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Learning by Subtraction: Hippocampal Activity and Effects of Ethanol during the Acquisition and Performance of Response Sequences

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Abstract

Learning is believed to be reflected in the activity of the hippocampus. However, neural correlates of learning have been difficult to characterize because hippocampal activity is integrated with ongoing behavior. To address this issue, male rats ($n=5$) implanted with electrodes ($n=14$) in the CA1 subfield responded during two tasks within a single test session. In one task, subjects acquired a new 3-response sequence (acquisition), whereas in the other task, subjects completed a well-rehearsed 3-response sequence (performance). Both tasks though could be completed using an identical response topography and used the same sensory stimuli and schedule of reinforcement. More important, comparing neural patterns during sequence acquisition to those during sequence performance allows for a subtractive approach whereby activity associated with learning could potentially be dissociated from the activity associated with ongoing behavior. At sites where CA1 activity was closely associated with behavior, the patterns of activity were differentially modulated by key position and the serial position of a response within the schedule of reinforcement. Temporal shifts between peak activity and responding on particular keys also occurred during sequence acquisition, but not during sequence performance. Ethanol disrupted CA1 activity while producing rate-decreasing effects in both tasks and error-increasing effects that were more selective for sequence acquisition than sequence performance. Ethanol also produced alterations in the magnitude of modulations and temporal pattern of CA1 activity, although these effects were not selective for sequence acquisition. Similar to ethanol, hippocampal micro-stimulation decreased response rate in both tasks and selectively increased the percentage of errors during sequence acquisition, and provided a more direct demonstration of hippocampal involvement during sequence acquisition. Together, these results strongly support the notion that

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Conflicts of Interest

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Author Consent

All authors participated in the gathering of data and assembly of the manuscript, and all agree to have their names listed as authors.

ethanol disrupts sequence acquisition by disrupting hippocampal activity and that the hippocampus is necessary for the conditioned associations required for sequence acquisition.

Keywords

CA1; micro-stimulation; multi-unit activity; repeated acquisition; rat

Introduction

The process of encoding experience into long-term memory, a vital aspect of learning, is believed to be reflected in the ongoing activity of the hippocampus. Recording hippocampal neurons in animals actively engaged in a learning task has proven a powerful tool in gaining insight into the learning process. There is evidence that the hippocampus is involved with the acquisition of relevant sensory and motor stimuli that are critical for learning, including the acquisition of odor cues (Eichenbaum et al., 1989), place learning (O'Keefe, 1976), and identifying contextual associations (Muller and Kubie, 1987; Bostock et al., 1991; Markus et al., 1995). Although the hippocampus may not be necessary for all forms of learning, a large body of evidence suggests the hippocampus is critical for declarative memory (for review, see Eichenbaum et al., 1992). In contrast to procedural memory, where information about objects or situations are characterized as distinct and inflexible representations, declarative memory permits representational flexibility, where information about objects or situations are characterized by their relationship among multiple items or events that can be re-appropriated to new circumstances. Thus, the hippocampus is presumed to be necessary for complex tasks that have a predictive relationship between a condition, a response, and a consequence, such as those involved in operant tasks.

Within an operant framework, learning (i.e., acquisition) is defined as transitional behavior that is progressing towards a steady-state (Sidman, 1960). In operant procedures involving animals, such transition states may reflect a variety of situations and behaviors. For example, a transition state can be: 1) the initial acquisition of a lever-press response, 2) the change in steady state behavior that occurs when a subject is moved from one schedule of reinforcement to another, or 3) the acquisition of more complex sequences of behavior. Boren (1963) described one of the first operant techniques developed to study the acquisition of complex sequences of behavior and called it 'repeated acquisition.' In many of the procedures that use this technique, subjects are required to respond in a predetermined sequence on some number of manipulanda, such as a lever or response key, with a reinforcer delivered at the end of one or more sequence completions. Repeated acquisition occurs as the subjects are required to learn a different sequence of responses each session. Over time, both the pattern of acquisition and number of errors reach a steady state from session to session (Boren and Devine, 1968). This steady state of transition states (Thompson, 1970, 1971) can be used as a baseline for evaluating the effects of different independent variables (e.g., stress, age, or drug administration). Two major advantages of a repeated-acquisition baseline are that it avoids the confound associated with "learning to learn" (Harlow, 1949) and allows for the use of individual-subject designs in situations where the inter-subject

variability associated with grouped data might obscure the true magnitude of an effect (cf. Thompson, 1978).

The technique of repeated acquisition also lends itself to a number of other procedural variations such as the direct comparison with a performance condition (Moerschbaeche et al., 1979). In procedures that include both a repeated-acquisition component and a performance component, subjects are required to learn a different sequence of responses each session, as well as emit a sequence of responses that is the same from session to session. Because both the acquisition and performance tasks occur within the same test environment, these procedures provide a powerful behavioral control for the nonspecific effects of the independent variables under study, as each task incorporates the same sensory stimuli and response topography. For drug studies, such procedures have helped dissociate the effects on learning from nonspecific effects, such as disruptions in locomotor activity, sedation, or changes in appetite (Thompson and Moerschbaeche, 1978; Thompson and Moerschbaeche, 1979; for review, see Cohn and Paule, 1995; Cohn et al., 1996). For investigations of hippocampal activity, these procedures could overcome a major impediment to understanding the hippocampal activity associated with learning; namely, the dissociation of learning from ongoing behavior (e.g., the nose-poke and related kinematics). Several studies have reported strong links between hippocampal activity and spatial location (O'Keefe and Conway, 1978; O'Keefe and Nadel, 1978), sensory inputs (Berger et al., 1986), and intended movement, such as speed, direction, and trajectory (Muller and Kubie, 1989; Eichenbaum and Cohen, 1988). Moreover, comparing neural activity obtained during acquisition of the new task to those observed during the performance of the well-known task may allow for a subtractive approach whereby the 'signature' indicating learning can be identified.

In addition to examining hippocampal activity during the acquisition and performance components, we also examined the effects of ethanol on hippocampal activity associated with each task. Ethanol is known to produce deficits in learning in a variety of behavioral tasks, including contextual avoidance (Melia et al., 1996), spatial learning (Gibson, 1985; White et al., 1997), non-spatial learning tasks (Givens and McMahon, 1995), and repeated acquisition (Leonard et al., 2009). Ethanol-induced behavioral disruptions have been attributed largely to its effects on a variety of neurotransmitter systems, including the GABAergic, glutamatergic, serotonergic, and cholinergic systems (Criswell et al., 1993; Crews et al., 1996). Furthermore, disruptions in these neurotransmitter systems alter the excitability and selectivity of activity from pyramidal cells and interneurons within the hippocampus (Steffensen and Henriksen, 1992; Matthews et al., 1996; Ludvig et al., 1998; White and Best, 2000), as well as afferent inputs to the hippocampus (Criado et al., 1994; Henn et al., 1998; for review, see White et al., 2000). These disruptions, in turn, contribute to the disruption of other neuronal processes related to learning, such as long-term potentiation (Givens and McMahon, 1995) and theta-associated activity (Givens, 1995). Despite the evidence that ethanol disrupts learning and alters hippocampal function, extraordinarily little is known about how it disrupts the activity associated with learning apart from the activity associated with ongoing behavior.

To date, we have identified sites within the CA1 that are differentially modulated by aspects of the procedure, such as the key position and schedule of reinforcement. Second, at those sites, acquisition behavior can be distinguished from performance behavior by the latency between activity and a response, and by the temporal shift in activity as acquisition of the response sequence proceeds. Third, ethanol disrupts CA1 activity and more potently disrupts acquisition behavior than performance behavior. Finally, high-frequency micro-stimulation of hippocampal sites, but not sites outside the hippocampus, produced larger disruptions of acquisition behavior than performance behavior. This last observation makes a powerful argument that the hippocampus is necessary for learning within this operant technique.

Materials and Methods

Subjects

All subjects and procedures were maintained in accordance with the Institutional Animal Care and Use Committee, Louisiana State University Health Sciences Center, and in compliance with recommendations from the National Research Council in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Six adult male Long-Evans hooded rats were purchased from a commercial vendor (Harlan Sprague Dawley, Indianapolis, IN, USA) and served as subjects (1285, 1337, 1339, 1340, 1341, and 1365). All subjects were maintained at 90% of their free-feeding weight (360–410 grams) throughout the course of the study. Subject body weight was maintained by the 45-mg pellets (Purina Mills TestDiet, Richmond, VA, USA) earned during the experimental sessions, and if necessary, rodent chow provided after the session. Subjects were housed individually on a 14L:10D light-dark cycle, and water was freely available throughout the study.

Behavioral Apparatus

A modular test chamber configured for rodents was fabricated from Plexi-glass (13 X 10 X 12 in). Located on the front panel of the chamber was a house light, electromechanical relay, pellet trough (centered 5.5 cm above the chamber floor and protruding 4.5 cm into the test chamber), and three response keys (8 cm apart, center to center, and 14.5 cm above the chamber floor). Each response key contained three Sylvania 28ESB indicator lamps, covered with a green, yellow, and red cap, which could be transilluminated to simultaneously present one color across all three keys. The test chamber was enclosed within a sound-attenuating cubicle (Coulbourn Instruments, Whitehall, PA), and white noise was used to mask extraneous sounds. The test chamber was operated by a nearby computer programmed with MED-PC/Medstate Notation, Version IV, and SoftCR software for Windows (MED Associated, Inc. and Thomas A. Tatham, St. Albans, VT, USA).

Training of Repeated Acquisition

Training of the procedure began after magazine training, shaping of the response (nose-poke) with food pellet delivery, and reinforcement for responding on each of the three response keys. After sessions in which subjects reliably responded on each key, a training step different from one described previously (Winsauer et al., 1995) was used to introduce subjects to the stimuli of the 3-response sequence over three consecutive days. On the first

day, subjects were allowed to respond on any key when all three keys were transilluminated yellow, the terminal stimulus of the response sequence. On the second and third day, subjects were still allowed to respond on any key, but the sessions began with the stimuli for the second (red) or first (green) response of the 3-response sequence, respectively. Each correct response activated the electromechanical relay, producing an audible click, and changed the key color. Completion of the 3-response sequence resulted in the delivery of a food pellet. Following reinforcement, the key lights were illuminated again. Key color presentation always followed a set order (i.e., green, red, yellow), and did not change between sessions.

Repeated-acquisition training began by restricting the ordering of responses to each of the colored stimuli. Specifically, the house light remained off and the three response keys were illuminated green for the first response in the sequence. A response on the correct key in the presence of the green stimuli activated the relay and changed the color of the key lights to red. A correct response in the presence of the red stimuli activated the relay again and changed the key lights to yellow. A correct response in the presence of yellow stimuli activated the relay, extinguished the stimulus lights for 0.4 seconds, and then reset the sequence by illuminating the keys with the green stimuli. Thus, the subject's task for a given session was to acquire the correct response in the presence of each of the three stimuli (e.g., keys green, L correct; keys red, C correct; keys yellow, R correct). Correct responses associated with a particular color did not change within a session. Incorrect responses or errors resulted in a 5-second timeout period, but did not reset the three-response sequence (i.e., the position of the correct response and color of the stimuli were the same before and after a timeout period). Responding was initially maintained by food pellet delivery after each completion of the 3-response sequence (i.e., a second-order, fixed-ratio one schedule). During the final step of preliminary training, every three completions of the sequence resulted in the delivery of a food pellet (i.e., a second-order fixed-ratio 3 schedule).

A steady state of repeated acquisition was established by changing the sequence from session to session. An example of sequences from five consecutive sessions was: C-L-R, L-R-C, C-R-L, R-L-C, and L-C-R. Across sessions, sequences were restricted on their ordering and selected to be equivalent in several ways. For example, each sequence was scheduled with equal frequency, and consecutive correct responses within a sequence were scheduled on different keys. However, on occasion, a correct sequence position for a given color was the same for two consecutive sessions (e.g., L-R-C and C-R-L).

Repeated acquisition of the three-response sequences continued until response rates varied by less than 20 responses per minute of the mean rate for the previous 10 sessions and less than 10 percent of the mean percentage of errors. At this point, a second task was added to the procedure. In this two-component procedure, subjects learned a different three-response sequence (i.e., the acquisition component) and emitted a well-rehearsed three-response sequence (i.e., the performance component) during each session.

During the performance components of the procedure, the house light and response keys were illuminated, and the sequence remained the same (R-C-L) for all test sessions. In all other aspects, the performance task was intended to mimic the acquisition task (e.g., color of

the stimuli for each response in the sequence, 3 sequence completions for food delivery, and 5-second timeout periods). Sessions typically began with an acquisition component and alternated with a performance component after 40 reinforcers or 20 minutes, whichever occurred first. The sequences for the acquisition and performance components were repeated throughout all components within that session. During the period of micro-stimulation testing, however, the first component of the session alternated from day to day. All sessions terminated after 200 reinforcers or 80 minutes, whichever occurred first. Throughout the experiment, sessions were generally conducted 5–6 days per week.

Alcohol

Ethanol (Decon Labs, Inc., King of Prussia, PA, USA) was diluted to 20% (v/v) using normal saline (0.9% NaCl/L). Fifteen minutes prior to beginning a test session, subjects received an intraperitoneal (i.p.) injection of ethanol in doses of 0.56, 0.75, 1.00, or 1.33 g/kg. Saline administration in similar injection volumes served as a control for the effects of ethanol. Doses of ethanol were administered in a mixed order, and each dose was administered at least twice. To avoid the development of tolerance, ethanol was only administered twice per week and responding was allowed to return to baseline levels before administration of the next dose.

Blood Ethanol Concentration

Blood was collected from three subjects at two time points, 30 minutes following ethanol administration or 5 minutes following the end of the test session for those sessions that lasted 80 minutes. Due to the disruption of testing for blood at the early time point, data from these sessions were not included in the behavioral and electrophysiological analysis. Blood ethanol concentration was analyzed using a Blood Alcohol Analyzer (Model GM7; Analox Instruments, Lunenburg, MA, USA), and each sample was analyzed at least twice with the mean value expressed in mg/dL.

Surgery

Electrode arrays were implanted when the aforementioned stability criteria were met for responding in each component. Subjects were surgically implanted with a fixed recording array consisting of 3–4 stereoelectrodes aimed at the CA1 subfield of the dorsal hippocampus using aseptic techniques. Stereoelectrodes were constructed by twisting together two pieces of Teflon coated 0.001-in platinum/iridium wire, as described by McNaughton et al. (1983), with typical impedances of 0.3–0.4 Mohm at 1 KHz. Subjects were anesthetized using a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg), and placed into a stereotaxic alignment system using blunt ear bars (Kopf Instruments, Inc., Tujunga, CA, USA). A small incision was made along the top of the head, and the skin and overlying fascia were retracted to reveal the top of the skull. Next, two pairs of holes were drilled (coordinates: 4.2 mm anterior to lambda and 2.5 mm lateral of the midline, and 3.2 mm anterior to lambda and 3 mm lateral of the midline, bilaterally). Cannulae, constructed from 3-mm lengths of 32-G stainless-steel tubing, were then lowered 1 mm into the brain and cemented to the skull with dental acrylic. To reinforce the bond with the skull, two 0–80 stainless-steel support screws were inserted bilaterally near the caudal end of the skull. A third 0–80 stainless-steel screw, which was in contact with the CSF, was inserted near the

rostral end of the skull and had bare 0.005-in Pt/Ir wire attached, and served as ground for the electrode array.

Using physiological guidance, stereoelectrodes were driven to target by stepping in 50–100 μm increments to reach the CA1 subfield. Each stereoelectrode was cemented in place, and the wire leads soldered to a connector strip. The entire array and bottom of the connector were then secured to the support screws using dental acrylic, and the wound on the top of the scalp was closed using surgical staples. Post-operative analgesic was administered (buprenorphine 1 mg/kg, every 8 hr) over the next 48 hr, and the scalp wound was treated periodically with antibiotic ointment. Following a 10-day recovery period, subjects resumed responding under the two-component procedure.

Recording Technique

Neural activity and behavioral responses were recorded from one subject at a time, and multiple subjects were tested serially throughout the day. During testing, the connector strip on the electrode array was connected to a head stage (Model HST/8o50-GX-xR; Plexon, Inc., Dallas, TX, USA) with a high-impedance unity gain follower, which was in turn plugged into a pre-amplifier (PBX Preamplifier, Plexon, Inc., Dallas, TX, USA). The cable connecting the head stage to the amplifier was highly flexible and allowed subjects to move freely in the testing environment. Recordings were amplified (1,000 X), filtered (0.001–10 KHz, 12 dB/octave), passed to an A/D converter, and digitalized at 25 KHz (Model NI USB-6212; National Instruments, Austin, TX, USA) for offline analysis. Changes in the input and output signals from the operant chamber were passed through the same A/D converter used to gather neural activity to synchronize neural activity and behavioral responses. Data was acquired using a computer programmed with LabView software (NIDAQ, National Instruments, Austin, TX, USA) and placed into a single data file.

Micro-stimulation

Following completion of recording sessions and ethanol administration, high-frequency micro-stimulations were conducted using one stereoelectrode from the recording array. Constant voltage pulses were generated using an electrical stimulator (Model S88; Grass Technologies, Natus Medical Inc., San Carlos, CA, USA) before being passed to a stimulus isolation unit, and finally to the stereoelectrode. Current intensity was restricted using a series of resistors and monitored using an ammeter. Monophasic pulses (100 Hz, 0.2 msec/pulse) were delivered using a single stereotrode pair, and pulses were delivered in 10-second trains every 5 minutes during the first half of a test session. Stimulations of different intensities (1–30 μA) were administered in a mixed order twice per week (most intensities were administered at least twice) and responding was allowed to return to baseline levels before the next stimulation.

Behavioral Measures

Response rate and percent errors served as dependent measures for acquisition and performance behavior. The percentage of errors was not included in the analyses when the response rate was less than 5 responses per minute or there were less than 10 responses emitted in a session. Both dependent measures were analyzed using a two-way repeated-

measures ANOVA, with ethanol dose and behavioral component serving as factors (SigmaPlot Software, SYSTAT Software, Inc., Point Richmond, CA). In addition, significant interactions were followed by one-way repeated measures ANOVA tests and Holm-Sidak post hoc tests. The within-session pattern of errors was also plotted for each task of the procedure. For this analysis, errors were grouped into bins, and each bin contained the cumulative number of errors that occurred during 60 consecutive responses (i.e., errors/60 responses). For micro-stimulation, where individual subject data were presented, an effect was defined as a mean intensity that fell outside of the control range.

Spike Extraction

Neural activity was analyzed using custom software routines written using MatLab (Version 2012a; Mathworks, Inc., Natick, MA, USA). Activity was discriminated by an algorithm that isolated spike events (2–5 KHz) whose amplitude was greater than 2.5 standard deviations above the noise (e.g., Figure 1, Panel A). This yielded activity at each site averaging ~10–200 Hz. This procedure was repeated for each stereoelectrode site to yield neural records. Based on these characteristics, spikes from multiple units (e.g., Figure 1, Panels B and C) were time-locked, summed in 1-msec bins, and stored on a separate data file.

Neural Measures

Neural activity and subject behavior in each behavioral component were compared along with discrete time intervals surrounding particular behavioral events, such as correct responses, errors, reinforcements, and periods between responding. For a more detailed analysis of neural activity and particular behavioral events, a 1–2 second interval surrounding a behavioral event was used (i.e., 500 to 1000 msec before and after the event). This interval duration was chosen to minimize overlap between separate behavioral events (e.g., two consecutive responses).

Spike Frequency

Spike frequency was determined by dividing the total number of spike events by the time within a component and expressed as spikes per second or hertz (Hz). Changes in spike frequency produced by ethanol (0.56–1.33 g/kg) at each recording site were examined, and an effective dosage for an individual subject was considered to be those doses with a mean frequency outside the control range.

Gaussian Filtered Activity

Spikes were assigned to 5-msec bins and convolved with a Gaussian filter ($\sigma = 50$ msec). Next, Gaussian filtered activity was examined across the test session and during discrete intervals of time surrounding individual behavioral events (e.g., ± 500 msec surrounding correct responses at the right key). Activity from multiple behavioral events (e.g., correct responses for an entire component) was then combined to create an averaged probability function surrounding a given behavioral event.

Modulations in activity were considered significant when 20 msec or more of activity were greater than or less than 3 standard deviations from the local mean. Such periods of

modulation were identified by constructing confidence intervals. Confidence intervals were created by summing 1-msec bins of activity from a given recording site, synchronizing the bins to an event of interest (e.g., right key response), and averaging those bins into 20-msec bins to construct a histogram. Next, to identify signals associated with these histograms, the original 1-msec histograms were shuffled in 1000 simulations to obtain a mean of activity and the related standard deviations of those shuffles, similar to Bootstrap methods (Efron and Tibshirani, 1993). Shuffling 1000 times produced a distribution well described by a Gaussian, such that 6 stdu's (± 3 standard deviations) encompassed greater than 99% of the distribution. The observed 'place' of Gaussian filtered activity within the distribution of the shuffled results (i.e., within the confidence intervals) allowed for a direct interpretation of probability. Reported probabilities of < 0.002 (2-tailed) indicate the observed result never occurred among the shuffled simulations.

Modulations in Activity

Significant modulations in activity, that is, the area of the Gaussian filtered activity falling outside the confidence intervals created from the shuffled results, were compared between behavioral events (e.g., right key response vs. center key response) and tasks (acquisition and performance) using a similar shuffling procedure. In this procedure, Gaussian filtered activity in the 1-second interval surrounding particular behavioral events (e.g., all correct responses on the right key during the first acquisition component) was pooled and then randomized. Next, five events were pulled from the randomized pool and combined to create an averaged sample function. The area of modulations from this sample function was then compared to other averaged sample functions (e.g., right key response vs. center key response). The area of the modulation from the sample function that best fit the area of modulation from the probability function created from an entire sample pool won the comparison (e.g., the area of modulation from a right-key sample function better represented the area of modulation from the probability function created from the entire pool of right-key responses than did a center-key sample function). This shuffle procedure was conducted in 1000 simulations to determine a p-value (e.g., if the right key won the comparison between a right key and center key 900 times, the associated p-value would be $p=0.01$).

Histology

Upon completion of the study, animals were deeply anesthetized with sodium pentobarbital (100–160 mg/kg, i.p.) and perfused with a formalin solution diluted to 10% (v/v) using normal saline. Whole brains were then fixed for several days in the formalin solution. Brains were blocked and mounted on the stage of a microtome (Model 860; American Optical Corp., Buffalo, NY, USA) before being frozen and sliced in 40-um coronal sections. Slices were mounted on gelatin-coated slides, dried, and stained for Nissl substance using a Cresyl Violet staining solution. Finally, slides were dehydrated and cover-slipped for microscopic inspection (e.g., Figure 1, Panel D).

Results

CA1 Activity is Modulated by Behavior

Figure 2 (Panel A) shows a remarkable cadence in CA1 activity as the subject (1285, site 1) worked nearly errorlessly across a 10-minute time span. A more detailed section of this activity (Panel B) shows that during bouts of responding activity increased and appears phasically linked to responding (circles and triangles above the activity indicate correct responses and reinforcements, respectively). In addition, the first response of each bout typically had the greatest burst of activity. A peak and trough (6–8 sec) in activity was also associated with reinforcements and the typical ('pre-ratio') pause that followed reinforcement. Panel C shows the largest increase in activity was aligned with the first response of each bout (0 sec), with a peak occurring ~0.2 seconds prior to responding. This peak was not an artifact due to response alignment (results from synchronizing activity to other responses are not shown). Panel D shows a peak associated with reinforcement (0 sec) and the subsequent depression of activity associated with the pause in responding. For this and subsequent figures, shaded regions indicate significant increases or decreases in activity (greater than or less than 3 s.d.'s above the shuffled mean; $p < 0.002$, 2-tailed).

Analysis of the activity at sites closely associated with response bouts indicated the magnitude of modulations (shaded region) differed depending on key position. For example, mean activity surrounding correct responses at the site depicted in Figure 2 was similar in both the first acquisition and performance components, but differed by position. Figure 3 shows the mean activity associated with correct responses (0 sec) at each of the three key positions (left, center, right) during the initial acquisition and performance components. Activity associated with left- and right-key responses was characterized by a peak in activity just prior to the response, whereas activity associated with center-key responses was characterized by a trough prior to the response.

The magnitude of the modulations (shaded region) associated with correct responses also differed depending on the serial position of that response within the schedule of reinforcement. Figure 4 shows activity surrounding correct responses on the right key (0 sec), when this key position was the first correct response for both the acquisition and performance components. The panels show activity surrounding the first, fourth, and seventh responses, which represented the first response of the first (top), second (middle), and third (bottom) sequence, respectively. Activity was similar for both the acquisition and performance tasks; however, activity preceding the first response was the largest relative to the fourth ($p < 0.001$ in acquisition, $p < 0.001$ in performance) and seventh responses ($p = 0.007$ in acquisition, $p < 0.001$ in performance). There was no significant difference in activity associated with the fourth and seventh responses ($p = 0.96$ in acquisition, $p = 1$ in performance).

In the time immediately surrounding a response, both correct and incorrect responses produced similar signatures of CA1 activity, displaying similar modulations depending on the key position and serial position of a response within the schedule of reinforcement (not shown). Incorrect responses were only differentiated from correct responses by the increase in activity that occurred at some sites during the 5-second timeout period following each

incorrect response (e.g., Figure 10, middle trace). Across CA1 sites, activity and behavior were heterogeneous during timeouts (superstitious responding and exploration are common during timeouts), making discrete comparisons difficult.

Although activity associated with particular responses was similar across acquisition and performance behaviors, the latency between peak activity and correct responses could shift during the acquisition task, but not the performance task, providing a potential signature of learning. The upper panels of Figure 5 show the accumulation of activity that occurred for 15 correct responses at the beginning of each component. In this case, the correct response was the seventh response in the schedule of reinforcement and it was emitted on the left key. The bottom panels show the last 15 occasions that this response was emitted during the first acquisition and performance components. In each panel, the activity associated with the first response of the 15 is on the bottom, and this activity increased with subsequent responses.

During early acquisition for example (top left panel), summing the activity for the first 6 responses created a peak in activity at -230 msec. This peak begins to shift to the right, however, as the next 9 responses are summed. For clarity, a vertical line has been inserted at -200 msec to reflect the cumulative peak for all 15 responses. This activity contrasted with that obtained near the end of the same acquisition component (bottom left panel). Again, the peak activity was summed over the 15 responses, but there was no difference from the cumulative peak at -110 msec. These data indicated that peak activity shifted 90 msec closer to the response as the subject acquired the sequence, and that activity associated with responses in performance was nearly identical during early and late sampling periods (top and bottom right panels, respectively), with a peak in activity at -110 msec. Further, the peak in activity that occurred late in acquisition was temporally similar to that for the peak in both early and late performance.

Figures 2–5 highlight the types of modulations observed among CA1 sites. Of the 14 sites analyzed, not all were modulated, and among those modulated, not all were as robustly modulated as shown in these examples. Nonetheless, 12 sites showed significant modulations in activity ($p < 0.002$, two-tailed), with similar effects to those in the examples. Table 1 summarizes whether activity was modulated (increased or decreased) by key position (left, center, or right), serial position of a response within the schedule of reinforcement, or a shift during response bouts relative to the pauses in responding.

Ethanol Alters CA1 Activity and Behavior

Following saline administration (shaded region of Figure 6), the mean response rate ranged from 30.2–36.4 responses per minute during acquisition (top left panel) and from 31.4–34.1 responses per minute during performance (top right panel). Percent errors were typically higher in acquisition (bottom left panel) than in performance (bottom right panel), and a notable decrease in acquisition errors occurred during the session. This within-session error reduction, which defines sequence acquisition for this procedure, was most evident between the first and second acquisition components (ACQ1, $17.37 \pm 1.76\%$; ACQ2, $11.96 \pm 1.48\%$; ACQ3, $13.04 \pm 2.11\%$). In contrast, percent errors during performance remained relatively stable across components (PERF1, $5.64 \pm 1.62\%$; PERF2, $6.82 \pm 1.67\%$).

Ethanol (0.56–1.33 g/kg) significantly decreased response rate for all subjects in both the acquisition ($F(4,57)=7.41$, $p<0.001$) and performance ($F(4,40)=3.253$, $p=0.021$) components after 1 g/kg ($p=0.04$ in acquisition, $p=0.041$ in performance) and 1.33 g/kg ($p<0.001$ in acquisition, $p=0.025$ in performance). Ethanol also uniformly increased percent errors during the first acquisition component (ACQ1) after all doses of ethanol, but had no significant effect on performance errors. This selective effect of ethanol on acquisition errors was indicated by a main effect of component ($F(2,52)=17.225$, $p<0.001$). In addition, 1.33 g/kg of ethanol significantly increased percent errors during all three acquisition components. This was indicated by a main effect of dose ($F(4,52)=3.13$, $p=0.022$). There were no significant main effects and no interaction (component $\times$ dose) for percent errors in the performance components.

As shown by the shaded region in Figure 7 (top panel), after saline administration (shaded region), cumulative errors in the acquisition component rapidly increased at the beginning of the session and then asymptoted during the session as fewer errors occurred. In contrast, the slope of the cumulative-error curve after ethanol administration increased more linearly, indicating that there was less within-session error reduction. This was particularly evident after 1.33 g/kg, which not only increased errors across all acquisition components, but also markedly decreased response rate and overall number of responses emitted.

During the performance component (bottom panel), total errors emitted after both saline and ethanol administration was always smaller than in the acquisition components. In addition, ethanol was less disruptive to cumulative performance errors than acquisition errors. This was indicated by the fact that only 1.33 g/kg increased performance errors above the control range throughout the session. As in acquisition, 1.33 g/kg also decreased the total number of responses emitted.

Ethanol administration also dose-dependently increased blood ethanol concentration (B.E.C.), and it was higher during the first 15 minutes of the session than at the end of the session (Figure 8). Overall, the magnitude of behavioral disruption also corresponded with the B.E.C., and the recovery of both response rate and response accuracy coincided with the decrease in B.E.C. that occurred toward the end of the session.

Figure 9 shows the within-session pattern of responding and the concomitant CA1 activity for one subject after saline administration and two intermediate doses of ethanol (0.75 and 1 g/kg). The pattern after saline (top record) was comparable to each subject's daily, non-injection or baseline, response pattern. During these sessions, subjects typically acquired the three-response sequence during the initial acquisition component shortly after the session began. A large number of errors occurred during the first 5–10 minutes of this component, but responding quickly transitioned to a pattern where fewer errors occurred and a large number of consecutive errorless sequences were emitted (as indicated by the shift in slope from shallow to steep). After the sequence was acquired, the pattern of responding in the acquisition component was similar to the pattern of responding in the performance component, with few errors and a large number of consecutive errorless sequences. Here, each component also terminated after 40 reinforcements rather than time (20 minutes). Associations of CA1 activity and behavior were heterogeneous in that three distinct

frequencies of activity were observed at the three recording sites. For sites 1 to 3, the average activity was 50, 8, and 66 Hz, respectively (vertical calibration = 20 Hz), and the pattern of activity appeared relatively stable at each site across the session.

The format of the middle (0.75 g/kg) and bottom (1 g/kg) records is identical to those in the top record. Note that the records are longer because the subject was unable to obtain the maximum number of reinforcers within the 80-minute session. Unlike saline administration, 0.75 g/kg of ethanol produced a relatively selective disruption of responding in the acquisition components compared with performance components. This was most evident from the initial pause in responding at the beginning the session, and from numerous smaller pauses after responding commenced; there was also a notable increase in errors during the subsequent acquisition components (indicated by the shallow slope and numerous errors). Despite the disruption in acquisition, subjects responded at a high rate with few errors during the performance components. Note that each performance component terminated prior to the maximum component duration. This dose also uniformly increased the activity at all three sites, irrespective of the baseline level of activity (i.e., increasing activity from 50 to 72 Hz at site 1, 8 to 18 Hz at site 2, and 66 to 99 Hz at site 3).

The 1-g/kg dose of EtOH (bottom row) virtually eliminated responding during the first 38 minutes of the session, a period that encompassed all of the first acquisition component and almost all of the first performance component. In addition, both the pattern of sequence acquisition and CA1 activity were disrupted when responding resumed. For example, the transition to relatively errorless responding (i.e., sequence acquisition) was slowed and CA1 activity at each site remained elevated. Conversely, responding during the second performance component was similar to that during control conditions, with few errors and a large number of consecutive errorless sequences. The subject was also able to obtain the maximum number of reinforcers during this component. Interestingly, although elevated, the activity at all three sites was notably different during the period of inactivity than during the period in which responding occurred. Site 1 shows a significant increase in activity throughout the session and shows a cadence associated with responding during the second half of the session. Site 2 shows patterns of activity similar to control conditions during periods of inactivity, but activity increased in association with responding. Activity at site 3 increased relative to saline, but showed little modulation associated with responding.

Figure 10 shows 90-second epochs of CA1 activity while a subject (1339) was in a plastic container (14 X 10 X 15 in) containing woodchip bedding (i.e., a non-contextual environment), and in the experimental chamber after either saline or ethanol (1.33 g/kg) administration. Spike frequencies associated with exploring the non-contextual environment (top trace) were similar to spike frequencies following saline administration (middle trace) during the first acquisition component, but this recording lacked the distinct modulations of activity surrounding behaviorally-contingent responding. Following saline, activity showed a clear modulation during the 5-second timeout period following an error (black squares), and these modulations were considerably greater than anything observed in the 'spontaneous' trace in the non-contextual recording. By comparison, activity during bouts of responding (circles and squares) showed little modulation and was similar to the non-contextual recordings. Although 1.33 g/kg ethanol (bottom trace) eliminated responding during this 90-

second epoch, spike frequency was increased above 20 Hz and there were fewer modulations compared to saline administration. Together, these tracings show that CA1 activity associated with contingent responding differed from both the activity associated with non-contingent exploration, and the activity following ethanol administration.

Entries in Table 2 reflect the mean frequency observed at each recording site during the first 20 minutes of a test session and do not attempt to account for modulations associated with bouts of responding or periods between response bouts. During control conditions, spike frequencies at each recording site varied both between individual subjects and across sites. Ethanol administration produced heterogeneous changes in spike frequencies across CA1 sites. Eight sites showed an increase in spike frequency, one site decreased, and the remaining sites showed little or no change. An effect in this case was defined as mean activity that was outside the control range for each subject.

Ethanol also dose-dependently disrupted modulations in activity associated with correct key positions in the acquisition (Figure 11, top) and performance (bottom) components. For example, although the overall pattern of activity was preserved for correct left-key responses, increasing doses of ethanol increased the duration of the modulation and shifted the peak. Following saline or 0.56 g/kg of EtOH a modest peak was evident in the acquisition component ~170 msec prior to the response. In contrast, following 0.75 g/kg of EtOH, the modulation was significantly larger ($p = 0.002$ vs. saline) and the peak in activity occurred at -210 msec. After 1 g/kg EtOH, the modulation was even larger ($p < 0.001$ vs. saline) and the peak in activity occurred earlier, -360 msec. The effects were more subtle in the performance component, but the same pattern of disruption was observed. Whether this difference was due to differences in the complexity of the task, or simply, the decrease in BEC is unknown, and could not be determined statistically because performance components were always scheduled after acquisition components at this point in the study.

As mentioned previously, ethanol did not produce these types of systematic modulations at all sites. Following ethanol administration, 10 sites showed an increase in the magnitude of modulations in activity during bouts of responding, while four sites showed a decrease. Distinct alterations in epochs between bouts of responding were also evident, with five sites showing an increase in magnitude of modulation and nine sites showing a decrease.

Hippocampal Micro-stimulation Selectively Disrupts Acquisition

In the two micro-stimulation sessions depicted for subject 1339, which began with an acquisition and performance component respectively (Figure 12, middle and bottom records), micro-stimulation decreased response rate and increased errors compared with responding during baseline sessions. Note also that the micro-stimulations were more disruptive for acquisition behavior than performance behavior. This was evident from the large number of errors emitted and the delay in within-session error reduction during the first acquisition component. With respect to the performance component, more pausing and a larger number of errors were emitted after micro-stimulations than under baseline conditions, but long bouts of correct responding were still evident. Following the cessation of micro-stimulation, subject response rate recovered and a typical pattern of responding resumed.

Compared to baseline responding, increasing hippocampal micro-stimulation (1–30 uA) decreased response rate in an intensity-dependent manner for subjects 1339 and 1365 (top graphs). In subject 1339, for example, decreases occurred with stimulations greater than 1 uA in acquisition and 3 uA in performance. With respect to response accuracy (bottom graphs), hippocampal micro-stimulations increased percent errors to a greater extent in the acquisition component than the performance component in both subjects, albeit at different intensities. The lowest effective intensity on percent errors was 5 uA in subject 1339, and 3 uA in subject 1365. The increased sensitivity of responding in the acquisition component compared to the performance component was also evident in the magnitude of the error-increasing effects (e.g., at 30 uA for subject 1339, errors increased 17.6% in acquisition, but only 3.02% in performance).

Unlike micro-stimulation of the hippocampus, stimulation of the ventral orbital cortex in subject 1339 (filled and unfilled squares) did not affect percent errors in either component. Further, micro-stimulation of the ventral orbital cortex only decreased response rate at the highest current (30uA).

As shown in Figure 14, increasing micro-stimulation intensity in the hippocampus displaced the cumulative-error curves upwards, indicating that sequence acquisition occurred more slowly compared to responding without stimulation (shaded area). This was particularly evident after the 30 uA current for subject 1339 and 3 uA current for subject 1365, which increased errors throughout the acquisition component and markedly decreased response rate and overall number of responses emitted. This contrasted with the effects on errors in the performance component where micro-stimulation was less disruptive for both subjects. For example, current intensities greater than 15 uA were required to increase performance errors above the control range for large portions of the session for subject 1339.

Discussion

The results of the present study indicated that CA1 activity was differentially modulated by key position and serial position of a response within the schedule of reinforcement. In addition, CA1 activity shift temporally during the acquisition of a response sequence, whereas activity remained relatively stable during the completion of the performance sequence. Ethanol administration produced dose-dependent rate-decreasing and error-increasing effects in both the acquisition and performance components in all subjects, and these disruptions were closely associated with disruptions in CA1 activity. Similar to ethanol, hippocampal micro-stimulation produced rate-decreasing and error-increasing effects that were more selective for responding in the acquisition component than the performance component. The involvement of the hippocampus in ethanol's disruptive effects was also suggested by micro-stimulation of a site in the ventral orbital cortex, which produced only small decreases in response rate and did not affect response accuracy in either component.

The activation of hippocampal circuitry is known to have striking task-related functional correlations, and across different sites to encode nearly every aspect of an experience (Ergorul and Eichenbaum, 2009), including spatial location (O'Keefe and Dostrovsky,

1971), intended movement (Eichenbaum et al., 1986), and relational cues (O'Keefe and Speakman, 1987; Wiener et al., 1989). In the present study, the observation that some CA1 activity was differentially modulated by key position and serial position of a response within the schedule of reinforcement is not surprising (Ranck, 1973). Unique multi-unit signatures representing at least one key position (center) was identified from several recording sites (e.g., Figure 3). A multi-unit signature representing the first serial position in the sequence was also identified and the magnitude of this modulations was the furthest in proximity to reinforcement; that is, as subjects advanced through the response sequences (e.g., Figure 4). These results are consistent with other studies indicating that unit activity can reflect specific aspects of the environment (Muller and Kubie, 1987) and can display sensitivity to multiple aspects of the same environment (for review, see Eichenbaum and Cohen, 1988). Certainly, research from Eichenbaum and colleagues have shown that rats use a combination of spatial and contextual cues to distinguish, as well as bind, information of what, where, and when events occur (Ergorul and Eichenbaum, 2009; Eichenbaum and Fortin, 2009).

Differential modulation of CA1 activity by key position and serial position of a response provides strong evidence that hippocampal activity reflects aspects of the task apart from the behavioral (i.e., kinematics and sensory stimuli associated with responding) and attentional requirements. For example, if animals were only learning different, sequence-specific motor patterns (kinematics), identical activity signatures for particular key positions would seem unlikely. Moreover, a depression in activity occurred for correct center-key responses (Figure 3) regardless of the position of that response within the different sequences (i.e., CRL, LCR, RCL, LRC, etc.) in the acquisition component. This suggests that the activity was the same whether that key position was approached from the right or the left, and irrespective of whether it was the first, middle, or last response in a motor sequence. Furthermore, the likelihood that a depression in activity would be reflective of a motor movement 500 msec prior to a microswitch closure seems small given that there were no obvious visual differences in the topography of the responses at each key position and all of the subjects were initially trained to nose poke similarly.

The fact that responding in both acquisition and performance components had the same signature also indicates that the activity contained elements that were independent of attentional demand. If CA1 activity was only dependent on attentional demands, the activity during the acquisition component should have been be markedly different from that during the performance component where learning is not required. The serial position of a response within the schedule of reinforcement may also serve as an excellent example of how neural activity within the hippocampus can encode information regarding the demands of the task, even when ongoing behavior and spatial and temporal information are taken into account (Wood et al., 2000; Howard and Eichenbaum, 2015). As shown by Howard et al. (2014), spatial and temporal computation by the hippocampus cannot be fully accounted for by the integration of immediately available sensory information, movement, or velocity.

In the time immediately surrounding a response, CA1 activity was similar for correct and incorrect responses, and similar modulations occurred depending on the key position and serial position of a response. Incorrect responses were most easily differentiated from correct responses by the increase in activity that occurred at some sites during the 5-second

timeout period following each incorrect response (e.g., Figure 10, middle trace). These modulations may reflect changes in stimuli (all stimulus lights are extinguished) or motor activity (superstitious responding or exploration during timeouts). CA1 activity and subject behavior during timeouts was heterogeneous across subjects, making discrete comparisons difficult. These findings are similar to a study by Deadwyler et al. (1996), who found that there were no differences in CA1-CA3 ensemble activity during the non-match phase for correct and incorrect responses in a nonmatch-to-sample procedure. The authors attributed the incorrect responses to improper encoding during the sample phase, which is one possible explanation for the current study. However, the authors examined only a short duration of activity surrounding a response, making comparisons between studies difficult. In the current study, incorrect responses may have resulted from the retrieval of a previously established pattern of activity. For example, a subject may emit a response from a previously acquired sequence. Incorrect retrieval would also seem more likely for information under weak stimulus control (i.e., acquisition) compared to information under strong stimulus control (i.e., performance) (Thompson, 1978).

The utilization of a subtractive approach revealed flexibility among the temporal pattern of activity during sequence acquisition. The temporal shift in peak activity that occurred during the acquisition component was unique to sequence acquisition (e.g., Figure 5, left panels). This shift in latency between the activity and a response was not observed during the performance component, as this latency was largely the same at early and late time points in the session (e.g., Figure 5, right panels). Tort et al. (2009) reported similar shifts in hippocampal activity in rats learning item-context associations. In their study, several CA3 local field potentials were examined for associations while rats learned to discriminate between two environmental contexts. Coupling among different sites was found to increase as animals learned, and the amount of coupling strongly correlated with an increase in accuracy. In addition, during overtraining sessions where subjects were exposed to the same contextual associations repeatedly across several test sessions, a high level of coupling was observed from the onset of the session and varied little. Similar to the present study, these results show that a temporal shift in hippocampal activity occurred during learning, whereas activity during performance varied little.

One explanation for the temporal shift in activity associated with sequence acquisition may be that neural populations within the hippocampus become coupled or synchronized to facilitate the transfer of information to efferent brain regions. The importance of temporal coordination has been advanced by several researchers. Proper coordination of neural activity is known to play an integral role in learning and long-term potentiation (Larson et al., 1986; Arai and Lynch, 1992; Markram et al., 1997), and appears vital for encoding within the hippocampus and subsequent transfer of information to other brain regions (Buzsaki, 1996; Dragoi and Buzsaki, 2006; Sirota et al., 2008; for review, see Buzsaki and Draguhn, 2004). The disruption of coordinated hippocampal activity has been proposed as a mechanism underlying the deleterious effects of drugs, such as THC, on behavior (Robbe et al., 2006; Goonawardena et al., 2011). If coordinated activity is required for the transfer of information, the disruption of this coordination could explain the selective disruptions of learning produced by many drugs on repeated acquisition (Leonard et al., 2009; Winsauer et al., 2011).

Several studies have shown that patterns of activity observed during learning are reinstated by the hippocampus during memory retrieval (for review see, Treves and Rolls, 1994). In a study by Tanaka et al. (2014), neocortical and hippocampal neurons were monitored simultaneously in subjects during a contextual-fear-conditioning procedure. The authors found that the hippocampus and neocortex produce patterns of activity during the initial learning of the contextual associations that were similar to patterns observed when subjects were re-exposed to the conditioned context. However, inactivation of hippocampal neurons diminished the cortical representations upon re-exposure and impaired subject memory recall, strengthening the idea that the hippocampus reactivates specific memory representations during retrieval. Reactivation might also help explain differences between things that are newly learned versus things that are simply re-acquired as with repeated acquisition. New learning would occur much more slowly because there is no pattern to re-activate, whereas the reorganization of an existing pattern or a pattern associated with a previous experience would occur much more quickly (McKenzie et al., 2013). Such a reorganization would fit with data indicating that the major function of the hippocampus is to integrate information about events and the context in which they occur, including information related to the sequence of events and spatial location of those events (Fortin et al., 2002; Eichenbaum et al., 2007; McKenzie et al., 2014).

Along with the decreases in response rate in both components, ethanol produced dose-dependent increases in the percentage of errors in the acquisition component, but not the performance component (Figure 6, bottom panels). These selective increases in errors during acquisition were most evident during the first acquisition component. The decreases of response rate and increases in errors in the acquisition component were not surprising, as high doses of ethanol are known to decrease locomotion and induce motor impairment in rodents (Criswell et al., 1994) and to disrupt learning in a variety of experimental procedures, including contextual avoidance (Melia et al., 1996), spatial learning (Gibson, 1985; White et al., 1997; Matthews et al., 2002), non-spatial learning (Givens and McMahon, 1995), and repeated acquisition (Leonard et al., 2009). The deleterious effects of ethanol on learning show many similarities to those produced by lesions of the hippocampus (for reviews, see Matthews et al., 1996 and Silvers et al., 2003). Thus, these results provide additional behavioral evidence for the deleterious effects of ethanol on hippocampal function, as subjects were more sensitive to the error-increasing effects during the acquisition of response sequences than the performance of the well-known sequence.

The elimination of ethanol generally follows a linear function (Doherty and Gonzales, 2015). In the present study, the behavioral and neural effects of ethanol were closely associated with blood ethanol concentrations (Figure 8). That is, alterations in CA1 activity showed a similar time course of disruption and recovery as that for response rate, response accuracy, and blood ethanol concentration (e.g., Figure 9). These alterations were absent on days between doses of ethanol, and activity reflected the same associations (i.e., key position or serial position of a response) observed prior to ethanol administration. Other studies have reported similar patterns of disruption and recovery following ethanol administration (Matthews et al., 1996). Together, these results provide strong evidence that the observed changes in behavior and CA1 activity are attributable to the dosage of ethanol and that acute

ethanol administration produce little or no lasting changes in CA1 activity associated with this procedure.

The effects of ethanol on neural activity can range from suppression to excitation in specific brain regions (for review, see White et al., 2000). These effects may be explained in part by ethanol's capacity to affect multiple receptor systems. For instance, ethanol is a positive allosteric modulator of GABA_A receptors (for review, see Grobin et al., 1998), which largely affect hippocampal interneuron activity. There is also evidence that GABAergic interneurons promote changes in the synaptic plasticity of hippocampal principal cells. These interneurons also can affect NMDA receptor-dependent changes in synaptic plasticity linked to long-term potentiation (Markram et al., 1997), and play a critical role in gating information flow through the hippocampus (Paulsen and Moser, 1999). The GABAergic system plays an integral role in learning and this has been demonstrated in numerous studies by the administration of other positive allosteric modulators of GABA_A receptors, such as the benzodiazepines and barbiturates. In fact, drugs from these classes have been shown to produce rather selective disruptions of repeated-acquisition behavior (Campbell et al., 2004; Quinton et al., 2005).

Ethanol is a negative allosteric modulator of NMDA receptors (Lovinger et al., 1990; Criswell et al., 1993), which are believed to be essential for the induction of long-term potentiation (Collinridge et al., 1983; for review see, Morris, 2013). Ethanol has been shown to block long-term potentiation in freely-behaving rats (Givens and McMahon, 1995). Several investigators have demonstrated that NMDA receptor antagonists block long-term potentiation, while simultaneously impairing performance in hippocampal-dependent memory tasks and leaving non-hippocampal forms of learning, as well as previously established memory, intact (Morris et al., 1986; Morris et al., 1989; Morris et al., 1990). In addition, administration of NMDA receptor antagonists has been shown to affect acquisition to a greater extent than performance in animals responding under a procedure similar to that used in the present study (France et al., 1991).

The variety of changes in the frequency of CA1 activity produced by ethanol administration (Table 2) is consistent with other studies. Ethanol has been shown to increase the excitability of hippocampal interneurons (Steffensen and Henriksen, 1992), which can affect the activity of numerous principal cells (Cobb et al., 1995). In contrast, the activity of hippocampal pyramidal neurons has been reported to both increase and decrease activity following ethanol administration (Matthews et al., 1996; Weiner et al., 1997; White and Best, 2000). Although activity was increased at many sites following ethanol administration, these increases in activity did not necessarily reflect an increase in the basal firing rate, but rather an increase in the activity associated with a specific behavior (e.g., Figure 11). Other studies have shown that the effects of ethanol can depend on the specialization of the affected cells. For example, ethanol has been shown to decrease place cell specificity without increasing the overall activity of these neurons (Matthews et al., 1996; White and Best, 2000). Such a loss of specificity may have occurred in the present study; although the pattern of behaviorally-associated CA1 activity was often preserved following ethanol administration, the magnitude of modulation and latency of activity was altered (e.g., Figure 11). Despite the disruptions in CA1 activity, accuracy in performance was minimally disrupted,

highlighting ethanol's capacity for selectively disrupting learning and the importance of the hippocampus in sequence acquisition. These results support the notion that ethanol disrupts sequence acquisition by disrupting hippocampal activity.

Many of the correlations presented above suggest that the hippocampus plays a role in this operant procedure. However, these correlations did not offer direct evidence for the necessity of the hippocampus in this procedure. Micro-stimulation provided a potential means to determine the necessity of the hippocampus beyond correlative measures. In addition, a two-component schedule that incorporates both an acquisition and performance task can dissociate circuits required for sequence acquisition from those required for sequence performance.

Hippocampal micro-stimulation selectively increased acquisition errors and produced similar decreases in response rate in the acquisition and performance components (Figure 13). These disruptions were also intensity-dependent, with larger intensities producing larger reductions in response rate in both components and increases in errors in the acquisition component. Larger currents likely affected larger cell populations, impacting larger portions of the hippocampus. Nevertheless, there was little or no effect on response accuracy during the performance component, again indicating how responding in this component serves as a good non-specific control for learning. The effect of micro-stimulation was also similar to the effects of ethanol, because it decreased response rate non-selectively, while increasing percent errors during acquisition selectively. Moreover, micro-stimulation of a site outside the hippocampus (i.e., the ventral orbital cortex) did not affect response accuracy in either component, strongly suggesting an association between the hippocampus and sequence acquisition. These data are also reminiscent of the data in a paper by Campbell et al. (2004) where response-independent tail shock was periodically presented to squirrel monkeys responding under a repeated-acquisition procedure. Under these circumstances, response rate during sequence acquisition was not disrupted, but there was a marked increase in errors. Together with the present study, these data suggest that both peripheral and central stimulation could be increasing acquisition errors by disrupting attention, which may be required more for sequence acquisition than sequence performance. Lastly, the fact that subject response rate and the percentage of errors returned to baseline on days between micro-stimulations also indicated that micro-stimulation did not produce irreversible effects on the subjects' ability to learn.

In summary, results of the present study provide evidence that: (1) CA1 multi-unit activity was closely associated with subject behavior and differentially modulated by key position and the serial position of a response within the schedule of reinforcement; (2) there was a temporal shift between activity and responding during sequence acquisition, but not during sequence performance; (3) ethanol produced dose-dependent rate-decreasing and error-increasing effects that were more selective for responding in the acquisition component than performance component, and these disruptions were closely associated with alterations in CA1 activity; (4) ethanol produced dose-dependent alterations in the magnitude of modulations and temporal pattern of activity; and (5) the disruptive effects of ethanol on sequence acquisition were similar to those of hippocampal micro-stimulation and markedly different from those of stimulations of a site outside the hippocampus. This last observation

makes a powerful argument that the hippocampus is necessary for the conditioned associations required for sequence acquisition in this procedure.

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Figure 1.

Example of electrode placement location, trace of raw neural activity, and isolated neural units. **(A)** Trace of raw neural activity (~90 msec) recorded from one stereoelectrode site depicting several neural units (1 and 2). **(B)** Overlapping plots of several isolated waveforms from two neural units identified from the same recording site depicted in Panel A. **(C)** Three-dimensional plot comparing waveform characteristics (amplitude, falling slope, rising slope) of the neural units depicted in Panel B, which reveals two distinct clusters (putatively isolating single neurons). **(D)** Cresyl Violet-stained section showing a representative electrode placement, targeted to the CA1 subfield of the hippocampus.

Figure 2.

Example of Gaussian filtered CA1 activity from one recording site (1285, site 1) where a relationship between subject behavior and activity was transparent. **(A)** Remarkable cadence of activity as the subject worked errorlessly across a 10-minute time span during the first performance component. **(B)** A 90-second epoch of activity from Panel A highlights the close association of activity and behavior; concomitant correct responses (circles) and reinforcements (triangles) are depicted above the trace of activity. **(C)** Activity associated with the first response (0 sec) for each bout of responding, averaged from multiple response bouts. **(D)** Activity associated with reinforcement (0 sec) and the pause that preceded the next bout of responding, averaged from multiple reinforcements. Shaded regions indicate significant increases or decreases in activity (less than or greater than 3 standard deviations from shuffled mean; $p < 0.002$, 2-tailed).

Figure 3.

Gaussian filtered CA1 activity associated with correct responses at each key position (left, center, right) from one recording site (1285, site 1) during the first acquisition and performance components. Activity represented is an average of the 1-second epochs (+/- 500 msec) surrounding correct responses (0 msec) from five test sessions following saline administration. Shaded regions indicate significant increases or decreases in activity (less than or greater than 3 standard deviations from shuffled mean; $p < 0.002$, 2-tailed).

Figure 4.

Gaussian filtered CA1 activity that was associated with the first response in each of the three sequence completions required for reinforcement (i.e., the first, fourth, and seventh responses) at one recording site (1285, site 1). Each graph depicts responses on the right key (0 msec) when the first correct response in the acquisition and performance sequence was a right-key response (i.e., R-L-C and R-C-L, respectively). Activity represented is an average of the 1-second epochs (± 500 msec) from two test sessions following saline administration. The pattern of activity surrounding a response was not significantly different between acquisition and performance components. Activity associated with the first

response on the right key was significantly larger than the fourth ($p < 0.001$ in acquisition, $p < 0.001$ in performance) and seventh responses ($p = 0.007$ in acquisition, $p < 0.001$ in performance), whereas activity associated with the fourth and seventh was not significantly different ($p = 0.96$ in acquisition, $p = 1$ in performance). Shaded regions indicate significant increases or decreases in activity (less than or greater than 3 standard deviations from shuffled mean; $p < 0.002$, 2-tailed).

Figure 5.

One-second epochs (± 500 msec) of Gaussian filtered CA1 activity surrounding correct left-key responses (0 msec) emitted during the third sequence required for reinforcement at one recording site (1285, site 1). The upper and lower panels show the accumulating activity associated with the initial 15 responses (top panels) and last 15 responses (bottom panels) of the first acquisition and performance components. The first response of each 15 responses is on the bottom, whereas the last is on the top. The vertical bar to the left is calibrated to the 15th response, and the vertical line overlying activity indicates the cumulative peak.

Figure 6.

Effects of increasing doses of ethanol on the mean response rate (upper panels) and percentage of errors (lower panels) for subjects ($n=5$) responding during each of the acquisition (left panels) and performance (right panels) components. Shaded regions represent the effects of saline (control) administration (± 1 SEM). Symbols with vertical lines represent group means (± 1 SEM) for response rate in responses/min or percentage of errors. Crosses and brackets indicate main effects of dose (1 and 1.33 g/kg) on response rate in the both acquisition ($p=0.04$ and $p<0.001$, respectively) and performance ($p=0.041$ and $p=0.025$, respectively) components. There was also a main effect of dose (1.33 g/kg) on percent errors in acquisition ($p=0.022$). The asterisk and brackets indicate that all of the doses of ethanol uniformly increased percent errors during the first acquisition component ($p<0.001$). Numerical values in parentheses and adjacent to a symbol indicate the number of subjects represented by that point (e.g., some subjects did not reach a third acquisition component due to the rate-decreasing effects of ethanol).

Figure 7.

Within-session pattern of errors for five subjects following saline (shaded region, ± 1 SEM) or ethanol administration in the acquisition (top panel) and performance (bottom panel) components. Symbols with vertical lines represent group means (± 1 SEM) at different doses of ethanol (0.56–1.33 g/kg), and data are plotted as the cumulative number of errors per bin of 60 consecutive responses. Symbols without vertical bars represent instances in which the variability is encompassed by the point.

Figure 8.

Mean blood ethanol concentrations for three subjects following ethanol administration. B.E.C. samples were obtained either 15 minutes after the start of the session or 5 minutes after the session ended. Samples for the early time point were collected 30 minutes following acute ethanol administration, which was 15 minutes after the initiation of the behavioral session. Symbols with vertical lines represent group means (± 1 SEM) for the different doses of ethanol (0.56–1.33 g/kg). Symbols without vertical bars represent instances in which the variability is encompassed by the point.

Subject 1285 **Figure 9.**

Within-session pattern of responding for one subject (1285) following the administration of saline (top record) or two intermediate doses of ethanol (0.75 and 1 g/kg, middle and bottom records respectively). Each cumulative record shows the pattern of responding during the acquisition (A) and performance (P) components of the two-component operant procedure. As illustrated by the top insert to the left of the cumulative record, the response pen (top line) stepped upward with each correct response and was deflected downward following each completion of the three-response sequence. The slope of the stepping pen (top line)

reflects the rate of correct responding. Errors are indicated by the event pen (bottom line), which was deflected for 5 seconds during each timeout period. The end of a component (40 reinforcers or 20 minutes) reset the response pen, and all sessions terminated after 200 reinforcers or 80 minutes, whichever occurred first. Acquisition and performance components alternated across sessions. Below each cumulative record are traces of Gaussian filtered CA1 activity from three recording sites during those sessions. The insert to the left of the cumulative record depicts a vertical bar calibrated to 20 Hz that can be applied to each trace of activity.

Non-Contextual

Saline

1.33 g/kg EtOH

Figure 10.

Representative traces of 90 seconds of Gaussian filtered CA1 activity from one recording site (1339, site 1) obtained during exposure to a novel environment (i.e., a plastic container with wood-chip bedding, top trace) or following saline (middle trace) or ethanol administration (1.33 g/kg, bottom trace) in the operant chamber. Recordings in the novel environment (i.e., non-contextual) occurred several hours after operant test sessions and subjects were free to explore the environment for several minutes. Traces obtained following saline or ethanol administration were taken at equal time points during the first acquisition component. Concomitant correct responses (white circles), errors (black squares), and reinforcement (black triangles) are displayed above the traces.

Figure 11.

Dose-dependent effects of ethanol on CA1 activity from one recording site (1285, site 1) during the initial acquisition and performance components of a session. Each line depicts an average of Gaussian filtered activity reconstructed from 1.5 seconds (± 750 msec) surrounding correct left-key responses (0 msec) following the administration of saline or increasing concentrations of ethanol (0.56 – 1 g/kg). Modulations falling outside the confidence intervals (horizontal lines) indicate significant increases or decreases in activity (less than or greater than 3 standard deviations from shuffled mean; $p < 0.002$, 2-tailed). Comparisons of the area outside the confidence intervals (constructed from saline sessions) between saline and each dose of ethanol are indicated by the p-values.

Subject 1339 **Figure 12.**

Within-session pattern of responding for one subject (1339) during baseline conditions (top record) and during hippocampal micro-stimulation (15 uA) when the session started with either an acquisition (middle record) or a performance (bottom record) component. Each cumulative record shows the pattern of responding during acquisition (A) and performance (P) components of the two-component operant procedure. For additional details regarding the cumulative records of responding, see Figure 9. Micro-stimulations (rate: 100 Hz; pulse: 0.2 msec), which are indicated by the arrows, were delivered in 10 second trains every 5 minutes during the initial acquisition and performance components.

Figure 13.

Effects of increasing micro-stimulation current intensity (1–30 uA) on the overall response rate and percentage of errors in two subjects (1339 and 1365) responding during the acquisition (unfilled symbols) and performance (filled symbols) components. Although micro-stimulation was only delivered during the first two components, the data for the entire session are plotted. Symbols with vertical lines represent the mean and range for response rate in responses/min (upper panels) or percentage of errors (lower panels) during control conditions and following hippocampal (circles) or ventral orbital cortex (squares) micro-stimulation. Control ranges were created from six or more baseline sessions, and most micro-stimulation intensities were administered at least twice. Asterisks indicate an effect of micro-stimulation (mean outside control range). Symbols without vertical bars represent either single determinations for that particular intensity or instances in which the variability is encompassed by the point.

Figure 14.

Effects of increasing micro-stimulation current intensity (1–30 uA) on the within-session pattern of errors for two subjects (1339 and 1365) responding in the acquisition (top panel) and performance (bottom panel) components. Shaded regions represent the range of the baseline pattern of errors. Symbols with vertical lines represent the mean and range for different micro-stimulation intensities, and data are plotted as the cumulative number of errors per bin of 60 consecutive responses. Control ranges were created from six or more baseline sessions, and most micro-stimulation intensities were administered at least twice. Symbols without vertical bars represent either single determinations for that particular intensity or instances in which the variability is encompassed by the point.

Table 1

Summary of modulation of multi-unit activity during the operant procedure at each recording site.

Subject	Site	Modulated by Task	Key Position	Key Sequence
1285	1	+	+	+
	2	+	-	+
	3	-	-	-
1337	1	+	+	+
	2	+	+	+
	3	+	+	-
1339	1	+	-	+
	2	+	+	-
1340	1	+	+	+
	2	+	+	+
	3	-	-	-
1341	1	+	+	+
	2	+	+	+
	3	+	-	+

A plus (+) indicates that activity was differentially modulated by behavior, and a dash (-) indicates that activity was not differentially modulated by behavior.

Table 2

Effects of increasing doses of ethanol on the spike frequency (Hz) at each recording site during the first 20 minutes of a test session.

		Dose EtOH (g/kg)					
Subject	Site	Saline	0.56	0.75	1	1.33	
1285	1	63.3	65.5	80.6	81.3	69.1	
	2	18.1	18.7	17.7	15	14.6	
	3	71.1	78.6	86.9	97.5	101.0*	
1337	1	26	26.8	21.5	31.4	90.7*	
	2	24.9	25.5	21.9	31	67.3*	
	3	31.8	30	31.5	32.5	39.6*	
1339	1	62.8	80.6	63	125.2*	131.8*	
	2	21.3	21	15.7	13.8	10.9*	
1340	1	54.5	83.5	29.9	62.7	43.1	
	2	73.7	64.8	86.2	69.7	99.0*	
	3	41	42.3	35.3	44.7	42.1	
1341	1	70.3	75.8	79	82	120.6*	
	2	17.7	16.5	13.4	16	25.4*	
	3	49.1	45.1	54.8	50.6	60.2	

Asterisks (*) indicate an effect (increase or decrease) on spike frequency, as defined by a mean frequency outside the control range.