EFFECTS OF TEMPORARY INACTIVATION OF DORSAL HIPPOCAMPUS ON EXPLICITLY NONSPATIAL, UNIMODAL, CONTEXTUAL FEAR LEARNING

By

Teresa Camille Parsons

A thesis submitted to the

Graduate School-New Brunswick
Rutgers, The State University of New Jersey
in partial fulfillment of the degree requirements
for the degree of
Master of Science
Graduate Program in Psychology
written under the direction of
Timothy Otto, Ph.D
And approved by

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New Brunswick, New Jersey

January 2008
Several studies have reported that dorsal hippocampal damage attenuates the acquisition (Kim et al., 1993; Phillips & LeDoux, 1992; Young et al., 1994) or expression (Anagnostaras et al., 1999; Holt & Maren, 1999) of recently acquired contextual fear conditioning. "Context" is often operationalized as the conditioning chamber in which CS-US pairings occurred. However, the hippocampus is known to participate in spatial learning, presenting interpretative difficulties regarding the role of dorsal hippocampus in learning and memory. The current study examined the effects of temporary inactivation of DH on freezing, rearing, ambulating, grooming, and whisking behavior in an explicitly nonspatial contextual fear conditioning paradigm, where olfactory stimuli served as temporally and spatially diffuse contexts. Results indicate that temporary inactivation of DH produced both anterograde and retrograde deficits in contextually conditioned freezing, while sparing the acquisition and expression of freezing to a discrete auditory CS. Further, animals with DH inactivation froze modestly and similarly to the unsafe and safe contextual stimuli, while intact animals froze robustly to the unsafe, but not the safe, contextual stimulus. These data indicate that there is a decidedly nonspatial component to the role of DH in contextual conditioning, and suggest that olfactory contextual conditioning is a fruitful means of further exploring this function.
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1. Introduction

For organisms from rodents to primates, the ability to associate events with one another in time and space appears to rely heavily on the hippocampus (Anagnostaras, Gale, & Fanselow, 2001; Eichenbaum, Otto, & Cohen, 1994; Kim & Fanselow, 1992). Mounting evidence suggests that within this large bilateral structure, dissociable regions exist with respect to their cytoarchitecture and connections (Brun et al., 2002; Eichenbaum et al., 1994; Pitkänen, Pikkarainen, Nurminen, & Ylinen, 2000). Based on these considerations, the hippocampus may be divided along its septotemporal axis into ventral and dorsal portions (Hargreaves, Rao, Lee, & Knierim, 2005; Ishikawa & Nakamura, 2006; Kusljic & van den Buuse, 2004; Moser & Moser, 1998) that may subserve unique or overlapping memory processes (Moser & Moser, 1998; Richmond et al., 1999).

Among the many fruitful behavioral paradigms used to identify neural substrates of learning and memory is Pavlovian fear conditioning. In one version of this paradigm known as delay fear conditioning, presentations of a neutral stimulus (henceforth referred to as a conditioned stimulus, or CS) co-terminate with presentations of a stimulus such as footshock (an unconditioned stimulus, or US). Repeated CS-US pairings eventually result in the development of a learned association between the CS and US. Use of this paradigm has led many researchers to conclude that while the hippocampus is normally not critically involved in temporally discrete CS-US associations (Kim & Fanselow, 1992; Phillips & LeDoux, 1992), it does participate in the acquisition of the context in which tone-shock pairings take place (Kim, Rison, & Fanselow, 1993; Phillips & LeDoux, 1992; Young, Bohenek, & Fanselow, 1994). Further, while lesions of the entire hippocampus generally produce some disruption in contextual but not auditory fear conditioning
(Kim & Fanselow, 1992; Otto & Poon, 2006; Philips & LeDoux, 1992), attempts to clarify the respective roles of ventral hippocampus and dorsal hippocampus in contextual fear conditioning have been inconclusive. In some cases, electrolytic or excitotoxic dorsal hippocampus lesions made prior to contextual fear conditioning training were found to interrupt acquisition of contextual fear conditioning (Kim et al., 1993; Phillips & LeDoux, 1992; Young et al., 1994), while other studies reported that dorsal hippocampus lesions produced no impairment in the acquisition of contextual fear conditioning (Maren, Aharonov, & Fanselow, 1997; Richmond et al., 1999).

In the event of permanent damage to a structure, surrounding areas may be compromised, recruited or otherwise affected, leading some researchers to adopt instead of lesions the reversible technique of temporary inactivation. The fact that most hippocampal neurons contain the GABA\(_\alpha\) receptor (Chan-Palay, 1978) makes the GABA\(_\alpha\) agonist muscimol a potentially useful means for temporarily inactivating components of the hippocampus. Using muscimol to reversibly inactivate the dorsal hippocampus, several researchers have found dorsal hippocampus to be critically involved in the acquisition or retrieval of contextual fear memory and context-specific fear expression in paradigms such as latent inhibition and facilitated extinction, without affecting contextual discrimination or performance of the freezing response (Corcoran & Maren, 2001; Holt & Maren, 1999; Matus-Amat, Higgins, Barrientos, & Rudy, 2004).

Almost without exception, studies investigating the role of dorsal hippocampus in contextual learning have defined “context” as the behavioral chamber where CS-US pairings took place. However, it is well established that the dorsal hippocampus participates in spatial conditioning (Bannerman et al., 2002; Eichenbaum et al., 1994; Moser, Moser, & Andersen, 1993). Thus, equating space with context presents a potential confound. Recently, Bannerman, Rawlins, and
Good (2006) referred to as “context” the features of an environment that collectively define the place or situation in which the animal is located at the time learning occurred. Conceptual definitions of context typically emphasize the nature of the temporal and spatial relationship between the CS and other aspects of the environment (see Balsam & Tomie, 1985). Stimuli ranging from background color of a response key (Thomas, 1985), the presence or absence of unimodal stimuli (Otto & Poon, 2006), tones of specific pitch (Hulse, Cynx, & Humpal, 1994), and internal states resulting from drug administration or food deprivation (Davidson & Jarrard, 1993; Hock & Bunsey, 1998; Overton, 1964) have been experimentally manipulated as contextual variables. In the light of evidence that dorsal hippocampus participates in certain aspects of contextual conditioning, such variety within the class of events that may function as contextual stimuli underscores the importance of examining neural substrates of explicitly nonspatial forms of contextual conditioning.

Recently, Otto and Poon (2006) described a unique paradigm for dissociating the role of dorsal hippocampus in contextual fear conditioning from its involvement in spatial conditioning. In an olfactory contextual conditioning procedure, the presence of strawberry odor and 15% pyridine, presented in alternation, constituted different “safe” and “unsafe”, temporally and spatially diffuse contexts. During a single delay fear conditioning session in one behavioral chamber, tone-CS/footshock-US pairings occurred during presentations of the “unsafe” context. At the time of testing 24 hr later, intact rats froze significantly during presentation of the “unsafe” but not the “safe” contextual stimulus, while animals with lesions of dorsal hippocampus were dramatically impaired in freezing during the “unsafe” contextual stimulus (but froze normally with respect to the CS). Even as these results evince a function for dorsal hippocampus in a form of contextual fear conditioning that is explicitly nonspatial, the permanent nature of the dorsal hippocampus damage precluded examination of the
role of dorsal hippocampus in olfactory contextual fear memory retrieval independent of its involvement in acquisition. To this end, the current study replicates use of the olfactory contextual conditioning paradigm (Otto & Poon, 2006) while adopting temporary inactivation of dorsal hippocampus at various time points, affording examination of the role of dorsal hippocampus during acquisition and retrieval of fear conditioned to contextual and discretely presented stimuli. Further, because both muscimol and hippocampal lesions may produce motor deficits or hyperactivity, respectively (see Anagnostaras et al., 2001), the present study examined a host of other behavioral responses in addition to the more commonly measured freezing and immobility (Fanselow, 1980; Fendt & Fanselow, 1999).
2. Materials and Methods

All procedures were approved by Rutgers University’s Institutional Animal Care and Use Committee.

2.1. Subjects

Subjects were 60 naïve male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 225-249g on arrival, individually housed in plastic tubs on a 12-hr light/dark cycle in a Rutgers University (New Brunswick, NJ) psychology department colony room. Animals had access to food and tap water ad libitum, and were handled 2 min daily for 5 days prior to surgery. All procedures took place during the light phase of the cycle.

2.2. Apparatus

Contextual fear conditioning was conducted in a behavioral chamber (30 X 24 X 27 cm) enclosed in an aluminum sound-attenuating box (56 X 41 X 42 cm). Transparent Plexiglas constituted one pair of opposing walls and the ceiling, and aluminum composed the other pair of opposing walls. The chamber floor consisted of 16 stainless steel rods (diameter 5 mm) equally spaced by 1.9 cm. Rods were wired to a shock generator (H13-15, Coulbourn Instruments, Allentown, PA) and delivered scrambled footshock (0.5 mA). A sawdust-filled tray was placed under the grid floor. When appropriate, a computer-generated tone (3.9 kHz, 80 dB) was presented through a speaker mounted outside one of the aluminum chamber walls, and a single light bulb (29V, 0.04A) was located 24.5 cm above the floor. A motion detector (model H24-61, Coulbourn Instruments, Allentown, PA) situated on top of the behavioral chamber allowed detection of movement via a hole drilled through the chamber ceiling.

Olfactory stimuli were presented during the experiment via ports in the chamber ceiling, using a procedure described in previous olfactory fear conditioning
studies (Cousens & Otto, 1998; Herzog & Otto, 1997, 1998; Otto, Cousens, & Rajewski, 1997). Operation of a solenoid valve caused clean air (1.5 L/min) to be pumped to a 20-ml bottle containing 3 ml of either 15% pyridine in propylene glycol or strawberry extract (McCormick, Hunt Valley, MD). Odorized air was then directed to the conditioning chamber through Tygon tubing (1/8 in inner diameter) connected to a ceiling outlet port. An exhaust fan mounted on the chamber provided ventilation, directing the odorized air out to a vacuum pump; odor was eliminated from the inner behavioral chamber within 20s of the solenoid closure. Training chambers were cleaned between sessions with cage cleaner.

The testing apparatus consisted of a separate behavioral chamber in a distal room. While it contained dimensions identical to those of the training chamber and was capable of presenting olfactory and auditory stimuli, the testing apparatus also contained the distinguishing features of a diagonally striped back wall and a solid black Plexiglas floor. A video camera was positioned in a corner of the sound-attenuating outer chamber. Alcohol wipes were used to clean the inner testing chamber, further differentiating it from the chamber used during training.

2.3. Procedure

2.3.1. Surgery

Subjects were anesthetized with a Ketamine/Xylazine solution (80 ml/kg Ketamine, 12 ml/kg Xylazine, ip). The subject’s head was then shaved and mounted in a stereotaxic frame (Kopf Instruments, Tujunga, CA), and the scalp cleaned with 20% Nolvasan solution. Bupivicaine (0.15 ml) was injected in multiple subcutaneous sites along the scalp midline. The scalp was incised, retracted, and fascia was removed from the skull. Bregma and lambda were located, and the position of dorsal hippocampus (A/P -3.8, M/L +2.5 from bregma) was marked in bilateral locations on the skull. Six burr holes were drilled through the skull, and screws were inserted and
tightened in four of the holes. A double guide cannula (Plastics One, Roanoke, VA) was implanted into the two bilateral holes above dorsal hippocampus, reaching a depth of 2.2 mm ventral to dura. Dental acrylic and cement were applied to secure the cannula. After the acrylic/cement structure was secure, the incision was sutured with stainless-steel surgical staples. An obturator was inserted into the guide cannula. After surgery subjects were placed in home cages where they were closely monitored for two days.

2.3.2. Drug infusion

Subjects were randomly assigned to one of four groups. Each group was assigned to receive either saline or muscimol 30m prior to a training session, and either saline or muscimol prior to two subsequent test sessions on consecutive days. Thus, each group received infusions on three occasions, separated by 24hr. The first group (SAL-SAL, n = 15) received saline prior to both training and testing sessions. Group MUS-SAL (n = 15) received muscimol infusions prior to training, and saline infusions prior to testing sessions. A third group (SAL-MUS, n = 15) received saline prior to training and muscimol prior to testing. A fourth group (MUS-MUS, n = 15) received muscimol prior to both training and testing sessions.

Between surgery and the onset of behavioral procedures, each subject was brought to the infusion room for 2 min every 2 days, and the pump was run in the background in order to acclimate subjects to the infusion room and noise associated with infusion. Infusions began 10 days after surgery, when subjects were brought individually in clear plastic boxes to the infusion room. The obturator was removed and replaced with 30-gauge injection cannula, attached by polyethylene tubing (PE-10) to 10-μL Hamilton syringes mounted in an infusion pump (Harvard Apparatus, South Natick, MA). Bilateral microinfusion of saline (0.9%, pH=7.4) or muscimol (1μg/μL dissolved in 0.9% saline; Sigma Aldrich, St. Louis, MO) occurred over a 1.5-
min period. Each bilateral infusion introduced volumes of 0.25\(\mu\)L saline or muscimol per hemisphere, for a total volume of 0.5\(\mu\)L into dorsal hippocampus. Rats were held during the infusion period to prevent dislodging tubing, and held for an additional 2 min to allow diffusion of the drug or vehicle prior to removal of the injection cannula. Dummy cannula and cap were then replaced, and the subject was returned to the holding box for 28 min before transporting to the training or testing room so that 30 min separated infusions from training or testing sessions.

2.3.3. Olfactory contextual delay fear conditioning

Training took place 10 days after surgery in a single session, in one of two identical behavioral chambers (A and B) in the same room. Whether an animal received training in Chamber A or B was counterbalanced for each group. Each subject received an infusion of either saline or muscimol 30 min prior to training (see 2.3.2., Drug infusion). During the 35-min training session, two olfactory contexts were presented in alternation, each for 5 min and separated from each other by 1-min inter-context-intervals. Thus, each context was presented three times. During context periods referred to as “safe”, no auditory or footshock stimuli were presented; during each presentation of the “unsafe” context, subjects were exposed to three pairings of a 3.9 kHz-pure tone (~80dB, 20s) co-terminating with a footshock (2 sec, 0.5mA). Successive CS-US pairings were separated by a 1 min ITI. Therefore, a total of nine CS-US pairings was presented during training, each against the background of an “unsafe” unimodal olfactory contextual stimulus (see Fig. 1). Strawberry extract and pyridine, 1.5 L/min flow rate, served as olfactory contextual stimuli and were counterbalanced for all groups.

2.3.4. Testing
Testing began 24 h after training in a separate room and chamber, as described in Section 2.2 (Apparatus) above. Thirty min after an infusion of either saline or muscimol, subjects were placed in the testing chamber for 10 min. In each testing session, one contextual stimulus was presented during Min 3, and the tone CS was presented during Min 10. No other stimuli were explicitly presented. Responses to unsafe and safe contextual stimuli were assessed on consecutive days, with unsafe day counterbalanced for each group (see Table 1). That is, for a subject in group SAL-MUS assigned to unsafe test Day 1, muscimol infusion first occurred prior to unsafe context testing on Day 1. Muscimol was infused 24 hr later on test Day 2, and the safe context was tested. Each subject therefore received two testing sessions separated by 24 hr, with each test session preceded by the same drug infusion. For all test sessions, behavioral measures were scored by a human observer, other data were compiled by a computer, and a videotape was made for offline analysis.

2.3.5. Behavioral measures

2.3.6. Primary measure of conditioned fear

The primary dependent measure of fear was freezing behavior, scored continuously by a human observer blind to the infusion condition of each subject. To record the onset and offset of freezing (the adoption of rigid posture unaccompanied by body, extremity, head, or whisker movement except that required for respiration), the observer held a hand switch sampled by the computer each second. Depression of the switch recorded freezing onset, while release of the switch constituted offset. For each minute, freezing observations were transformed to a percentage of total observations, yielding the percent time spent freezing during each minute. Simultaneously, an infrared motion detector (see section 2.2, Apparatus) registered
immobility and was sampled by the computer controlling stimuli presentation, yielding the percent time spent immobile during each minute of the testing session.

2.3.7. Secondary behavioral measures

Previous studies have found a high and positive correlation between freezing behavior assessed by human subjects and immobility registered by computerized means (Anagnostaras et al., 2001; Yoon & Otto, 2001), and freezing behavior is generally accepted to be a valid measure of conditioned fear (Fanselow, 1980; Maren, 1998). However, interpretations of freezing data can potentially be confounded by performance accounts (Anagnostaras et al., 2001; Holt & Maren, 1999). The testing paradigm used in the current study allowed the observation of behavior conditioned to a “safe” context on a day different from the “unsafe” test, perhaps introducing other relevant dependent variables. In addition to freezing, the occurrence of whisking, grooming, ambulating, and rearing behavior were measured during test sessions. Data were collected offline from videotapes using a momentary time-sampling technique. Briefly, tapes were scored by trained human observers, who categorized behavior during each 5-s epoch of a session as one of the following: rearing (standing on hind legs with the upper limbs above midline), ambulating (consecutive grossmotor movements that resulted in displacement of the rat in the horizontal plane), whisking (visible movement of vibrissae in the air or contacting surfaces), grooming (body part to body part contact), or freezing (rigid and motionless posture, except for respiration-related movement). These data were coded by an observer unaware of the condition of subjects, and were converted into percentage of time spent engaged in freezing, rearing, ambulating, whisking or grooming during relevant testing periods including pre-context, context, post-context, pre-CS, and CS presentation for analysis.

2.3.8. Histology
Following the last testing session, each subject was anesthetized with sodium pentobarbital (100 mg/kg, ip) and perfused transcardially with 0.9% saline and 10% buffered formalin solution. Brains were removed and held in 30% sucrose solution (wt/vol) for at least 48 hr, then frozen and sliced into coronal sections of 50 µm thickness. Slices were mounted on glass slides, stained with cresyl violet, and examined visually via a light microscope for verification of cannula placement in dorsal hippocampus.

2.3.9. Statistical analysis

Freezing behavior and immobility during testing sessions were statistically analyzed using two-way repeated measures analyses of variance (ANOVAS), with treatment group as the between-subjects factor and testing minute as the within-subjects factor. Subsequent multiple pairwise comparisons were conducted using Student-Newman-Keuls (SNK) post-hoc tests. A one-way ANOVA was used to analyze treatment groups’ difference between conditioned freezing to safe versus unsafe unimodal contextual stimuli, and subsequent post-hoc analyses were conducted using Dunnett’s test (α=0.05). Data from offline video analysis, including rearing, ambulating, grooming, whisking, and freezing, were analyzed using separate two-way ANOVAs, with treatment group as the between-subjects factor and testing period the within-subjects factor. A one-way ANOVA was performed on unsafe test data of the control group (SAL-SAL), to analyze effects of testing the unsafe contextual stimulus on Day 1 versus Day 2. Likewise, a one-way ANOVA was performed within the SAL-SAL group on unsafe odor, to analyze the effects of different olfactory contextual stimuli (strawberry versus pyridine odor) on contextually conditioned freezing.

3. Results

3.1. Cannula placement
Three subjects were excluded from statistical analyses due to post-surgical complications. Muscimol is expected to spread at least 1 mm below the site of infusion (Martin, 1991); thus, subjects were retained for statistical analyses only if cannula tips were localized within the dorsal hippocampus, above or within (but not below) area CA3. Following histological examination of the location of cannula tracks in dorsal hippocampus, four subjects were removed from group SAL-SAL and three subjects were removed from the MUS-SAL, SAL-MUS, and MUS-MUS groups, resulting in final group sizes of 11 subjects in each group for statistical analyses. Figure 1 illustrates cannula placement in the dorsal hippocampus for each group.

3.1.2. Correlation between hand-scored freezing behavior and computer-detected immobility

Correlational analyses compared freezing data obtained by human observers to the computerized detection of immobility during the contextual conditioning test. That is, for relevant periods during testing, the number of seconds that each subject was scored as “freezing” by a human observer was examined against the number of seconds during which the motion detector recorded immobility. Two correlation coefficients were obtained. First, the unsafe context correlation coefficient was 0.91 (p < 0.0001), between the number of seconds recorded by a human observer as freezing and the number of seconds recorded as immobile by the computerized motion detection system, during the unsafe context presentation. Second, the safe context correlation coefficient was 0.76 (p = 0.20), between the human observer-scored freezing and computer-scored immobility during the safe contextual stimulus presentation. In general, these correlations are similar to those previously reported in the literature comparing human observer scores of freezing; the figures within the text to follow show freezing data only.

3.1.3. Effects of particular contextual stimuli and day of unsafe context test
As mentioned previously, the odor used as unsafe context was counterbalanced within groups. In order to determine the effects of particular odor (strawberry odor versus pyridine odor) as unsafe contextual stimulus on conditioned contextual freezing, a one-way ANOVA was performed on freezing during unsafe context (odor). For group SAL-SAL, results of this one-way ANOVA revealed no difference between subjects contextually conditioned with strawberry, and subjects for whom the unsafe contextual stimulus was pyridine (F(1,9) = 0.88, p > 0.05). Therefore, subsequent analyses collapsed data across unsafe contextual stimulus (odor). Also counterbalanced within groups was unsafe context testing day, with some subjects tested for unsafe contextual conditioning on Day 1, and others on Day 2. To evaluate the effects of unsafe context testing day on contextual freezing for SAL-SAL subjects, a one-way ANOVA was performed for freezing on unsafe context testing day one versus unsafe context testing day 2. This analysis did not reveal a difference between unsafe context testing days (F(1,9) = 0.49, p > 0.05). Data are thus collapsed across unsafe context test day.

### 3.1.4. Freezing to unsafe olfactory contexts and auditory CS

Data from the unsafe context testing session 24 or 48 hr after training are presented in Figure 2. The effects of infusions of muscimol into dorsal hippocampus on contextually conditioned freezing were analyzed using a two-way repeated-measures ANOVA with one repeated measure (Min). Neither the main effect of condition (F(3,40) = 0.699, p > 0.05) nor the minute by condition interaction (F(27,360) = 1.102, p > 0.05) reached significance. There was a significant main effect of minute on freezing during the unsafe test session (F(9,360) = 38.042, p < 0.0001). Pairwise comparisons (Student-Newman-Keuls) showed that regardless of infusion condition, all groups froze similarly to the auditory CS (p>0.05).
Figure 3 shows freezing during the safe contextual test session. The safe contextual stimulus was presented during min 3, and the auditory CS was presented in min 10. A two-way repeated measures ANOVA of freezing during the safe context test revealed a significant main effect of minute ($F(9,360)=45.49, p<0.0001$). Neither the main effect of group ($F(3,40)=0.497, p>0.05$) nor the group-by-minute interaction ($F(27,360)=0.582, p>0.05$) reached significance.

Although there was considerable variability within the SAL-SAL group with respect to freezing during the unsafe context, subjects in this group froze considerably more during the unsafe context than the safe context. In contrast, subjects in groups MUS-SAL, SAL-MUS, and MUS-MUS froze modestly and similarly to both contexts. In order to further explore differences between groups during the contextual stimulus tests, the effect of infusion condition on the difference between freezing to unsafe versus safe contextual stimuli was explored using a one-way ANOVA. Difference scores were obtained for each subject by subtracting the percent of time spent freezing to the safe contextual stimulus from the percent of time spent freezing during the unsafe contextual stimulus, in the relevant contextual stimulus presentation period. Figure 4 depicts the difference between freezing during unsafe versus safe contextual stimuli for the groups SAL-SAL, MUS-SAL, SAL-MUS, and MUS-MUS. A one-way ANOVA revealed a significant effect of infusion condition ($F(3,40) = 3.08, p = 0.0383$) on the difference between freezing during unsafe and safe contextual stimuli. Subsequent post-hoc comparisons (Dunnett's test, $p < 0.05$) of treatment groups with the control group showed that MUS-SAL, SAL-MUS, and MUS-MUS groups all differed significantly from group SAL-SAL.

3.1.5. Other behaviors recorded during test sessions

Other behaviors occurring during relevant periods during the unsafe context test are illustrated in Figure 5. Experienced observers, blind to the infusion condition
of subjects, scored videotapes using a momentary time-sampling technique, scoring each 5-sec epoch of the 10-min sessions as rearing, ambulating, grooming, whisking, freezing, or other. For analysis, each 10-min session was divided into the following five periods: baseline (the first two minutes of the testing session, during which no stimuli were presented); the unsafe period (during which the unsafe contextual stimulus was presented); the post-unsafe period (the two minutes following the presentation of the unsafe contextual stimulus); the pre-CS period (the minute prior to CS presentation); and the CS period, during which the auditory CS was delivered. Separate two-way repeated measures ANOVAs were performed for rearing, ambulating, grooming, whisking, freezing, and other behavior.

A two-way repeated measures ANOVA on freezing data collected using the time-sampling method, with infusion group as the between-subjects factor and test period as the within-subjects factor, revealed significant main effects of both group (F(3,30) = 6.32, p = 0.0019) and period (F(4,120) = 90.54, p < 0.0001). A significant interaction was also observed between group and period (F(12,120) = 3.27, p = 0.0004). Post-hoc comparisons (SNK) showed that each muscimol-infused group differed from group SAL-SAL during the unsafe context period (p < 0.05). Freezing was the only behavior that depended on the infusion condition of subjects.

A two-way repeated measures ANOVA on rearing revealed a main effect of period (F(4,120) = 11.599, p < 0.0001). Neither the main effect of group (F(3,30) = 0.721, p > 0.05) nor the interaction between group and period (F(12,120) = 0.925, p > 0.05) reached significance. For ambulating, a two-way repeated measures ANOVA revealed a main effect of period (F(4,120) = 6.33, p = 0.0001), but not group (F(3,30) = 1.25, p > 0.05), and there was not a significant interaction between group and period (F(12,120) = 1.22, p > 0.05). A two-way repeated measures ANOVA over whisking behavior revealed a main effect of period (F(4,120) = 10.6, p < 0.0001), but
neither the main effect of group (F(3,30) = 0.346, p > 0.05) nor the group-by-period interaction (F(12,120) = 0.895, p > 0.05) reached significance. Finally, a two-way repeated measures ANOVA over grooming behavior revealed no main effects of group (F(3,30) = 0.192, p > 0.05) or period (F(4,120) = 1.57, p > 0.05), and no significant interaction (F(4,120) = 0.479, p > 0.05).
4. Discussion

The present results suggest that the temporary inactivation of dorsal hippocampus, whether occurring prior to training, prior to subsequent testing, or both, dramatically impairs contextual conditioning. In contrast, inactivation of dorsal hippocampus spared subjects’ abilities to acquire and express conditioned freezing to an explicit auditory CS and had no effect on ambulation, grooming, rearing, and whisking behavior. The selective deficit in olfactory contextual conditioning is likely not attributable to stimulus, means of dorsal hippocampus inactivation, day of testing, or the specific odor used as the “unsafe” contextual stimulus. These data provide further evidence that dorsal hippocampus participates in the association of a US with contextual stimuli that are relatively ambiguous and temporally and spatially diffuse. That is, when context is operationalized as a unimodal cue both temporally and spatially diffuse from the US, dorsal hippocampus is critically involved in the acquisition and expression of contextual fear.

The role of dorsal hippocampus in contextual fear conditioning has been examined using both temporary inactivation (Corcoran & Maren, 2001; Holt & Maren, 1999) and multiple means of producing permanent damage (Maren 1999; Maren & Fanselow, 1997; Otto & Poon, 2006). Methods of producing both temporary (Bellgowan & Helmstetter, 1995) and permanent (Douglas & Isaacson, 1964; Maren & Fanselow, 1997) hippocampal damage have been reported to affect rats’ ability to perform the freezing response. In a related vein, McNish, Gewirtz, and Davis (1997) have found discrepancies in the effects of hippocampal damage on freezing versus fear-potentiated startle. Therefore, the current study employed multiple measures both in order to rule out potential performance accounts of any deficits observed in animals with dorsal hippocampal inactivation, and to assess whether behaviors other than freezing were systematically related to contextual fear memory. These data
show no group differences with respect to rearing, ambulating, grooming, or whisking, indicating that the ability of animals to physically execute the responses of interest was not likely compromised by inactivation of dorsal hippocampus. This conclusion is strengthened by the fact that even for groups that did not appreciably freeze during presentation of contextual stimuli, robust freezing occurred later in the same test session, during CS presentation.

It might legitimately be questioned whether the pattern of results seen here could be attributed to the specific modality of contextual stimuli used in the present paradigm. However, recent work by Otto and Poon (2006) has shown that while lesions of dorsal hippocampus impaired freezing to an olfactory contextual stimulus, the identical olfactory stimulus elicited freezing when serving as a discrete, temporally punctate CS. Thus, stimulus modality alone cannot account for the failure of animals with dorsal hippocampal damage to acquire contextual fear conditioning. Furthermore, the current experiment counterbalanced odors used as the unsafe context across subjects. Regardless of the specific stimulus serving as the unsafe contextual stimulus during training, subjects effectively acquired contextual fear conditioning when dorsal hippocampus was intact during training and testing, while subjects infused with muscimol did not.

Dissociating the potentially functional difference of spatial learning and contextual fear conditioning has been complicated by the fact that context is typically defined as the chamber in which conditioning occurred (Kim, Rison, & Fanselow, 1993; Phillips & LeDoux, 1992; Maren & Fanselow, 1997; Young et al., 1994), although it is widely acknowledged that stimuli other than spatially defined cues can serve as effective contextual variables (Otto et al., 1997; Otto & Poon, 2006; Overton, 1964; Randrich & Ross, 1985). But are spatial and olfactory contexts functionally dissociable? Recent research by Waxler and Otto (2004) suggests that
they are: in their study, hippocampal place fields were observed to remap during exploration of a novel spatial environment—but not to novel odors—and to remain stable during olfactory contextual conditioning even though subjects acquired freezing responses to an “unsafe” but not a “safe” context within the same space. Similarly, other researchers have suggested that a neural representation of space is distributed across the hippocampus, with certain place fields remapping completely when an animal enters a novel spatial environment (Muller, Kubie, & Ranck Jr., 1987), while other fields only “partially remap” when aspects of the environment other than space are changed (Jeffrey, Anderson, Hayman, & Chakraborty, 2004). Results of the current study are consistent with the idea that spatial and other forms of contextual conditioning may be dissociable processes (Bannerman et al., 2004; Richmond et al., 1999) with overlapping neural substrates (Anderson et al., 2006; Barry et al., 2006).

While hippocampal damage typically impairs spatial learning (Jarrard, 1983; Morris, Schenk, Tweedie, & Jarrard, 1990; Olton, Becker, & Handelmann, 1979; Sutherland et al., 1983), research suggests that the mere exploration of a novel environment does not depend on an intact dorsal hippocampus (Gaskin, Chai & White, 2005). This possibility is supported by results of the current study, in which testing occurred in a separate room and chamber, positioned at a different orientation, and containing tactile, visual, and olfactory stimuli different from those used during training. If an intact dorsal hippocampus is required for the exploration of novel spatial contexts, behavioral correlates of exploration such as rearing (Anderson et al., 2006; Gaskin et al., 2005; Lever, Burton, & O’Keefe, 2006) would be expected to be impaired by inactivation of dorsal hippocampus. In fact, neither rearing nor ambulating (see Fig. 5) was impaired during testing sessions, which occurred in a novel spatial environment. It is suggested that spatial and olfactory
contexts are not only topographically but also functionally dissociable, and that dorsal hippocampus integrity may participate differently in their processing.

The results of several studies suggest that dorsal hippocampal lesions prior to training may impair contextual learning (Maren & Fanselow, 1997; Phillips & LeDoux, 1992), although other studies have detected only retrograde—and not anterograde—impairments in contextual learning after such damage (Anagnostaras et al., 2001; Maren et al., 1997; Richmond et al., 1999). Discrepancies such as these have led some to conclude that contextual fear conditioning is more susceptible to damage inflicted between training and testing than to lesions performed before training (Bannerman et al., 2004; Richmond et al., 1999). Attempting to explain cases in which an anterograde deficit was observed following dorsal hippocampal damage, it has been suggested that fibers of passage between ventral and dorsal hippocampus may have been responsible (Bannerman et al., 2004; Maren et al., 1997). The current study speaks specifically to this controversy, in that reversible inactivation of dorsal hippocampus produced both anterograde and retrograde deficits in one form of contextual fear conditioning.

In studies of temporary inactivation, one precondition for interpreting effects is first to rule out state-dependent learning (Bannerman et al., 2004; Overton, 1964). To this end, the present experiment included one group (MUS-MUS) receiving muscimol prior to both training and testing. If acquiring olfactory contextual fear conditioning depends on being in the same drug state at the time of original conditioning and subsequent testing, group MUS-MUS would be expected to effectively acquire and express contextual fear conditioning (much like that seen in group SAL-SAL, Fig. 2). However, the present results indicate an actual and dramatic impairment in group MUS-MUS, suggesting state-dependent learning cannot account for effects.
The current paradigm afforded the assessment of conditioned responses to the safe and the unsafe contextual stimuli on separate testing days. Freezing evoked by two separate olfactory stimuli could be compared without potential confounds that might have resulted from evaluating both stimuli in a single test session. Of course, this design raises the possibility that for subjects presented with one contextual stimulus on the first testing day, extinction could account for reduced freezing to the second tested stimulus presented on day two. Importantly, an analysis of freezing to the unsafe contextual stimulus as a function of test day indicated that for group SAL-SAL, freezing was unrelated to day of testing.

The conditioning paradigm employed in the current study departs from that typically used to assess conditioned responses to stimuli (Kim & Fanselow 1992). Here, conditioning to contextual stimuli was assessed on consecutive days, with the CS presented during the final minute of each session. Results of CS presentation are consistent with the existing body of literature suggesting that auditory fear conditioning is not disrupted by inactivation of dorsal hippocampus or hippocampal damage (Kim & Fanselow, 1992; Otto & Poon, 2006), irrespective of both testing day serial order and additional stimuli presented during the test session.

Anagnostaras et al. (1999) report that lesions of dorsal hippocampus severely affected recently acquired, but not remote, (spatial) contextual fear memory. The current study assessed olfactory contextual fear either 24 or 48 hr after original training, therefore not addressing the time-course of dorsal hippocampal involvement in contextual conditioning. A crucial and interesting follow-up to this study concerns the participation of dorsal hippocampus in memory for olfactory contextual fear conditioning at multiple time points after training.

Even as mounting evidence suggests that the hippocampus is involved in encoding contextual representations (Frankland, Cestari, Filipkowski, McDonald, &
Silva, 1998; Good et al. 1998; Kim and Fanselow 1992; Maren et al. 1997; Phillips & LeDoux 1992; Rudy & O’Reilly 2001), the extent to which dorsal hippocampus normally participates in discrimination between contexts remains unclear (Frankland et al., 1998; Holt & Maren, 1999). Holt and Maren (1999) demonstrated that in contrast to saline-infused rats, in which the expression of latent inhibition to a tone after pre-exposure was context-specific, animals with dorsal hippocampus inactivation exhibited low levels of freezing to a tone independent of where tone pre-exposure had occurred. This result seems incongruent with evidence that animals with inactivation of dorsal hippocampus discriminated between spatial contexts, freezing more in spatial contexts which had been associated with shock than in neutral spatial contexts. To explain this discrepancy, the authors suggest that muscimol disrupts contextual retrieval only in cases involving ambiguous cues or contexts (Holt & Maren, 1999). In fact, there is some precedent for the idea that hippocampal damage may reduce the ability of subjects to discriminate between contexts. The computational model presented by O’Reilly and Rudy (2001) predicts a degree of context generalization in subjects with hippocampal damage. Possible evidence for a tendency to generalize between contexts was observed in the current study in that animals with inactivation of dorsal hippocampus froze modestly and similarly to both the unsafe and safe contextual stimuli, while control animals froze robustly to the unsafe but not to the safe contextual stimuli (see Fig. 4). Assessing the different contextual stimuli in the same spatial environment eliminated the ability of subjects to utilize spatial cues to disambiguate the meaning of olfactory contextual stimuli. Taken together, these results indicate that there is a decidedly nonspatial component to the role of the dorsal hippocampus in contextual conditioning, and that olfactory contextual conditioning is a fruitful means of examining such a function.
While several studies have concluded that selective damage of dorsal hippocampus does not produce anterograde deficits in contextually conditioned freezing (Richmond et al., 1999; Maren et al., 1997), compelling evidence suggests that space is not the only contextual variable encoded by the hippocampus (Anderson et al., 2006; Otto & Poon, 2006). Results of the present study support this notion, and further suggest that theories of hippocampal function need to account for forms of explicitly nonspatial forms of learning. The time-course and exact nature of dorsal hippocampus’ normal participation in such explicitly nonspatial forms of contextual learning remains to be seen.
5. Appendix
<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-training infusion</th>
<th>Unsafe contextual stimulus</th>
<th>Pre-testing infusion</th>
<th>Contextual stimulus tested</th>
<th>Pre-testing infusion</th>
<th>Contextual stimulus tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL-SAL</td>
<td>Saline</td>
<td>Pyridine or strawberry, counter-balanced within groups</td>
<td>Saline</td>
<td>Unsafe or safe, counter-balanced within groups</td>
<td>Saline</td>
<td>Unsafe or safe, counter-balanced within groups</td>
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<td>(N=11)</td>
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<tr>
<td>MUS-SAL</td>
<td>Muscimol</td>
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<td>Saline</td>
<td>Muscimol</td>
<td>Muscimol</td>
<td>Muscimol</td>
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<td>(N=11)</td>
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<td>30m</td>
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<tr>
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<td>Muscimol</td>
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<td>MUS-MUS</td>
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Table 1. Experimental design.
Figure 1. Cannula placement in dorsal hippocampus for each group.

Fig 1a. Group S-S (n=11)  
Fig 1b. Group M-S (n=11)  
Fig 1c. Group S-M (n=11)  
Fig 1d. Group M-M (n=11)
Figure 2. Freezing behavior during unsafe context test. The "unsafe" contextual stimulus was presented during min three, the CS was presented in min 10, and no additional stimuli were presented. While neither the main effect for condition ($F(3,40)=0.699, p>0.05$) nor the minute by condition interaction ($F(27,360)=1.102, p>0.05$) reached significance, the two-way repeated measures ANOVA revealed a significant main effect for minute on freezing during the unsafe test session ($F(9,360)=38.042, p<0.0001$). Subsequent post-hoc analyses (Student-Newman-Keuls) revealed that groups did not differ from one another during the auditory CS test.
Figure 3. Freezing behavior during safe context test. The "safe" contextual stimulus was presented during min three, the CS was presented in min 10, and no additional stimuli were presented. A two-way repeated measures ANOVA of freezing during the safe context test revealed a significant main effect of minute (F(9,360)=45.49, p<0.0001). Neither the main effect of group (F(3,40)=0.497, p>0.05) nor the group-by-minute interaction (F(27,360)=0.582, p>0.05) reached significance.
A one-way ANOVA revealed a significant effect of infusion condition \((F(3,40)=3.08, p=0.0383)\) on the difference between freezing to unsafe and safe contextual stimuli. Subsequent post-hoc comparisons (Dunnett) of treatment groups with the control group showed that MUS-SAL, SAL-MUS, and MUS-MUS groups all differed significantly from group SAL-SAL \((p<0.05)\).
Figure 5a-b. Behavior allocation during baseline and unsafe context presentation. Separate two-way repeated measures ANOVAs for freezing, rearing, ambulating, grooming, and whisking revealed a significant interaction between infusion condition (group) and testing period only for freezing behavior (F(12,120) = 3.27, p = 0.0004). Subsequent post-hoc comparisons (Student-Newman Keuls) revealed that for freezing behavior, group SAL-SAL differed from group MUS-SAL, group SAL-MUS, and group MUS-MUS during the unsafe context presentation (Fig. 5b).
Figure 5c-d. Behavior allocation during pre-CS period (return to baseline) and CS presentation.
References


