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Cholinergic Modulation of Pavlovian Fear Conditioning: Effects of Intrahippocampal Scopolamine Infusion

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ABSTRACT: Cholinergic neurotransmission has been implicated in the acquisition of a variety of tasks, including Pavlovian fear conditioning. To more precisely define the role of cholinergic modulation in this process, the effect of site-specific cholinergic antagonism was assessed. Male Long-Evans rats were implanted with chronic, bilateral cannulae aimed at the dorsal hippocampus. Infusions of scopolamine hydrobromide (50 μg bilaterally) or phosphate-buffered saline (PBS) were made immediately prior to a signaled Pavlovian fear conditioning procedure. On consecutive days following training, all rats were given independent tests assessing freezing to both the training context and the tone conditional stimulus (CS). Relative to PBS infused controls, rats that received intrahippocampal infusions of scopolamine showed a significant attenuation of contextual freezing but comparable levels of freezing to the tone CS. Neither shock sensitivity nor general activity levels differed between rats infused with scopolamine or PBS. These findings suggest that fear conditioning to context, but not discrete CS, requires intact cholinergic neurotransmission in the hippocampus. *Hippocampus 2001;11:371–376.*

KEY WORDS: muscarinic; hippocampus; acquisition; freezing

INTRODUCTION

Pavlovian fear conditioning, in which an aversive unconditional stimulus (US) is paired with a neutral conditional stimulus (CS), has been a useful paradigm for examining the neural substrates of learning and memory. Following this type of experience, rats will show a characteristic freezing response to both the discrete CS and the contextual cues paired with US presentation. Substantial evidence suggests that this response reflects an associative memory for the training episode (Blanchard et al., 1976; Fanselow, 1980, 1986) that positively correlates with the magnitude of the unconditioned stimulus and amount of training (Fanselow, 1982; Young and Fanselow, 1992). Thus, freezing provides a reliable and sensitive index of the strength of conditioning.

Recent evidence suggests that the neural substrates necessary for the acquisition of fear-related conditional responses to contextual and discrete CS are partially dissociable. Lesions of the hippocampal region have been shown to significantly attenuate the acquisition of long-term but not short-term contextual fear. In contrast, amygdala lesions produce significant deficits in both short-term and long-term contextual fear, as well as long-term fear to discrete CS (Kim et al., 1993; Anagnostaras et al., 1999a; Phillips and LeDoux, 1992; Kim and Fanselow, 1992; Maren et al., 1996). Such dissociations have led to the view that nuclei of the amygdala subserve the acquisition of fear-related behaviors, while the role of the hippocampus in fear conditioning is primarily one of contextual representation (Fansleow et al., 1993; Young et al., 1994; Rudy and O'Reiley, 1999).

While substantial progress has been made in delineating the neural circuitry critical for Pavlovian fear conditioning, less is known about the contribution of individual neurotransmitter systems to this process. The cholinergic system has been widely implicated in learning and memory processes, particularly in the acquisition of new information (Van der Zee and Luiten, 1999). A recent report, employing a signaled Pavlovian fear conditioning paradigm, demonstrated a dose-dependent disruption of contextual fear conditioning following systemic administration of the muscarinic receptor antagonist scopolamine (Anagnostaras et al., 1999b). Importantly, the dose response curves for contextual and tone freezing were not parallel. Contextual freezing was more susceptible to disruption than tone freezing. The differential sensitivity of contextual and tone freezing to scopolamine suggests that acquisition to these two stimuli may depend on different neural regions.

Given the widespread distribution of cholinergic receptors and the dependence of fear conditioning on multiple brain structures, the specific site of action for the observed effects on contextual conditioning following systemic scopolamine administration is unclear. Since there is substantial cholinergic innervation of the hippocampus, it is possible that this effect stems, in part, from disruption of normal cholinergic neurotransmission in the hippocampus. The cholinergic projections to the hippocampus directly contribute to hippocampal theta rhythm, facilitation of which significantly enhances contextual fear conditioning and hippocampal long-term potentiation (LTP) (Maren et al., 1994). In addition, disruption of theta rhythm by cholinergic antagonists attenuates hippocampal plasticity in vitro, providing a possible mechanism for the obeserved effects of cholinergic antagonists on memory (Vanderwolf and Robinson, 1981; Heurta and Lisman, 1995).

The nature of cholinergic modulation of hippocampal activity, along with the selective effects of a variety of

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hippocampal manipulations on contextual fear conditioning (Phillips and LeDoux, 1992; Kim and Fanselow, 1992; Maren et al., 1994), suggests that cholinergic neurotransmission in the hippocampus may be critical to the normal acquisition of contextual fear memories. To assess this possibility, we examined the effects of hippocampal cholinergic antagonism on the acquisition of freezing behavior. Rats received either intrahippocampal infusions of either scopolamine or vehicle, or sham surgery prior to Pavlovian fear conditioning.

METHODS

Subjects

Twenty-five male Long-Evans rats, bred at the UCLA Department of Psychology vivarium, served as subjects. All rats were individually housed 1 week prior to the beginning of the experiment, had free access to food and water, and were maintained on a 14:10-h light-dark cycle.

Surgery

One week after housing, rats received surgical implantation of a guide cannula aimed at the dorsal hippocampus or sham surgery. Rats were first anesthetized with sodium pentobarbital (50 mg/ml) and mounted in a stereotaxic frame. Guide cannulae (26-gauge, 7 mm; Plastics One) were then lowered to the dorsal hippocampus (3.8 mm posterior to bregma, 2.5 mm lateral to bregma, and 1.8 mm ventral to dura). Dental acrylic was used to fix the cannula to the skull, and dummy cannulae (33-gauge, 7 mm) were inserted into the guide cannulae. Rats receiving sham surgery underwent a similar procedure, except that no cannulae were implanted and the scalp incision was closed with stainless steel wound clips.

Apparatus

Training and contextual fear testing were conducted in a pair of identical observation chambers (28 × 22 × 21 cm; Lafayette Instruments, Lafayette, IN) housed in an isolated room. Illumination was provided by two overhead fluorescent light bulbs. Background noise (70 dB) was provided by shock scramblers and ventilation fans housed next to each chamber. The floor of each chamber consisted of 17 steel rods spaced 1.0 cm center-to-center wired to the shock generator for the delivery of the footshock US. Speakers mounted in the wall of each chamber allowed for tone CS presentation during training. Prior to training and testing, each chamber was cleaned with a 5% ammonia hydroxide solution. A video camera mounted directly in front of the chambers allowed for behavioral observations and recording of the training session for off-line analysis.

Testing of the discrete CS was conducted in a pair of modified observation chambers (28 × 21 × 22 cm; Lafayette Instruments). These chambers were housed in a separate room illuminated by a 15-W red bulb. Background noise (70 dB) was provided by a white-noise generator. The floor of each chamber consisted of 17 vertically staggered steel rods spaced 1.5 cm apart. Two white Plexiglass panels were inserted at approximately 60° angles with the floor in each chamber. Prior to testing, all chambers were cleaned with a 1% acetic acid solution. A video camera mounted directly in front of the chambers allowed for behavioral observations during testing.

Procedure

Prior to the fear conditioning procedure, rats with cannula implants received bilateral infusions of either scopoloamine hydrobromide (50 μg/μl; n = 9) or PBS (n = 7). Rats were manually restrained while injection cannulae (33-gauge; 8 mm), connected to 10-μl Hamilton syringes with PE-20 polythylene tubing (Plastics One) and mounted on a microinfusion pump (Harvard Apparatus, South Natick, MA), were inserted into the guide cannulae. A total volume of 1 μl of either scopolamine or PBS was infused at a rate of 0.25 μl/min. Injection cannulae were left in place for an additional minute to facilitate drug/vehicle diffusion. Dummy cannulae were then reinserted and rats were placed in the conditioning chambers. Sham lesioned animals (n = 9) were left in the transportation vehicle for an equivalent period of time prior to being placed in the conditioning chambers.

Three minutes after placement in the conditioning chambers, rats received three tone presentations (2 kHz, 90 dB, 10 s), each coterminating with footshock (1 mA, 2 s) with an intertrial interval of 60 s. Two minutes following the last footshock, rats were removed from the chambers and returned to their home cages.

Because Pavlovian fear conditioning procedures typically result in the acquisition of freezing to both the training context and the tone CS, independent tests were conducted to assess contextual and tone freezing. To assess contextual freezing, 1 day following training, rats were reexposed to the observation chamber for an 8-min extinction test to assess levels of contextual fear. No infusions were made prior to this session. Behavior was sampled once every 8 s, and occurrences of freezing behavior, defined as the cessation of all movement except that necessitated by respiration, were counted. To assess freezing to the tone CS, on the following day, rats were again transported to the laboratory but were placed in a novel observation chamber. The use of a novel conditioning chamber to asses tone freezing precludes any contribution of contextual freezing to this measure. Two minutes after placement in the chambers, rats received a 6-min presentation of the CS. Each rat's behavior was again sampled once every 8 s and scored for freezing.

Histology

To assess cannula placements, rats were perfused transcardially with 0.9% saline followed by 10% formalin solution. Brains were removed from the skull and placed in a 10% formalin/30% sucrose solution for 3 days prior to sectioning. Coronal sections (40 μm thick) were taken throughout the extent of the cannula track and mounted on gelatinized slides. Sections were stained with thionin (0.25%) and injection sites were reconstructed.

Behavioral Data Analysis

Ambulatory crossovers

As a measure of general activity, the number of ambulatory crossovers were observed during the 3-min period prior to the first trial during the training session. A crossover was defined as the rat's hind paws crossing the midline of the observation chamber.

Activity burst analysis

To assess shock sensitivity, the unconditional response (UR) to footshock during the conditioning session was quantified from videotape. Using a PowerMac 8100/80, video from the 2-s period during footshock and the 2-s period prior to tone onset were digitized, using NIH image 1.61 software. The location of the rat in each frame (X-Y coordinate) was then calculated for each of 20 consecutive 100-ms frames. The change in position, defined as the change in X-Y coordinates, between consecutive frames was converted into distance traveled, and average velocity (cm/s) for each 2-s period sampled was calculated.

Freezing

For both the context and tone tests, behavioral observations were made by a blind observer and were converted to percent freezing by dividing the number of observations scored as freezing by the total number of observations for 2-min blocks during each test. Freezing percentages during the tone test were calculated for the 2-min baseline period and the 6-min CS presentation.

RESULTS

All rats had damage to the overlying neocortex but no damage to the hippocampus or thalamic areas. Based on guide cannulae and injector tracks, all rats had placements within the dorsal hippocampus. While the extent of infusion was not directly determined, previous reports suggest that hippocampal infusions of comparable volume diffuse approximately 1 mm from the injector tip (Myers et al., 1971). Based on this estimate, the site of action for scopolamine was predominantly restricted to the dorsal hippocampus. Figure 1 depicts the location of injection sites for all rats.

As can be seen in Figure 2A, pretraining infusions of either scopoloamine or PBS vehicle did not significantly increase activity relative to sham-operated controls. General activity prior to the first conditioning trial, as assessed by ambulatory crossovers across the 3-min period, did not differ between groups ($F(2,22) = 2.13$, $P > 0.1$). Additionally, a significant effect of time was observed ($F(2,44) = 7.61$, $P < 0.01$), suggesting that all rats show a comparable decrease in activity across the preshock period. The group $\times$ time interaction was not significant ($F(2,22) = 0.836$, $P > 0.5$).

Pretraining infusions did not effect the UR to footshock. Analysis of the UR to the first footshock during the conditioning session revealed no significant group differences ($F(2,22) = 1.17$, $P > 0.3$). The effect of period was significant ($F(1,22) = 209.84$, $P < 0.0001$). Rats showed a significant increase in velocity between the baseline period and the US period. No significant group $\times$ period interaction was observed ($F(2,22) = 1.23$, $P > 0.3$; Fig. 2B).

Analysis of freezing behavior observed prior to footshock and during the 1-min periods following each of the three shocks during training revealed a significant effect of group ($F(2,22) = 12.21$, $P < 0.001$; Fig. 3A). Pretraining infusion of scopolamine significantly attenuated freezing relative to both PBS-infused and sham-operated rats. The effect of trial was also significant ($F(3,66) = 31.41$, $P < 0.0001$), suggesting a significant increase in freezing across the session.

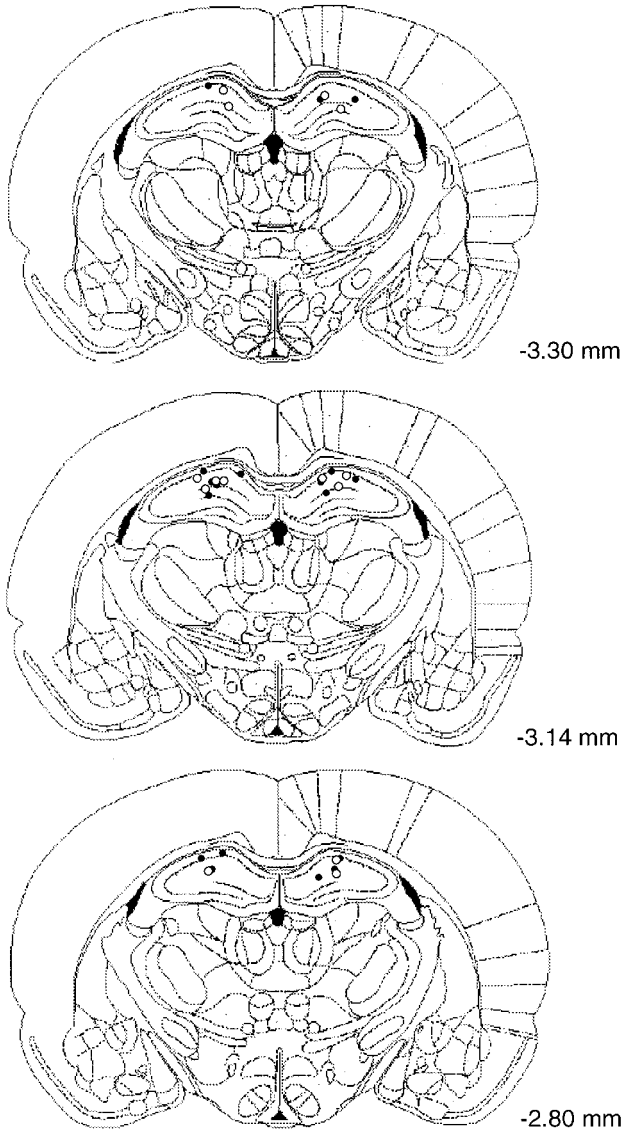

FIGURE 1. Location of injection sites in dorsal hippocampus for scopolamine (solid circles) and PBS (open circles)-infused rats. Coronal sections were taken from atlas of Paxinos and Watson (1996). Numbers indicate distance from bregma.

Figure 3B depicts freezing percentages for the context test conducted 1 day after conditioning. Pretraining infusion of scopolamine into the dorsal hippocampus significantly attenuated freezing to the training context relative to both PBS-infused and sham-operated rats. A significant effect of group was observed ($F(2, 22) = 4.89$, $P < 0.05$). The effect of time was not significant ($F(3,66) = 1.27$, $P > 0.2$), and neither was the group $\times$ time interaction ($F(6,66) = 0.54$ $P > 0.7$).

In contrast to the effects of intrahippocampal infusions of scopolamine on contextual fear conditioning, scopolamine did not produce a significant attenuation of conditioning to the tone CS. Figure 3C depicts percent freezing during the 2-min period prior to CS onset and during the 6-min CS presentation. Freezing during the pre-CS period was negligible for all groups. Freezing across the CS test session revealed no effect of group ($F(2, 22) = 1.58$, $P > 0.2$) or a group $\times$ time interaction ($F(6, 66) = 0.631$, $P > 0.7$).

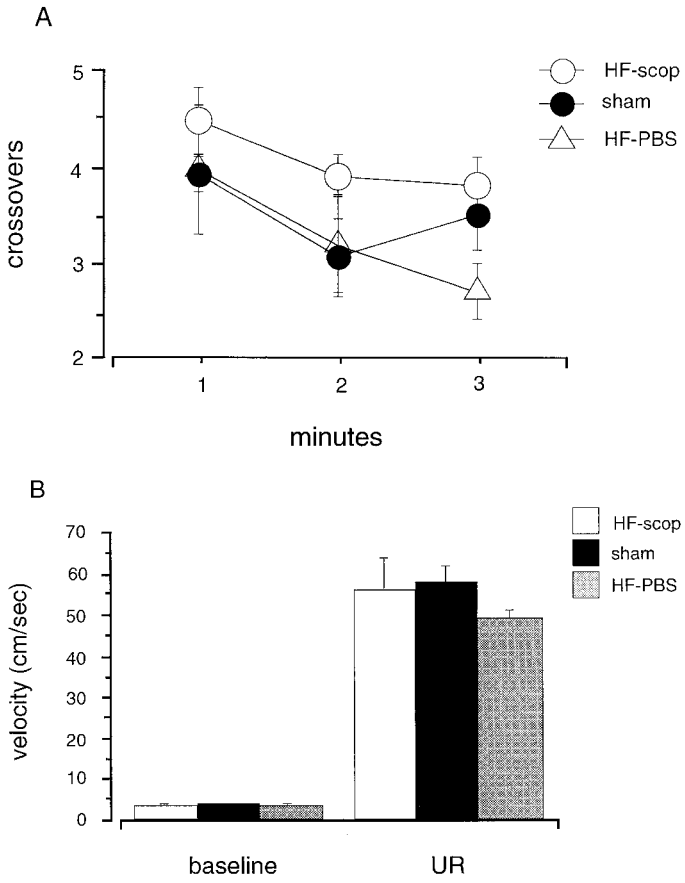

FIGURE 2. A: Mean number of ambulatory crossovers during 3-min period preceding first training trial. B: Unconditional response to first footshock during training. Error bars represent standard error of the mean.

DISCUSSION

The major finding of the current study is a dissociation of the effects of intrahippocampal scopolamine infusion on the acquisition of conditional freezing to contextual and discrete CS. Specifically, pretraining scopoplamine infusions attenuated the acquisition of contextual freezing but not the acquisition of freezing to the discrete CS. Importantly, rats showed no freezing in the nonshocked context prior to tone testing, suggesting that freezing observed during the context extinction tests was associatively mediated and not due to sensitization. Additionally, because rats infused with scopolamine before training show normal shock sensitivity, the observed conditioning deficits cannot be attributed to attenuated US processing.

While the pattern of results suggest that pretraining intrahippocampal scopolamine infusions selectively disrupt the acquisition of contextual fear conditioning, all rats in the current experiment were tested off-drug, leaving the data open to interpretation in terms of state-dependency. By this view, the deficits observed in contextual conditioning may simply reflect generalization decrement between the training state (on-drug) and testing state (off-drug). Comparison of freezing levels during the training session with freezing during the context test appear to be inconsistent with this possibility. Rats infused with scopolamine before training showed low freezing levels during the training session (on-drug), and during the context test 24 h later (off-drug). If the deficits observed during testing were the result of generalization decrement, then normal levels of freezing might be expected during training; however, deficits in contextual fear were observed both during training and during testing. Additional aspects of the data also appear to be inconsistent with a state-dependency interpretation. In the present study, the observed freezing deficits were selective for contextual freezing. When tested off-drug, the same rats

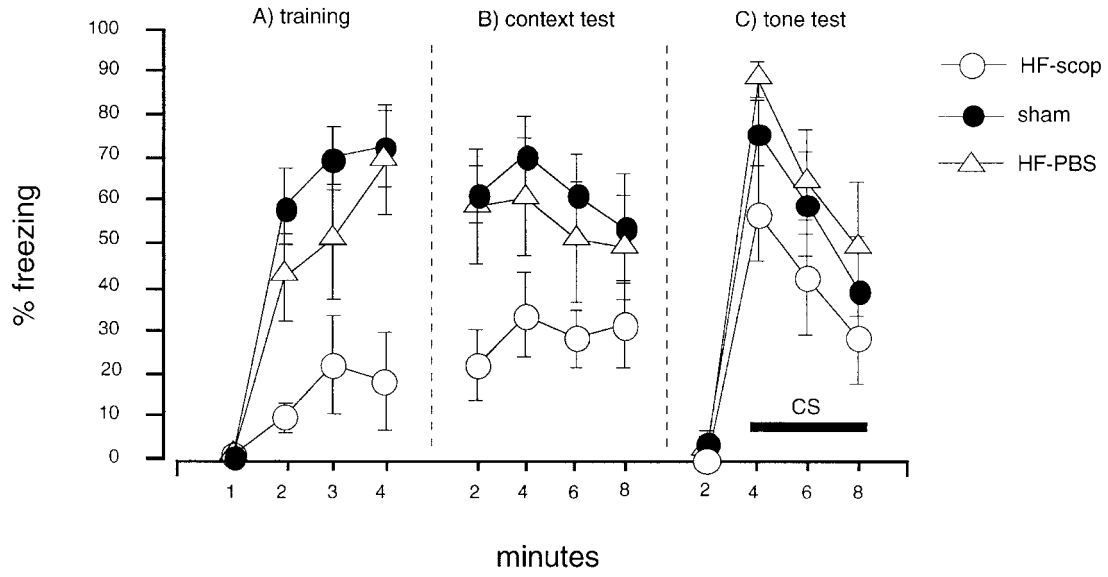

FIGURE 3. Percent freezing behavior across training and test sessions. A: Freezing behavior during 1-min interval prior to first shock and after each of three shocks during training session. B: Freezing during 8-min context extinction test. Data points represent mean freezing percentage for each 2-min block. C: Freezing during CS extinction test. Bar indicates minutes during which CS was presented. Error bars represent standard error of the mean.

that displayed impaired contextual freezing also displayed normal freezing to the CS. If state-dependency contributed to the freezing deficits observed during the context test, then similar deficits to the tone CS might also be expected. However, this assumes that both contextual and tone freezing are equally susceptible to state-dependent effects. Even though there is no direct evidence inconsistent with this possibility, the fact that tone freezing reliably generalizes across contexts may indicate a relative insensitivity of this behavior to changes in state.

An alternative account of the selective contextual conditioning deficit is that the training parameters produced stronger conditioning to the tone CS relative to the context. By this view, acquisition to both contextual and tone CS may be impacted by hippocampal cholinergic antagonism, but a selective contextual conditioning deficit was observed due to a ceiling effect for tone conditioning. Comparison of freezing levels for control rats across the context and tone extinction tests do not support this interpretation. Nearly identical levels of freezing were observed in these rats during context and tone extinction tests. In addition, the training parameters employed produced freezing levels well below those produced by additional training. Thus, as observed in previous studies with permanent lesions (Kim and Fanselow, 1992; Anagnostaras et al., 1999a), the selective contextual freezing deficits observed are likely not due to differences in the strength of contextual and tone conditioning.

Pretraining intrahippocampal scopolamine infusions produced no effects on general activity levels as measured by ambulatory crossovers. In contrast, several studies demonstrated increased activity levels following permanent hippocampal lesions (Roberts et al., 1962; Douglas and Isaacson, 1964; Blanchard et al., 1977; Kim et al., 1993). Recent studies from our laboratory demonstrated both hyperactivity and contextual fear conditioning deficits in rats with electrolytic hippocampal lesions (Maren and Fanselow, 1997; Maren et al., 1997). In light of these findings, it has been suggested that the attenuation of contextual freezing observed following permanent hippocampal lesions may be the result of hyperactivity rather than an associative deficit (McNish et al., 1997). Using a measure of activity similar to that used by Maren et al. (1997), intrahippocampal scopolamine infusion in the present study had no effect on activity levels, yet produced significant deficits in contextual freezing during the test session. A similar pattern was observed following pretraining APV administration (Kim et al., 1992). Thus, the current data join a body of evidence suggesting that deficits in the acquisition of contextual conditioning following hippocampal lesions are not the result of changes in general activity. It is important to note that the hyperactivity produced by electrolytic lesions may reflect indirect effects mediated by extrahippocampal regions. Because elevated activity levels are typically observed following electrolytic lesions, but not after excitotoxic lesions, both the hyperactivity and the resulting contextual conditioning deficits produced by electrolytic lesions may stem from disruption of fibers passing through the dorsal hippocampus. It has been suggested that subiculo-accumbens projections may be responsible for these effects (Maren et al., 1997). These fibers traverse the dorsal hippocampus (Canteras and Swanson, 1992), and pharmacological manipulations in the nucleus accumbens impair acquisition of contextual fear conditioning (Westbrook et al., 1997). Regardless of the source of conditioning deficits following electrolytic lesions, the current data provide a clear dissociation between activity levels and acquisition of contextual fear conditioning, supporting a direct hippocampal involvement in contextual representation.

Consistent with the selective effects of intrahippocampal scopolamine infusion on contextual conditioning, several reports have demonstrated effects of hippocampal manipulations on the acquisition of contextual fear conditioning but not conditioning to discrete cues (Phillips and LeDoux, 1992; Kim and Fanselow, 1992; Maren et al., 1994). This dissociation stands in contrast to the commonly observed effects of amygdala manipulations on the acquisition of fear conditioning. Both permanent lesions (Phillips and LeDoux, 1992; Maren et al., 1996) and pharmacological manipulations (Wilensky, Schafe, and LeDoux, 1999) of the amygdala prior to conditioning produce substantial deficits in the acquisition of freezing to both contextual and discrete CS. Taken together, these results provide further support for dissociable contributions of the hippocampus and amygdala in fear conditioning and implicate hippocampal cholinergic neurotransmission in the processes underlying contextual fear conditioning.

It has been suggested that the sensitivity of contextual fear conditioning to hippocampal manipulations reflects a selective role of this structure in the sensory processing of the training context (Young et al., 1994). By this view, the hippocampus is thought to integrate the unimodal sensory information of the context into a unified representation that can be associated with the US. Disruption of hippocampal function prevents the development of this representation, producing contextual conditioning. The observation of impaired freezing during the training session and the subsequent context test, but normal freezing to the discrete CS, in the current study is consistent with this conceptualization. While these data support a role for cholinergic neurotransmission in the development of contextual representations, cholinergic activity does not appear to be critical for all spatial learning. Hippocampal cholinergic deafferentation, produced by 192 IgG-saporin lesions of the medial septum, did not significantly affect the ability of rats to acquire place discriminations in the water maze (Baxter et al., 1995). As performance of this task depends on a spatial representation of the environment, this finding appears to be inconsistent with a general role for hippocampal cholinergic transmission in representing spatial relationships.

However, evidence from fear conditioning studies suggests that under some circumstances, extrahippocampal regions may be sufficient to mediate acquisition of contextual conditioning. Hippocampal lesions made prior to training typically produce mild impairments in contextual conditioning, while lesions made after training produce robust deficits (Maren et al., 1997). These data suggest that, when intact, the hippocampus is necessary for contextual representation, but in the absence of the hippocampus, other regions assume this function. Permanent cholinergic deafferentation may also promote the involvement of extrahippocampal areas, allowing for the normal acquisition of spatial tasks. Alternatively, the differential effects of cholinergic manipulations on the acquisition of these two tasks may reflect a selective contribution of hippocampal cholinergic receptors to the development of contextual representations required for fear conditioning. Further studies comparing the effects of pretraining hippocampal cholinergic deafferentation and permanent hippocampal lesions on contextual fear conditioning will be necessary to resolve this issue.

While the mechanisms by which the hippocampus mediates contextual conditioning are currently unknown, the current data along with previous findings suggest a role for cholinergic and NMDA receptors in this process (Anagnostaras et al., 1999b; Kim et al, 1992). With respect to cholinergic transmission, one possibility is that these receptors are critical for normal sensory transmission, and acquisition deficits reflect an inability of hippocampal cells to respond to sensory input. While the current design does not rule out this possibility, it seems unlikely, given that scopolamine has little effect on evoked unit responses in the hippocampus (Luntz-Leybman et al., 1992). Another possibility is that interactions between cholinergic and NMDA receptors contribute to the hippocampal plasticity underlying contextual representation. Recently it was demonstrated that m1 cholinergic receptors and NMDA receptors are colocalized on hippocampal pyramidal cells (Marino et al., 1998). This suggests a functional link between cholinergic and glutamatergic transmission in the regulation of hippocampal pyramidal cell activity. Given the substantial cholinergic innervation of the hippocampus (Frotscher and Leranth, 1985), the dependence of hippocampal theta rhythm on cholinergic innervation, and the contribution of theta rhythm and NMDA receptors to hippocampal plasticity (Huerta and Lisman, 1994), the observed attenuation of contextual fear conditioning following scopolamine infusion may reflect a disruption of NMDA-mediated hippocampal plasticity resulting from attenuated hippocampal theta rhythm.

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