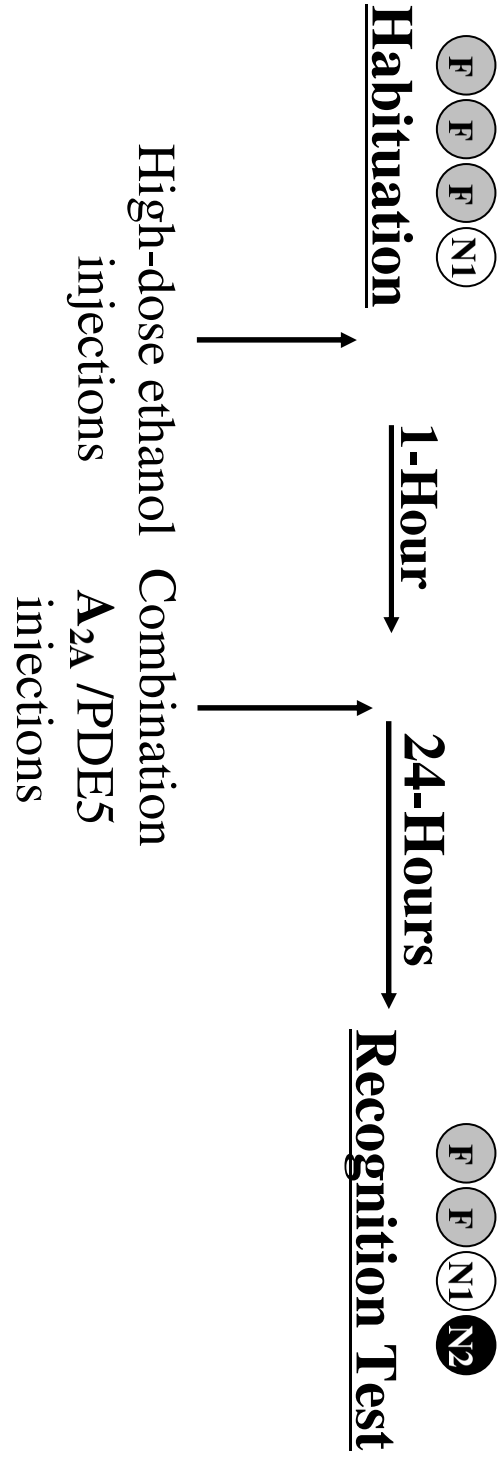


Figure A2.12 Anisomycin consolidation procedure

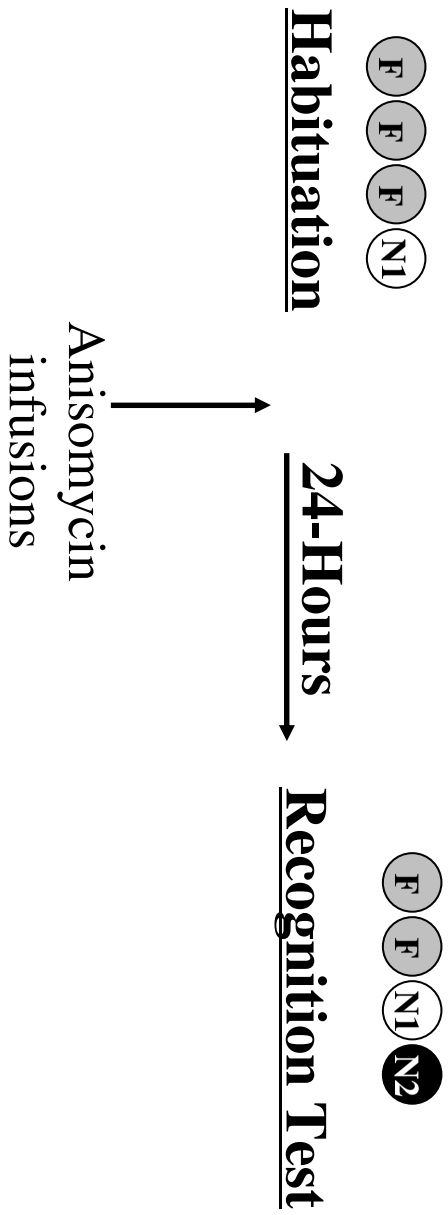


Figure A2.13 Anisomycin reconsolidation procedure

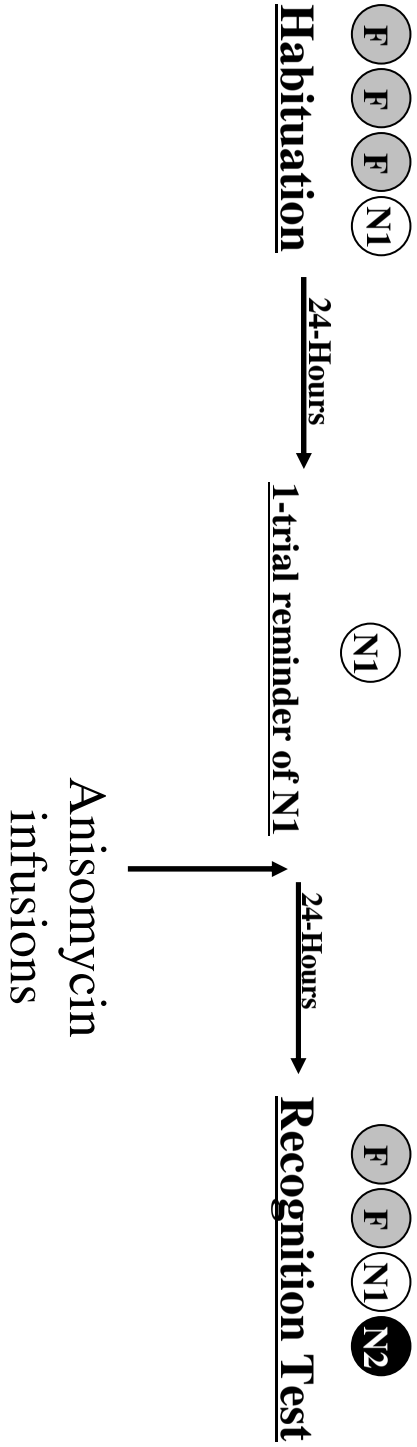


Figure A2.14 Ethanol reconsolidation procedure

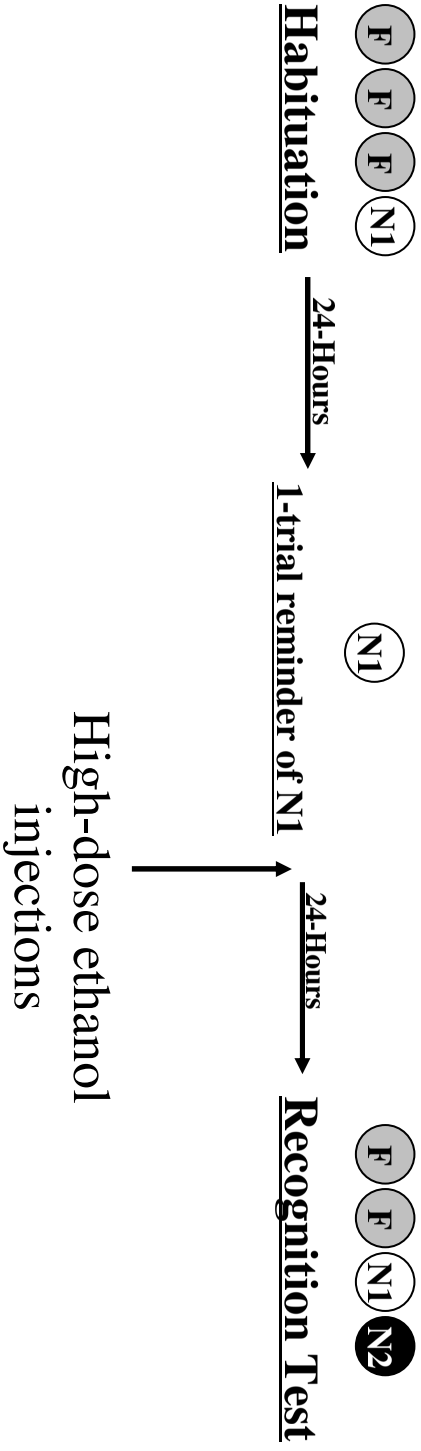


Figure A2.15 Non-Reactivated control procedure

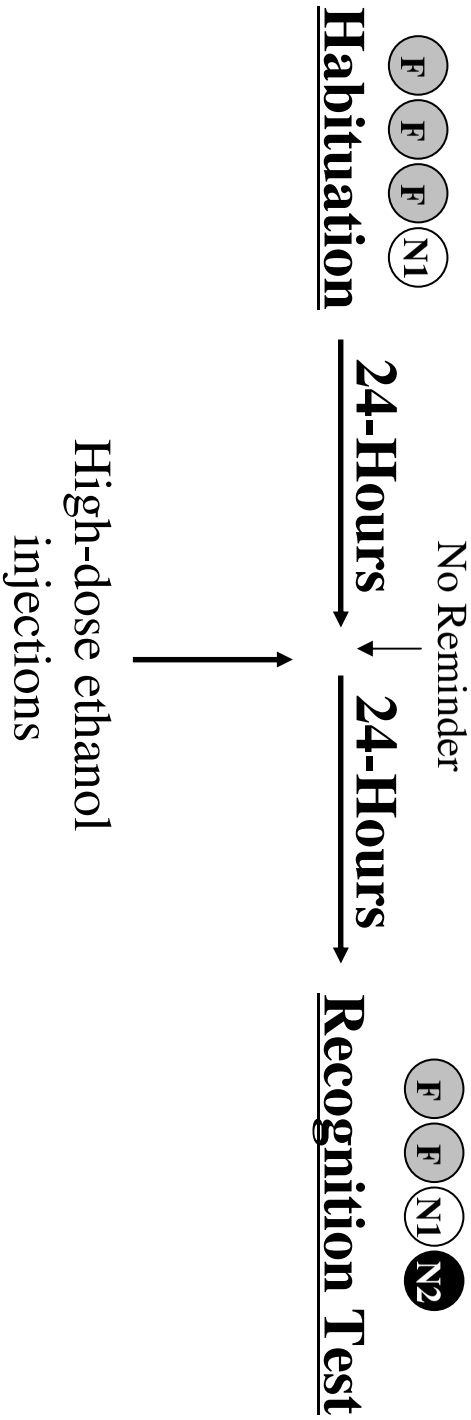


Figure A2.16 Ethanol reconsolidation hangover procedure

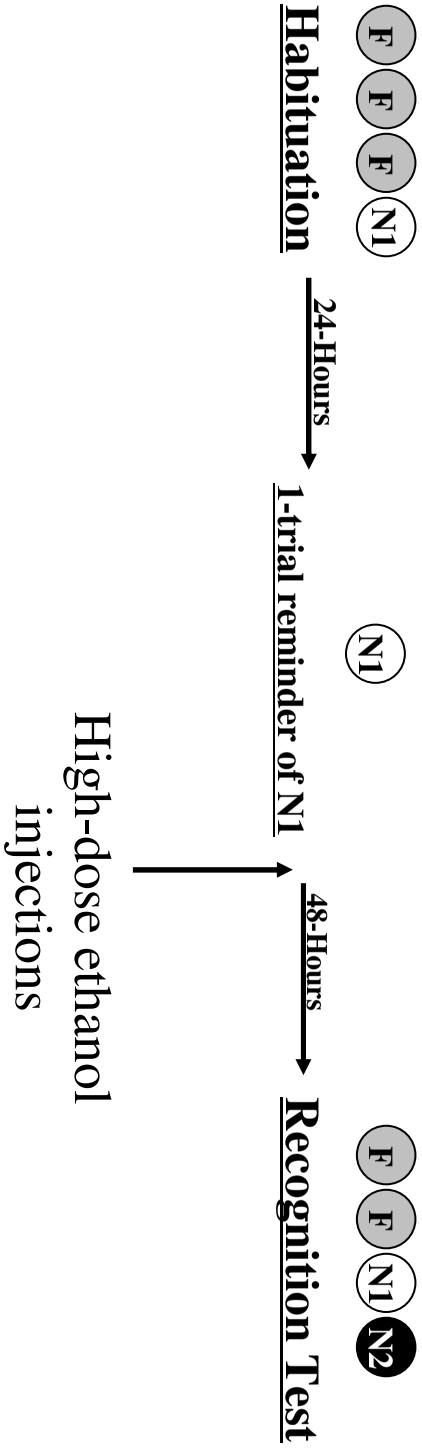
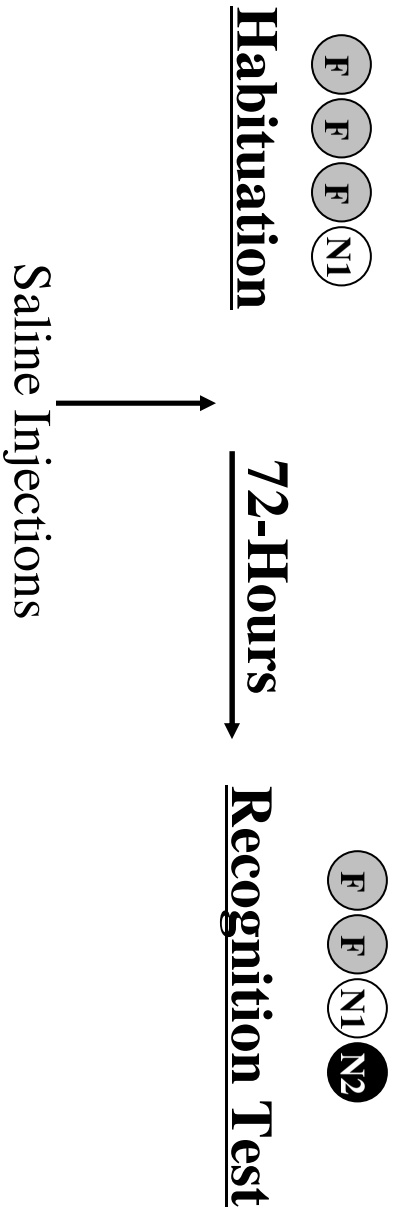


Figure A2.17 72-hour control procedure



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Vitae

Michael John Spinetta was born in the city of San Diego California. The world seemed to celebrate his arrival with fireworks, although he was informed later that this is accurately referred to as the "Bicentennial". His parents stuck with him through the often-tumultuous first 18 years of his life and were rewarded with the man that he is today. He earned both a B.A. in Philosophy and a B.S. in Psychology from Santa Clara University in 1999. He then left California to start his adventure in Texas at The University of Texas at Austin in 2001 and has enjoyed all of the wonderful friends he has made in the time since then. He also met his Love Melissa in Austin and is eternally thankful for that.

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