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Long-term stability of fear memory depends on the synthesis of protein but not mRNA in the amygdala

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Abstract

Synaptic modification supporting memory formation is thought to depend on gene expression and protein synthesis. Disrupting either process around the time of learning prevents the formation of long-term memory. Recent evidence suggests that memory also becomes susceptible to disruption upon retrieval. Whether or not the molecular events involved in the formation of new memory are the same as what is needed for memory to persist after retrieval has yet to be determined. In the present set of experiments, rats were given inhibitors of protein or messenger ribonucleic acid (mRNA) synthesis into the amygdala just after training or retrieval of fear memory. Results showed that blocking mRNA or protein synthesis immediately after learning prevented the formation of long-term memory, while stability of memory after retrieval required protein, but not mRNA, synthesis. These data suggest that the protein needed for memory reconsolidation after retrieval may be transcribed from pre-existing stores of mRNA.

Keywords

anisomycin; fear conditioning; local protein synthesis; rat; transcription

Abbreviations

ACSF, artificial cerebrospinal fluid; ACT-D, actinomycin-D; ANI, anisomycin; CREB, cyclic AMP response element-binding protein; CS, conditioned stimulus; DMSO, dimethylsulfoxide; DRB, 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside; ITI, intertrial interval; mRNA, messenger ribonucleic acid; SPRCs, synapse-associated polyribosome complexes; UCS, unconditioned stimulus

Introduction

The synthesis of new protein has long been recognized as critical for memory formation in many behavioral tasks (Davis & Squire, 1984), including fear conditioning (Abel *et al.*, 1997; Schafe *et al.*, 1999). Previous studies have shown that memory for fear conditioning is dependent on the synthesis of new messenger ribonucleic acid (mRNA) and protein in the amygdala (Bailey *et al.*, 1999; Schafe & LeDoux, 2000). The memory impairments caused by protein and mRNA synthesis inhibitors are thought to be due to disruption of a consolidation-like process due to the fact that these drugs spare short-term memory and are only effective if given within a relatively restricted time window near the time of training (Bourtchouladze *et al.*, 1998; Schafe *et al.*, 1999).

A number of reports have demonstrated that consolidated memories are susceptible to disruption around the time of retrieval with various forms of interference (e.g. Misanin *et al.*, 1968). Recently, there has been renewed focus on the idea that memory becomes amenable to disruption following retrieval of a previously learned event (Sara, 2000). A report (Nader *et al.*, 2000) showed that infusion of the protein synthesis inhibitor anisomycin (ANI) into the amygdala following fear memory reactivation resulted in a disruption of memory when tested later, suggesting that upon retrieval memory goes through a reconsolidation process that requires the production of new protein.

One key question with regard to memory reconsolidation is whether or not it shares the exact same molecular mechanisms as initial consolidation. The available data indicate that memory reconsolidation relies on the synthesis of new protein (e.g. Nader *et al.*, 2000). However, these data are inconsistent with regard to the role of mRNA synthesis in memory reconsolidation. One study (Kida *et al.*, 2002) found that cyclic AMP response element-binding protein (CREB)-mediated transcription is necessary for both the consolidation and reconsolidation of fear memory. Other work has argued that *zif268*, a transcription factor, is selectively involved in memory reconsolidation (Hall *et al.*, 2001; Lee *et al.*, 2004). Work by Taubenfeld *et al.* (2001) found that downregulation of the CCAAT enhancer-binding protein β , a transcription factor, prevented the consolidation of memory for an inhibitory avoidance task, but had no effect when given after reactivation of the memory.

More recent work has directly examined the role of mRNA synthesis in memory reconsolidation. Work in invertebrates demonstrated a putative requirement for mRNA synthesis in the reconsolidation of memory for an operant task (Sangha *et al.*, 2003). Similarly, other research has demonstrated a role of hippocampal mRNA synthesis in the reconsolidation of contextual fear memory (Lee *et al.*, 2004). These studies suggest that memory reconsolidation is a process dependent on both translation and transcription. However, questions still remain with respect to the involvement of transcription in memory reconsolidation. The following set of experiments was designed to examine the contribution of amygdala mRNA and protein synthesis to fear memory formation and memory reconsolidation after retrieval by infusing inhibitors of protein or mRNA synthesis into the amygdala after training or after memory retrieval.

Materials and methods

Male Long–Evans rats obtained from Harlan (Madison, WI, USA) served as subjects. All rats were housed individually in stainless steel cages, and had free access to water and rat chow throughout the experiment. The colony room was maintained on a 14 : 10 h light : dark cycle. All procedures were approved by the University of Wisconsin–Milwaukee institutional animal care and use committee, and carried out in accordance with the NIH guidelines for using animals in experimental procedures.

The rats weighed between 325 and 400 g at the start of the experiment, and were handled for 2 days prior to surgery. Each rat was prepared with 26-gauge bilateral cannulae (Plastics One, Roanoke, VA, USA) aimed at the amygdala using stereotaxic coordinates (AP = $-2.8/L = \pm 5.0/V = -7.2$) relative to bregma (Paxinos & Watson, 1986). After behavioral testing, rats were overdosed with an i.p. injection of a sodium pentobarbital–ethanol solution and perfused transcardially with saline followed by 10% buffered formalin. Frozen 40- μ m sections were taken throughout the amygdala, mounted on slides and stained with Cresyl violet. Injection sites were determined with the aid of a rat brain atlas (Paxinos & Watson, 1986).

After recovery from surgery, all subjects were exposed to the restraint and injection procedure for the 3 days immediately prior to the training. During this time each rat was transported to

the laboratory, wrapped in a small towel and gently restrained by hand for several minutes. The infusion pump to be used for the intracranial injections was activated during these periods.

Conditioning took place in a set of four observation chambers ($28 \times 20.5 \times 21$ cm) constructed of Plexiglas and stainless steel. The floor of each chamber consisted of stainless steel rods spaced 1.5 cm apart through which the footshock could be delivered, and each chamber was illuminated by a 7.5 W white light bulb. Ventilation fans provided a constant background noise of approximately 60 dB. Testing to the auditory cue took place in a separate set of chambers that had a slanted wall on one side and rounded wall on the other. The floors were made of Plexiglas and fans provided a background noise of approximately 58 dB. All chambers were housed in sound-attenuating boxes. The training chambers were cleaned with a 10% ammonium hydroxide solution before each set of animals, while the boxes where auditory testing took place were cleaned with a 5% acetic acid solution.

In all cases rats received bilateral infusions (0.5 μ L/side) into the amygdala over 60 s. The injection cannulae, which were cut to extend approximately 0.5 mm beyond the guide cannulae, remained in place for an additional 90 s to ensure diffusion. Rats were returned to their home cages after infusions. ANI (125 μ g/ μ L; Sigma) was dissolved in HCl and diluted with artificial cerebrospinal fluid (ACSF). A small amount of NaOH was added to bring the pH to approximately 7.4. Actinomycin-D (ACT-D; 5 ng/ μ L or 20 ng/ μ L; Sigma) was diluted with ACSF. 5,6-Dichlorobenzimidazole 1- β -D-ribofuranoside (DRB; 20 or 200 ng/ μ L; Sigma) was diluted with 100% dimethyl sulfoxide (DMSO).

In Experiment 1, rats were placed in the training chambers where they received a series of three 1.6-mA/s scrambled AC footshocks [20 s intertrial interval (ITI)] 4 min into the session. Rats were removed from the chambers 3 min after the final shock and immediately infused with ANI, ACT-D or ACSF. The next day all rats were placed back in the training chambers for an 8-min context test, which served as both a memory test and a memory retrieval session. Immediately following this test, one group that received ACSF after training was infused with ANI (ANI post-retrieval, $n = 6$), another was given ACT-D (ACT-D post-retrieval, $n = 6$) and the other group was given ACSF (ACSF, $n = 10$). Rats that received either ANI (ANI post-training, $n = 7$) or ACT-D (ACT-D post-training, $n = 6$) after training were given ACSF following the 24-h test. Rats were given another context test (8 min) the following day. Four days following the last test session all animals were retrained and retested. All of the behavioral testing was recorded on videotape.

For training in Experiment 2, rats were placed in the training chambers and exposed to four pairings of white noise (10 s/72 dB) and shock (1 s/1.0 mA; 90 s ITI). Rats were removed from the chambers 4 min after the final shock and taken into an adjacent room and infused with DRB ($n = 7$) or DMSO ($n = 8$). Approximately 24 h after training rats were tested for retention of the white noise and context in a counterbalanced order. Rats were tested to the context for 15 min, while testing to the white noise consisted of 5-min presentation of the white noise in a different context 6 min into the session. The rats were removed 4 min after termination of the white noise and returned to their home cages.

Rats were trained in Experiments 3–5 as described in Experiment 2. For memory retrieval in Experiments 3 and 4, rats were placed in Context B the next day and given a 32-s presentation of the white noise 6 min after being placed into the chambers. The retrieval session was the same in Experiment 5, except that it occurred 6 days following training. In Experiment 3, rats were removed immediately after termination of the white noise and infused with ANI ($n = 7$), DRB ($n = 6$) or the vehicle. In the vehicle condition, half the rats were given ACSF ($n = 4$) and half DMSO ($n = 4$). The data for these animals were collapsed, as there were no differences between groups during any of the testing. For Experiment 4, rats were infused with ACSF (n

= 8) or ACT-D (5 ng/ μ L, n = 6; 20 ng/ μ L, n = 8). In Experiment 5, the rats were infused with DMSO (n = 6) or DRB (20 ng/ μ L, n = 5; 200 ng/ μ L, n = 6). The following day, all rats were tested to the white noise as described in Experiment 2.

For the autoradiography experiments, four male Long–Evans rats were prepared with bilateral cannulae aimed at the amygdala (AP = $-2.8/L = \pm 5.0/V = -7.2$). Two of the rats received infusions (0.5 μ L) of ACSF in one hemisphere and ANI (125 μ g/ μ L) in the opposite hemisphere (counterbalanced). Thirty minutes later these rats received 1.0 μ L/side of [U- 14 C]-leucine (50 μ Ci/mL, 306 mCi/mmol specific activity, Amersham Biosciences; Piscataway, NJ, USA) into both hemispheres. The other two rats received infusions (0.5 μ L/side) of 100% DMSO into one side of the amygdala or DRB (20 ng/ μ L) into the other. These rats were then given 1 μ L infusions of [U- 14 C]-uridine (50 μ Ci/mL, 474 mCi/mmol specific activity, Amersham Biosciences) into both hemispheres. One hour later, all rats were killed and transcardially perfused. Brains were stored overnight in sucrose formalin and sectioned (40 μ m) the next day. A section was taken every 0.16 mm beginning from the area $\sim$ 2 mm rostral to the most anterior part of the amygdala, continuing through the amygdala and $\sim$ 2 mm beyond. The tissue was mounted on slides and exposed to autoradiographic film (Hyperfilm MP Film; Amersham Biosciences) for 2 weeks. Densitometry and 3D volume estimations were performed on developed films using an automated system (MCID, Imaging Research, St. Catherine's Ontario, Canada).

Densitometry values were taken by digitally aligning the film with the appropriate Nissl-stained image and sampling within amygdala tissue. The 3D volume of tissue reflecting radiolabel incorporation was estimated using MCID software. The thickness of each slice was determined by calibrating the system with the following parameters: 1 mm was set to 0.04 mm, and the interval between slices was 0.16 mm. In-plane 2D areas for each autoradiogram were calculated based on an optical density threshold that remained constant for all images. Selected features (injection area, external capsule, etc.) were extracted from the autoradiographic and Nissl-stained images using image-processing software (Image J version 1.32 J, NIH), and these 2D renderings were then aligned and grouped in order to create a 3D representation of the relative effectiveness of the inhibitors (Blender version 2.33a, Stichtung Blender Foundation Frederiksstraat 12-2, 1054 LC Amsterdam, The Netherlands).

Data analysis

The strength of memory during the sessions was determined by the amount of time engaged in freezing behavior. Freezing was defined as the absence of all body movement except that required for respiration. All other behavior was scored as general activity. Each rat's behavior was scored as either freezing or not once every 4 s for the duration of all sessions. One-way analysis of variance (ANOVA), Fisher's LSD *post hoc* and *a priori* tests were used to test for differences between groups when necessary.

Results

Experiment 1 was designed to examine the contribution of protein and mRNA synthesis in the amygdala to the consolidation and reconsolidation of contextual fear memory. Figure 1A depicts the design of the experiment including timing of the test sessions and infusion schedule for each of the five groups. No group differences were observed during training (data not shown). Figure 1B depicts mean percent freezing during the first context test session for all groups. An ANOVA showed a significant main effect for Group ($F_{4,30} = 7.81$, $P < 0.001$). In particular, planned comparisons showed that the group that received ANI immediately after training differed significantly from the group that received ACSF at both time points ($t_{30} = 3.883$, $P < 0.001$). The group that received ACT-D immediately after training also differed significantly from the group that only got ACSF ($t_{30} = 4.024$, $P < 0.001$). This shows that

administration of either of these drugs disrupts the consolidation of fear memory when given into the amygdala after training.

After the first test session the rats received another set of infusions and were tested again 24 h later. Figure 1C depicts the mean percent freezing for all groups for this second context test session. An ANOVA showed a significant main effect for Group ($F_{4,30} = 5.829$, $P < 0.001$) during this test session. *T*-tests revealed that the groups that received either ANI ($t_{30} = 2.725$, $P < 0.05$) or ACT-D ($t_{30} = 3.059$, $P < 0.01$) after training still differed significantly from the control group 48 h later. Figure 1C also shows that animals given ANI after Context Test 1 showed less freezing than controls when tested the next day ($t_{30} = 2.802$, $P < 0.01$). Interestingly, animals given ACT-D after Context Test 1 showed no deficit when tested the next day ($t_{30} = -0.815$, $P > 0.05$). To examine the possibility that the deficits in performance were due to drug toxicity, all animals were retrained 4 days after the last test session. Levels of freezing did not differ significantly between any of the groups at any point during the retraining or retesting sessions (Fig. 1D and E), indicating that the effects of both ANI and ACT-D are fully reversible at the doses we used.

Experiment 2 was designed to establish the effectiveness of a different transcriptional inhibitor on the consolidation of fear memory. We gave infusions of DRB into the amygdala immediately after white noise-shock training (Fig. 2A). There were no differences between groups at any point during the training session (data not shown). However, animals given DRB after training froze significantly less than controls during the 15 min of the context test ($F_{1,13} = 8.32$, $P < 0.05$). Infusion of DRB into the amygdala after training also disrupted freezing to the auditory cue 24 h later ($F_{1,13} = 9.25$, $P < 0.01$; Fig. 2B). Importantly, there were no differences during the 6 min prior to the white noise presentation ($P > 0.3$). The low levels of freezing during this period demonstrate that the rats were clearly able to discriminate between Context A and B, and also argue that the deficits observed were not due to a general disruption in freezing behavior. These data are consistent with other published results showing that a similar dose of DRB disrupts memory consolidation (Igaz *et al.*, 2002).

In Experiment 3 we used DRB to test whether or not we would see effects on memory reconsolidation with a transcriptional inhibitor other than ACT-D. We also used an auditory stimulus to signal shock and serve as the retrieval cue in order to rule out the possibility that our previous results were somehow unique to contextual fear conditioning. Data from Experiment 3 are depicted in Fig. 3B. ANOVA showed no differences between groups at any point during training (data not shown) or during the memory retrieval session (Fig. 3B). However, there was a significant group effect during the 5-min test session the following day ($F_{2,18} = 5.14$, $P < 0.05$). Fisher's LSD *post hoc* analyses performed on this 5-min block of data showed that rats given ANI froze significantly less than the control group ($P < 0.05$; Fig. 3B). However, rats given DRB did not differ from the control group.

In order to rule out the prospect that the null results in Experiment 1 were due to insufficient blockade of mRNA synthesis, Experiment 4 used a higher dose of ACT-D to test the effects of a relatively large dose on memory reconsolidation. Data from Experiment 4 are shown in Fig. 3D. One-way ANOVA indicated that there were no differences between groups for any period during the training session (data not shown) or during the retrieval session 24 h later (Fig. 3D). When tested the final day, no differences were seen between groups during the 5-min test period (Fig. 3D).

The first set of experiments demonstrated that mRNA synthesis inhibitors, when given after retrieval, have no effect on subsequent performance. These data suggest that memory reconsolidation is dependent on pre-existing mRNA. One possible explanation of these data is that when the memory is reactivated at a short interval after training (i.e. 1 day) then memory

reconsolidation depends on protein synthesized from mRNA that was made during the training experience and still present in neurons. If this was the case, a positive result with transcription blockers should be observed if the memory is reactivated at a longer interval after training when, presumably, the mRNA made during training has degraded. In order to test this possibility, in Experiment 5 we infused two doses of DRB into the amygdala following a retrieval session that occurred 6 days after training (Fig. 4A). Data from this experiment are depicted in Fig. 4B. A one-way ANOVA on the data showed that freezing levels did not differ significantly between the groups at any point during training, retrieval or testing.

Data in Fig. 5 were collected to determine the effectiveness of ANI and DRB when they are infused into the amygdala. In these experiments animals were given infusions of these drugs into the amygdala on one side and their respective vehicles into the other side. After 30 min, ^{14}C leucine or ^{14}C uridine was infused into the amygdala on both sides to measure local rates of protein and mRNA synthesis, respectively. Data from these experiments demonstrate that the side of the amygdala that received ANI showed a 60% decrease in leucine incorporation rates relative to controls (Fig. 5A and C). Likewise, the side of the amygdala where DRB (Fig. 5B) was infused showed about a 53% decrease in uridine incorporation. Figure 5C shows the volume of the radiolabel infusion for a sample rat infused with ANI (bar graph on the left side) in one side of the amygdala and ACSF (right-most bar graph) on the other side. The image in Fig. 5C depicts the approximate spread of a 1- μL infusion in the amygdala, and also demonstrates a reduced volume of amygdala tissue incorporating ^{14}C leucine and a diminished intensity of signal following ANI infusion.

Discussion

The data from this study suggest that the reconsolidation of fear memory after retrieval does not rely on mRNA synthesis in the amygdala. This was the case regardless of the type of fear training (context or auditory), type of transcription inhibitor (ACT-D or DRB), dose or interval between training and retrieval. The finding that the reconsolidation of contextual fear memory requires protein synthesis in the amygdala complements previous work showing that the reconsolidation of auditory fear memory requires protein synthesis in this same brain structure (Nader *et al.*, 2000). This work also replicates previous studies showing that fear memory formation depends on both protein and mRNA synthesis in the amygdala (Bailey *et al.*, 1999; Schafe & LeDoux, 2000; Maren *et al.*, 2003). Collectively, the pattern of data in this study suggests that while initial consolidation requires both transcription and translation in the amygdala, the reconsolidation of fear memory after retrieval is dependent on protein synthesis, but independent of mRNA synthesis.

One simple explanation of our null results with transcription blockers is that the drugs may not be blocking mRNA synthesis enough to disrupt memory reconsolidation. This is unlikely given that both ACT-D and DRB were effective in blocking consolidation when given into the amygdala after training, and the fact that the dose of DRB used in our behavioral experiments decreased rates of ^{14}C uridine incorporation by about 55% compared with controls. This magnitude decrease is similar to what our lab has seen before with ACT-D infusions (Bailey *et al.*, 1999).

A key question with regard to memory reconsolidation is whether or not it shares the same biochemical mechanisms as initial consolidation. Our data argue that the reconsolidation of memory after retrieval differs from memory consolidation in terms of its dependence on transcription. A recent study (Taubenfeld *et al.*, 2001) lends support to this conclusion. This report showed that intrahippocampal infusions of antisense against the CCAAT enhancer-binding protein β prevented original memory formation for an inhibitory avoidance task, but had no effect on subsequent performance when given after retrieval. In the same study,

infusions of ANI into the hippocampus after reactivation did not impair memory when tested later. This would seem to indicate that either CCAAT enhancer-binding protein β -dependent transcription is not necessary for memory reconsolidation or the hippocampus is not involved in the reconsolidation for this task, or perhaps both (Taubenfeld *et al.*, 2001).

In contrast to our findings, a handful of reports have indicated a role for mRNA synthesis in memory reconsolidation. One prior report (Kida *et al.*, 2002) showed that CREB-mediated transcription is necessary for memory formation during training, and for memory stability during retrieval of auditory and contextual fear memory. However, the knockout technique used in this study created a mouse that was CREB deficient in several forebrain regions making it difficult to tell which structure(s) contributed to the deficit. Although this may be unlikely given the central role of the amygdala in fear conditioning, it could be the case that the reconsolidation deficits are due to disruption of CREB activity in structures other than the amygdala or that this effect requires CREB to be absent in several brain areas simultaneously. Additionally, in our experiments the disruption of transcription took place immediately following retrieval, while the CREB knockout was induced 6 h prior to retrieval and persisted from that point on. It is possible that in order to prevent reconsolidation with transcriptional inhibitors, active inhibition must be taking place while the animals are retrieving the memory or perhaps at time points further after the retrieval session, which may not have been covered by our infusions.

Two recent studies have directly examined the role of mRNA synthesis in memory reconsolidation. Results from one study (Sangha *et al.*, 2003) showed that snails given ACT-D after memory retrieval showed decreased performance on an operant task when tested the next day. Interpretation of this study is difficult due to the fact that the retrieval session included presentation of the reinforcing stimulus making the procedure tantamount to a training trial. Several studies have demonstrated differences in the biochemical properties of memory during procedures that do and do not involve reinforcement, such as extinction (Cain *et al.*, 2002) or typical reconsolidation experiments (Taubenfeld *et al.*, 2001; Lee *et al.*, 2004). Especially interesting is one study (Lin *et al.*, 2003) showing that while acquisition of fear-potentiated startle depended on mRNA synthesis in the amygdala, extinction did not.

Another study has demonstrated a role of hippocampal mRNA synthesis in the reconsolidation of contextual fear memory (Lee *et al.*, 2004). This study showed that hippocampal protein and mRNA synthesis is necessary for memory to persist following a retrieval session. One factor that could explain the discrepant results is the fact that the dose (4 $\mu\text{g}/\mu\text{L}$) of ACT-D used in the previous study (Lee *et al.*, 2004) was exponentially higher than the doses we used (5 ng and 20 ng/ μL) in our experiments. Published reports indicate that doses from 5 ng and up can permanently damage brain tissue (Gomi *et al.*, 1999). In our hands, we see observable toxicity with ACT-D infusions at 50 ng/ μL (unpublished data), but not at the doses we used in this study. There was no indication whether or not toxicity was observed with 4 $\mu\text{g}/\mu\text{L}$ of ACT-D (Lee *et al.*, 2004).

One potentially confounding aspect of many reconsolidation studies, including the current one, is the fact that the reminder session typically involves presenting the conditioned stimulus (CS) in the absence of the unconditioned stimulus (UCS; i.e. extinction trials). Research has shown that extinction does not simply involve the erasure of the original CS–US association, but involves learning that the CS predicts the absence of the UCS (Bouton & Bolles, 1979). If the animals were learning extinction during the retrieval session you would expect an outcome opposite to that predicted by the reconsolidation hypothesis (i.e. enhancement of responding). Indeed, a number of reports have demonstrated blockade of extinction learning with infusion of protein synthesis inhibitors following extinction trials (Berman & Dudai, 2001; Vianna *et al.*, 2001). It has been proposed that these discordant findings may be explained by the fact

that there was significant extinction within the retrieval session in the experiments that reported blocking of extinction, while there was no within-retrieval session extinction in the reconsolidation studies (Nader, 2003). A recent report (Pedreira & Maldonado, 2003) lent experimental support to this notion. Briefly, this study examined the difference between reconsolidation and extinction by varying the duration of the retrieval session. The results showed that with a short retrieval session animals given a protein synthesis inhibitor showed less responding when tested the next day. However, in the animals that were exposed to a longer retrieval session, the same treatment preserved responding (i.e. prevented extinction).

The extent to which extinction factors into the current experiments is unknown, being that we did not explicitly control for it. However, with the exception of Experiment 1, the reminder sessions were limited to 32 s in order to minimize extinction learning. In Experiment 1 we used a longer reminder session (8 min) in order to adequately assess the manipulations that followed training. It is possible that this longer reminder session induced some extinction, as there appears to be less responding during Context Test 2 than there was during the initial test. However, pilot data from our lab using the exact same training and testing parameters showed that there was no difference in responding to the training context on the second context test between groups exposed to the training context or a shifted context during the initial test. These data argue that under the parameters used in Experiment 1, any extinction learning taking place is likely small and is not confounding the results.

The freezing deficits seen with post-training administration of mRNA and protein synthesis inhibitors could be considered partial in that drug-treated rats still show some freezing behavior during the test sessions in some of our experiments (e.g. Experiments 1 and 2). An argument could be made that if these processes are critical for memory formation, then the animals should not freeze at all. The residual freezing seen in our experiments could be due to the fact that the drug infusions are regionally restricted and to achieve a total blockade of freezing, protein and mRNA synthesis needs to be shut down simultaneously in several brain regions important for fear memory. Also, several studies have shown that fear-related behaviors can be observed as a result of non-associative processes (e.g. Bellgowan & Helmstetter, 1996; Quinn *et al.*, 2002). Therefore, drug-treated rats might also show some non-associative freezing behavior that is not affected by protein synthesis blockade.

The data in the current set of experiments demonstrate that mRNA synthesis in the amygdala is not necessary for the reconsolidation of fear memory. However, the data are consistent with prior published work showing that protein synthesis is necessary (Nader *et al.*, 2000; Debiec *et al.*, 2002; Kida *et al.*, 2002) for fear memory reconsolidation. These findings suggest that memory reconsolidation must be supported by a mechanism in which new proteins are produced in the absence of mRNA synthesis. The idea that proteins can be synthesized at specific synaptic sites stems from the discovery of synapse-associated polyribosome complexes (SPRCs) at postsynaptic sites in the dendrites of neurons (Steward & Levy, 1982). Subsequent work has demonstrated that these SPRCs contain all the elements necessary to support protein synthesis, and a number of mRNAs that are dendritically localized have been identified (for review, see Steward & Schuman, 2001). A number of these mRNA present in dendrites are types that had been previously identified as being involved in activity-dependent synaptic modifications and memory formation, including *Arc*, brain-derived neurotrophic factor (BDNF) and CaMKII α (Jiang & Schuman, 2002). Given that many of these mRNA may also be somatically located, it is difficult to examine the function of protein made specifically in dendrites. Furthermore, studying the role of local protein synthesis in behaving animals is difficult due to the fact that traditional procedures used to selectively disrupt dendritic translation are often invasive. However, recent studies have made strides to overcome these restraints (e.g. Miller *et al.*, 2002). More work is needed to determine if memory reconsolidation, as our results suggest, is supported by local protein synthesis.

The results from these experiments demonstrate that initial consolidation of fear memory differs from reconsolidation in its dependence on mRNA synthesis. The fact that the data from these experiments found no disruption of reconsolidation with different doses and types of transcription blockers, and that these same drugs prevented initial memory formation, strongly suggest that reconsolidation can occur in the absence of mRNA synthesis. Still, relatively little is known about the molecular biology supporting acquisition, reconsolidation and extinction. Determining the extent to which these processes interact and overlap will be a major step in understanding the intracellular events that support memory formation.

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Fig. 1.

The effects of protein and mRNA synthesis inhibition of memory consolidation and reconsolidation. (A) Design for Experiment 1 indicating the timing of the test sessions and when infusions occurred (arrows). Infusions of anisomycin (ANI) and actinomycin-D (ACT-D) into the amygdala prevented the consolidation of contextual fear memory (B) Infusions of ANI after retrieval disrupted future memory tests (C), however, ACT-D did not. No differences were seen in the 3-min period immediately after training (D) or during the 8-min context retest (E) the next day. Bars show mean freezing levels across the 8-min test session ($\pm$ SEM). $*P < 0.05$, compared with artificial cerebrospinal fluid (ACSF)-treated rats.

Fig. 2. Blockade of mRNA synthesis in the amygdala with DRB disrupts fear memory consolidation. (A) Depiction of the experimental procedure (arrows indicate time of infusion). (B) Rats infused with DRB (gray) froze less ($*P < 0.05$) during both the context test and auditory conditioned stimulus (CS) test than DMSO (black)-treated rats. Mean freezing levels are depicted ($\pm$ SEM) during the white noise and context tests.

Fig. 3.

Reconsolidation of auditory fear memory is dependent on protein, but not mRNA, synthesis. Design for Experiments 3 and 4 (A, C). Rats received infusions of vehicle (VEH, black), anisomycin (ANI, white) or 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside (DRB, gray) into the amygdala after memory retrieval (arrows). (B) Only rats given ANI froze less than controls during the test session (* $P < 0.05$). Infusion of two different doses of actinomycin-D (ACTD, gray and hatched) after retrieval had no effect on responding to the white noise 24 h later (D). Mean freezing levels are depicted ($\pm$ SEM) during the retrieval session and white noise test.

Fig. 4.

Blockade of transcription in the amygdala following a delayed retrieval session does not affect memory reconsolidation. (A) Basic procedure for Experiment 5 including when the testing and infusions (arrows) took place. Infusion of 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside (DRB, 20 or 200 ng; gray and hatched bars) into the amygdala after retrieval did not affect performance to the auditory CS the following day (B). Mean freezing levels are depicted ($\pm$ SEM) during the retrieval session and auditory CS test.

Fig. 5.

Anisomycin (ANI) and 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) reduce rates of protein and mRNA, respectively. Optical density measurements were taken from amygdala tissue and the data were graphed as a mean percentage of control. (A) Infusion of ANI (white) into the amygdala resulted in reduced rates of protein synthesis as measured by ^{14}C leucine incorporation rates relative to the side treated with ACSF ($n = 2$). Similarly, infusion of DRB (gray) into the amygdala resulted in lower rates of mRNA synthesis compared with the DMSO-treated side ($n = 2$) when ^{14}C uridine incorporation was measured. (B) The image depicts a 3D rendering of a rat brain infused with ANI in the amygdala on one side and ACSF in the other, and conveys the approximate spread of a 1-μL infusion in the amygdala and also demonstrates decreases in the spread of the radiolabel and strength of the signal following ANI infusion (C).