CONSOLIDATION AND RECONSCILATION OF CONTEXTUAL FEAR MEMORY REQUIRES MAMMALIAN TARGET OF RAPAMYCIN-DEPENDENT TRANSLATION IN THE DORSAL HIPPOCAMPUS

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Abstract—The mammalian target of rapamycin (mTOR) pathway is important for regulating protein translation. The present study characterized the role of mTOR-dependent translation in the dorsal hippocampus (DH) during the consolidation and reconsolidation of contextual fear memory. We first showed that fear conditioning resulted in increased phosphorylation of p70s6 kinase (p70s6K) in the DH and that infusion of the mTOR inhibitor rapamycin (RAP) into the DH immediately after training disrupted formation of long-term contextual fear memory. Additionally we showed that p70s6K was activated after retrieval of a previously stored fear memory, and inhibition of mTOR by DH infusion of RAP blocked the reconsolidation of contextual fear memory. Together these results demonstrate that within the DH translational control through the mTOR pathway is important for consolidation as well as the stability of fear memory after retrieval. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: fear conditioning, mTOR pathway, hippocampus, reconsolidation, consolidation, rapamycin.

Pavlovian fear conditioning, a procedure in which a novel stimulus paired with an aversive event comes to elicit a fear response, has been extensively used to study memory consolidation. The formation of a fear memory in this paradigm requires de novo gene transcription and protein synthesis in several brain areas (for review see, Helmstetter et al., 2008). Reconsolidation refers to the finding that a consolidated memory again becomes sensitive to disruption after a brief reminder treatment (Misanin et al., 1968; Nader et al., 2000). This phenomenon has been demonstrated in multiple species in a variety of behavioral paradigms and is reliant upon protein synthesis (for review see, Tronson and Taylor, 2007). Evidence for the importance of protein synthesis in memory reconsolidation comes from studies showing disruption of fear memory with delivery of protein synthesis inhibitors around the time of memory retrieval (Nader et al., 2000; Debiec et al., 2002; Suzuki et al., 2004; Parsons et al., 2006a). Recent work has suggested that specific signaling pathways controlling translation may play a pivotal role in the initial consolidation process (Kelleher et al., 2004; Costa-Mattioli et al., 2005; Parsons et al., 2006b; Bekinschtein et al., 2007; Blundell et al., 2008) as well as reconsolidation following memory retrieval (Parsons et al., 2006b; Blundell et al., 2008; Glover et al., 2010).

The mTOR pathway is one such signaling pathway and it regulates mRNA translation through its downstream targets p70S6 kinase (p70s6K) and the elongation factor binding protein 4EBP1 (for review see Hoeffer and Klann, 2010). Measurement of phosphorylated p70s6K and/or phosphorylated 4EBP1 is often used as an indicator of mTOR activity. Several studies have shown that mTOR signaling is critical for activity-dependent synaptic plasticity in a variety of systems (Casadio et al., 1999; Tang et al., 2002; Gong et al., 2006). A number of recent reports indicate that components of the mTOR pathway are engaged following learning (Parsons et al., 2006b; Bekinschtein et al., 2007; Slipczuk et al., 2008; Belelovsky et al., 2009; Glover et al., 2010; Qi et al., 2010). Many of these same studies and others (Blundell et al., 2008) have shown that rapamycin (RAP), an inhibitor of the mTOR pathway, disrupts contextual fear memory consolidation when the drug is given around the time of learning.

Translational regulation by mTOR might also be involved in the reconsolidation of memory following retrieval. Prior work from our laboratory showed that delivery of RAP into the amygdala after fear memory retrieval disrupted performance on subsequent tests (Parsons et al., 2006b). Other work has shown that systemically administered RAP given just after retrieval disrupts reconsolidation (Blundell et al., 2008; Glover et al., 2010) and that this disruption is long lasting (Blundell et al., 2008). Finally, mTOR signaling in the dorsal hippocampus (DH) might also be involved in reconsolidation of contextual fear memory, as targeted infusions of RAP disrupt the reconsolidation of hippocampal-dependent object recognition memory (Myskiw et al., 2008).

Here we addressed whether the mTOR pathway is activated in response to hippocampally dependent contextual fear learning and retrieval. We showed that rats trained using contextual fear conditioning had enhanced phosphorylation of p70s6K in the DH 60 min later, and that blockade of mTOR activity in the DH after fear conditioning disrupted the formation of contextual, but not cued, fear memory. Animals that were trained and subsequently retrieved a contextual fear memory showed increased phosphorylated p70s6K in the DH. Finally, we show that RAP
infused into the DH prior to retrieval disrupted memory when tested the following day. These findings suggest that mTOR signaling is normally required within the DH during consolidation and reconsolidation of contextual fear memory.

**EXPERIMENTAL PROCEDURES**

### Subjects

Naive male Long Evans rats (n=114) obtained from Harlan (Madison, WI, USA) weighing approximately 300–350 g served as subjects. All animals were individually housed in stainless steel hanging cages in a room maintained on a 14:10 h light/dark cycle. Food and water were available ad libitum. Animals were handled prior to all experiments over 3 days. All procedures were carried out with approval of the Institutional Animal Care and Use Committee.

### Surgery

Animals were handled for several days prior to surgery. Rats that underwent surgery (n=51) were implanted with bilateral cannulae aimed at the dorsal hippocampus (AP -3.5, L ±2.6, V -3.0). Coordinates were chosen based on a rat brain atlas (Paxinos and Watson, 1998). Before surgery animals were anesthetized with systemic injections of ketamine HCl (100 mg/kg body weight) and sodium pentobarbital (2.5 mg/kg/rat). The cannulae were anchored to the skull using stainless steel screws and acrylic cement. To prevent blockade of the cannulae 33-gauge obturators remained in the guide cannulae when not in use. Animals were habituated to the handling and transport procedure for 2 min each day for 4 days.

### Apparatus

Fear conditioning was conducted in Context A which was made of Plexiglas and stainless steel observation chambers illuminated with white light and housed in sound attenuating chambers. The floor was comprised of 18 stainless steel bars 5 mm in diameter spaced 12 mm apart and connected to a shock generator. Ventilation fans produced 62–64 decibel (dB) of background noise. Each chamber was equipped with a speaker centered in the middle of one end of the chamber. Before training or testing of each animal, Context A was cleaned with a 5% ammonium hydroxide solution.

The novel chamber (Context B) had a different shape, olfactory cue (2% acetic acid solution) and flooring (Plexiglas) than Context A. Fans provided background noise (~58 dB) and the chambers were enclosed in a sound attenuating box illuminated with white light.

### Drug preparation and infusion procedure

Prior to behavioral testing animals with cannulae in the DH were habituated to transport and the microinjection procedure for 4 days. Each rat was restrained in a towel for several minutes, their obturators were removed and their scalp was cleaned. During this time the infusion pump to be used during the experiment was activated in order to habituate the animals to the sound it produces. After this was complete, the obturators were replaced and the animal was returned to its home cage.

Rats received a bilateral infusion into the DH (1 µl/side) given over 60 s. The injection cannulae remained in place for an additional 90 s to ensure diffusion away from the injector tip. The injection cannulae were cut to extend approximately 0.5 mm beyond the guide cannulae to ensure they infused into fresh tissue. Animals either received rapamycin (Calbiochem, San Diego, CA, USA) dissolved in 100% Dimethyl Sulfoxide (Sigma, St. Louis, MO, USA) to 1, 2 or 5 µg/µl or the same volume of 100% DMSO. Rats were returned to their home cage after infusions.

### Behavioral procedures

Animals shocked immediately after placement into a context do not learn contextual fear conditioning (Fanselow, 1990). This finding is referred to as the immediate shock deficit. Using the immediate shock deficit group as a control that received the same shock exposure and time in the context as the experimental group, we compared phosphorylated p70s6K expression in animals shocked (1.3 mA, 2 s) immediately (IMM; n=6) to animals shocked after a 2 min delay (DLY; n=7) after placement in the context. Both groups of animals remained in Context A for a total of 3 min. A homecage (HC) group (n=6) remained in the vivarium. IMM and DLY animals received a lethal dose of sodium pentobarbital (100 mg/kg) 60 min after training. HC animals were euthanized throughout the day along with IMM and DLY animals. Brains were removed and stored at ~80 °C until DH tissue could be collected and prepared for Western blot analysis.

To verify that rats did not learn with the immediate shock parameters we used in Experiment 1, we tested whether immediate shock resulted in memory formation in a separate experiment (n=19). In Experiment 2 we took advantage of the finding that the immediate shock deficit can be reversed in an animal exposed to the training context the day before training (Rudy et al., 2002). We pre-exposed animals to either Context A (A/IMM; n=10) or Context B (B/IMM; n=9) for 8 min. 24 h later all animals received immediate shock (2.0 mA, 1 s) in Context A and remained in Context A for a total of 3 min. The following day all animals were tested for 5 min in Context A.

For Experiment 3 all rats (n=42) were placed in the training chambers and after 6 min they were given four pairings of white noise (72 dB, 10 s) and shock (1.3 mA, 1 s) 90 s apart. Immediately after training animals were infused with DMSO (n=16), 1 (n=12), 2 (n=6) or 5 (n=8) µg/µl of RAP into the DH. Approximately 24 h after training rats were tested for retention of the white noise cue and training context in a counterbalanced order. The testing sessions were separated by approximately 4 h for all animals. For context testing, rats were placed in the training chambers (Context A) for 15 min. For auditory cue testing, rats were placed into the novel Context B and exposed to the white noise for 5 min after a 6 min baseline.

Experiment 4 was done to examine the expression of phosphorylated p70s6K after the retrieval of a contextual fear memory (n=23). In this experiment one group of animals was trained with shocks (+) in Context A and re-exposed to Context A 24 h later (A+/A, n=5). Three control groups were included in this experiment to verify that any difference in protein expression was due to retrieval of the contextual fear memory rather than to some other aspect of the procedure. These control groups included a group exposed to Context A without shock and re-exposed to Context A on day 2 (A/A, n=7); a group that was trained with shocks but exposed to the novel Context B on day 2 (A+/B, n=5); and a home cage group (HC, n=6).

After 4 min baseline animals in groups A+/A and A+/B received three shocks (1.3 mA, 1 s) separated by 20 s and were returned to their home cage after a 3 min post-shock period. Rats in group A/A were placed into Context A for an equivalent period of time as A+/A and A+/B but did not receive shock. 24 h later animals were exposed to Context A (A+/A, A/A) or Context B (A+/B) for 90 s. Brains were removed 60 min later and stored at ~80 °C until DH tissue could be collected and prepared for Western blot analysis.

Experiment 5 was done to determine whether contextual fear memory reconsolidation was sensitive to disruption of the mTOR pathway using RAP (n=9). Animals were trained as in Experiment 4. 24 h later animals were returned to Context A for a 90-s retrieval
session. 30 min prior to the retrieval session rats were brought into an adjacent room and given 5 µg/µl of RAP (n=4) or DMSO (n=5) into the DH (1 µl/side). Drug was infused 30 min prior to retrieval based on previous work showing that drug infusion at this time point disrupted reconsolidation (Suzuki et al., 2004; Doyère et al., 2007). 24 h after retrieval animals were returned to Context A for an 8 min test.

Western blot procedure

DH tissue was dissected out and homogenized in buffer (in 100 ml deionized distilled water; Tris–hydrochloride 0.605 g; Sodium Dodecyl Sulfate 0.25 g; Sodium Chloride 0.876 g; Ethylenediaminetetraacetic acid 0.038 g; Sodium Fluoride 0.0042g; phenylmethylsulfonyl fluoride 1 g/ml; leupeptin 1 g/ml; 10% Sodium Dodecyl Sulfate 10 ml; sodium orthovanadate 1 mM, Sigma) and immediately placed on dry ice. Samples were stored at −80 °C until further processing. Samples were thawed and then centrifuged at 4k rpm for 20 min; the supernatant was removed and measured using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples were normalized and 50 µg/µl of protein from each sample was loaded onto a 6% SDS/PAGE gel. Proteins were transferred to a membrane using a semidry transfer apparatus (Bio-Rad Laboratories). Membranes were incubated in blocking buffer for 2 h and then incubated overnight at 4 °C in primary antibody for phosphorylated p70s6K (Thr 412) (1:1000, Millipore, Billerica, MA). Non-phosphorylated-p70s6K (1:1000, Millipore) was used to verify equal amounts of protein that were added to each lane. Total p70s6K has been shown to be unaffected by memory formation at the time point used here (Qi et al., 2010; Slipczuk et al., 2009). Following primary antibody exposure, all membranes were incubated in secondary antibody (Millipore, 1:5000) for 90 min. Membranes were washed thoroughly, placed in a chemiluminescence solution for 3 min (Santa Cruz Biotechnology) and exposed to autoradiographic film (Hyperfilm MP). An image of the film was taken and the appropriately sized band was measured in gray scale and subtracted from a background measurement from the same film resulting in the relative optical density (ROD) score. The ROD was derived by dividing each rats ROD by the average of the HC group ROD and multiplying it by 100. This percentage of control score was then statistically analyzed using one-way ANOVA and Fisher’s LSD post-hoc tests.

Histology

Animals were euthanized with an injection of sodium pentobarbital (100 mg/kg). Animals were transcardially perfused with saline followed by 10% buffered formalin solution. Heads, with cannulae intact, were placed in 10% formalin solution for at least 24 h. The brains were then extracted from the skull and placed in a 30% sucrose formalin solution until they were ready to section. Frozen sections (40-µm) were collected throughout the hippocampus, mounted on slides, and stained with Cresyl Violet. Injection sites were then determined with the aid of a rat brain atlas (Paxinos and Watson, 1998).

RESULTS

Experiment 1: contextual fear conditioning increases the phosphorylation of p70s6K in the hippocampus after training

Experiment 1 examined whether the mTOR pathway was activated after contextual fear conditioning. We measured phosphorylated p70s6K as an indicator of mTOR pathway activation. Rats were trained with a single shock delivered immediately (IMM) or 2 min after (DLY) placement into the training chamber. One-way ANOVA performed on the level of phosphorylated p70s6K in DH tissue after training revealed a significant group difference (F(2,15)=4.020, P<0.05). Fisher’s LSD post-hoc tests indicated that phosphorylated p70s6K immunoreactivity was significantly increased for the DLY group compared to HC and IMM groups (P’s<0.05, Fig. 1A). The same samples were also analyzed for total p70s6K (Fig. 1B) as a loading control to measure any differences in total protein loaded. One-way ANOVA showed no significant difference in total p70s6K (F(2,15)=.014, P>0.05).

Experiment 2: immediate shock does not result in formation of contextual fear memory

To verify that our immediate shock paradigm did not lead to conditioning in the immediate shock group, a separate cohort of animals was pre-exposed to either the context in which they were to be shocked (Context A, A/IMM) or a novel context (Context B, B/IMM). The following day all animals were trained with immediate shock in Context A and tested 24 h later. A two-sample t-test showed that animals pre-exposed to Context B (B/IMM) froze significantly less than animals pre-exposed to the Context A (A/IMM) when given an immediate shock (t(10)=2.587,
Experiment 4: retrieval of contextual fear conditioning increases activation of p70s6K

Since phosphorylation of p70s6K in the DH was selectively increased after acquisition of a contextual fear memory we next asked whether the same would be true after memory retrieval. In this experiment one group of animals was trained with shocks (+) in Context A and re-exposed to Context A 24 h later (A+/A); a second group exposed to Context A without shock and re-exposed to Context A on day 2 (A/A); a third group was trained with shocks but exposed to a novel Context B on day 2 (A+/B); and a fourth group remained in the HC.

A significant difference was seen in freezing behavior during training ($F(2,14)=32.421, P<0.05$). As shown in Fig. 4A (left), Fisher LSD post-hoc analysis showed that animals that were shocked (A+/A, A+B) froze significantly more than those that were not (A/A) ($P<0.01$). 24 h later groups A+/A and A/A were returned to Context A while group A+/B was placed in Context B. A one-way ANOVA on behavioral data from the retrieval test (Fig. 4A, right) showed a significant effect of group ($F(2,14)=24.297, P<0.05$). Follow-up LSD post-hoc analysis showed that the A+/A group showed significantly more freezing behavior ($P<0.01$) than groups A+/B or A−/A during the 90 s retrieval test.

One-way ANOVA on data from Western blot analysis showed a significant group difference in phosphorylated p70s6K expression after retrieval ($F(3,19)=3.452, P<0.05$, Fig. 4B). Fisher LSD post-hoc tests showed that group A+/A had increased phosphorylated p70s6K in the DH compared to all other conditions ($P<0.05$). As shown in Fig. 4C no differences were found in expression of total p70s6K in the same samples indicating...
Bars represent mean percentage freezing behavior (±SEM) after presentation of shock during the training session. Animals that were trained with shocks (A+/A, white bar; A+/B, light gray bar) showed significantly more freezing during training than those not shocked (A/A, dark gray bar). (A, right) Freezing behavior during retrieval was significantly higher for animals that were previously shocked and tested in Context A the following day (A+/A) compared to animals trained in Context A and tested in Context B or animals that were never shocked (A/A). (B, C) Bars represent mean optical density values (±SEM) expressed as a percentage of home cage control. (B) Western blots conducted on rats sacrificed 60 min after retrieval of a contextual fear memory showed increased phosphorylated p70s6K compared to HC (black bar), A+/B and A/A conditions. (C) As loading control membranes were exposed to antibody against total p70s6K and no differences were observed. A representative Western blot image is pictured below graphs of Western blot data. * P<0.05.

Experiment 5: blocking mTOR prior to retrieval disrupts reconsolidation

The increase in phosphorylated p70s6K after retrieval suggests that mTOR-dependent translation in DH neurons is activated in response to retrieval of a contextual fear memory. In our final experiment we tested whether this increase was necessary for maintaining the stability of memory after retrieval. As shown in Fig. 5 no significant differences in freezing behavior were seen between groups during training (t-test, P>0.05) or retrieval (t-test, P>0.05). However, animals given an infusion of RAP into the DH 30 min prior to contextual fear memory retrieval showed attenuated freezing at test 24 h later (t=4.196, P<0.05) compared to vehicle controls. These findings provide support for the idea that the increase in mTOR pathway activation in the DH is necessary for maintaining memory stability. The finding further supports a potential role for mTOR signaling in reconsolidation of a contextual fear memory.

**DISCUSSION**

Our results add to a growing body of work indicating mTOR signaling is critical for synaptic plasticity and memory formation. We showed that phosphorylated p70s6K was increased 60 min after contextual fear conditioning in the DH of animals that received delayed presentation of shock. The immediate shock control condition shows that the increase in phosphorylated p70s6K was not due to some non-associative effect of training such as placement into the context, amount of time in the context, or receipt of the shock. We next showed that RAP selectively disrupts reconsolidation of a contextual fear memory when infused into the DH immediately after fear conditioning without affecting the ability of the animal to show fear behavior to an auditory cue. Intact auditory fear conditioning argues against a general deficit in freezing behavior in RAP treated rats, and underscores the importance of the DH in contextual, but not cued, fear conditioning.

Next we assessed whether phosphorylated p70s6K is increased after retrieval of a contextual fear memory. Animals trained and then tested the following day in the same context showed an increase in phosphorylated p70s6K 60 min after retrieval compared to controls. The control conditions included in this experiment speak about two important issues. First, simply training animals does not lead to activation of the mTOR pathway 24 h later, as indicated by group A+/B. This argues against the possibility that in our final experiment we disrupted ongoing memory consolidation 24 h after training with our infusion of RAP prior to retrieval. Also, although several markers of plasticity are increased in the DH between 12 and 24 h after training (e.g. Bekinschtein et al., 2010), mTOR does not appear to be one of these, as DH infusions of rapamycin do not block the protracted cellular consolidation events that are thought to underlie persistence of memory (Bekinschtein et al., 2008).

Second, the fact that we found no increase in phosphorylated p70s6K after re-exposing rats to a context they
had explored the previous day suggests that exploration of an environment and replacement into that same environment the following day does not engage the same sort of cellular processes as exposure to a fearful context. This is consistent with work by Biedenkapp and Rudy (2004) showing that anisomycin infused into the DH after retrieval did not disrupt reconsolidation of a basic contextual memory. It also agrees with the finding that expression of zif268 mRNA in the DH correlates best to retrieval of contextual fear memories and not simply re-exposure to a context (Hall et al., 2001). However, these data should not be taken as evidence that the DH is involved in context-shock associations, as there is considerable evidence indicating that the DH is involved in the formation of contextual memory, but is not necessary for the association between context and shock (e.g. Matus-Amat et al., 2004). Thus, the increase in phosphorylated p70S6K expression after retrieval in the A+/A group might reflect the fact that there is new learning because the context is presented without the shock during retrieval. Further, the finding of no change in p70S6K activity in the A/A group may be due to animals learning nothing new during retrieval. These two possibilities cannot be ruled out with the present data.

Recent studies have begun to tackle how the mTOR pathway becomes engaged and what the downstream targets are through which it affects synaptic plasticity and memory. BDNF is a crucial mediator of synaptic plasticity and memory (Rattiner et al., 2005; Alonso et al., 2002; Tyler et al., 2002) and is known to activate the mTOR pathway (Takei et al., 2004; Schratt et al., 2004). One recent study showed that BDNF likely acts through mTOR to facilitate the synaptic expression of GluR1 containing AMPA receptors during memory consolidation (Slipczuk et al., 2009). The effect that RAP has on memory might also involve the regulation of other synaptic proteins including AMPA receptors to translation initiation machinery to gate induction. Anim Learn Behav 18:264 –270.

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CONCLUSIONS

To date only a limited number of studies have demonstrated the involvement of the mTOR pathway in memory. Our current findings demonstrate that mTOR signaling in the DH is engaged after consolidation, retrieval and reconsolidation of contextual fear memory. Along with previous published works, our results highlight the burgeoning understanding of mRNA translation and its role in memory at the synaptic and behavioral level.

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