Brief Communication

# mTOR inhibition impairs extinction memory reconsolidation

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**Fear-motivated avoidance extinction memory is prone to hippocampal brain-derived neurotrophic factor (BDNF)-dependent reconsolidation upon recall. Here, we show that extinction memory recall activates mammalian target of rapamycin (mTOR) in dorsal CA1, and that post-recall inhibition of this kinase hinders avoidance extinction memory persistence and recovers the learned aversive response. Importantly, coadministration of recombinant BDNF impedes the behavioral effect of hippocampal mTOR inhibition. Our results demonstrate that mTOR signaling is necessary for fear-motivated avoidance extinction memory reconsolidation and suggests that BDNF acts downstream mTOR in a protein synthesis-independent manner to maintain the reactivated extinction memory trace.**

**[Supplemental material is available for this article.]**

Repeated or prolonged nonreinforced recall may induce extinction of consolidated memories, a form of learning involving the formation of a new association that inhibits the expression of the original one (Bouton 2004). On the contrary, brief re-exposure to retrieval cues may destabilize consolidated memories, which must then be reconsolidated to persist (Przybyslawski and Sara 1997; Nader et al. 2000). Psychotherapy based on extinction enhancement or reconsolidation disruption might reduce the intrusive recollection of aversive events and help in the treatment of post-traumatic stress disorder (PTSD), a prevalent mental health condition characterized by the persistent avoidance of places, people, and objects resembling traumatic experiences (Ressler et al. 2004; Schwabe et al. 2014; Dunbar and Taylor 2017; Bryant 2019). Therefore, considerable effort has been lately dedicated to analyze the properties and potential interactions of fear memory extinction and reconsolidation. In this regard, it has been reported that these processes are mutually exclusive (Merlo et al. 2014), and that extinction training during the reconsolidation time window enhances extinction learning and prevents the recovery of fear (Monfils et al. 2009). Moreover, we have previously shown that recall renders fear-motivated avoidance extinction memory susceptible to amnesia, indicating that this memory type is prone to reconsolidation when active and suggesting that targeting extinction memory reconsolidation can be a feasible treatment strategy for PTSD (Rossato et al. 2010; Rosas-Vidal et al. 2015). However, the neurochemical basis of extinction memory reconsolidation has seldom been analyzed.

Mammalian target of rapamycin (mTOR) is a 289-kDa phospho-inositide 3-kinase (PI3K)-related serine-threonine protein kinase that functions as a key element of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) signaling modules to regulate protein synthesis through the phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase (p70S6K) (Hay and Sonenberg 2004). A well-known mediator of cell growth and proliferation (Hall 2008; Ryskalin et al. 2017), mTOR involvement in synaptic plasticity was first suggested by studies showing that rapamycin (RAPA), a macrolide that selectively inhibits mTORC1 signaling by interacting with the chaperone FKBP12 and binding to mTOR FKBP12–RAPA-binding domain, impairs long-term facilitation in Aplysia as well as long-term potentiation (LTP) in the rat hippocampus (Casadio et al. 1999; Tang et al. 2002). Interestingly, avoidance memory consolidation and recall need mTOR signaling in the dorsal hippocampus (Bekinschtein et al. 2007; Pereyra et al. 2018), as it also happens with the reconsolidation and extinction of several other memory types (Myskiw et al. 2008; Gafford et al. 2011; Zubedat and Akirav 2017; Jarome et al. 2018; Lee et al. 2018; Yang et al. 2019). Here, we examined whether reconsolidation of fear-motivated avoidance extinction memory requires mTOR activity in the CA1 region of the dorsal hippocampus. To do that, we used 3-mo-old, 300- to 350-g, male Wistar rats ($n = 320$), housed in groups of five with free access to water and food in a holding room at 22°C–23°C on a normal light cycle (12 h light:12 h dark; lights on at 6.00 a.m.). Animals were implanted with 22-gauge guides aimed at the CA1 region of the dorsal hippocampus (Supplemental Fig. S1, stereotaxic coordinates in millimeters: anteroposterior, −4.2; laterolateral, ±3.0; dorsoventral, −3.0), as previously described (Radiske et al. 2015), and allowed to recover from surgery for 10 d before being handled by the experimenter once per day for 2 d. One day later, the animals were trained in a one-trial step-down inhibitory avoidance (SDIA) task, an aversive learning paradigm in which stepping down from a platform is paired with a mild footshock. Briefly, the SDIA training box (50 × 25 × 25 cm) was made of Plexiglas and fitted with a grid floor through which scrambled electric shocks could be delivered to the rat's feet. Over the left end of the grid floor there was a 5-cm-high,

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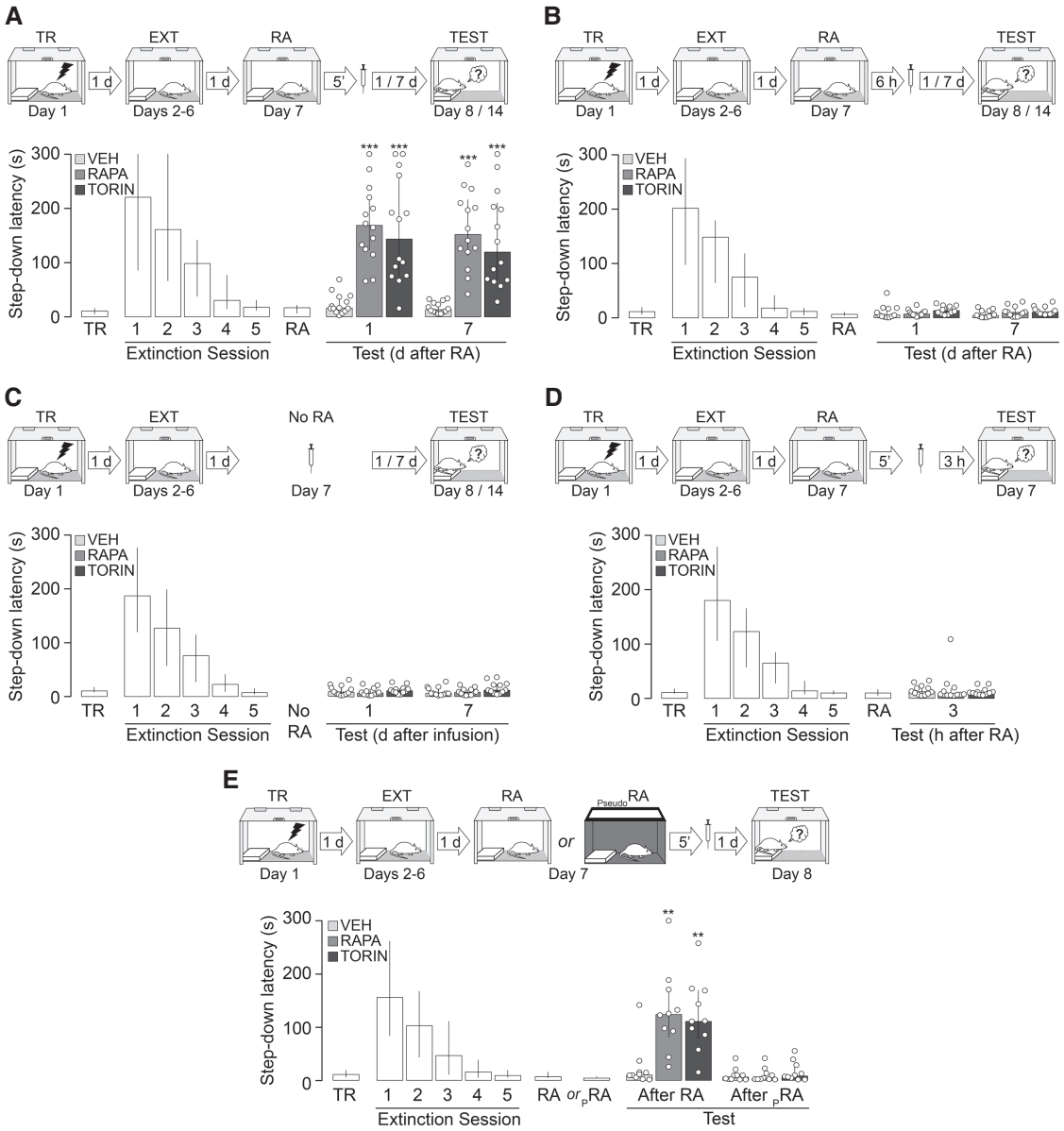


**Figure 1.** mTOR is required for fear-motivated avoidance extinction memory reconsolidation. (*A*) Animals were trained in SDIA (TR; 0.4 mA/2 sec) and beginning 24 h later submitted to one daily extinction session for five consecutive days (EXT). Twenty-four hours after the last session, extinction memory was reactivated (RA) and, 5 min thereafter, the animals received bilateral intradorsal CA1 infusions of vehicle (VEH; 5% DMSO in saline), rapamycin (RAPA; 0.02 µg/side) or TORIN (0.20 µg/side). Retention was assessed 1 and 7 d later (Test). (*B*) Animals were treated as in A except that they received intra-CA1 infusions of VEH, RAPA, or TORIN 6 h after RA. (*C*) Animals were treated as in *A*, except that they received VEH, RAPA, or TORIN in dorsal CA1 24 h after the last extinction session in the absence of RA (No RA). (*D*) Animals were treated as described in *A*, except that VEH, RAPA, or TORIN were given 5 min after RA and retention was assessed 3 h later. (*E*) Animals were treated as in *A*, except that a subgroup of animals received VEH, RAPA, or TORIN 5 min after an extinction pseudoreactivation session in an avoidance training box rendered nonaversive for SDIA-trained animals. The nonaversive box was similar in dimensions to the SDIA training box, but it was made of dark gray wood and had a Plexiglas platform. ($_P$RA) Pseudoreactivation session. Data are expressed as median ± IQR. (**) $P < 0.01$, (***) $P < 0.001$ versus VEH in Dunn's multiple comparisons after Kruskal–Wallis test.

8-cm-wide, 25-cm-long wooden platform. For training, the animals were individually placed on the platform facing the left rear corner of the training box and, when they stepped down and placed their four paws on the grid, received a 2-sec, 0.4-mA scrambled footshock, whereupon they were immediately withdrawn from the training box. This training protocol induces a long-lasting, hippocampus-dependent, fear-motivated avoidance memory expressed as an increase in step-down latency at test (Bernabeu et al. 1995; Paratcha et al. 2000; Katche et al. 2013). However, repeated testing in the absence of the footshock causes clear-cut extinction (Cammarota et al. 2005; Rossato et al. 2006; Bonini et al. 2011). Therefore, to extinguish the learned avoidance response, we submitted SDIA trained rats to one daily unreinforced test session for five consecutive days. To that end, we put the animals back on the training box platform until they stepped down to the grid. No footshock was given, and the animals were allowed to freely explore the training apparatus for 30 sec after stepping down. During this time, the animals stepped up onto the platform

and down again several times. This procedure induces an SDIA extinction memory immune to spontaneous recovery, reinstatement and renewal that lasts for at least 14 d and requires NMDA receptor activation as well as protein synthesis and gene expression in dorsal CA1 to consolidate (Cammarota et al. 2003; Rossato et al. 2010; Radiske et al. 2015). One day after the last extinction session, extinction memory was reactivated by placing the animals on the training box platform until they stepped down from it. Five minutes or 6 h later, the animals received bilateral intradorsal CA1 infusions (1 µL/side) of vehicle (VEH; 5% DMSO in saline), RAPA (0.02 µg/side) or the selective ATP-competitive inhibitor of mTOR, TORIN2 (TORIN; 0.20 µg/side). RAPA and TORIN were dissolved in DMSO and diluted to working concentration in sterile saline (<5% DMSO). The doses used were determined based on pilot experiments and previous studies showing the behavioral and biochemical effects of each compound (Bekinschtein et al. 2007; Revest et al. 2014; Renard et al. 2016; Lee et al. 2018). Retention was evaluated at different times after extinction memory reactivation by placing the animals on the training box platform and measuring their latency to step down. Because of the 300-sec ceiling imposed on test latency, step-down data were expressed as median ± IQR and analyzed using the Kruskal–Wallis test followed by Dunn's post hoc comparisons. We found that animals that received VEH recalled SDIA extinction memory normally regardless of the time elapsed between reactivation and test sessions. Conversely, RAPA and TORIN given 5 min, but not 6 h, after SDIA extinction memory reactivation impaired retention of extinction and induced reappearance of the SDIA response 1 d and 7 d later (Fig. 1A, 1 d after RA: $H = 24.42$, $P < 0.001$; $P < 0.001$ for VEH vs. RAPA, $P < 0.001$ for VEH vs. TORIN; 7 d after RA: $H = 26.85$, $P < 0.001$; $P < 0.001$ for VEH vs. RAPA, $P < 0.001$ for VEH vs. TORIN in Dunn's multiple comparisons after Kruskal–Wallis test; Fig. 1B, 1 d after RA: $H = 4.510$, $P = 0.1049$; 7 d after RA: $H = 4.606$, $P = 0.0999$ in Kruskal–Wallis test). Neither RAPA nor TORIN affected SDIA extinction memory when administered 24 h after the last extinction session in the absence of extinction memory reactivation (Fig. 1C, 1 d after infusion: $H = 2.141$, $P = 0.3428$; 7 d after infusion: $H = 4.086$, $P = 0.1296$ in Kruskal–Wallis test) or when given 5 min post-reactivation but retention was evaluated 3 h thereafter (Fig. 1D, $H = 1.654$, $P = 0.4375$ in Kruskal–Wallis test). Moreover, RAPA and TORIN had no effect on extinction memory retention if injected in dorsal CA1 5 min after an extinction pseudoreactivation session carried out in an avoidance training box rendered non-aversive for SDIA-trained animals (Fig. 1E, After RA: $H = 13.86$, $P = 0.001$; $P < 0.01$ for VEH vs. RAPA, $P < 0.01$ for VEH vs. TORIN; After $_{Pseudo}$RA: $H = 0.7503$, $P = 0.6872$ in Dunn's multiple comparisons after Kruskal–Wallis test; Supplemental Fig. S2). mTOR activity is regulated by phosphorylation at different sites (Watanabe et al. 2011). Phosphorylation at Ser2448 is mediated by p70S6K, occurs mainly to mTOR associated with mTORC1 (Chiang and Abraham 2005; Holz and Blenis 2005; Akcakanat et al. 2007), enables mTOR binding to regulatory-associated protein of mTOR (RAPTOR), and correlates with mTORC1 activation (Rosner et al. 2010). On the contrary, Ser2481 is an autophosphorylation site insensitive to acute rapamycin treatment that is phosphorylated only when mTOR makes part of mTORC2 complexes (Peterson et al. 2000; Copp et al. 2009). To analyze mTOR phosphorylation levels, we performed immunoblotting on total homogenates from the CA1 region of the dorsal hippocampus. Samples were not pooled. Equal amounts of proteins (15 µg) were fractionated by SDS-PAGE and transferred to PVDF membranes. Blots were blocked for 1 h, incubated overnight at 4°C with anti-pSer2448 mTOR (1:10,000; RRID:AB_330970), anti-pSer2481 mTOR (1:10,000; RRID: AB_2262884), or anti-mTOR (1:10,000; RRID:AB_330978), and then incubated for 2 h at room temperature with HRP-coupled anti-IgG secondary antibody. Immunoreactivity was detected using the Amersham ECL Prime Western Blotting Detection Reagent and the Amersham Imager 600 system. Densitometric analyses were performed using the ImageQuant TL 8.1 analysis software (GE Healthcare). We found that pSer2448 mTOR levels peaked 5 min after SDIA extinction memory reactivation and returned to control values within 30 min (Fig. 2, $F_{(5,20)} = 2.805$, $P = 0.0446$; $P < 0.05$ for 5 min vs. No RA in Dunnett's multiple comparison test after repeated measures ANOVA). No changes in pSer2481 mTOR or total mTOR levels were found up to 6 h post-reactivation (Fig. 2, pSer2481 mTOR: $F_{(5,20)} = 1.241$, $P = 0.3274$; mTOR: $F_{(5,20)} = 1.208$, $P = 0.3411$ in repeated measures ANOVA; Supplemental Fig. S3). mTORC1 activation stimulates brain-derived neurotrophic factor (BDNF) production in hippocampal neurons (Jeon et al. 2015), which in turn may induce mTOR-dependent activation of dendritic mRNA translation (Takei et al. 2004). Previously, we reported that hippocampal BDNF maintains fear-motivated avoidance extinction memory after recall (Radiske et al. 2015). In agreement with this finding, coinfusion of recombinant BDNF (0.25 µg/side) after SDIA extinction memory reactivation impeded the recovery of the avoidance response provoked by RAPA (Fig. 3, 1 d after RA: $H = 27.52$, $P < 0.001$; $P < 0.001$ for VEH vs. RAPA, $P < 0.001$ for BDNF vs. RAPA, $P < 0.05$ for RAPA vs. RAPA + BDNF; 7 d after RA: $H = 26.76$, $P < 0.001$; $P < 0.001$ for VEH vs. RAPA, $P < 0.001$ for BDNF vs. RAPA, $P < 0.01$ for RAPA vs. RAPA + BDNF in Dunn's multiple comparisons after Kruskal–Wallis test).

Our results show that dorsal CA1 mTOR inhibition during a short post-recall time window persistently impairs retention of SDIA extinction memory and causes avoidance reappearance. This effect took time to develop, was time-dependent, concomitant with SDIA extinction memory reactivation, and occurred after the administration of mTOR inhibitors with different mechanisms

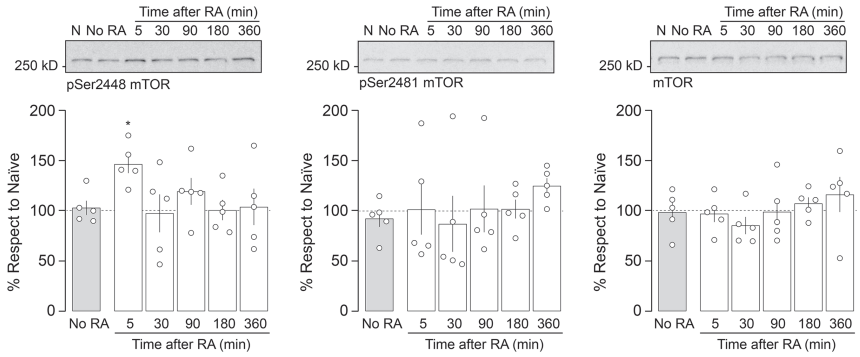


**Figure 2.** Reactivation of fear-motivated avoidance extinction memory increases mTOR phosphorylation at Ser2448, but not at Ser2481, in the CA1 region of the dorsal hippocampus. Animals were trained in SDIA (0.4 mA/2 s) and beginning 24 h later submitted to one daily extinction session for 5 consecutive days. Twenty-four hours after the last session, extinction memory was reactivated (RA) and the animals killed by decapitation at different post-reactivation times (5–360 min). The CA1 region of the dorsal hippocampus was dissected out, homogenized, and used to determine of pS2448 mTOR, pS2481 mTOR, or mTOR levels by immunoblotting. (N) Naïve animals, (No RA) animals trained in SDIA that were submitted to five daily extinction sessions and killed 24 h after the last extinction session. Data are expressed as mean ± SEM. (*) $P < 0.05$ versus No RA in Dunnett's multiple comparison test after repeated measures ANOVA.

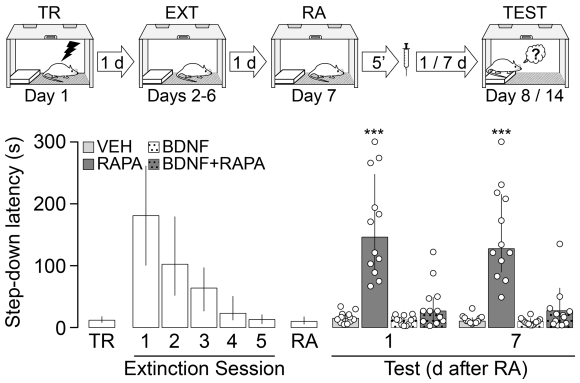


**Figure 3.** Coinfusion of recombinant BDNF reverses the effect of RAPA on fear-motivated avoidance extinction memory reconsolidation. Animals were trained in SDIA (TR; 0.4 mA/2 sec) and beginning 24 h later were submitted to one daily extinction session for five consecutive days (EXT). Twenty-four hours after the last session, extinction memory was reactivated (RA) and 5 min later the animals received bilateral intradorsal CA1 infusions of vehicle (VEH; 5% DMSO in saline), rapamycin (RAPA; 0.02 µg/side), BDNF (0.25 µg/µL), or RAPA plus BDNF (RAPA + BDNF). Retention was assessed 1 and 7 d later (Test). Data expressed as median ± IQR. (***) $P < 0.001$ versus VEH in Dunn's multiple comparisons after Kruskal–Wallis test.

of action, suggesting that it was not spontaneous or caused by nonspecific pharmacological interactions but due to bona fide impairment of an active mTOR-dependent reconsolidation process. This conclusion is further supported by findings showing that SDIA extinction memory reactivation rapidly and transiently increased mTOR phosphorylation at Ser2448, a post-translational modification customarily used as a proxy for mTOR activation (Reynolds et al. 2002; Guertin and Sabatini 2007; Rivas et al. 2009; Guo et al. 2017; Dong et al. 2018; Rosa et al. 2019). Most findings indicate that BDNF modulates protein synthesis through mTOR (Takei et al. 2001, 2004). In fact, BDNF controls hippocampal synaptic mRNA translation by regulating mTORC activation state (Briz et al. 2013; Leal et al. 2014), which seems to be necessary for SDIA memory consolidation (Slipczuk et al. 2009). However, in agreement with previous findings that BDNF is sufficient to restabilize a reactivated extinction memory trace, even when hippocampal protein synthesis and gene expression are inhibited (Radiske et al. 2015), our results show that mTOR acts upstream BDNF during the reconsolidation of extinction, and suggest not only that BDNF is a key protein synthesis product for this process but also that its actions are not mediated by mTOR-dependent mRNA translation. Indeed, mTOR signaling controls BDNF activity-dependent dendritic translation (Baj et al. 2016), and several protein synthesis-dependent plastic mechanisms, including late-LTP and memory consolidation, are rescued by BDNF when protein synthesis is impaired (Pang and Lu 2004; Moguel-González et al. 2008; Martínez-Moreno et al. 2011; Ozawa et al. 2014). Exogenous BDNF becomes quickly available for activity-dependent secretion, rapidly replacing the endogenous biosynthetic pathway after its administration (Santi et al. 2006). Thus, the rapid modulation of hippocampal high-frequency transmission produced by this neurotrophin is unaffected by protein synthesis inhibitors (Gottschalk et al. 1999; Tartaglia et al. 2001) and BDNF administration may induce the lasting structural reorganization and potentiation of hippocampal synapses in an mRNA synthesis and protein translation-independent manner (Martínez-Moreno et al. 2020), perhaps through a mechanism involving PKMζ activity regulation (Mei et al. 2011). In fact, hippocampal PKMζ acts downstream BDNF to control AMPAR synaptic insertion through a protein synthesis-independent mechanism during declarative memory reconsolidation (Rossato et al. 2019).

In conclusion, our results confirm that extinction does not erase the SDIA response but generates an inhibitory memory that coexists with it and controls its expression. The data also corroborate that avoidance extinction memory enters a labile state when reactivated by recall and needs to be reconsolidated through a mechanism involving hippocampal mTOR/BDNF signaling activation to maintain its dominance over the aversive trace. Finally, though not less important, our findings emphasize the necessity of understanding the dynamics of memory competition in order to develop better therapeutic strategies for PTSD treatment.

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