Effects of Acute Ethanol on Memory Encoding, Retrieval, and the Theta Rhythm

THESIS

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By

Kristin S. Edwards

Graduate Program in Psychology

The Ohio State University

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Master's Examination Committee:

Dr. Ben Givens, Advisor

Dr. John Bruno

Dr. Derick Lindquist
Abstract

In the set of experiments within this document, the effects of acute ethanol intoxication on memory encoding, retrieval, and the theta rhythm were explored. We adapted a common working memory operant task (DNMTP) into a serial reversal paradigm that assesses both short-term and long-term processes of encoding and retrieval of memory. Using this task, we found that animals are able to reliably learn match and non-match to position rules, and are readily able to switch between the two rule conditions. The single switch paradigm produced a dose-dependent impairment in memory encoding for task rules demonstrated by decreased overall accuracy for the post-switch session. The double switch paradigm showed that ethanol produced a slight improvement in the second switch performance relative to controls, suggesting less interference due to a failure to encode the new contingency on the intervening first switch day. These results support the hypothesis that ethanol disrupts memory encoding for task rules on switch days, and that these effects are not due to a general cognitive impairment. By recording neurophysiological activity while the animals engaged in a rule-switch session, we were able to gain insight into the effects of alcohol on the rapid encoding and retrieval processes associated with the sample and choice phases of the task respectively. In awake, behaving rats we observed local field potentials that showed high activity in the
theta band frequency (4-10 Hz). The overall power of the theta rhythm was reduced in a
dose-dependent manner. In the encoding phase of the task when waveform averages
were gated to the light onset, we found clear and consistent synchrony across anatomical
recording sites including the entorhinal cortex, the dentate gyrus of the hippocampal
formation, and the anterior cingulate region of the prefrontal cortex. In the present
studies, we did not find conclusive evidence in support of a theta reset phenomenon or
stronger synchronization between the entorhinal cortex and the dentate gyrus during the
rapid encoding of relevant sensory information; however we believe that further research
is needed to clarify these findings. We found significant differences in the pre-stimulus
vs. post-stimulus waveform, potentially indicating an encoding-stimulus evoked
enhancement of the overall theta rhythm. In the retrieval phase of the task when
waveform averages were gated to the onset of the tone, we found preliminary evidence in
support of the hypothesis that the synchronization between the dentate gyrus and the
anterior cingulate cortex increases preferentially during retrieval events. The effect of
ethanol on memory encoding, retrieval and the theta rhythm remains important and
interesting directions for additional research.
This thesis is dedicated to my parents: Don and Carol Edwards, for their infinite support and for always reminding me that something positive will happen every day.
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Vita

2007……………………………………..B.S., Psychology,
   Bowling Green State University

2007……………………………………..Distinguished University Fellow,
   The Ohio State University

2008-Present…………………………..Graduate Teaching Associate,
   The Ohio State University

2010……………………………………..Graduate Associate Teaching Award Winner,
   The Ohio State University

2010……………………………………..Louise B. Vetter Teaching Award Winner,
   The Ohio State University

Publications


**Fields of Study**

Major Field: Psychology
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Chapter 1: Introduction

This thesis will describe experiments designed to investigate the effects of ethanol on encoding and retrieval of memory by exploring the \textit{in vivo} neurophysiology related to the theta rhythm.

1.1. Harm associated with alcohol consumption

Alcohol is undoubtedly one of the most widely used and abused drugs, but it is also a drug with profound potential for numerous detrimental consequences. The Independent Scientific Committee on Drugs, an expert panel of researchers from a variety of disciplines, used a multi-criteria decision analysis model to assess the relative harm of many psychoactive compounds typically used for recreational purposes. Based on a combination of factors including physical, psychological, and social harm to both the individual and to others, this panel scored drugs on a 100-point weighted scale where zero was no harm and 100 was the most harmful. Somewhat surprisingly, alcohol was scored, by far, as the “most harmful drug” with a combined score of 72, followed by heroin with a combined score of 55, crack cocaine with a combined score of 54, and methamphetamine with a combined score of 33 (Nutt et al., 2010). The potential harm of alcohol consumption is indisputable and thus continued research on the specific
consequences, particularly the physiological and psychological effects and their underlying neural mechanisms, is an essential strategy for improving public health.

1.2. Synaptic mechanisms of ethanol
The field of ethanol research has progressed significantly in their understanding of synaptic mechanisms of ethanol. For example, it is well understood that ethanol increases the activity of \( \gamma \)-amino butyric acid (GABA), the chief inhibitory neurotransmitter in the brain, and decreases the activity of Glutamate, a main excitatory neurotransmitter. Effects on GABA and glutamate transmission occur both through actions on postsynaptic receptors (Weight, 1992; Criswell and Breese, 2005) and presynaptic release mechanisms (Siggins et al., 2005). Ethanol acts on many different brain regions, but the hippocampus is particularly vulnerable. Ethanol disrupts hippocampal activity by direct interactions with ligand gated ion channels at synapses in the hippocampus, affecting GABA\(_A\) receptor subtypes particularly on interneurons, and by altering cellular activity in afferent brain regions such as the medial septum, ventral tegmental area, and raphe nuclei (for review see White et al., 2000). Although these mechanisms inform us about the synaptic effects of ethanol, it is difficult to translate these effects into psychological phenomena, like impaired working memory, observed following alcohol consumption. Therefore, to understand the neural mechanisms of ethanol, it is necessary to incorporate physiological mechanisms that are relevant to \textit{in vivo} neural circuits implicated in impaired cognitive functions.
1.3. Overview of working memory

Alcohol is associated with detrimental effects on many aspects of cognition, but perhaps none are more intriguing than memory impairments. Memory research has a long history and has traditionally been a prolific field for many theorists and researchers. Memory is often not thought of as a unified system but instead is subdivided into several different systems each with distinct stages, psychological characteristics, and neural circuitry. Memory systems are classified based on their capacity, or how much information they hold, and their duration, how long they can hold information. The first memory system, sensory memory, has a very limited capacity and typically lasts less than 1-2 seconds. Short-term memory has a larger capacity and lasts on the order of seconds to minutes. Working memory is one type of short-term memory that involves active manipulation by the individual (Eichenbaum and Cohen, 2001). In other words, working memory is the memory that is actively being used “in the moment”. In the context of a behavioral task, working memory is defined as a representation of a cue over a delay period in which the cue is not present, to make a subsequent response (Honig, 1978). Another interpretation of working memory is a short-term memory for an object, stimulus, or location that is used within a testing session, but not typically between sessions (Dudchenko, 2004). In the rodent literature, several tasks are used to assess working memory function, notably including delayed alternation, radial arm and water mazes, delayed non match to sample with objects and odors, as well as operant tasks (Dudchenko, 2004). Working memory is often contrasted with reference memory which refers to the rules for a given task that do not change between sessions.
1.4. Overview of episodic-like memory

Reference memory is an example of the third memory system, long-term memory. Long-term memory has a virtually limitless capacity and can last anywhere from minutes to years. One of the most well-known types of explicit long-term memory is episodic memory. Episodic memory refers to autobiographical events, or episodes, in one's life that one can declare and consciously recall (Tulving, 1983). Originally, many believed that only humans were capable of episodic memory functions, but a landmark study in cache recovery by scrub jays posited the existence of episodic-like 'what-where-and when' memories in animals (Clayton and Dickinson, 1998). The set of background features that surround an event such as spatial context (where), temporal context (when), and internal context such as motivational state or reinforcement (what) contribute to the episodic-like characteristics of behavioral tasks (Ferbinteanu et al., 2006). Several tasks, notably including multiple reversal learning tasks, as well as delayed matching and non-matching tasks that have an 'episodic-like' character are used to model long-term episodic-like memory in animals (Morris, 2001). It has been suggested that one of the most powerful strategies for understanding the neurophysiological mechanisms of episodic memory is to record activity in brain regions homologous to structures involved in human episodic memory while animals perform tasks with episodic-like characteristics (Ferbinteanu et al., 2006).
1.5. The three stages of memory

Each memory system has three stages: encoding, consolidation, and retrieval (Eichenbaum, 2001; Morris, 2001; Ferbinteanu et al., 2006; Moscovitch et al., 2007). The stages of memory are distinct processes that explain how information gets into memory and gets back out again for later use. Encoding is the process by which a memory representation is initially acquired. Consolidation is the process by which the brain forms a physical representation of a memory and subsequently stores that memory over time. Retrieval is a process in which stored memories are accessed and actively reconstructed in the current moment. In tasks assessing working memory, encoding and retrieval happen in very close temporal proximity to each other. However, in long-term memory tasks, encoding and retrieval can be separated by a varying amount of time. Different neural circuits are associated with encoding, consolidation, and retrieval and they have been found to be differentially susceptible to the effects of ethanol.

1.6. Differential effects of ethanol on memory.

Pharmacodynamic and neurobiological mechanisms of encoding, consolidation, and retrieval remain incompletely understood, but it is clear that ethanol differentially affects these stages of memory. In particular, in both working memory and episodic-like memory, the initial encoding of information appears to be especially vulnerable to ethanol-induced impairment. Ethanol preferentially blocks memory formation without impairing previously formed memories (Wixted, 2005). Hippocampal-dependent learning via an associative task and experience-dependent activation of the hippocampus
as measured by immediate early genes are preferentially disrupted by ethanol (Melia et al., 1996). In humans, alcohol produces greater accuracy impairment during tasks that require the encoding and maintenance of stimulus sequences than in tasks that require other working memory functions such as spatial array recognition or focused attention (Saults et al., 2007). Low doses of ethanol produce selective dose-, delay- and time-dependent impairments in spatial working memory that are not indicative of general cognitive decrement (Givens, 1995).

A large body of research indicates that ethanol has contradictory effects on anterograde encoding and retrograde retrieval aspects of memory. Ethanol often causes anterograde amnesia in which individuals could “blackout” and experience total memory loss for events that occur while he or she is intoxicated, but ethanol is also associated with retrograde facilitation in which it is easier to remember material learned just before intoxication (Wixted, 2005). Alcohol also has different effects on encoding and retrieval depending on the memory task, the type of memory involved, and the limb of the Blood Alcohol Concentration (BAC) curve (Söderlund et al., 2005). These results and many others suggest that different aspects of memory are differentially susceptible to modulation via acute ethanol intoxication. If ethanol affects some aspects of memory more than others, then it is likely that different pharmacological and neurobiological mechanisms underlie the separate stages and systems of memory.
1.7. **Oscillatory patterns as a neurobiological mechanism of memory**

Current trends in neurophysiological research indicate gross synchronized electrical activity in the brain as a possible mechanism of action for synchronizing many cognitive functions, including memory (Buzsáki, 2006). Once thought of as an artifact or an epiphenomenon, oscillatory patterns within the brain have emerged as an area of considerable interest and have been described as a potential “phenotype of cognition” (Başar and Güntekin, 2008). Most neural oscillators are relaxation oscillators that exhibit distinct phases of input when they can easily be perturbed by environmental input, and output when they are refractory (Sirota and Buzsáki, 2007). Oscillations are an efficient way for large groups of neurons to be in states of maximal depolarization when information arrives.

1.8. **The theta rhythm**

A specific oscillatory brain wave pattern known as the theta rhythm has received considerable attention as a possible neurophysiological mechanism for cognitive functions. The theta rhythm is the largest extracellular synchronous signal in the mammalian brain detectable by electroencephalographic (EEG) techniques (Bland, 1986; Vertes et al., 2004). Theta rhythms exist in many mammals, but research has emphasized rodent and human findings. The theta rhythm is typically a relatively large (1-2 mV) amplitude oscillation of 4-10 Hz in the awake, behaving rat or 4-8 Hz in humans. There are two distinct types of theta activity. Type I theta is associated with voluntary movement, like running. Type II theta is at a slightly lower frequency and is correlated
with sensory processing, immobility, or involuntary motor behaviors like licking, shivering, or scratching. Type I theta is resistant to impairment from atropine, a muscarinic acetylcholine antagonist, but Type II theta is abolished by atropine thus suggesting that Type II is mediated by cholinergic mechanisms, but Type I theta is not (Bland and Oddie, 2001; Givens, 1996).

Theta is typically strongest in the hippocampal formation, but subcortical pathways contribute to the synchronization and maintenance of the theta rhythm. Neurons in the reticular pontine oralis nucleus project tonically to the supramammillary nucleus. The supramammillary nucleus projects to the medial septum / vertical diagonal band of Broca where the tonic excitation is converted to phasic, rhythmic firing (Vertes et al., 2004). The medial septal region is often thought of as a pacemaker that helps coordinate hippocampal theta activity and activates intrinsic theta neurons (Givens et al., 2000). The medial septum communicates with the hippocampus via cholinergic and GABA-ergic projections. Ascending cholinergic pathways terminate predominately on principal cells within the hippocampus and GABA-ergic projections terminate predominately on hippocampal interneurons (Bland and Oddie, 2001). When the hippocampus transitions from large amplitude irregular activity to synchronized theta activity, the medial septum initiates two cellular changes that occur approximately 500 milliseconds before the transition and help synchronize theta activity in the hippocampus. The medial septum inhibits hippocampal theta-off cells, which provides tonic depolarizing inputs that initiate membrane potential oscillations in hippocampal phasic theta-on cells which in turn
synchronize membrane potential oscillations of hippocampal phasic theta-on cells and discharge hippocampal tonic theta-on cells (Bland et al., 1999).

1.9. **Theories of theta activity coordination of memory function**

The precise mechanism by which theta activity coordinates memory function is subject to debate with many proposed roles, including binding together activity from different cortical areas, providing temporal context for neuromodulation, or acting as a clock signal to time action potentials in pyramidal cells (Burgess, 2005). It is clear that theta activity has a prominent role in information processing and memory (Winson, 1972; Givens and Olton, 1994; O’Keefe and Burgess, 1999; Seager et al., 2002; Yamaguchi et al., 2004). In humans, it has been found that observed increases in theta power during encoding predicted subsequent successful recall (Klimesch et al., 1996; Sederberg et al., 2003). Vertes (2005) has proposed that theta serves as a “significance signal” or “tag” for information arriving to the hippocampus such that information arriving with theta would be stored but information arriving in the absence of theta would not be encoded.

1.10. **Theta Reset**

One possible instantiation of an information “tag” is found in theta activity reset. The first known report of theta reset found that CA1 hippocampal theta was reset by a visual stimulus early in the stages of learning a visual discrimination task, but disappeared once performance plateaued in cats (Adey, 1967). Recording of theta activity in rodents demonstrates that the onset of visual or auditory stimuli in a working memory task causes
ongoing theta rhythm activity to reset such that it becomes temporally phase-locked to the sensory stimuli (Givens, 1996; Williams & Givens, 2003). In other words, mnemonically relevant stimuli reset the theta oscillation to the peak phase of activity. The purpose of hippocampal theta resetting may be to ensure that the hippocampal formation is in a maximal state of depolarization at the time of incoming sensory information from afferent projection sites. Theta rhythm activity showed stimulus-evoked reset in rats performing a working memory task, but not in rats performing a reference memory task (Givens, 1996). Theta reset follows the presentation of stimuli across different sensory modalities, suggesting theta reset as a viable mechanism for mnemonic processing across modalities. Consequently, theta reset in the hippocampus produces optimal conditions for long-term potentiation (LTP) (McCartney et al., 2004). Theta reset has also been observed in human participants via intracranial electroencephalogram recordings during a spatial working memory task (Rizzuto et al., 2003). Combined, this suggests that theta synchronization may be a plausible model for neurophysiological correlates of animal and human mnemonic function.

1.11. Neuroanatomical regions associated with the theta rhythm synchrony
A number of brain areas including the hippocampus, the entorhinal cortex, and the anterior cingulate region of the prefrontal cortex are consistently associated with activity in the theta range (Williams and Givens, 2003). The theta rhythm is often most prominent in the hippocampal formation and thus is associated with hippocampally-dependent memory functions. The hippocampal-entorhinal axis has population dynamics
that allow populations of neurons to discharge in close temporal synchrony and thus communicate rapidly and effectively (Chrobak and Buzsáki, 1998). During consolidation and retrieval of episodic information, hippocampal theta activity is known to synchronize with prefrontal circuits (Siapas et al., 2005; Paz et al., 2008). It is clear that the transfer of information from hippocampal circuits to the medial prefrontal cortex mediates aspects of memory retrieval and consolidation (Wiltgen et al., 2004). The medial prefrontal cortical sites that receive afferents from the hippocampus appear to be particularly involved in task switching (Rich and Shapiro, 2007). In human studies that used subdural recording nets and found theta activity during encoding predicted successful recall, theta activity was clustered in the right temporal lobe and the frontal cortex (Sederberg et al., 2003). Studying neurophysiological changes in theta rhythm at the entorhinal cortex, dentate gyrus of the hippocampus, and anterior cingulate region of the prefrontal cortex will help clarify the role of these neuroanatomical sites in memory and under the influence of ethanol.

Previous research from this lab has found that the neural circuit including the entorhinal cortex, the hippocampus, and the anterior cingulate region of the pre-frontal cortex are involved in theta reset. The entorhinal cortex receives converging sensory input from polymodal association areas in the cortex through its connections with the perirhinal cortex (Suzuki et al., 1997) and relays this information to the hippocampus via the perforant path. Theta reset is found in the entorhinal cortex preferentially during encoding, in the anterior cingulate preferentially during retrieval, and in the dentate hilus.
of the hippocampus during both encoding and retrieval phases of an operant task (Williams and Givens, 2003). Other research from this lab has indicated that theta rhythm activity is highly susceptible to modulation via ethanol. Research demonstrating the ethanol-induced vulnerability of hippocampal neurons ranges from single neuron activity (Givens et al., 1998) to multi-unit activity (Givens et al., 2000). According to a change in choice accuracy in a delayed alternation task, ethanol suppresses hippocampal theta activity in the same doses that caused impairments in working memory (Givens, 1995). Ethanol produces a loss of hippocampal responsivity to oscillating drive from the medial septal area, and thus reduces the ability of the hippocampus to encode and retrieve memory information via theta activity (Givens et al., 2000). However, there is a need for more research to determine if and how alcohol affects encoding and retrieval of memory via the mechanism of differential theta reset modulation.

1.12. Expected effects of alcohol on memory and theta rhythm

The specific aims of this thesis are to investigate if and how alcohol affects encoding and retrieval of working memory and episodic like-memory via alterations in theta rhythm activity. First, this thesis will investigate the dose-dependent effects of ethanol on theta reset mechanisms of rapid encoding and retrieval processes associated with working memory. Secondly, this thesis will attempt to ascertain the dose-dependent effects of ethanol on mechanisms of memory encoding, consolidation, and retrieval via alterations in theta rhythm synchrony across brain regions. It is hypothesized that alcohol will disrupt theta rhythm synchronization of cortical inputs to the hippocampus. This will
result in impaired stimulus encoding and will be measured by reduced memory performance. Theta synchronization between the entorhinal cortex and the hippocampus is expected during encoding phases of the task, and this synchrony will be disrupted in a dose-dependent fashion by alcohol. It is also hypothesized that synchrony will be observed between the hippocampus and the anterior cingulate cortex during retrieval phases, and that ethanol will not or will minimally affect synchronization between hippocampal outputs to the cortex and so memory retrieval will be intact.

1.13. Experiment overview

In order to advance our understanding of the neural basis for ethanol-induced cognitive impairments associated with working memory, it is necessary to develop and use valid animal models of these cognitive processes. Researchers have developed several behavioral tasks to study working memory in rodents. Some of the most widely used paradigms include maze tasks such as delayed alternation, the radial arm maze, or the Morris water maze, matching paradigms with objects or odors, and operant tasks such as the delayed (non) match to position (DNMTP) task (Dudchenko, 2004). All of these tasks conform to the definition of working memory as a type of short-term memory that involves active manipulation by the individual (Eichenbaum & Cohen, 2001). This lab and others have used a delayed non-matching-to-position (DNMTP) task to model working memory in rats (for further details on this task please see the general methods section). Advantages of this task include multiple trials over which to average the physiological data, precise timing of stimulus onset, precise timing of behavioral
responses, and separate encoding and retrieval epochs (McCartney et al., 2004). The DNMTP task also allows neurophysiological recordings from many neuroanatomical sites and there is minimal potential for locomotive confounds. This lab has previously used DNMTP tasks to elicit theta resets (McCartney et al., 2004; Williams and Givens, 2003) in response to stimuli in the encoding sample phase but not the retrieval choice phase. The sample phase of the task corresponds to encoding into working memory and the choice phase of the task corresponds to retrieval.

Although the DNMTP task allows separate analysis of encoding and retrieval processes associated with short-term working memory, these epochs are in immediate succession of each other and therefore it is difficult to attribute an ethanol-induced change in behavioral responses to impairments in one specific stage of memory. To determine potentially different impacts of ethanol on encoding and retrieval via theta reset and synchrony, it was necessary to develop a behavioral task that temporally separates encoding and retrieval into discrete times for ethanol experimentation. We have adapted the task to include serial rule reversals so that rats are trained on both delayed match and non-match to position paradigms, D(N)MTP. Switching between the rules requires long-term memory and involves more episodic-like characteristics.
1.14. Specific Hypotheses and significance of proposed experiments

There is an immediate need for additional clarifying research regarding neurophysiological mechanisms of how ethanol differentially modulates encoding versus retrieval. Thus far, the majority of investigations into the physiological mechanisms of action of alcohol have tended to focus on the microscopic neurochemical interactions at the level of the individual neuron or synapse. Although the field of ethanol research understands a great deal about the pharmacology of ethanol in the brain, it is difficult to translate these synaptic mechanisms into the psychological phenomena observed in impaired cognition. Therefore, to understand the neural mechanisms of ethanol modulation of encoding and retrieval, it is necessary to incorporate physiological mechanisms that are relevant to in vivo neural circuits. The theta rhythm is one of the most promising neurophysiological findings associated with differential roles in encoding and retrieval. Through investigation of ethanol modulation of theta activity in the neural circuit including the entorhinal cortex, the hippocampal formation, and the anterior cingulate cortex, we expect to find dose-dependent effects on memory performance, theta reset to stimuli, and theta synchronization across distal brain regions.

Our specific hypotheses address the validity of the task, the effects of ethanol on memory, and the occurrence and synchronization of the theta rhythm. If the delayed (non-) match to paradigm is a valid tool to assess impairments in memory, then there should not be a lasting significant difference between the rules in terms of time to criterion or session accuracy. If acute ethanol impairs memory encoding, then levels of
accuracy relative to controls should be impaired in the post-switch session of a single reversal paradigm, and improved when the rule is reverted in the double switch paradigm. If theta rhythm activity is present in a recording site, then the power spectrum should show peaks between 4 and 10 Hz. If overall theta activity is vulnerable to ethanol, then power spectrum peaks for all anatomical recording sites should decrease according to dosage. If there is synchronization during anatomical sites in response to encoding or retrieval relevant stimulus onsets, then there should be a high correlation and a predictable relationship across the waveform averages.

The proposed experiments will enhance our understanding of the neural basis of ethanol modulation of encoding and retrieval. This information could be useful in explaining deficits in learning and memory, and may eventually contribute to effective treatments. Additionally, these experiments will explain detailed effects of alcohol on cognitive functions relevant to short-term and working memory. This may yield valuable information on mnemonic contributors to the consumption of alcohol as well as behavioral and cognitive functions before, during, and after acute intoxication. Information about the acute effects of alcohol on memory could contribute to an understanding and possible prevention of injuries and fatalities associated with situations such as driving under the influence of alcohol. Furthermore, studying the acute effects of alcohol on encoding and retrieval may lead to a better understanding of the neural structures vulnerable to chronic effects and possible treatment benefits or prevention strategies for alcohol abuse and alcoholism.
Chapter 2: Methods

2.1. Subjects
Subjects for all experiments were adult male Long-Evans rats (shipped as adolescents from Charles River Laboratories International, Inc. Wilmington, Mass) weighing 300-400 g at the time of the experiment. All subjects were single-housed in clear plastic cages (38.1 cm x 20.32 cm x 17.78 cm) with corncob bedding in a humidity- and temperature-controlled (25° C) vivarium. Lights were maintained on a 12-hour light-dark cycle with lights on at 6:00 AM. All testing occurred during the animal’s light cycle. All rats had free access to food but restricted access to water. Rats received water rewards during daily training sessions and additionally as needed to maintain at least 85% of normal age-adjusted body weight. All underwent regular general health examinations and were handled daily outside of training or testing contexts to minimize stress. All animal procedures were approved by the Ohio State University Institutional Animal Care Use Committee and were performed in accordance with NIH guidelines.

2.2. Behavioral Apparatus
All behavioral experiments took place in operant conditioning chambers developed by Med Associates Inc. (St. Albans, VT). All behavioral training took place in standard operant chambers (28 X 20.5 X 21 cm) housed in a ventilated light and sound attenuating
shell (56 X 50 X 34.5 cm). The operant chamber consisted of two sidewalls of .5 cm clear Plexiglas, front and back walls of stainless steel, and a floor consisting of parallel stainless steel rods (1 cm apart). The chamber was illuminated by a houselight (2.8 Watts) located on the back panel. The front panel contained two signal lights (2.8 Watts, 2.5 cm diameter), two retractable stainless steel response levers, and a recessed port with a water dispenser (40-80 µl per drop, 20 cc dispenser capacity). The operant chamber used for electrophysiology was identical to the initial training chambers except for higher side panels (42 cm) and a larger water port to accommodate the preamplifier/cable system that was attached to the rats’ head stage during recording sessions. Both types of operant chambers were controlled by personal computers interfaced with hardware and software developed by Med Associates Inc (East Fairfield, VT).

2.3. Operant Conditioning Task Training

Rats were trained to perform the delayed (non-) match to position operant task through a series of four gradual shaping steps: habituation, sample phase training, training on full task with correction trials, and then full task without correction trials. During the habituation step, both levers were inserted into the box, all signal cues remained off, and rats received a water reward for lever presses. To prevent the development of a side bias, the total number of presses on one lever could not exceed the total of the other lever by more than five. If this imbalance occurred, then subsequent responses to the more frequently pressed lever were no longer rewarded or counted until the other lever was pressed. The habituation session continued until there were 50 rewarded presses on each
lever, for a total of 100 rewarded presses. After successful completion of habituation, rats were advanced to the second step of training consisting of the sample phase portion of the task. Both levers were inserted into the box and one of two signal lights was illuminated randomly and remained illuminated until a lever was pressed. Rats only received water rewards for lever presses underneath the illuminated signal light. Any lever press extinguished the signal light and initiated a 10 second inter-trial interval. Each session lasted 150 trials and training continued until rats demonstrated criterion performance greater than or equal to 75% accuracy for three consecutive sessions. When a subject reached criterion performance in the sample phase of training, the rat was progressed to the full version of the task with corrections. A trial began with a sample phase, described above, that was followed by a 5 second delay period with no signal cues, then an auditory tone for one second that signified the start of the choice phase. Only trials that resulted in a rewarded response in the sample phase proceeded to the choice phase. During the choice phase, a correct response varied according to the rules of a session, either delayed match to position of the sample phase response (DMTP) or delayed non-match to position (DNMTP). In DMTP sessions, a correct response was defined as pressing the lever that corresponds to the side where the signal light was previously illuminated. In DNMTP sessions, a correct response was defined as pressing the lever in the location that was opposite of the previously illuminated signal light. An incorrect response initiated up to five correction trials in which the same light was illuminated during the sample phase. Correction trials were not included in the total trial count or overall accuracy. A correct response in the sample phase or during a correction
trial yielded a water reward of 0.04 seconds (approximated 0.05 ml) while a correct response in the choice phase of the task yielded a water reward of 0.08 seconds (approximated 0.1 ml). Rats were tested for one session, 6-7 days per week. Each session lasted for 90 minutes or 150 trials, whichever came first. Criterion performance was defined as greater than or equal to 75% accuracy for three consecutive sessions. If a rat did not reach criterion within a session, the rule continued for the next daily session(s) until it reached criterion. When a rat reached criterion performance for one rule, the visual discrimination rule was switched from DMTP to DNMTP or vice versa. Training with correction trials persisted until the rat demonstrated criterion performance for each rule two times (4 rounds). After completion of the fourth round, correction trials were removed and a session lasted for 150 trials or up to 90 minutes. Serial rule switches continued throughout the duration of the experiment and criterion for mastery of a rule was defined as greater than or equal to 70% accuracy for three consecutive sessions. See Figure 1 for a schematic representation of the D(N)MTP task.

2.4. Single Rule Reversals

Rats continued training on Rule A (e.g. DMTP) until they reached the performance criterion described above. Once criterion was reached, then the subsequent session included alcohol or saline injections (if applicable) and the task was switched to the other rule, generically defined as Rule B. In the single reversal paradigm, after the switch then Rule B was maintained until criterion. Behavioral and neurophysiological data were
collected and analyzed for Day 1 (Pre-Switch, Rule A), Day 2 (Switch, Injection, Rule B), and Day 3 (Post-Switch, Rule B).

2.5. Double Rule Reversals

Rats continued training on Rule A (e.g. DMTP) until they reached the performance criterion described above. Once criterion was reached, then the subsequent session included alcohol or saline injections (if applicable) and the task was switched to Rule B. On the next session, the rule was reverted back to Rule A and was maintained until criterion. Behavioral and neurophysiological data were collected and analyzed for Day 1 (Pre-Switch, rule A), Day 2 (Switch, Injection, Rule B), and Day 3 (Post-Switch, Return to Rule A).

2.6. Acute Systemic Alcohol Injections

All systemic injections were given intraperitoneally 10 minutes before the animal was placed in the operant conditioning chamber. We mixed absolute ethanol with physiological saline in a 10% (weight/volume) solution. Injections were given at doses of 0.0 (1.0 ml/kg saline vehicle), 0.25, 0.50, 0.75, or 1.0 g/kg body weight. We created two dosage groups to minimize the number of injections each animal received. Dosage group “A” received saline, 0.25, or 0.75 g/kg body weight. Dosage group “B” received saline, 0.5, or 1.0 g/kg body weight. We randomized the order of injections and timing of injections across subjects with a Latin Square Design. At least five days separated each injection to minimize any potential residual effects or tolerance.
After being injected with 1.0 g/kg of ethanol, the lateral tail vein was nicked for blood collection to verify blood ethanol content. Samples of approximately 20-µL were obtained every 15 minutes after the injection over an hour for a total of four samples per animal. Blood samples were spun in a microcentrifuge and the plasma was frozen in a negative 80 °C freezer for further analysis with an alcohol oxidase reaction in an AM-1 alcohol analyzer (Analox Instruments USA, Lunenburg, MA).

2.7. Behavioral Analysis

All behavioral analyses were done on personal computers using data acquisition software developed by Med Associates (East Fairfield, VT) and statistical analysis software developed by SPSS (Chicago, IL). We recorded behavioral events simultaneously with neurophysiological data. Behavioral manipulations include rule (Match or Non-Match to Position), trial block, day (pre-switch, switch, or post-switch), and dosage of alcohol. Behavioral dependent measures included the number of trials to criterion for each training rule, total lever presses, number of incorrect responses, and overall choice accuracy. Data was analyzed via repeated measures within subjects ANOVAs and paired sample t-tests when applicable.

2.8. Electrode Construction

Recording electrodes were constructed using a plastic 8-channel [10 pin] male connector, .050 manufactured by Omnetics Connector Corporation (Minneapolis, MN). Two ground wires measuring .005 mm, made of stainless steel with Teflon coating (California
Fine Wire Company, Grover Beach, CA) were cut and soldered into the Omnetics connector. Three recording wires measuring .003 mm, made of stainless steel with Teflon coating (California Fine Wire Company, Grover Beach, CA) were cut and soldered into the Omnetics connector. Connectivity was verified using an electrical multimeter (Fluke Corporation, Everett, WA). All connections were insulated and sealed with 5-minute epoxy and then dental acrylic (Reliance Dental Manufacturing, Worth, IL).

2.9. Electrode Implantation Surgery

All surgeries were performed with aseptic procedures. Each rat was anesthetized with isoflourene delivered via a snout mask and adjusted as needed to maintain stable respiratory rates but prevent corneal, hindlimb, or tail reflexes. Body temperature was monitored and maintained at $37 \pm 0.5^\circ$C using a deltaphase isothermal pad (Braintree Scientific, Inc. Braintree, MA). The rat’s head was shaved, sterilized, and then positioned in a small animal stereotaxic apparatus with blunt ear bars (Kopf instruments, Tujunga, CA). Teflon-coated stainless steel recording electrodes were implanted unilaterally into the dentate gyrus (4.0 mm posterior to Bregma; 2.5 mm lateral to midline; 2.7-3.0 mm ventral to the dural surface), the anterior cingulate region of the medial prefrontal cortex (0.7 mm anterior to Bregma; 0.6 mm lateral to midline; 2.0-3.0 mm ventral to the dural surface) and the entorhinal cortex (8.8 mm posterior to Bregma; 4.5 mm lateral to midline; 4.7-5.5 mm ventral to the dural surface). One Teflon-coated, stainless steel ground-wire was lowered approximately 1 mm into the cortex and served as a reference ground, another ground-wire was stripped and wrapped around a skull screw
and served as an animal ground. All wires were connected to the plastic Omnetics connector at the time of implantation. Four jeweler’s screws were inserted to help keep the headstage fixed to the skull. All wires were secured to the skull and screws with dental acrylic (Reliance Dental Manufacturing, Worth, IL). Post-operatively, all animals were injected subcutaneously with 1.0 ml of physiological saline, and treated with topical antibiotic ointment (Neosporin, Johnson & Johnson Consumer Companies, Inc.).

2.10. Neurophysiological Recording

The implanted connector was attached to a high-impedance head stage with 8 channels, .050 mm spacing, and 1x gain (Plexon, Inc., Dallas, TX). The signal was carried by a cable from the preamplifier to a commutator and a differential AC amplifier ((1000x)/filter 0.1-500Hz, A-M Systems, Sequim, WA), to an A-D board for digitization (Power 1401, Cambridge Electronic Design (CED), Cambridge England), and then to a personal computer with data acquisition and analysis software (Spike 2 version 7.06, CED).

2.11. Neurophysiological Analysis

Neurophysiological analyses took place on personal computers with data acquisition and analysis software developed by CED (Spike 2 version 7.06, Cambridge, England). Initial recordings included a wide-bandwidth, but were filtered with a finite impulse response (FIR) digital filter for subsequent analyses. An FIR settles to zero at a specified time, whereas an infinite impulse response filter does not. The FIR filter used a low pass band
filter to select frequencies between 5 and 12 Hz, with a transition gap of 2.5 Hz.

Physiological recordings took place on rule-switch sessions. All files were also manually scanned and filtered to eliminate instrumental noise and movement artifacts. Noise, artifacts, or interference were collectively defined as any irregular activity that was not part of the normal ongoing oscillatory rhythm. Specifically, intervals containing potentials that exceeded 5 millivolts were deleted from all waveforms.

First, power spectrum analyses were performed on the waveform channels representing activity in the entorhinal cortex, the dentate gyrus, and the anterior cingulate cortex for the duration of a full recording session. A power analysis is a representation of a frequency variable as determined by time. Power analyses are computed by using a Fast Fourier Transform (FFT) to convert the waveform data into a power spectrum. The FFT transforms a block of data between a waveform and an equivalent representation as a set of cosine waves. The output of a power analysis is the amount of energy at a given frequency. Power analyses are useful for determining the frequency content of a signal and help identify repeated frequency patterns within the data. For example, theta rhythm occurs between 4 and 10 Hz. If theta rhythm is present in a waveform recording, then the power analysis should show a peak within this frequency range. The power analyses for each anatomical recording site were then compared for any dose-dependent differences. Behavioral events from the operant task including onset and offset of all lever presses, light stimuli, tone stimuli, and water rewards were simultaneously recorded with the neurophysiological waveforms as square wave pulses. Separate channels were created to
mark the onsets of events such as the onset of the light stimulus for the encoding phase of the task and the onset of the tone stimulus for the retrieval phase of the task. These stimulus onset markers for encoding and retrieval were used as triggers for subsequent physiological analyses. The triggers defining onset of either light or tone stimuli were identified as point zero on all time scales.

Peri-stimulus waveform averages were performed on the recording channels for each anatomical site (entorhinal cortex, dentate gyrus, and anterior cingulate cortex) triggered by encoding then separately by retrieval events. Waveform averages were calculated by sweeping a specified time interval around the nearest trigger event and then calculating the arithmetic mean of all the sweeps over the span of the time window. For example, in a session in which all 150 trials were completed, a waveform average triggered by either encoding or retrieval events would include a sweep of 150 trials since each event happens once per trial. For these analyses, all waveform averages included a 1 second offset prior to the trigger, as well as 1 second after the trigger. The mean of each of the waveform averages for each anatomical recording site and each dosage of alcohol was calculated for further analysis.

Peri-stimulus event histograms were created to identify the frequency of lever pressing behavior corresponding with the exact timing of the peri-stimulus waveform averages. Peri-stimulus event histograms were generated for both encoding and retrieval event triggers and counts of bar presses were collected in bins representing activity over 0.025
seconds. The total number of presses for the left bar and the right bar at each condition were summed together and then averaged for further analysis.

\[ \text{2.12. Lesion and Histological Procedures} \]

Final recording sites were marked with a small electrolytic lesion (± 150 µA for 20 seconds at each current direction). At the end of each series of experiments, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.), transcardially perfused first with heparinized saline and then with formalin. The brains were extracted, cryoprotected with a 30% sucrose solution for two days, frozen, sliced into coronal brain sections 50 µm thick with a cryostat, mounted on gelatin-coated microscope slides, and stained with cresyl violet for verification of anatomical placement of the electrodes. Photomicrographs were obtained with an Olympus AX70 microscope.
Figure 1. Delayed (Non) Match to Position Operant Conditioning Task Schematic. Schematic representation of sample phase (encoding) and choice phase (retrieval) for the Delayed (Non-) Match to Position (D(N)MTP) operant task.
Chapter 3: Results

3.1: Acquisition of Non-match and Match Rules

One cohort of animals (n=8) was trained on up to 5 rounds each of Match and Non-Match to Position variants of the operant task. The initial rule was counterbalanced across subjects. The mean number of daily training sessions needed to acquire criterion (≤ 70% overall session accuracy, 150 trials per session), and overall session accuracy on rule switch days and post-switch days were calculated to determine quantitative differences in the acquisition of each rule. All of these animals were able to achieve at least criterion performance, and many achieved higher levels of accuracy, up to 95% in some cases. All animals were also able to repeatedly switch between non-match and match rules.

The mean number of daily sessions needed to acquire criterion for each rule ranged between 7 and 13. As shown in Figure 2 (Time to Criterion by Rule) the first instantiation animals experienced each rule, non-match took significantly more sessions to reach criterion than match (One-tailed, paired sample t-test, p<0.05). However, for all subsequent instantiations, the number of sessions needed to acquire criterion did not significantly differ by rule (p > 0.05). The mean number of sessions needed to acquire criterion performance regardless of rule was between 9 and 10 sessions.
Although the time to criterion did not vary significantly for most instantiations of the rule, this does not account for performance differences in the initial acquisition of the rule contingencies, so mean session accuracy on the first and second day a rule was experienced was calculated (See Figure 3 Session Accuracy by Rule). On the first day of training, the mean accuracy for match was 42% correct versus 33% correct for non-match. This difference in accuracy was statistically significant (two-tailed paired sample t-test, p<0.05). By the second day of training, both groups improved substantially and improved to mean accuracy of 58% for match and 54% for non-match. While the mean level of accuracy for match was still slightly higher than for non-match, this difference was no longer statistically significant (two-tailed paired sample t-test, p > 0.05) on day 2. The mean accuracy for match and non-match conditions continued to be not significantly different (two-tailed paired sample t-test, p > 0.05) for all subsequent days of training until criterion was reached.
Figure 2. Time to Criterion by Rule
Comparison of sessions needed to achieve criterion ($\leq 70\%$ overall session accuracy) by rule. Time to criterion is significantly longer for non-match on the first instantiation, but this difference disappears over time. ($^* = p < 0.05$) Error bars indicate SEM.
Figure 3. Session Accuracy by Rule
Comparison of accuracy for switch session (Day 1) and post-switch session (Day 2) by rule. The accuracy is significantly higher for match than non-match on the first day, but this difference disappears by the second day (* = p < 0.05). Error bars indicate SEM.
3.2 Effects of ethanol on single rule switches

Five animals were trained on the delayed (non-) match to position task and tested with injections of ethanol in both the single rule switch and double rule switch paradigms. Rules corresponding to ethanol injections were counterbalanced across animals. The effects of ethanol on single rule switches were determined by looking at dose-dependent changes in overall accuracy and within-session performance.

Data from three time points were of particular interest in the single rule switch paradigm: the pre, switch, and post sessions. The pre session demonstrated criterion-level performance of the previously learned rule and occurred approximately 24 hours before the switch session. The animals were injected with a dose of ethanol 10 minutes before commencing the switch session in which they were placed in the operant chamber and reinforced for the opposite rule they previously learned (e.g. match would switch to non and vice versa). Importantly, ethanol was only on board during the switch session. The post switch session occurred approximately 24 hours after the switch session and continued reinforcing the new rule contingency to which the animal previously switched. Each animal experienced each time point (pre, switch, post) for each dosage of ethanol (none, 0.25 g/kg EtOH, 0.50 g/kg EtOH, 0.75 g/kg EtOH, and 1.00 g/kg EtOH), so the experimental design for the single-switch paradigm was completely within-subjects.

The effects of ethanol on single rule switches were first assessed by looking at the overall performance and calculating the mean percent correct for each dosage-group at each time
point of interest (See Figure 4: Effects of Ethanol on Single Rule Switch). Data were analyzed with a repeated measure ANOVA. There was a significant main effect of time ($F_{2, 8} = 72.19, p < 0.001$), but there was not a significant main effect of dosage ($F_{4, 16} = 1.179, p > .05$). There was also a significant time by dose interaction ($F_{8, 32} = 50.08, p < 0.001$).

Post-Hoc t-tests were performed at each time point comparing the no ethanol control to each of the other four doses. All conditions performed at similarly high levels of accuracy during the pre-switch session (two tailed t-test, $p > 0.05$). All conditions showed similar declines in accuracy on the switch session (two tailed t-test, $p > 0.05$). Interestingly, the post-switch session showed varying levels of accuracy depending on the dose of ethanol received 24 hours prior. The lowest dose of ethanol was not significantly different from the control condition (two tailed t-test, $p > 0.05$), but the 0.50 g/kg EtOH, 0.75 g/kg EtOH, and 1.00 g/kg EtOH conditions showed significantly lower mean accuracy percentages than the control condition (two tailed paired sample t-test, $p < 0.05$).

To further explore the dose-dependent changes in the post-switch session, the mean difference in overall accuracy between the post-switch session and the switch session was calculated for each dosage condition (See Figure 5: Improvement in Performance between Post session and Switch Session). The no ethanol control showed the highest improvement, and the mean difference in overall percent correct declined in a dose-dependent fashion. In a repeated measures ANOVA, the main effect of was not
significant but indicated a trend towards significance ($F_{4,16} = 2.362$, $p = 0.097$). In a subsequent one-tailed paired sample t-test to compare the control and the highest dose of ethanol, the 1.0 g/kg dose of ethanol showed significantly less difference in overall percent correct between the two sessions, indicative of less improvement over time.

The effects of ethanol on within-session performance for the pre, switch, and post sessions were also assessed. Each session consisted of 150 trials that were divided into 15 blocks of 10 trials each. The mean accuracy for each block was computed and expressed as a ratio out of 10. The first five blocks for each session were collapsed into an “early” category and also expressed as a ratio of correct responses out of 10. Blocks 6-10 were collapsed into a “mid” category, and blocks 11-15 were collapsed into a “late” category (See Figure 6: Effects of Ethanol on Within-Session Performance).

Data were analyzed with a repeated measures ANOVA that compared the three time points of interest (pre, switch, and post), the three block periods (early, mid, late), at each dosage condition (none, 0.25 g/kg EtOH, 0.50 g/kg EtOH, 0.75 g/kg EtOH, and 1.00 g/kg EtOH). There were significant main effects of time ($F_{2,8} = 181.15$, $p < 0.001$), dosage ($F_{4,16} = 6.91$, $p < 0.01$), and block ($F_{2,8} = 47.002$, $p < 0.001$). There were also significant two-way interactions of time by dose ($F_{8,32} = 2.895$, $p < .05$), time by block ($F_{4,16} = 32.208$, $p < 0.001$). The interaction of dose by block was not statistically significant ($F_{8,32} = 0.802$, $p > 0.05$). The three-way interaction of time by dose by block was significant ($F_{16,64} = 1.969$, $p < 0.05$).
The early, mid, and late block analysis within the session revealed some noteworthy trends in the data. During the pre-switch session, all conditions performed similarly well in the early and mid blocks, but exhibited a noticeable drop-off during the late block. This drop-off is likely due to satiety from a high number of water rewards in the previous blocks. During the switch session, the highest proportion of correct responses for all three blocks was found in the 0.25 g/kg EtOH condition, and the lowest proportion of correct responses was the 0.75 g/kg EtOH condition. In the early block of the post-switch session, the control, 0.25 g/kg EtOH, and 0.50 g/kg showed an equivalent or greater proportion of correct responses than they showed in the late block of the switch session a day prior. However, the 0.75 g/kg EtOH and 1.00 g/kg EtOH conditions showed a decrease in their respective proportions of correct responses in the early block of the post-switch session compared to the late block of the switch session. All conditions showed comparable improvement in the mid and late blocks of the post-switch session. The dose-dependent effects on the proportion of correct responses for each of the 15 blocks of 10 trials on the switch session and the post-switch session is shown in Figure 7. A and B.
Figure 4. Effects of Ethanol on Single Rule Switch
Comparison of the effects of Ethanol on pre-switch, switch, and post-switch time points of a single rule switch paradigm. All rats showed similar high performance in the pre-switch session and comparable impairments on the switch + injection session. Ethanol on a switch session dose-dependently impaired accuracy on the post switch session the following day. (* = p <0.05 difference from control) Error bars indicate SEM.
Figure 5. Improvement in Performance between Post session and Switch Session
Mean difference between post-switch session accuracy and switch session accuracy. Control doses show the highest level of difference, indicating more improvement from switch day to post-switch day. Rats with 1.00 g/kg showed the lowest improvement on the post-switch session.
Figure 6. Effects of Ethanol on Within-Session Performance
Dose-dependent changes on performance within pre, switch, and post sessions. Early represents the mean of the first five blocks of 10 trials each, mid represents the second five blocks of 10 trials each, and late represents the last five blocks of 10 trials each. Error bars indicate SEM.
Figure 7. Within-Session Performance for Switch and Post-Switch Days
The within session performance during the switch + injection session and the post-switch session that occurred 24 hours after injection. Sessions were divided into 15 blocks of 10 trials each and the mean correct out of 10 was computed for each sequential block.
3.3 Effects of ethanol on double rule switches

Data from three time points were of particular interest in the double rule switch paradigm: the pre, switch 1+injection session, and the switch2 session. The pre session demonstrated criterion-level performance of the previously learned rule and occurred approximately 24 hours before the first switch session. The animals were injected with a dose of ethanol 10 minutes before commencing the first switch session in which they were placed in the operant chamber and reinforced for the opposite rule they previously learned (e.g. match would switch to non and vice versa). Similar to the single rule switch paradigm, ethanol was only on board during the first switch session. The second switch session occurred approximately 24 hours after the switch session and reverted back to the rule that was at criterion during the pre-switch session. The double rule switch paradigm was piloted with the no ethanol control compared to the 1.00 g/kg dose of ethanol.

As shown in Figure 8, all rats showed similar high performance in the pre-switch session for Rule A. When rats received ethanol on the first switch day, then they demonstrated slightly improved performance on the second switch day back to the original rule. There were significant main effects of time ($F_{2,8}=31.78$, $p < 0.05$) and dosage ($F_{1,4}=11.50$, $p < 0.05$). The interaction between time and dosage was not statistically significant ($F_{2,8}=2.11$, $p > 0.05$). Post-hoc paired sample t-tests comparing the control with the 1.0 g/kg EtOH dose at each time point revealed a significant difference in only the second switch sessions ($p < 0.05$). In the double reversal paradigm, ethanol on the first switch day
actually improved overall performance in the session 24 hours later that reverted back to
the original rule.
Figure 8. Effects of Ethanol on Double Rule Switch
Comparison of the effects of Ethanol on pre-switch, switch1+injection, and switch2 rule reversion time points of a double rule switch paradigm. All rats showed similar high performance in the pre switch session and comparable impairments on the switch1+injection session. Ethanol on the first switch session improved accuracy on the session the following day that reverted back to the original rule. ( * = p <0.05 difference from control) Error bars indicate SEM.
3.4 Histology

A small subset of animals was electrolytically lesioned and perfused as described previously in the methods section. As shown in the photomicrographs in Figure 9, Cresyl violet staining identified electrolytic lesions near the anterior cingulate cortex, the dentate gyrus of the hippocampus, and the entorhinal cortex. Targeted electrode placement diagrams are from the Paxinos and Watson Brain Atlas.
Figure 9. Representative Histology and Targeted Electrode Placement

In the left panel, arrows indicate observed electrolytic lesions. In the right panel, dashed circles represent the targeted region of electrode placement.
3.5 Neurophysiology

Nine rats were surgically implanted with recording electrodes according to methods described previously. One animal experienced post-operative health complications and two animals belonging to the group that included 0.25 and 0.75 were affected by technical difficulties during experimentation, so those groups were not included in the analyses below. Six animals yielded viable neurophysiological recordings and were distributed across all six original dosage groups. All recording sessions took place on the day of a rule switch, either without an injection, or 10 minutes after an intraperitoneal injection of 1.00 mL physiological saline, 0.50 g/kg ethanol, or 1.00 g/kg ethanol. Local Field Potentials (LFPs) were simultaneously recorded from the anterior cingulate cortex (AC), the dentate gyrus (DG), and the entorhinal cortex (EC) of each animal.

3.6 Power of frequencies in the Theta band

A power spectrum analysis was performed on all available wave forms to yield the amount of energy at a given frequency. To compute the power spectrum, we used an FFT size of 2048 (2.048 seconds) to show frequencies from 0 to 500 Hz in 1024 bins with a resolution of 0.4883 Hz. To avoid sharp discontinuities at the end of one block and the start of the next, the FFTs used standard Hanning windows, also known as raised cosines, for tapering the start and end of each data block to zero so that they join smoothly. The mean power spectrum for all recording sites for control conditions is shown in Figure 10, and the mean power spectrum for conditions under acute ethanol is shown in Figure 11. Across all anatomical recording sites, and across all dosage
conditions, there was pronounced activity within the 4-10 Hz range associated with the Theta Rhythm. In Figure 12, the mean power spectrum for each anatomical recording site was compared by dose. The highest power is found in the control conditions, whereas the 0.50 g/kg EtOH and 1.00 g/kg EtOH conditions show dose-dependent decreases in overall power. For further quantification of this difference, the area under the curve was computed and compared across anatomical recording sites and dosages (see figure 13). Subsequently, a two-tailed paired sample t-test was used to compare differences between the no injection and the 1.00 g/kg EtOH dose at each anatomical site. In the entorhinal cortex, the difference was not significant ($t = 3.648$, p > 0.05), but in both the dentate gyrus and the anterior cingulate cortex regions, there was a significant difference ($t = 17.168$ and $t = 15.19$, p < 0.05) between the overall power in the control conditions and the highest dose of alcohol.
Figure 10. Mean Power Spectrum for all Recording Sites under Control Conditions

Representation of energy per unit time at each frequency (0-20 Hz displayed) calculated by Fast Fourier Transform (FFT) Analysis. Activity in the theta band (4-10 Hz) was found in all anatomical recording sites and under all dosage conditions.
Figure 11. Mean Power Spectrum for all Recording Sites with Ethanol

Representation of energy per unit time at each frequency (0-20 Hz displayed) calculated by Fast Fourier Transform (FFT) Analysis. Activity in the theta band (4-10 Hz) was found in all anatomical recording sites and under all dosage conditions.
Figure 12. Mean Power Spectrum for each Recording Site by Dose

Representation of energy per unit time at each frequency (0-20 Hz displayed) calculated by Fast Fourier Transform (FFT) Analysis. Activity in the theta band (4-10 Hz) was found in all anatomical recording sites and under all dosage conditions.
Figure 13. Gross Power Spectrum Difference across Recording Site and Dose

The area under each mean power spectrum curve for each anatomical recording site at each dose was calculated to compare total activity within the theta band range. Control conditions showed the greatest levels of total theta activity, ethanol dose-dependently decreased the levels of total theta activity.
3.7 Effects of acute ethanol on the theta rhythm and behavior during encoding

The short-term encoding stage of the task was characterized by the onset of the light stimulus in the sample phase. According to methods described previously, the onset of the light was used to create waveform averages over all trials within a session that represented 1 second windows before and after the light was illuminated. The onset of the light stimulus is represented as zero on the time scale of all ensuing figures.

The resulting mean waveform averages for control conditions are displayed in Figure 14 and the mean waveform averages for acute ethanol conditions are shown in Figure 15. In each of the dosage conditions, there is a clear and consistent timing relationship between the peaks and troughs of the average waveforms across all of the anatomical recording sites. The reliability of the synchronization across anatomical regions was assessed by performing partial correlations on the data. Partial correlations were used to measure the strength of connection between two random variables controlling for the factor of time. At each dosage condition, the three anatomical regions showed significant ($p < 0.001$) similarity with correlation coefficients ranging from 0.711 to 0.965, with 1 being a perfect correlation. The hypotheses that the dentate gyrus would correlate more strongly with the entorhinal cortex than the anterior cingulate cortex during encoding phases of the D(N)MTP task, and that this correlation would be disrupted dose-dependently by alcohol, were not consistently supported by the presently available data.
It was observed that the theta rhythms after the onset of the light stimulus appeared more synchronized and showed higher amplitude than the ongoing rhythm prior to stimulus onset. Data for all of the waveform averages at each recording site for each dose were separated into pre-stimulus and post-stimulus conditions, and compared with two-tailed paired sample t-tests. All pre-stimulus vs. post stimulus comparisons were significantly different (p < 0.05), except for the entorhinal cortex and dentate gyrus under the no injection condition and for the anterior cingulate under the 0.50 and 1.00 g/kg ethanol conditions. These results suggest that the onset of the light correlated with a stimulus-evoked enhancement of the theta rhythm in most conditions.

The mean waveform averages for each dosage condition were temporally aligned with peri-stimulus lever press event histograms to link neurophysiological data with behavioral activity (See Figures 16-19). The histogram represents the mean frequency of combined left and right bar presses. All presses prior to the onset of the light stimulus were counted as intertrial interval responses, but were not rewarded. The first press after the onset of the light was considered the animal’s response and was only rewarded if it corresponded with the location of the illuminated light cue. In all conditions, the peri-stimulus event histogram showed a distinct spike in frequency of lever presses approximately 50 milliseconds before the recorded trigger for stimulus onset.

When comparing peri-stimulus lever press event histograms, increasing doses of ethanol appeared to suppress or inhibit lever pressing behavior compared to the saline and no
injection control conditions (Figure 20). Two-tailed paired sample t-tests confirmed that 1.00 g/kg ethanol was significantly different from control conditions (p < 0.001), but 0.50 g/kg ethanol was not significantly different from the controls (p > 0.05).
Figure 14. Synchronization across Sites under Control Conditions during Encoding

Mean waveform averages over 1 second sweep before and after onset of light stimulus. Light stimulus onset is indicated by 0.0 seconds. Stimulus-evoked changes in the theta rhythm include increased amplitude and synchrony.
Figure 15. Synchronization across Recording Sites under Ethanol during Encoding

Mean waveform averages over 1 second sweep before and after onset of light stimulus. Light stimulus onset is indicated by 0.0 seconds. Stimulus-evoked changes in the theta rhythm include increased amplitude and synchrony in the 0.50 g/kg EtOH condition but not the 1.00 g/kg EtOH condition.
Figure 16. Mean Encoding Waveforms and Lever Pressing for No Injection

Mean waveform averages over 1 second sweep before and after onset of light stimulus. Light stimulus onset is indicated by 0.0 seconds. The first lever press after the light onset was considered the animal’s response, and was rewarded if it was under the illuminated light. Incorrect responses initiated a correction trial in which the same light was illuminated.
Figure 17. Mean Encoding Waveforms and Lever Pressing for Saline

Mean waveform averages over 1 second sweep before and after onset of light stimulus. Light stimulus onset is indicated by 0.0 seconds. The first lever press after the light onset was considered the animal’s response, and was rewarded if it was under the illuminated light. Incorrect responses initiated a correction trial in which the same light was illuminated.
Figure 18. Mean Encoding Waveforms and Lever Pressing for Moderate Ethanol

Mean waveform averages over 1 second sweep before and after onset of light stimulus. Light stimulus onset is indicated by 0.0 seconds. The first lever press after the light onset was considered the animal’s response, and was rewarded if it was under the illuminated light. Incorrect responses initiated a correction trial in which the same light was illuminated.
Figure 19. Mean Encoding Waveforms and Lever Pressing for High Ethanol

Mean waveform averages over 1 second sweep before and after onset of light stimulus. Light stimulus onset is indicated by 0.0 seconds. The first lever press after the light onset was considered the animal’s response, and was rewarded if it was under the illuminated light. Incorrect responses initiated a correction trial in which the same light was illuminated.
Figure 20. Comparison of Encoding Phase Lever Press Frequency by Dose

Light stimulus onset is indicated by 0.0 seconds. The first lever press after the light onset was considered the animal’s response, and was rewarded if it was under the illuminated light. Incorrect responses initiated a correction trial in which the same light was illuminated. The highest rate of overall responding was in the control conditions and 1.0 g/kg EtOH reduced the overall number of lever presses.
3.8 Effects of acute ethanol on the theta rhythm during retrieval

The onset of the auditory tone signal in the choice phase corresponded to the short-term retrieval memory stage of the behavioral task. Similar to the light stimulus onset triggering in the encoding stage, the onset of the auditory tone signal was marked as an event trigger for subsequent analyses. Contrary to the light cue which was terminated upon response, the tone cue persisted for one full second and then the first lever press after tone offset was considered the animal’s response and was rewarded only if correct according to the previously described rules of the task.

The mean waveform averages triggered by tone onset for the control conditions are presented in Figure 21 and mean waveform averages for each or the ethanol conditions are shown in Figure 22. Compared to the waveform averages triggered by encoding events, these waveforms are noticeably more variable with less alignment between peaks and troughs of the rhythm across anatomical recording sites. Partial correlation analyses confirmed that there was more variability in the tone-triggered waveforms. Correlation coefficients ranged from 0.289 (dentate gyrus with the entorhinal cortex, 1.00 g/kg EtOH condition) to 0.834 (dentate gyrus with anterior cingulate cortex, saline condition). In the saline, 0.50, and 1.00 g/kg ethanol conditions, the correlation coefficients for the dentate gyrus with the anterior cingulate are higher than the coefficients for the dentate gyrus with the entorhinal cortex. This data is congruent with the hypothesis that synchronization between the anterior cingulate cortex and the dentate gyrus of the hippocampus occurs preferentially during retrieval events.
The tone-onset-triggered waveforms were then separated into pre-stimulus and post-stimulus onset components for analysis via two-tailed paired sample t-tests. The only condition that showed significant differences between the pre-stimulus and post-stimulus waveforms was the saline condition (p < 0.05). No other conditions showed a retrieval stimulus-evoked change in the overall theta rhythm. This suggests that the stimulus-evoked enhancement of the theta rhythm observed in the encoding condition may be unique to either the light stimulus or the process of encoding.

As was done in the encoding stage, the mean waveform averages for each dosage condition were temporally aligned with peri-stimulus lever press event histograms to link neurophysiological data with behavioral activity (See Figures 23-26). None of the lever presses in the time window shown were rewarded; all were recorded as presses occurring during the fixed 5 second + 1 second tone delay interval. The tone continued for one second and the first press after the offset of the tone was considered the animal’s choice. Mean lever pressing frequency in the peri-stimulus window around the tone cue was less overall than in the window around the light cue. Furthermore, the event histograms did not show any noticeably distinct peaks of activity, immediately around the tone onset or otherwise.

In the retrieval condition as well as the encoding condition, ethanol dose-dependently suppressed the overall lever pressing behavior (See Figure 27). Two-tailed paired sample
t-tests revealed that 1.00 g/kg ethanol had significantly less lever presses than any other dosage condition (all p < 0.0001). This inhibition of lever pressing behavior was also found in the 0.50 g/kg ethanol condition (p < 0.0001) relative to saline and non-injected controls.
Figure 21. Synchronization across Sites under Control Conditions during Retrieval

Mean waveform averages over 1 second sweep before and after onset of tone stimulus. Tone stimulus onset is indicated by 0.0 seconds and continues playing for 1 second.
Figure 22. Synchronization across Recording Sites with Ethanol during Retrieval

Mean waveform averages over 1 second sweep before and after onset of tone stimulus. Tone stimulus onset is indicated by 0.0 seconds and continues playing for 1 second.
Figure 23. Mean Retrieval Waveforms and Lever Pressing for No Injection

Mean waveform averages over 1 second sweep before and after onset of tone stimulus. Tone stimulus onset is indicated by 0.0 seconds and persists for the duration of the waveform average. None of the responses were rewarded but were counted as delay responses.
Figure 24. Mean Retrieval Waveforms and Lever Pressing for Saline

Mean waveform averages over 1 second sweep before and after onset of tone stimulus. Tone stimulus onset is indicated by 0.0 seconds and persists for the duration of the waveform average. None of the responses were rewarded but were counted as delay responses.
Figure 25. Mean Retrieval Waveforms and Lever Pressing for Moderate Ethanol

Mean waveform averages over 1 second sweep before and after onset of tone stimulus. Tone stimulus onset is indicated by 0.0 seconds and persists for the duration of the waveform average. None of the responses were rewarded but were counted as delay responses.
Figure 26. Mean Retrieval Waveforms and Lever Pressing for High Ethanol

Mean waveform averages over 1 second sweep before and after onset of tone stimulus. Tone stimulus onset is indicated by 0.0 seconds and persists for the duration of the waveform average. None of the responses were rewarded but were counted as delay responses.
Figure 27. Comparison of Retrieval Phase Lever Press Frequency by Dose

Tone stimulus onset is indicated by 0.0 seconds. The tone continues for 1 second and the first lever press after the tone offset (not shown) was considered the animal’s response, and was rewarded if it was correct according to the rules of the session. Incorrect responses initiated a fixed 10 second intertrial interval. None of the lever presses shown were rewarded. The highest rate of overall responding was in the control conditions and 1.0 g/kg EtOH showed the lowest overall number of lever presses.
Chapter 4: Discussion

4.1. General discussion
The focus of this project was to ascertain the dose-dependent effects of acute ethanol on memory encoding, retrieval, and the theta rhythm. Using a novel serial rule switch paradigm of the delayed (non-)match to position operant task, we were able to train and switch animals repeatedly, determine that ethanol dose dependently impairs encoding for a new rule, decreases overall theta power, and inhibits lever pressing behavior. The validity of the novel adaptation of the D(N)MTP task, the implications of the behavioral effects of ethanol on memory encoding and retrieval, the effects of ethanol on the theta rhythm, and future directions for additional research are discussed in more detail below.

4.2 Validity of the novel serial reversal paradigm of the D(N)MTP task
Since the origination of the delayed match to position operant task by Dunnett (1985), delayed match- and non-match to position (or sample) tasks have been an important tool in the behavioral assay repertoire of many researchers interested in memory. Delayed match-and non-match tasks have been customized in a variety of ways by many researchers over time (For review, see Dudchenko, 2004). For example, these tasks have been consistently tested in rodents, non-human primates, as well as humans under a wide
variety of drug and or lesion manipulations. Some adaptations include matching or non-matching for objects, odors, or cues within an operant box.

The delayed non-and match to position paradigms are thought to be particularly dependent on the hippocampus because of the salient component of the cue to be remembered. The importance of the hippocampus to delayed non-matching tasks was emphasized by Morris (2001), “Delayed matching and non-matching to place are exquisitely sensitive to hippocampal lesions and to the selective disruption of hippocampal synaptic plasticity.” If the hippocampus is critical for performance in this task, then hippocampal activity must exhibit a representational code for task-relevant events and that when this representation is not present, is distorted, or is diminished, then there is a corresponding decrement in task performance. These questions were explored by Deadwyler and colleagues by recording hippocampal ensemble activity during a two lever spatial delayed-non-match-to-sample performance in rats very similar to the task described in the present experiment (Deadwyler et al., 1996). Results indicated that small numbers of hippocampal neurons, particularly within the CA1 and CA3 fields, could effectively represent “conjunctions” of task-relevant features. The relevant aspect of this to our interests is that the DNMTP family of tasks is relevant to the hippocampal formation, and has the potential to show subtle variations in physiological activity such as the changes in theta rhythm.
In general, this task is well-suited for studying the effects of ethanol on encoding and retrieval and memory. The key factor that makes the DNMTP family of tasks relevant to memory is that the behavioral response is controlled by events that are no longer present at the time of response execution. Variables that have been found to affect the acquisition and maintenance of performance in similar operant tasks are intertrial interval duration, sample modality/dimension, exposure time, response requirement, delay duration (van Hest & Steckler, 1996) and motivational state (Echevarria et al., 2004).

There are many advantages of this task, including: capacity for systematic modification of task variables such as intertrial intervals and delay periods, ability to study the retention of information over time, enabling the dissociation between mnemonically relevant events, having multiple trials over which to average the physiological data, controlling precise timing of stimulus onset, recording precise timing of behavioral responses, and dissociating the task into separate short-term encoding and retrieval phases (van Hest & Steckler, 1996; Robinson et al., 2000; McCartney et al., 2004). The DNMTP task also allows neurophysiological recordings from many neuroanatomical sites and there is minimal risk for large locomotive confounds. The major disadvantage of the task is that training to a stable criterion performance is more time-consuming than many other behavioral tasks that are used to assess memory function.

Although variants of the D(N)MTP tasks are commonly used in many areas of research, very few, if any, studies have been conducted in which animals are trained to switch
between both non- and match rule contingencies. In one study, rats lesioned in either the hippocampus or the prefrontal cortex were trained to switch to a non-match contingency for one round, and both lesion groups demonstrated ability to learn the new rule (Sloan et al., 1996). Adapting the task to include serial rule switches enables us to test the effects of acute ethanol on longer-term encoding and retrieval processes related to rule retention from day to day. The novel double rule switch paradigm shows great potential as a behavioral assay to assess failure to encode information by yielding improved performance during the next session. It is believed that our results indicate the validity of the D(N)MTP switching protocol, and that this task has the relatively unique potential to yield important information about the effect of experimental manipulations, particularly ethanol, on both short-term and long-term memory encoding.

4.3 Behavioral effects of ethanol

When ethanol was administered on a rule switch session, we found significant dose-dependent effects on accuracy in the session 24 hours after the injection. The likely interpretation is that ethanol dose-dependently impaired encoding of the experience of the new rule contingency on the switch day. When animals were retested on the following day, animals with higher doses of alcohol did not have the benefit of experience from the previous day and thus took longer to provide correct responses and demonstrated significantly lower overall accuracy. In the double switch condition, the ethanol-induced failure to encode the experience of the first switch day reduced interference with the original rule, and so ethanol actually improved performance when the contingency was
reverted to the original rule. These results are consistent with a large body of research showing that ethanol causes impairments in memory encoding for new information. For example, it is known that ethanol preferentially blocks memory formation without affecting previously formed memories (Wixted, 2005).

An interesting finding that emerged from the effects of ethanol on the single rule switch was that the highest performance in both the switch session and the post-switch session was found in the group that received 0.25 g/kg ethanol 10 minutes prior to the switch session. Although this performance was not significantly different from that of the control condition, it was a consistent trend throughout the data. Although it is counterintuitive that very small doses of ethanol can correlate with improvements in performance on a memory task, it is not necessarily incongruent with existing data. Similar facilitative effects in humans on recognition memory for pictures have been found in doses comparable to the low doses used in the present study (Parker et al., 1981). In a study looking at the effects of ethanol on human performance in a battery of cognitive tests, it was found that alcohol’s specific effects on memory depend on the task, the memory process needed, the method of consumption, and the ascending versus descending limb of the blood alcohol curve (Söderlund et al., 2005). Three possible explanations for memory enhancement after consuming alcohol include alcohol having a short-term stimulating effect on consolidating neural networks, an overall depressive effect impairing the ability to acquire interfering information after something has learned, or reducing retroactive interference (Tyson & Schirmuly, 1994). Facilitative effects of
low doses of ethanol were not a main hypothesis of this study, but they may be an interesting area for additional exploration in the future.

One of the other significant behavioral findings related to ethanol was a suppression of overall lever pressing behavior, for both encoding and retrieval events, at the highest dose (1.00 g/kg) of ethanol. This effect is likely due to the well-known sedative-hypnotic effects of alcohol. Overall locomotion was likely reduced, and this decrease in activity resulted in fewer lever presses.

4.4. Effects of Ethanol on theta rhythm

The overall power of the signal at the theta frequency was significantly reduced in the 1.0 g/kg Ethanol condition. This loss of energy could be indicative of the sedative hypnotic effect associated with reduced lever presses described above, or this loss of energy could be associated with memory impairments. In humans, it has been found that intracranial recordings during a memory task showed that increased power during encoding predicted subsequent successful recall (Sederberg et al., 2003). Conversely, if power is reduced by ethanol, then that reduction may correlate with subsequent failures to encode or retrieve information, which are observed under the influence of ethanol.

In waveform averages surrounding the light cue that was part of the encoding phase of the task, we observed synchronization across the anterior cingulate cortex, the dentate gyrus of the hippocampus, and the entorhinal cortex. We also observed stimulus-evoked
enhancement of the theta rhythm following the onset of the light stimulus. We did not find evidence in support of the hypothesis that the entorhinal cortex and the dentate gyrus synchronize with each other preferentially during encoding, nor did we find clear evidence of theta reset. However, we are not ready to reject those hypotheses because we believe that an increased number of subjects and improved methodology will increase our likelihood of finding these subtle variations in the ongoing theta rhythm activity.

The waveform averages surrounding the tone cue that was part of the retrieval phase of the task still showed synchronization between the anatomical recording sites, but this relationship was not as clean as the sweeps representing the encoding condition. We did find preliminary evidence in support of the hypothesis that the anterior cingulate cortex and the dentate gyrus of the hippocampus synchronize with each other preferentially during retrieval, but further exploration on this topic is still needed.

### 4.5 Future Directions

The effects of acute ethanol intoxication on memory encoding, retrieval, and the theta rhythm remain an important and interesting question. This specific line of research will continue to be pursued with methodological improvements and additional manipulations. The in vivo neurophysiological approach has the potential to allow studying effects of other substances, interactions with other waveforms, connections to models of psychopathological conditions.
Methodological improvements will improve the likelihood of seeing theta rhythm synchrony across anatomical regions and theta reset. One such methodological improvement for the future is to record field potential activity during surgery. This will allow optimization of the strength of the theta rhythm signal as the electrode is lowered into the regions of interest. Another methodological improvement for the future is to adjust the programming code for the D(N)MTP task to create finite response windows for the sample and choice phases of the task. This will allow for even more precise alignment between the behavioral events and the physiological signals. Implementing finite response windows will also improve the opportunity to look at physiological differences between correct, incorrect, and omitted trials.

For further elucidation of the effects of ethanol on memory stages, it will be necessary to inject animals at varying time points. In the studies described above, all animals were injected 10 minutes prior to a rule switch session. Futures studies will include injections 10 minutes after a pre-switch session in which behavioral criterion was reached, 10 minutes before a rule switch session, 10 minutes after completion of a rule switch session, and 10 minutes before a post-switch session. Adding these additional time points of injection will allow us to tease out the differential effects of varying doses of acute ethanol on encoding, consolidation, and retrieval of rule-related information.

It would also be interesting to use the previously described behavioral and in vivo neurophysiological methodologies to study other psychoactive compounds and see how
these compare with alcohol. Because caffeine and alcohol are increasingly consumed in conjunction with each other, it would be interesting to explore whether or not caffeine attenuates any of the memory deficits that we have found to be vulnerable to alcohol. In a rodent study involving a novel-odor habituation task, caffeine has preventative properties against ethanol-induced retrograde amnesia (Spinetta et al., 2008). Caffeine may not only be interacting with alcohol to influence subjective impressions of intoxication, but also be minimizing the typically aversive amnestic effects of alcohol, possibly by attenuating detrimental effects on neurophysiological activity associated with learning and memory processes. It would be interesting to explore the behavioral and neurophysiological differences between control conditions, alcohol only conditions, caffeine only conditions, and alcohol plus caffeine conditions. It would be hypothesized that the greatest memory deficits would occur in the highest dosage of the alcohol only condition, caffeine in conjunction with alcohol would attenuate these deficits, and caffeine alone would have minimal detrimental effect and may be facilitative.

Although the present research focuses exclusively on the theta rhythm, there are several other oscillatory patterns of activity in the brain that are of interest for complex patterns of behavior and cognition. In particular, the connections between the theta rhythm and high frequency gamma activity are of interest in connection to cognitive disorders such as schizophrenia. Changes in coherence between these different oscillatory patterns may be indicative of dysfunctional neuronal network dynamics (Jones, 2010). Patients with
schizophrenia show decreased synchrony and evoked coherence between theta and gamma frequency (Başar and Güntekin, 2008).

### 4.6 Conclusions

Our preliminary results support the validity of using serial reversals of the delayed (non-)match to position operant task to study the effects of acute ethanol on encoding, retrieval, and the theta rhythm. Our results support the hypothesis that alcohol impairs memory encoding, inhibits lever pressing behavior, and impacts the overall power of the theta rhythm. The preliminary research is inconclusive regarding theta rhythm synchrony and theta rhythm reset across the entorhinal cortex, dentate gyrus, and anterior cingulate of the hippocampus during encoding and retrieval conditions. Further research is needed to clarify the effects of ethanol on both mnemonic and neurophysiological processes.

This information could be useful in explaining deficits in learning and memory, and may eventually contribute to effective treatments. Additionally, future experiments will explain detailed effects of alcohol on cognitive functions relevant to short-term and working memory. This may yield valuable information on mnemonic contributors to the consumption of alcohol as well as behavioral and cognitive functions before, during, and after acute intoxication. Information about the acute effects of alcohol on memory could contribute to an understanding and possible prevention of injuries and fatalities associated with situations such as driving under the influence of alcohol. Furthermore, studying the acute effects of alcohol on encoding and retrieval may lead to a better
understanding of the neural structures vulnerable to chronic effects and possible
treatment benefits or prevention strategies for alcohol abuse and alcoholism.
References


