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Research report

Memory for context is impaired by injecting anisomycin into dorsal hippocampus following context exploration

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Abstract

Pre-exposure to the context facilitates the small amount of contextual fear conditioning that is normally produced by immediate shock. This context pre-exposure facilitation effect provides a convenient way to study the rat's learning about context. We recently reported that anterograde damage to dorsal hippocampus prevents this facilitation. The present experiments strengthen this conclusion by showing that the protein synthesis inhibitor, anisomycin, injected bilaterally into the dorsal hippocampus following context pre-exposure also significantly reduces the facilitation effect. The same treatment given immediately after immediate shock, however, had no effect on facilitation. These results support theories that assume that, (a) contextual fear involves two processes, acquiring and storing a conjunctive representation of a context and associating that representation with fear; and (b) the hippocampus contributes to contextual fear by participating in the storage of the memory representation of the context. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Since the initial reports that damage to the hippocampus selectively impairs contextual fear conditioning but does not impair conditioning to a phasic auditory-cue also paired with that shock [12,19], the study of contextual fear conditioning has been a major concern for many researchers interested in the role of the hippocampus in memory. Subsequent work, however, has revealed that the contribution the hippocampus makes to contextual fear conditioning is not as straightforward as originally supposed. This is because there are a number of reports that damage to the hippocampus prior to conditioning does not always impair contextual fear conditioning, and the nature of the lesion (electrolytic or neurotoxic) is important. Anterograde electrolytic damage to dorsal hippocampus impairs contextual fear conditioning, but anterograde neurotoxic damage does not [15,20,23].

Such results question the idea that the hippocampus contributes to contextual fear conditioning by providing a memory substrate. However, there are other data that more strongly support the idea. First, retrograde damage to dorsal hippocampus produced by either electrolytic or excitotoxic means impairs contextual fear conditioning [12,15]. Second, it has been reported that the injection of the protein synthesis inhibitor, anisomycin, directly into dorsal hippocampus following conditioning prevents the consolidation of the memory for contextual but not auditory fear conditioning [1].

There are reasons to believe that the hippocampus makes a contribution to the memory substrate for contextual fear. Nevertheless, it is also clear that contextual fear conditioning can be supported by extra-hippocampal formation brain regions. From one point of view, it is not surprising that identifying the contribution the hippocampus makes to contextual fear conditioning is not straight forward. This is because several theoretical frameworks linking the hippocampus to memory assume that the background of cues that provide a space or context for experience can be represented in two ways, (a) a *features view* which assumes that the context is represented as a set of

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independent features or elements that each can enter into association with other events (e.g. shock); and (b) a *conjunctive or mapping view* which assumes that the individual features are combined into a unitary representation that is different from the sum of its parts [4,21,22,29,33]. In these frameworks, the hippocampus is thought to support the acquisition of the conjunctive representation, and extra-hippocampal areas are assumed to support the representation of the features and their linkages.

In such frameworks there is no a priori reason to suppose that damage to the hippocampus must impair contextual fear conditioning because the independent features of the context themselves could associate with shock and support conditioned fear. The finding that anterograde damage to the hippocampus does not always impair contextual fear conditioning is consistent with this view. That retrograde damage to the hippocampus or injecting a protein synthesis inhibitor into the hippocampus after conditioning severely compromises contextual fear however, is extremely important because it provides the strongest evidence that the hippocampus is involved in contextual fear conditioning.

Central to a conjunctive theory analysis of the contribution the hippocampus makes to contextual fear conditioning is the idea that it involves two independent processes [7,24,26,34]. When the rat is placed into the novel context it, (a) constructs a unitary representation of its features; and (b) associates that representation with the system that generates fear. It is the first process that depends on the hippocampus. There is considerable support for the view that learning about context is independent of associating it with shock. It comes from a range of experiments that have demonstrated what we will call the *context pre-exposure facilitation effect*—the fact that pre-exposure to the conditioning context the day before a context-shock experience can enhance low levels of fear contextual conditioning that otherwise would occur [4,21,22,29,33].

We have recently provided evidence that, (a) the context pre-exposure facilitation effect is the result of the rat acquiring a conjunctive representation of the context during the pre-exposure phase [27]; and (b) that the hippocampal formation is critically involved in this learning [25]. The latter finding provides the motivation for the present study. In that study [25], we employed the immediate shock paradigm and reported that context pre-exposure significantly enhanced the low level of contextual fear conditioning produced by immediate shock but that anterograde damage to the dorsal hippocampal formation produced by the neurotoxin, NMDA, eliminated this effect.

The finding that anterograde damage to the dorsal hippocampal formation significantly attenuates the context pre-exposure facilitation effect is consistent with the view that the hippocampus participates in contextual fear conditioning by providing the neural basis for a conjunctive representation. However, there are reasons to view this interpretation with caution. This is because anterograde damage to the hippocampus produces an animal that is not normal either at the time of the learning experience or at the time of testing. Consequently, in principle, the impairment could be the result of altering either of the processes essential to proper encoding of the environment at the time of learning [14], or the rat could be impaired during testing because the lesion altered the processes by which fear is expressed [17]. Rudy, Barrientos & O'Reilly's [25] findings weakened the fear expression hypothesis by showing that damage to dorsal hippocampus did not influence the acquisition of contextual fear conditioning under standard training parameters. However, they did not directly address the other alternatives.

Thus, the purpose of the present experiments was to provide additional evidence for the view that the hippocampus contributes to contextual fear conditioning by storing a conjunctive representation of the context. The ideal strategy for making this case is to use a methodology in which the rat is normal at the time of the learning experience and at the time of testing. To do this, we took advantage of the fact that there have been several reports that the consolidation of memory for contextual fear conditioning can be prevented by the injection of the protein synthesis inhibitor, anisomycin, either into the brain prior to conditioning [31], peripherally following conditioning [2] or into dorsal hippocampus following conditioning [1].

2. Methods

2.1. Subjects

Adult male Long–Evans derived rats (bred at the University of Colorado) weighing between 225 and 250 g at the beginning of the experiment were housed four to a cage at 25 °C on a 12 h light–12 h dark cycle (lights on at 07:00 h). Rats were allowed free access to food and water. All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee.

2.2. Surgery

Under Nembutal anesthesia (50 mg/kg), rats were placed into a Kopf stereotaxic apparatus and implanted with either one (lateral ventricle) or two (dorsal hippocampus) chronic stainless steel guide cannulae (Plastics One, Roanoke, VA). Using the Paxinos and Watson (1986) rat brain atlas [18] the following coordinates were used for bilateral dorsal hippocampal implantation: AP, −3.5 mm, ML, ±2.4 mm, DV: −3.0; and for lateral

ventricle implantation, AP: −0.9 mm, ML, ±1.5 mm, DV: −3.0 mm. Cannulae were secured with dental acrylic and were fitted with a dummy cannula that extended 1 mm beyond the tip of the guide cannulae (i.e. total length: 4 mm), to maintain patency. Rats were allowed to recover for 4 week.

2.3. Apparatus

Rats were pre-exposed in either a control or conditioning context. The control context was an individual opaque cage (26 L × 16 W × 12 H, cm) that was in a different room than the conditioning context. The conditioning context was one of two identical Igloo ice chests (54 L × 30 W × 27 H, cm) with white interiors. A speaker and an activated 24-V DC light bulb were mounted on the ceiling of each chest. A clear plastic window (30 × 18 cm) was cut into the door of the chest so that the rats could be observed. The conditioning chambers (26 L × 21 W × 24 H, cm) placed inside each chest were made of clear plastic and had window screen tops. The 2 s, 0.65 mA shock (as measured at the rod floor) was delivered through a removable floor of stainless steel rods 1.5 mm in diameter, spaced 1.2 cm center to center. Each rod was wired to a shock generator and scrambler (Lafayette Instruments Model 8240415-SS). Both chambers were cleaned with water before each animal was pre-exposed, shocked, or tested.

2.4. Behavioral procedures

In Experiment 1, rats were placed in the conditioning context for 2 min before the onset of one 2 s shock. They were taken out and given an ICV injection of either anisomycin or vehicle. A day later, they were re-placed in the conditioning chamber and observed 6 min for freezing behavior.

In the other experiments, rats were taken two at a time from their home cage and transported to either the conditioning or control context in a black ice bucket with the lid on so that they could not see where they were being taken. This procedure was used in order to establish an association between the contextual representation and the transport cues preceding placement of the rat in the context. Rats were placed in the context and allowed to freely explore for an allotted amount of time and then were transported back to their home cage where they remained approximately 40 s before the next pre-exposure. This procedure was repeated 6 times. Animals remained in the novel context for 5 min on the first exposure and for 40 s on the five subsequent exposures. The rats were transported in the black bucket each time that they returned to their home cage, but with the lid off. In Experiment 2, animals were microinjected with either the drug or vehicle immediately after the last pre-exposure and then returned to their home cage. In Experiment 3, animals were microinjected with either the drug or vehicle immediately after the immediate shock treatment.

Twenty-four h following context pre-exposure (in Experiments 1 and 2), each animal was taken from their home cage and transported to the conditioning context in the black bucket. There, they received one 2 s shock immediately after being placed in the context. They were then quickly taken out of the context and transported back to their home cage (except rats in Experiment 3 that received an injection before being taken back to their home cage).

Contextual fear was assessed 24 h following conditioning by placing the rat in the conditioning context for 6 min. Using a time sampling procedure, every 10 s each rat was judged as either freezing or active at the instant the sample was taken. Freezing, the rats dominant defensive fear response, is an immediate suppression of behavior that is accompanied by immobility, shallow breathing, and a variety of other autonomic changes including an increase in heart rate and pilo-erection [6]. Freezing in these experiments was defined as the absence of all visible movement, except for respiration. Scoring began approximately 10 s after the animal was placed into the chamber. The two scorers had no knowledge of the rat's experimental condition, and inter-rater reliability exceeded 97% for all experiments.

2.5. Microinjections

Microinjections were carried out after pre-exposure to a context (Experiments 1 and 2) or after immediate shock (Experiment 3). Rats were gently wrapped in a soft towel, and a 33-gauge microinjector (Plastics One) attached to PE50 tubing was inserted through the indwelling guide cannula. The distal end of the PE50 tubing was attached to a 100 μl Hamilton syringe that was attached to a Kopf micro-injection unit (Model 5000) that accurately dispensed the desired volume. For experiments employing bilateral intrahippocampal injections 0.5 μl was injected into each side, and for intracerebroventricular (ICV) injections 5.0 μl were injected into either the left or right (randomly assigned) lateral ventricle.

2.6. Drugs

Anisomycin (Sigma) was dissolved in equimolar HCL, diluted with ACSF and adjusted to pH 7.4 with NaOH for a final concentration of 125 μg/μl. The intrahippocampal dose was 62.5μg/0.5 μl per side. The ICV dose was 625.0 μg/5.0 μl. Controls received equivolume of the vehicle (pH 7.4).

2.7. Histology

Animals in Experiment 1 were cannulated in the lateral ventricle. A standard saline drip procedure was used, as previously described [32] at the time of surgery to verify that the cannula was correctly placed in the ventricle. To verify cannulae placement in all other experiments, rats were anesthetized with Nembutal and decapitated. Brains were removed and frozen in cold isopentane. Coronal sections (40 μm thick) were sliced through the hippocampus with a cryostat at −19 °C, and every third section was mounted. Sections were stained with cresyl violet and examined by light microscopy to visually verify the placement of the cannulae in the dorsal hippocampus. Rats were excluded from the statistical analyses if the cannulae track marks were found anterior to −2.80 mm or posterior to −4.15 mm; lateral to ±3.5 mm or medial to ±1.5 mm; dorsal to −3.0 mm or ventral to −4.0 mm.

3. Experiment 1

Other researchers have reported that an ICV injection of anisomycin either before [31] or after fear conditioning [2] impairs contextual fear conditioning. However, because we had never used this methodology, the purpose of Experiment 1 was to determine whether, in our hands, anisomycin injected ICV after conditioning would impair contextual fear conditioning. In this experiment rats were placed in the conditioning chamber for 2 min before receiving a single 2 s shock. Following removal from the chamber they were injected ICV with either anisomycin or its vehicle. The next day, all rats were tested for fear of the context.

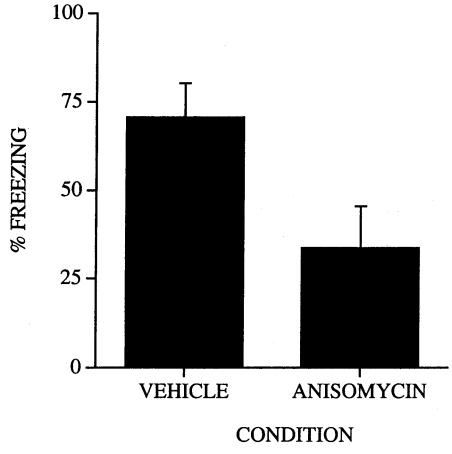

Fig. 1. Mean percent freezing during the contextual fear test. Rats were conditioned to a context followed by an ICV injection of either anisomycin or vehicle. Note that anisomycin-injected rats displayed little fear to the context compared with vehicle-injected control rats. Bars represent S.E.M.

As shown in Fig. 1, consistent with the literature [2,31] anisomycin-injected rats ($n = 7$) displayed significantly less fear to the conditioning context than vehicle-treated ($n = 7$) rats ($F(1,13) = 5.88$, $P < 0.05$. Thus, using somewhat different training conditions, we were able to replicate Schafe et al.'s finding that blocking protein synthesis impairs contextual fear conditioning. Schafe et al. [31] injected anisomycin prior to conditioning whereas we injected it after conditioning. Since anisomycin was injected ICV, this result could reflect protein synthesis-dependent consolidation processes in many parts of the brain, including the hippocampus and amygdala. In the following experiments however, injections were administered intrahippocampally to observe the effects of the drug specifically on hippocampal function.

4. Experiment 2

As noted, the context pre-exposure facilitation effect provides an ideal methodology for studying the contribution the hippocampus makes to a rat's construction of a representation of the context because it allows the rat to learn about the context independently of associating it with shock. The aim of this experiment was to determine if the memory for this learning depends on consolidation processes in the hippocampus.

Thus, rats were first exposed either to the conditioning context or to a very different control context. Immediately after context pre-exposure rats were injected with either anisomycin or its vehicle. The next day all rats were given an immediate shock in the conditioning context. On the third day, all rats were tested for fear of the context.

We expected that rats which were pre-exposed to the conditioning context and injected with the vehicle would display significantly more contextual fear conditioning than rats that were pre-exposed to the control context. This would constitute a context pre-exposure facilitation effect. However, if the hippocampus supports the storage of the context representation, and the consolidation of this memory depends on protein synthesis, then rats pre-exposed to the conditioning context and injected with anisomycin should not display a context pre-exposure facilitation effect and should not differ from the rats pre-exposed to the control context.

As shown in Fig. 2, rats injected with the vehicle showed a context pre-exposure facilitation effect: Rats pre-exposed to the conditioning context ($n = 10$) displayed significantly more fear than rats which were pre-exposed to the control context ($n = 5$). Rats injected with the anisomycin, however, did not display the facilitation effect: rats pre-exposed to the conditioning context ($n = 12$) did not differ from rats pre-exposed to the control context ($n = 4$). Consistent with this descrip-

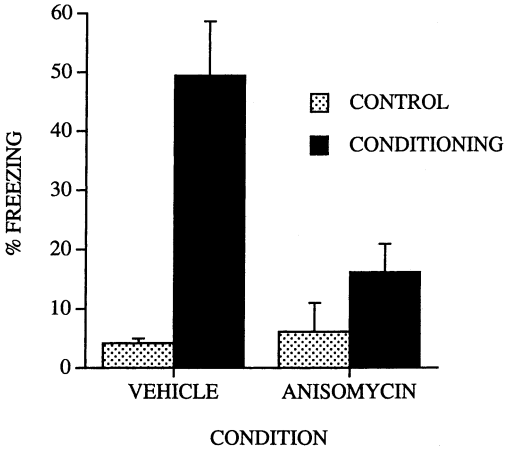

Fig. 2. Mean percent freezing during the contextual fear test. Rats were pre-exposed to either the conditioning or control context followed by an intrahippocampal injection of either anisomycin or vehicle. Note that in the vehicle group, rats pre-exposed to the conditioning context displayed more fear to the context than those pre-exposed to the control context. There was no difference between anisomycin-injected rats. Moreover, vehicle-injected rats pre-exposed to the conditioning context displayed more fear to the context than anisomycin-injected rats pre-exposed to the conditioning context. Bars represent S.E.M.

tion, a 2 factor ANOVA (Context Pre-exposure × Drug treatment) revealed a significant effect of Context Pre-exposure, $F(1,30) = 11.87$, $P < 0.005$, and a significant Context Pre-exposure × Drug interaction, $F(1,30) = 4.79$, $P < 0.05$. An analysis of the simple effects revealed that rats pre-exposed to the conditioning context and injected with the vehicle differed ($P < 0.05$) from rats in all the other conditions. No other differences were significant.

These results cannot be due to the effect of the drug on the rat's exploratory behavior because the rat was not injected with the drug until after context pre-exposure. They are unlikely due to the effect of the drug on the rat's ability to express fear because the rat was not under the influence of the drug at the time of testing. The results of this experiment are, however, consistent with the hypothesis that the acquisition of a representation of context depends on the neural processes supported by the hippocampus.

5. Experiment 3

As noted, contextual fear conditioning can be conceptualized as depending on two separable learning processes, (a) the acquisition of the representation of context, which depends on the hippocampus and (b) associating this representation with shock, which does not [7]. The results of Experiment 2 support this view because the injection of anisomycin following context pre-exposure virtually eliminated the context pre-exposure facilitation effect. In Experiment 3, we used anisomycin to evaluate another implication of the two-process model. Specifically, instead of injecting anisomycin into the hippocampus following the context pre-exposure phase, we injected it following the immediate shock phase. Note that the view that the hippocampus is essential to learning about the context but not to associating the context with shock predicts that inhibiting protein synthesis in the hippocampus following immediate shock should not influence the context pre-exposure facilitation effect. This is because rats pre-exposed to the context should have consolidated the hippocampal-dependent context representation and the associative process linking that representation is not in the hippocampus. Thus, disrupting protein synthesis in the hippocampus following immediate shock should have no effect on the context pre-exposure facilitation effect.

As shown in Fig. 3, immediately-shocked rats pre-exposed to the conditioning context (vehicle, $n = 8$; anisomycin, $n = 7$) displayed more contextual fear than immediately-shocked rats pre-exposed to the control context (vehicle, $n = 6$; anisomycin, $n = 6$). This context pre-exposure facilitation effect was not influenced by injecting anisomycin following immediate shock. Consistent with this description, a two-factor ANOVA (Context Pre-exposure × Drug) revealed a significant main effect of context pre-exposure, $F(1,30) = 11.87$, $P < 0.005$, but neither the Drug main effect or the Context Pre-exposure × Drug interaction was significant.

The two-process model of fear conditioning implies that the hippocampus is critical to the acquisition of a

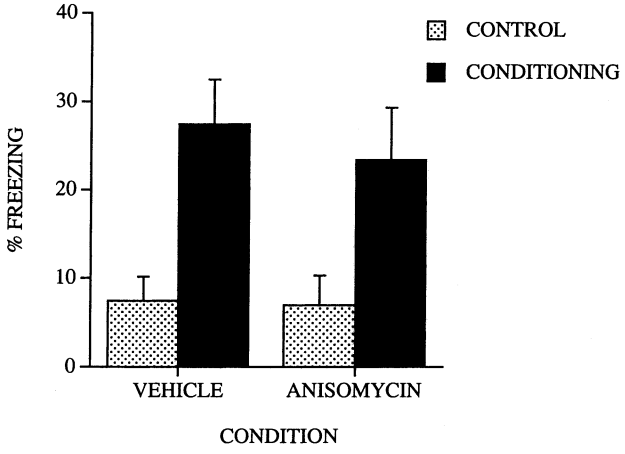

Fig. 3. Mean percent freezing during the contextual fear test. Rats were pre-exposed to either the conditioning or control context. Rats received an intrahippocampal injection of either anisomycin or vehicle following the immediate shock on day 2. Note that in the vehicle group, rats pre-exposed to the conditioning context displayed more fear to the context than those pre-exposed to the control context. The same was the case for anisomycin-injected rats. Moreover, vehicle-injected rats pre-exposed to the conditioning context did not differ from anisomycin-injected rats pre-exposed to the conditioning context. Bars represent S.E.M.

conjunctive representation of context but not for the association of that representation with shock. Consequently, it predicted that blocking protein synthesis in the hippocampus following the immediate shock should have no effect on contextual fear conditioning because rats would have already acquired the representation of context prior to the immediate shock experience. The results of Experiment 3 are entirely consistent with this prediction.

6. General discussion

Several researchers have proposed that contextual fear conditioning is a product of two independent learning processes, (a) the construction of a conjunctive representation of the independent features of the context; and (b) associating this unitary representation with the aversive event. In such accounts, the conjunctive process is assumed to depend critically on circuitry provided by the hippocampal formation but the associative process does not, but likely involves the amygdala [5,7,26,34].

There is now strong support for this view. First, studies of the context pre-exposure facilitation effect firmly establish that rats can learn about the context independent of associating it with shock [4,21,24,26,27,33]. Second, Rudy & O'Reilly [27] demonstrated unequivocally, that as a consequence of exploring the context, the rat automatically acquires a conjunctive representation of its features. Third, Rudy et al. [25] reported that the conjunctive learning that occurs during context pre-exposure depends on neural circuitry in the dorsal hippocampal formation.

The present results are important because they used a different methodology to strongly demonstrate that the hippocampal formation provides critical support for the processes that support the consolidation of the contextual representation that is generated when the rat explores a novel environment. In the key experiment (Experiment 2), the protein synthesis inhibitor, anisomycin, known to block the consolidation processes needed to establish a long-term memory [2,31] was injected into dorsal hippocampus immediately following the context pre-exposure phase. This retrograde treatment evidently blocked the consolidation of the contextual representation because rats that received anisomycin did not display the usual context pre-exposure facilitation effect. Since the rats that received this treatment were normal (a) at the time they explored the context, during the pre-exposure period, and (b) at the time of the contextual fear test, it is difficult to attribute the reduced context pre-exposure facilitation effect to altered encoding processes during either pre-exposure or testing. Moreover, this methodology also rules out interpreting the impairment as a product of an inability of the rat to express the fear response [8].

There is, however, another related explanation of these results that should be considered. In order to cue the recall of the memory of the context, our pre-exposure procedures were designed to establish the transport bucket as a retrieval cue for the memory of the context. Consequently, one might argue that anisomycin interfered with the incorporation of the transport features to the representation of context and did not exert its effect by preventing the consolidation of the conjunctive representation of the context features per se. Since the features of the transport bucket were not incorporated into the representation they could not activate the memory of the context. This alternative cannot presently be ruled out.

The results of Experiment 3 are also consistent with the two-process framework. In this experiment, anisomycin was injected into the hippocampus following the immediate shock phase. The two-process model correctly predicted that this treatment would not reduce the context pre-exposure facilitation effect. This is because this model assumes that the hippocampus is only essential for the acquisition and consolidation of the contextual memory representation. Once this representation is established, it can be activated by some subset of the cues experienced during the pre-exposure period [4,27,33] and the activated representation should associate with the shock. Injecting anisomycin following immediate shock should not effect the acquisition or consolidation of this association because it likely occurs elsewhere (e.g. the amygdala, [3,5,13,16]). As predicted, injecting anisomycin into the hippocampus after immediate shock did not impair the context pre-exposure facilitation effect.

Experiment 3 also rules out another interpretation of Experiment 2. One might argue that the reduced context pre-exposure facilitation effect produced by injecting anisomycin after context pre-exposure was the result of some unintended structural damage to the hippocampus that would alter test performance. If this were the case then one would expect that injecting anisomycin into the hippocampus following the immediate shock conditioning phase should also impair performance, but it did not.

In several papers, O'Reilly and Rudy have proposed that the hippocampus contributes to memory by automatically and rapidly storing a conjunctive representation of the stimulus features that make an experience [27,28]. One advantage of our theoretical position is that it reveals previously unrecognized relationships between processes embedded in contextual fear conditioning and those operating in other domains, such as the study of habituation and incidental learning. There is an emerging literature indicating that rats automatically store conjunctive representations in a number of such tasks,

and that the hippocampus makes an important contribution to these phenomena [9–11,30]. They include studies of the habituation of exploratory behavior [30], habituation of the orienting response [11] and the context specificity effect observed in Pavlovian conditioning [9,10]. These studies all provide evidence that animals automatically store representations of stimulus conjunctions even though there is nothing about these tasks that require this learning. The automatic conjunctive learning was revealed by transfer tests that occurred following training, in which the relationship among the features was varied.

The present work together with our studies of the processes underlying the context pre-exposure facilitation effect [27,28] provide strong support for this general view of how the hippocampal formation contributes to memory. This body of work indicates that as a consequence of exploring a conditioning environment, the rat automatically stores a conjunctive representation of its features and that the memory for this representation depends critically on processes associated with the hippocampal formation.

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