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Disruption of alcohol-related memories by mTORC1 inhibition prevents relapse

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Relapse to alcohol abuse is an important clinical issue that is frequently caused by cue-induced drug craving. Therefore, disruption of the memory for the cue-alcohol association is expected to prevent relapse. It is increasingly accepted that memories become labile and erasable soon after their reactivation through retrieval during a memory reconsolidation process that depends on protein synthesis. Here we show that reconsolidation of alcohol-related memories triggered by the sensory properties of alcohol itself (odor and taste) activates mammalian target of rapamycin complex 1 (mTORC1) in select amygdalar and cortical regions in rats, resulting in increased levels of several synaptic proteins. Furthermore, systemic or central amygdalar inhibition of mTORC1 during reconsolidation disrupts alcohol-associated memories, leading to a long-lasting suppression of relapse. Our findings provide evidence that the mTORC1 pathway and its downstream substrates are crucial in alcohol-related memory reconsolidation and highlight this pathway as a therapeutic target to prevent relapse.

Alcohol abuse is a worldwide problem with concomitant medical, social and economic burdens[1] for which pharmacotherapeutic approaches are limited[2]. Most patients with alcoholism will relapse within the first year of abstinence[3], highlighting relapse as an important clinical issue. A main cause of relapse is cue-induced drug craving[4], a process in which a cue that was previously associated with the reinforcing effects of alcohol elicits craving for alcohol itself, thereby increasing the likelihood of relapse. Thus, erasure of the memory for the cue-drug association is expected to reduce or prevent cue-induced relapse.

Current conceptions of memory processes hold that after retrieval of a memory, it is reactivated and undergoes a process of destabilization followed by a process of reconsolidation. After destabilization, a temporary 'reconsolidation window' opens during which the memory becomes labile and can be strengthened or attenuated[5,6], for example, by administration of amnestic agents shortly after memory reactivation[5,7]. Disruption of the reconsolidation of memories associated with drugs of abuse has been proposed as a potential strategy to prevent relapse[4,8,9]. However, although the dependence of reconsolidation on *de novo* protein translation is established[10,11], the specific signaling molecules and proteins that are required for drug memory reconsolidation remain largely unknown, especially for alcohol.

The mTORC1-mediated signaling pathway is required for the translation of a subset of dendritic proteins[12] and is implicated in synaptic plasticity[12,13] as well as memory processes[12]. Interestingly, mTORC1 has been reported to contribute to memory processes that are involved in cocaine-conditioned place preference and cue-induced reinstatement[14,15], as well as to the reconsolidation of fear and spatial recognition memories[16–20], which raises the possibility that this pathway is involved in the reconsolidation of memories that are associated with drugs of abuse, including alcohol. Here we tested whether reconsolidation of alcohol-associated memories requires activation of mTORC1 and, if so, whether these memories can be disrupted by mTORC1 inhibition, resulting in prevention of relapse.

RESULTS

Retrieval of alcohol-associated memories activates mTORC1

To determine whether the mTORC1 signaling pathway is activated after retrieval (reactivation) of alcohol-related memories (that is, during memory reconsolidation), we trained rats to voluntarily consume excessive amounts of alcohol in their home cage for 7 weeks using the intermittent access to 20% alcohol two bottle–choice procedure[21,22]. This procedure generates an average blood alcohol concentration of ~81 mg%[23], which corresponds to the definition of binge drinking in humans according to the US National Institute on Alcohol Abuse and Alcoholism. We then trained the rats in operant chambers for 4–5 weeks to press a lever for 0.1-ml aliquots of a 20% alcohol solution in daily 30-min sessions followed by 10 d of alcohol abstinence in the home cage. We then reactivated alcohol-associated memories by a 5-min exposure to the behavioral context in which alcohol was received (conditioning chambers), as well as to a non–pharmacologically active alcohol prime (0.2 ml of 20% alcohol) that served as a compound odor-taste cue (**Supplementary Table 1**). Control rats received identical training except that the reactivation stage was omitted (see **Supplementary Fig. 1** for the schematic timeline). Thirty minutes after memory reactivation, we assessed mTORC1 activation

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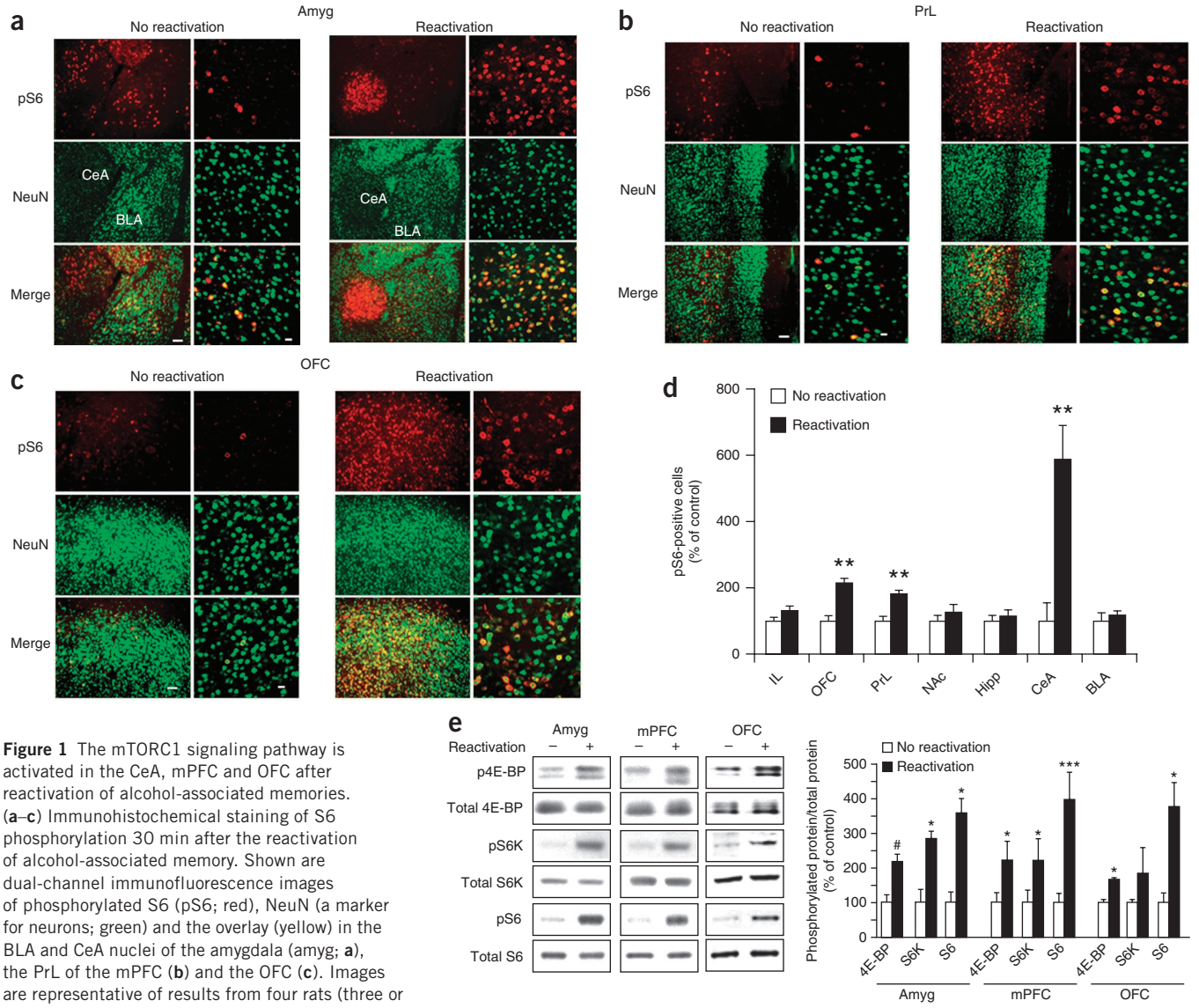

Figure 1 The mTORC1 signaling pathway is activated in the CeA, mPFC and OFC after reactivation of alcohol-associated memories. (**a**–**c**) Immunohistochemical staining of S6 phosphorylation 30 min after the reactivation of alcohol-associated memory. Shown are dual-channel immunofluorescence images of phosphorylated S6 (pS6; red), NeuN (a marker for neurons; green) and the overlay (yellow) in the BLA and CeA nuclei of the amygdala (amyg; **a**), the PrL of the mPFC (**b**) and the OFC (**c**). Images are representative of results from four rats (three or four sections per region per rat). Scale bars: left, 100 μm; right, 20 μm. (**d**) Quantification of the immunohistochemical staining of S6 phosphorylation. Data are shown as the mean ± s.e.m. and are expressed as a percentage of the no reactivation control group ($ts(6) > 4.17$; $^{**}P < 0.01$; $n = 4$ rats per group). IL, infralimbic cortex; hipp, dorsal hippocampus. (**e**) Western blot analyses of phosphorylated and total 4E-BP, S6K and S6 in the amygdala, mPFC and OFC. The immunoreactivity of phosphorylated 4E-BP, S6K and S6 was normalized to that of the total protein and is expressed as percentage of the control group (no reactivation). Data are shown as the mean ± s.e.m. ($ts(6) > 2.50$; $^{*}P < 0.05$, $^{***}P < 0.005$, $^{\#}P = 0.08$; $n = 4$ rats per group).

by measuring the phosphorylation levels of its downstream substrates, eukaryotic translation initiation factor-4E binding protein (4E-BP), S6 kinase (S6K) and the S6K substrate S6 (ref. 24).

We found that memory reactivation induced mTORC1 activation specifically in the central amygdala (CeA) and in the prelimbic region (PrL) and orbitofrontal region (OFC) of the prefrontal cortex (**Fig. 1**) but not in infralimbic cortex, nucleus accumbens (NAc), basolateral amygdala (BLA) or dorsal hippocampus (**Fig. 1** and **Supplementary Fig. 1**). Taken together these data show that reactivation of alcohol-associated memories activates the mTORC1 signaling pathway in the CeA, PrL and OFC.

Alcohol memory retrieval causes synaptic protein synthesis

mTORC1 controls the translation of 5′ terminal oligopyrimidine tracts, and all components of the mTORC1-dependent translational machinery are present at the synapse[12]. Thus, mTORC1 has an essential role in the local dendritic translation of mRNAs[12,25–27]. For example, the translation of the synaptic proteins Arc[28] and PSD-95 (refs. 29,30) and the AMPA and NMDA receptor subunits GluR1 (refs. 30,31) and NR1 (ref. 31), respectively, is mTORC1 dependent. Each of these proteins has an important role in synaptic plasticity and certain learning and memory processes[32–35]. Therefore we next tested the hypothesis that reactivation of alcohol-associated memories increases the amounts of key synaptic proteins whose translation is controlled by mTORC1.

Using the same reactivation procedure described above, we found that memory reactivation increased the amounts of Arc in the amygdala, OFC and medial prefrontal cortex (mPFC), as well as the amounts of GluR1 and PSD-95 in the amygdala and OFC (**Fig. 2**). We further found that the increase in the amount of Arc induced

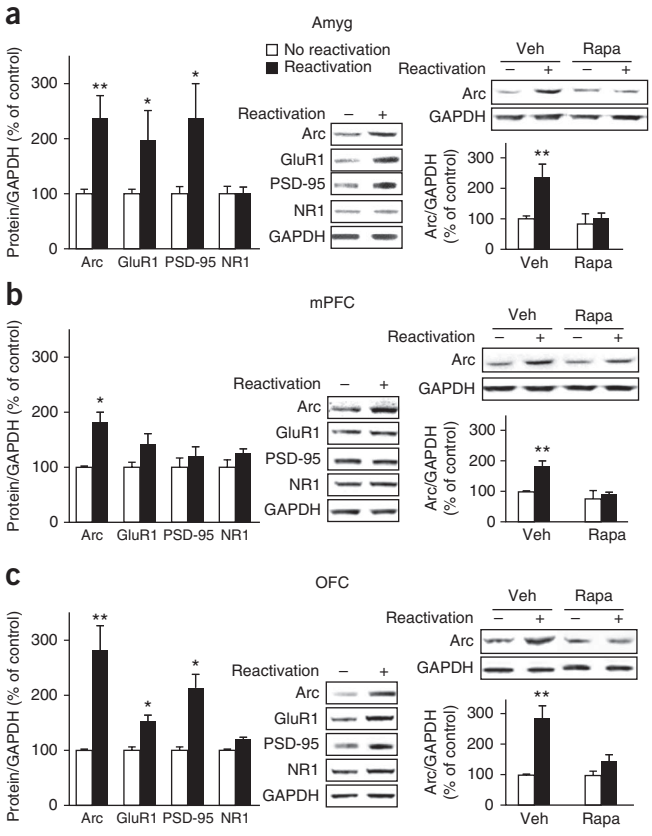

Figure 2 Reactivation of alcohol-associated memories increases the levels of synaptic proteins. (**a**–**c**) Immunoblotting of mTORC1-regulated proteins in the amygdala (**a**), mPFC (**b**) and OFC (**c**) 60 min after the reactivation of alcohol-associated memory. Left, the levels of Arc, GluR1, PSD-95 and NR1 were determined by western blot analysis and normalized to GAPDH. Right, the memory reactivation–induced increase in Arc immunoreactivity was blocked by rapamycin (rapa; 20 mg per kg body weight i.p.) administered immediately after memory reactivation. Data are shown as the mean ± s.e.m. and are expressed as percentage of the vehicle (veh)-treated control group (**a**–**c**, left: *t* test; ts(6) > 2.50; $^{*}P < 0.05$, $^{**}P < 0.01$; **a**–**c**, right: two-way analysis of variance (ANOVA); reactivation × treatment interaction, F(1,12) > 4.90, $P < 0.05$; *post-hoc* comparisons, $^{**}P < 0.01$; $n = 4$ rats per group).

by memory reactivation was abolished by mTORC1 inhibition in all three brain regions (**Fig. 2**) and the increase in the amounts of GluR1 and PSD-95 was reduced in the OFC (**Supplementary Fig. 2**). Taken together, these findings suggest that the consequence of mTORC1 activation during reconsolidation of alcohol-associated memories is the translation of specific synaptic proteins that take part in plasticity processes.

mTORC1 inhibition disrupts alcohol memory reconsolidation

If mTORC1 is essential for the reconsolidation of alcohol-associated memories, then inhibition of this pathway should disrupt this process, resulting in a subsequent reduction of relapse. To test this possibility, we trained rats to press a lever for alcohol, which we followed with a 10-d abstinence period as described above. We then administered the mTORC1 inhibitor rapamycin (20 mg per kg body weight intraperitoneally (i.p.)) or vehicle immediately after a 5-min reactivation session. We assessed relapse to alcohol seeking and drinking using retention[36] and reacquisition[37] tests 24 and 48 h, respectively, after the reactivation session (**Fig. 3a**). We found that mTORC1 inhibition after memory reactivation suppressed alcohol seeking and consumption 24 and 48 h later, respectively, as reflected in reduced numbers of active lever presses by rats receiving rapamycin compared to vehicle-treated rats (**Fig. 3b**,**c**). This finding indicates

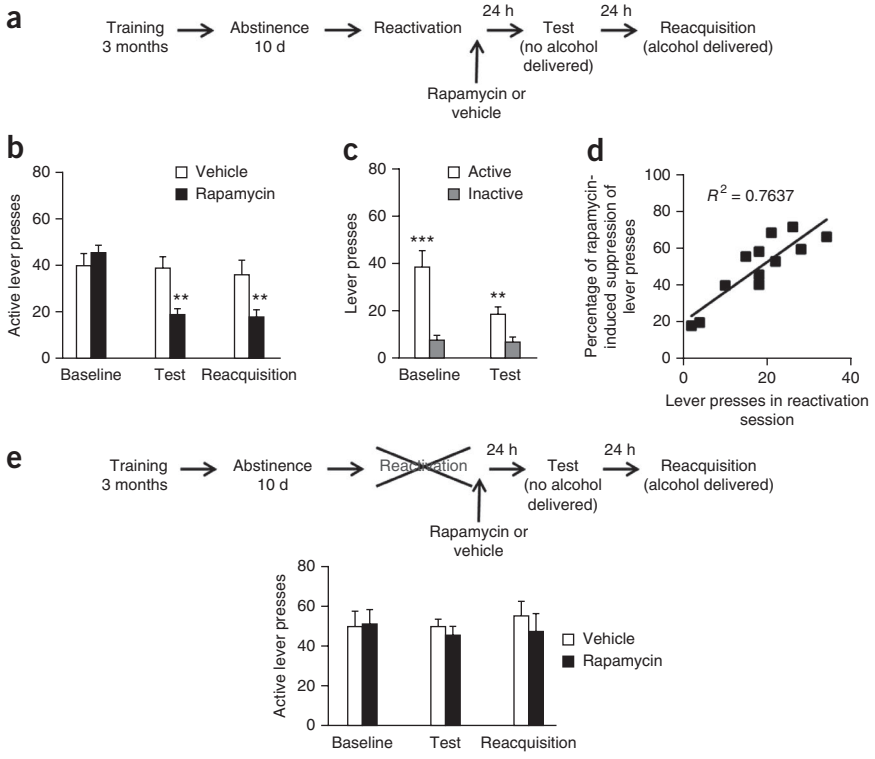

Figure 3 Inhibition of mTORC1 after reactivation of alcohol-associated memories attenuates relapse measured as instrumental responding for alcohol. (**a**) Schematic representation of the experimental procedure. (**b**) Effects of rapamycin (20 mg per kg body weight i.p.) or vehicle given immediately after memory reactivation using presentation of context and odor-taste cues on lever presses during the test and reacquisition periods (two-way ANOVA; stage × treatment interaction, F(2,22) = 6.38, $P < 0.01$; *post-hoc* comparisons, $^{**}P < 0.01$; $n = 12$ rats per group). (**c**) Active and inactive lever presses during the test stage (two-way ANOVA; stage × lever, F(1,22) = 27.57, $P < 0.001$; *post-hoc* comparisons, active compared to inactive lever presses, $^{**}P < 0.01$, $^{***}P < 0.0001$; $n = 12$ rats per group). (**d**) Correlation plot of the number of lever presses during the reactivation session and the percentage of rapamycin-induced suppression in lever presses during the test (calculated as (presses in test/presses in baseline) × 100 in the rapamycin group). (**e**) Effects of rapamycin (20 mg per kg body weight i.p.) or vehicle given 24 h before the test without a reactivation session on lever presses during the test and reacquisition periods (two-way ANOVA; stage × treatment interaction, F(2,18) = 0.53, $P = 0.59$; $n = 10$ rats per group). Data (**b**,**c**,**e**) are shown as the mean ± s.e.m. of active lever presses before abstinence (baseline) and during the retention test and reacquisition stages.

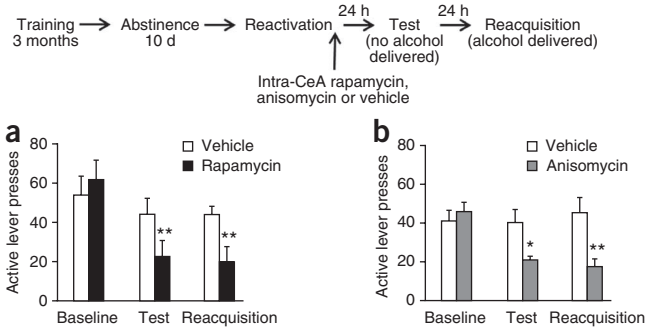

Figure 4 Infusion of rapamycin or anisomycin into the CeA after reactivation of alcohol-associated memories attenuates relapse. (**a**,**b**) Effects of rapamycin (**a**; 50 µg per side), anisomycin (**b**; 62.5 µg per side) or vehicle infused into the CeA immediately after memory reactivation on lever presses during the test and reacquisition periods. Data are shown as the mean ± s.e.m. of active lever presses before abstinence (baseline) and during the retention test and reacquisition stages (**a**: two-way ANOVA; stage × treatment interaction, $F(2,14) = 10.95$, $P < 0.005$; *post-hoc* comparisons, $^{**}P < 0.01$; $n = 8$ rats per group; **b**: two-way ANOVA; stage × treatment interaction, $F(2,11) = 8.59$, $P < 0.005$; *post-hoc* comparisons, $^{*}P < 0.5$, $^{**}P < 0.01$; $n = 6$–7 rats per group).

that mTORC1 activation is required for the reconsolidation of alcohol-related memories and that by inhibiting this pathway the memories can be attenuated and relapse can be reduced. Furthermore, the number of lever presses during the 5-min reactivation positively correlated with the suppressive effects of rapamycin on alcohol seeking 24 h later (**Fig. 3d**), suggesting that the more strongly the memory is reactivated, the more susceptible the memory becomes to mTORC1 inhibition.

Notably, in a control experiment, we found no effect when the reactivation session was omitted (that is, when we systemically administered rapamycin or vehicle 24 h before the test; **Fig. 3e**), showing that mTORC1 inhibition reduces relapse only if the memory is retrieved before the administration of rapamycin. This finding indicates that rapamycin disrupts memory reconsolidation rather than the motivation to respond or consume alcohol.

To test whether the effects of mTORC1 inhibition on memory reconsolidation are specific to memories that are associated with the reinforcing effects of alcohol, we tested the effects of administration of rapamycin after reactivation in rats trained to press a lever for a natural reward, sucrose (2% solution), rather than alcohol. We found that lever-press responses during both the retention and reacquisition tests were not different between the saline- and rapamycin-treated rats (**Supplementary Fig. 3**), indicating that mTORC1 inhibition is effective in disrupting memories associated with alcohol but not with other, natural reinforcers.

Because we found an increase in mTORC1 activation in the CeA after reactivation (**Fig. 1**), we reasoned that the reduction of alcohol relapse caused by rapamycin was mediated at least in part by the inhibition of mTORC1 activity specifically within the CeA. Thus we tested whether mTORC1 inhibition within the CeA disrupts memory reconsolidation. We found that infusion of rapamycin into the CeA (50 µg per side; **Supplementary Fig. 4**) focally inhibits the mTORC1 pathway, as reflected by reduced phosphorylation of S6, S6K and 4E-BP (**Supplementary Fig. 5**). Furthermore, infusion of rapamycin immediately after memory reactivation suppressed relapse to alcohol seeking and consumption on subsequent days (**Fig. 4a**). We also found that administration of the protein synthesis inhibitor anisomycin into the CeA produced similar behavioral effects to those observed after treatment of the CeA with rapamycin (**Fig. 4b**). Together these data suggest that mTORC1 activation within the CeA is required for the reconsolidation of alcohol-associated memories, a process that is probably mediated through mTORC1-dependent *de novo* protein synthesis.

Odor-taste cues evoke mTORC1-dependent reconsolidation

Alcohol is consumed orally, and therefore its odor and taste are potent cues for its reinforcing effects. We predicted that disrupting the association between these cues and alcohol reinforcement would attenuate relapse driven by these potent cues independently of specific contexts and other, more distal cues. We trained and tested rats using the same procedure as described above except that we conducted the reactivation session in the home cage with a 10-min exposure to two bottles: a water bottle and an empty bottle with the tip covered with

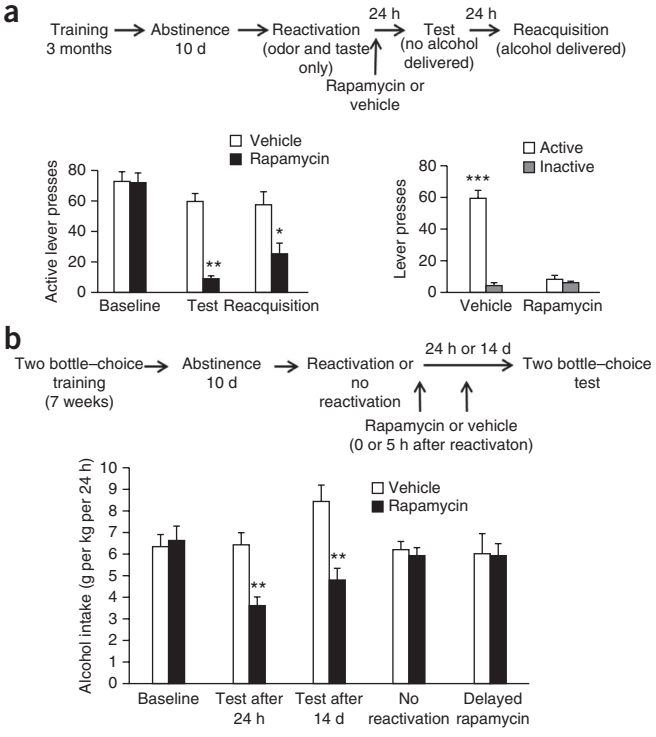

Figure 5 Inhibition of mTORC1 after reactivation of alcohol-associated memories in the home cage induces a potent, long-term suppression of relapse. (**a**) Effects of rapamycin (20 mg per kg body weight i.p.) or vehicle given immediately after memory reactivation using an alcohol odor-taste cue in the home cage on active lever presses during the test and reacquisition periods (left; two-way ANOVA; stage × treatment interaction, $F(2,26) = 14.51$, $P < 0.0001$; *post-hoc* comparisons, $^{*}P < 0.005$, $^{**}P < 0.001$; $n = 8$) and on active and inactive lever presses during the test stage (right; two-way ANOVA; stage × lever, $F(1,13) = 132.27$, $P < 0.0001$; *post-hoc* comparisons, active compared to inactive lever presses, $^{***}P < 0.0001$; $n = 8$ rats per group). Data are shown as the mean ± s.e.m. of lever presses. (**b**) Effects of rapamycin (20 mg per kg body weight i.p.) or vehicle given after memory reactivation on relapse to alcohol drinking in the two bottle–choice procedure. Data are shown as the mean ± s.e.m. of alcohol intake (g per kg per 24 h) during a 24-h two bottle–choice session in rapamycin- and vehicle-treated rats before abstinence (baseline), 24 h after reactivation, 14 d after reactivation, in the absence of reactivation and 24 h after reactivation with a delayed (5 h) administration of rapamycin (two-way ANOVA; condition × treatment interaction, $F(4,106) = 7.12$, $P < 0.0001$; *post-hoc* comparisons, $^{**}P < 0.001$; $n = 8$–12 rats per group).

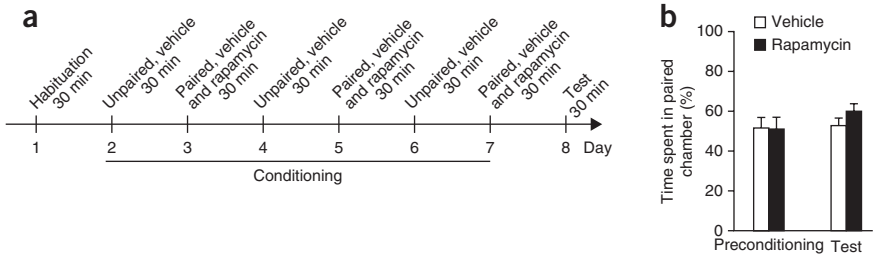

Figure 6 Rapamycin does not induce place aversion. (**a**) Design and schedule of the rapamycin place aversion experiment. Rats in the treatment group were systemically administered with rapamycin (20 mg per kg body weight) and vehicle 3 h before the 30-min conditioning paired and unpaired sessions, respectively. Rats in the control group received vehicle only. (**b**) Place preference and aversion for rapamycin expressed as the ratio ± s.e.m. of the time spent in the rapamycin-paired compartment divided by the time spent in both the paired and unpaired compartments (two-way ANOVA; treatment × conditioning interaction, $F(1,16) = 1.50$, $P = 0.24$; $n = 9$ rats per group).

a drop of alcohol (0.2 ml of 20% alcohol) serving as an odor-taste cue (an alcohol prime). We assessed relapse in the operant chambers, as described above, 24 and 48 h later. Notably, mTORC1 inhibition after memory reactivation substantially reduced relapse to alcohol seeking and drinking in this procedure, as indicated by low responses in the the retention and reacquisition tests as compared to vehicle-treated rats (**Fig. 5a**). The complete attenuation of relapse was also apparent in the lack of difference between the number of active and inactive lever presses in rapamycin-treated rats during the retention test (**Fig. 5a**). These findings demonstrate that the odor and taste of alcohol are potent cues that evoke memory reconsolidation independently of the training context, enabling a complete abolition of relapse to alcohol seeking by the inhibition of mTORC1.

To investigate the possibility that the odor-taste cue of alcohol is crucial for memory reactivation, we conducted an experiment entirely in the home cage using the intermittent access to 20% alcohol two bottle–choice procedure[37]. After 7 weeks of alcohol access and 10 d of abstinence, we reactivated alcohol-associated memories using the alcohol odor-taste cue presented in the home cage as described above (**Fig. 5b**). We found that systemic administration of rapamycin immediately after memory reactivation decreased relapse to alcohol consumption 24 h later as measured by intake from the home-cage bottle (**Fig. 5b**). Relapse to alcohol drinking was still suppressed when the test was conducted 14 d after the reactivation session (**Fig. 5b**), indicating that the rapamycin-induced relapse attenuation is long lasting. Notably, S6 phosphorylation was selectively increased in the CeA after reactivation (**Supplementary Fig. 6**), suggesting that activation of mTORC1 in the CeA underlies the retrieval of alcohol-related memories by the alcohol prime.

We showed that the reduction in home-cage alcohol consumption was due to disruption of memory reconsolidation. Specifically, administration of rapamycin with the omission of the reactivation session had no effect on alcohol intake (**Fig. 5b**), confirming that the effects of rapamycin on alcohol consumption 24 h later requires a previous reactivation of the memory. In addition, administration of rapamycin 5 h after memory reactivation had no effect on later alcohol consumption (**Fig. 5b**). These results suggest that the memory lability period after reactivation is limited to a few hours of the reconsolidation window, after which the memory reconsolidation process is completed, and the memories become stable[4,5] and resistant to mTORC1 inhibition. We found that when we trained rats to consume an alcohol solution and then a sucrose solution, rapamycin administration after the reactivation of alcohol-associated memories had no effect on subsequent sucrose consumption, indicating that the amnestic actions of rapamycin are specific to the reactivated memories, whereas other memories remain intact (**Supplementary Fig. 7**). We also found that rapamycin does not induce conditioned place aversion (**Fig. 6**), suggesting that the reduction in alcohol consumption we observed probably did not result from aversive effects of rapamycin causing either conditioned taste aversion or some other aversion-induced devaluation of the outcome.

DISCUSSION

We demonstrate that the most behaviorally relevant cues for relapse, the odor and taste of alcohol, are sufficient to elicit reconsolidation of alcohol-associated memories. Moreover, this process is correlated with activation of the mTORC1 signaling pathway in the CeA and specific cortical regions. We also show that the activation of mTORC1 leads to the translation of synaptic proteins that are important molecular contributors to memory processes[32–35]. Notably, we present data showing that the mTORC1 inhibitor rapamycin disrupts the reconsolidation of these memories, resulting in a long-lasting suppression of relapse.

Interestingly, mTORC1 activation in the BLA has been implicated in the reconsolidation of fearful[16,19,38] and object-recognition[17] memories; however, this signaling pathway was not activated in the BLA after the retrieval of alcohol-associated memories. The CeA has been implicated in behavioral responses to reward-predictive cues[39–41] and in incentive[42] and habit[43] learning, as well as incubation of cocaine and morphine cravings[44]. Our findings reveal a new role for the CeA in alcohol-cue memories and suggest that this region is crucial for reactivation of the association of the odor and/or taste of alcohol with its pharmacological effects. Interestingly, the CeA has also been implicated in anxiety and stress responses[45] and has been shown to have a role in the development of alcohol dependence through negative reinforcement mechanisms (alleviation of anxiety)[46]. Rats withdrawn from alcohol in the intermittent access to 20% alcohol procedure used here show a dopamine deficiency in the NAc, which is correlated with alcohol seeking and is alleviated by alcohol intake[21], implying the relevance of negative reinforcement mechanisms. It is thus plausible that retrieval of alcohol-associated memories after abstinence specifically reactivates affective (appetitive and/or aversive) aspects of memories, leading to mTORC1-dependent memory reconsolidation and synaptic protein synthesis. Inhibition of mTORC1 may disrupt these affective memories, resulting in disruption of the positive and/or negative reinforcement mechanisms that promote alcohol seeking.

Memory reactivation in the context of the alcohol self-administration chamber activated mTORC1 signaling in the PrL, OFC and CeA, whereas we found mTORC1 activation only in the CeA after memory reactivation in the home cage. Thus, additional associations related to the instrumental lever-press response and the contextual modulation of those associations are probably also retrieved, accounting for the activation of these cortical regions.

Some associations that support alcohol seeking probably remain after rapamycin treatment; when we assessed the effects of rapamycin on relapse after memory reactivation in the alcohol self-administration

chamber, the responses were attenuated but were still higher for the active lever compared to the inactive lever. In contrast, we found that relapse to alcohol seeking was completely abolished when mTORC1 was inhibited after retrieving the memory by presentation of the odor-taste cue in the home cage (that is, outside of the alcohol-associated context); hence, some instrumental associations may not be susceptible to reconsolidation disruption, which may account for the low number of responses that remained. Notably, even under conditions of reacquisition, when responding was again reinforced by alcohol, responding in the rapamycin-treated group was still considerably lower than in the control group, highlighting the potential utility of our approach for reducing relapse.

We found that mTORC1 inhibition after memory reactivation had no effect on relapse to consumption of a natural reward, sucrose. Specifically, sucrose intake was not altered by rapamycin administration after retrieval of sucrose-related memories or by administration of the inhibitor after reactivation of alcohol-associated memories. These findings suggest that the underlying mechanisms of memory processing are distinct for natural rewards as compared to alcohol. This possibility is not entirely surprising, as differential effects of various manipulations on behaviors reinforced by sucrose or alcohol reward have been previously reported (for example, see refs. 37,47,48).

The alcohol selectivity we demonstrate here has direct translational implications and may potentially enable selective interference with alcohol-related memories while leaving non-alcohol memories (for example, natural rewards memories) intact. Our findings that the reduction in relapse is observed even 14 d after the memory reactivation and that this effect cannot be attributed to taste aversion or devaluation of the outcome further highlight the translational potential of this relapse prevention approach.

We previously reported that alcohol exposure in pharmacologically relevant doses (2.5–6.5 g per kg body weight i.p. or voluntary consumption) activates the mTORC1 pathway in the NAc and that inhibition of this complex immediately before alcohol self-administration sessions reduced alcohol consumption[49]. In contrast, we found here that retrieval of alcohol-associated memories does not induce changes in mTORC1 activation in the NAc. A crucial difference between our two studies is the fact that we previously[49] compared alcohol-naive to alcohol-experienced rodents, whereas here we assessed the effects of retrieval of alcohol-associated memories on mTORC1 activation in rats that all had the same exposure to alcohol and that we tested after 10 d of abstinence. Hence, systemic administration of rapamycin has the potential to produce multiple effects on alcohol-seeking behaviors by acting on multiple neural circuits: an acute effect of alcohol exposure mediated by the NAc and an effect on later relapse driven by conditioned alcohol cues mediated by the CeA. These findings indicate that multiple alcohol-induced changes in neural function are mediated by mTORC1 signaling, adding further impetus to the investigation of the mTORC1 pathway for new therapeutic approaches to treat alcohol-use disorders.

Interference with the reconsolidation of memories had been proposed as a promising approach to attenuate or even erase memories, which could serve as a therapeutic strategy for several disorders that are associated with abnormally persistent memories, such as post-traumatic stress disorder[50] and substance abuse and dependence[4]. Experimental support for this approach was recently obtained in humans addicted to heroin[8]. The current findings suggest that disruption of reconsolidation could also be beneficial for alcohol-use disorders. Almost every behavioral experience with alcohol includes its odor and taste; thus a reconsolidation-based strategy for relapse disruption that focuses on these cues is a promising therapeutic approach. Our results show that effective reactivation of alcohol-associated memories is achieved by a brief presentation of the odor and taste of alcohol and that mTORC1 inhibition disrupts these memories and suppresses relapse. Thus, our results provide important translational implications for developing a new and potent strategy to prevent relapse in alcoholism through mTORC1-mediated disruption of memory reconsolidation mechanisms.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.B., J.N., P.H.J. and D.R. designed the research. S.B., F.L., Q.V.Y., S.B.H., J.N. and V.K. performed the research. S.B., F.L., S.B.H., Q.V.Y., J.N. and D.R. analyzed data. S.B., P.H.J. and D.R. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reagents. The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): anti-NMDAR1 (NR1; 4204, 1:2,000), pS6 ribosomal protein Ser235/Ser236 (2211, 1:1,000), total S6 (2217, 1:3,000), pS6K Thr389 (9234, 1:500), p4E-BP Thr37/Thr46 (2855, 1:1,000), total S6K (2708, 1:3,000) and total 4E-BP2 (2845, 1:2,000; 4E-BP2 is the main 4E-BP isoform in the brain[51,52]). Antibodies to GAPDH (sc-25778, 1:5,000), Arc (sc-17839, 1:1,000) and PSD-95 (sc-32290, 1:3,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GluR1 (06-306, 1:1,500) was purchased from Upstate (Billerica, MA). Mouse monoclonal anti–neuronal nuclei (NeuN) and nitrocellulose membranes were purchased from Millipore (Billerica, MA). The ethylenediaminetetraacetic acid (EDTA)-free Complete Mini Protease Inhibitors Mixture was purchased from Roche (11873580001, Indianapolis, IN). Phosphatase Inhibitors Mixtures 1 and 2, dimethylsulphoxide (DMSO) and anisomycin were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 594 donkey anti-rabbit (A21207) and Alexa Fluor 488 donkey anti-mouse (A21202) were purchased from Invitrogen (Eugene, OR). BCA (bicinchoninic acid) Protein Assay kits were purchased from Pierce (Rockford, IL). NuPAGE Bis-Tris precast gels were purchased from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence (ECL) Plus was purchased from GE Healthcare (Buckinghamshire, UK), and BioMax MR film was purchased from Kodak (Rochester, NY). Alcohol was purchased from Gold Shield Chemical (Hayward, CA). Rapamycin (R-5000) was purchased from LC Laboratories (Woburn, MA). Isoflurane was purchased from Baxter Health Care (Deerfield, IL).

Animals. Male Long-Evans rats (Harlan, Indianapolis, IN; 270–300 g at the beginning of training) were housed under a 12-h light, 12-h dark cycle (lights on at 7 a.m.) with food and water available *ad libitum*. All animal procedures were approved by the Gallo Center Institutional Animal Care and Use Committee and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

Preparation of solutions. Alcohol solution was prepared from ethyl alcohol absolute anhydrous (190 proof) diluted to 20% alcohol (vol/vol) in tap water. Rapamycin and anisomycin were dissolved in 100% DMSO.

Western blot analysis. Western blot analysis was conducted as previously described[49]. Briefly, brain regions were chosen according to the immunohistochemistry results (**Fig. 1a–c**). The amygdala, mPFC and OFC were rapidly dissected and immediately homogenized in a radioimmunoprecipitation assay (RIPA) buffer containing (in mM): 25 Tris-HCl, pH 7.6, 150 NaCl, 1 EDTA, 1% (vol/vol) NP-40, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and protease and phosphatase inhibitors. Protein concentration was determined using a BCA assay, and an equal amount of each sample (40 µg) was denatured with Laemmli buffer, boiled for 10 min, resolved on a 4–12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in 5% (wt/vol) nonfat milk in Tris-buffered saline and 0.1% (vol/vol) Tween 20 (TBST) and then incubated overnight at 4 °C with the appropriate antibody. After extensive washing with TBST, bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibody and visualized by ECL Plus. Membranes were then stripped for 30 min at 50 °C in buffer containing 100 mM 2-Mercaptoethanol, 2% (wt/vol) SDS and 62.5 mM Tris-HCl, pH 6.7, followed by extensive washing in TBST before re-blocking and re-probing with the appropriate total protein-specific antibody. The optical density of the relevant immunoreactive band was quantified using the NIH ImageJ 1.63 program. The optical density values of the phosphorylated protein signals were normalized to the signal of the total protein in the same sample. The optical density values of Arc, GluR1, PSD-95 and NR1 were normalized to GAPDH. Results are expressed as a percentage of the control group.

Immunohistochemistry. Immunofluorescent staining was conducted as previously described[22]. Briefly, free-floating paraformaldehyde-fixed 50-µm-thick sections were incubated with 50% ethanol for 20 min to permeablize the tissue, rinsed in PBS, blocked with 10% normal donkey serum in PBS for 30 min and then incubated for 48 h at 4 °C on an orbital shaker with a mixture of the two primary antibodies anti-pS6 (1:1,000) and anti-NeuN (1:500). Sections were then rinsed with PBS, incubated in 2% normal donkey serum for 10 min and then incubated for 12 h with the two secondary antibodies Alexa Fluor 594 donkey anti-rabbit (1:300) and Alexa Fluor 488 donkey anti-mouse (1:300). After staining, sections were rinsed in PBS, mounted on gelatin-subbed slides and coverslipped using Vectashield mounting medium (Vector Labs, Burlingame, CA). Images were acquired using a Zeiss LSM 510 META laser confocal microscope (Zeiss, Thornwood, NY) with the factory-recommended settings. Quantification was done by counting the number of pS6-positive cells and normalizing by area. All counts were performed blind with respect to treatment groups.

Intermittent access to 20% alcohol in the two bottle–choice drinking procedure. Intermittent access to alcohol was performed as previously described[21,22]. Briefly, rats were given 24 h of concurrent access to one bottle of 20% (vol/vol) alcohol in tap water and another bottle of water starting at 11 a.m. on Monday, Wednesday and Friday with 24 or 48 h of alcohol-deprivation periods between the alcohol-drinking sessions. The placement (left or right) of each solution was alternated between each session to control for side preference. The water and alcohol bottles were weighed after 24 h of access.

Operant alcohol self-administration after history of high voluntary alcohol consumption. The operant training began after rats achieved a stable baseline of alcohol consumption after 7 weeks of training in the intermittent access to 20% alcohol two bottle–choice drinking procedure as described above when rats maintained a stable baseline of alcohol consumption of 5.5–6 g per kg per 24 h. Rats were then trained to self-administer an alcohol solution in the operant self-administration chambers (Med Associates, Georgia, VT) as previously described[21], leading to a stable baseline of operant performance to obtain the delivery of 0.1 ml of a 20% alcohol solution under a fixed ratio 3 (FR3) schedule during 30-min sessions 5 d per week.

Operant-based memory reconsolidation procedure. Rats were trained in operant chambers to self-administer a 20% alcohol solution, as described above, or a 2% sucrose solution, as previously described[37]. After 4–5 weeks of training in FR3, when stable responses and alcohol consumption levels were obtained, rats were subjected to 10 d of abstinence from alcohol or sucrose in their home cage.

After completing 10 d of abstinence, rats were re-exposed to the alcohol- or sucrose-associated context and odor-taste cues. Specifically, rats were confined to the behavioral chamber for 5 min with the levers presented, and a non–pharmacologically active alcohol prime (0.2 ml of 20% alcohol) or sucrose prime (0.2 ml of 2% sucrose solution) was delivered immediately at the beginning of the session, which served as an odor-taste cue. Alcohol or sucrose was not delivered after lever presses in the remainder of the session. Control (no reactivation) rats were handled but were not presented with the context and cues. In experiments in which rapamycin or vehicle was administered, the injection was given immediately after the reactivation session.

Relapse to alcohol or sucrose seeking was assessed in a retention test stage that took place 24 h after the reactivation session. Rats were placed in the operant chambers for a 30-min session, similarly to the self-administration training sessions, except that no alcohol or sucrose was delivered after either lever was pressed. In addition, an alcohol or sucrose prime was noncontingently delivered at the beginning of the session, as in the reactivation session.

Relapse to alcohol or sucrose consumption was assessed in a reacquisition stage that took place 24 h after the test session (48 h after the reactivation session). This session was identical to the test stage except that alcohol or sucrose was delivered after lever presses (at FR3), as in the pretraining sessions. See **Figures 3–5** for a schematic timeline of experiments.

Non-operant memory reconsolidation in a two bottle–choice procedure. Rats were first trained for 7 weeks to voluntarily consume high levels of alcohol in their home cage, as described above. After obtaining a stable baseline alcohol consumption level (5.5–6.5 g per kg per 24 h; **Fig. 4**), rats were subjected to 10 d of abstinence from alcohol in their home cage. Rats were then re-exposed to the alcohol-associated odor-taste cues. Specifically, the *ad libitum* water bottle was taken out, and rats were presented for 10 min with two bottles in a similar manner to their two bottle–choice experience; however, now one bottle contained water, whereas the other bottle was empty with a 0.2-ml drop of alcohol applied on the tip to serve as an odor-taste cue. Control rats (no reactivation) were presented with two

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water bottles. In experiments in which rapamycin or vehicle was administered, the injection was given immediately or 5 h after the reactivation session as indicated.

Relapse to alcohol drinking was assessed by measuring alcohol and water intake in a 24-h two bottle–choice drinking session.

After 7 weeks of training in the intermittent access to 20% alcohol in the two bottle–choice environment, rats had access to a bottle containing sucrose solution (0.5% (wt/vol)) and a bottle of water for 3 weeks. Sucrose and water intake was monitored daily. After 10 d of access to water only (abstinence period), the alcohol-associated memory was reactivated in the home cage as described above, and rapamycin (20 mg per kg body weight i.p.) or vehicle was given immediately after memory reactivation. The next day, sucrose intake was tested in a 24-h two bottle–choice (sucrose and water) drinking session.

Surgery and microinfusion of rapamycin. Rats were anesthetized continuously with isoflurane. Guide cannulae (26 gauge; Plastics One) were aimed dorsal to the CeA (2.50 mm posterior to bregma, 4.1 mm mediolateral, 7.4 mm ventral to the skull surface) according to the Paxinos and Watson rat brain atlas. The coordinates for the CeA were chosen on the basis of the immunoreactivity of pS6 after reactivation of alcohol memories (**Fig. 1**). Microinjections began when self-administration responding returned to the pre-surgery baseline. Immediately after the memory reactivation session, rapamycin (50 µg/0.5 µl per side) or vehicle, or anisomycin (62.5 µg/0.5 µl per side) or vehicle, was infused over 1 min into the CeA of gently restrained rats through injection cannulae extending 0.5 mm beyond the guide cannula tip. Injection cannulae were left in place for an additional 2 min. The dose of rapamycin was based on previous studies[14] and an experiment demonstrating that at this dose, rapamycin infusion into the CeA inhibits the mTORC1 pathway (**Supplementary Fig. 5**). The dose of anisomycin was based on previous reconsolidation studies in which the inhibitor was infused into the amygdala[11].

Conditioned place preference apparatus and procedure. Rats were trained in identical three-chamber conditioned place preference boxes (Med Associates, Georgia, VT) consisting of a small gray middle chamber (12 cm × 21 cm × 21 cm) joined to two larger side chambers (28 cm × 21 cm × 21 cm) that differed in color, lighting and floor texture. Total time spent in each chamber and horizontal locomotor activity were automatically recorded by infrared beam breaks.

The place conditioning procedure was conducted as we previously described[21] and is illustrated in **Figure 6**. Briefly, rats were allowed to explore the entire apparatus for 30 min for habituation and to obtain baseline measurements (day 1, preconditioning session). The next day, the conditioning training started with one conditioning trial per day during 6 d (days 2–7). Rapamycin (20 mg per kg body weight) or vehicle was systemically administered 3 h before confinement of the rats for 30 min in the paired side chamber (days 3, 5 and 7). All rats were administered with vehicle before confinement in the unpaired side chamber (days 2, 4 and 6). On day 8, rats were allowed to explore the entire apparatus for 30 min (post-conditioning test session), as during habituation, and preference was scored by dividing the time spent in the paired compartment by the total time spent in the unpaired and paired compartments during this session (preference ratio). Three conditioning sessions were chosen, as three to four sessions are generally used to obtain robust placed preference or aversion to rewarding substances, including drugs of abuse, in rodents[53].

Histology. Locations of cannulae were verified in 50-µm coronal sections of paraformaldehyde-fixed tissue stained with thionin. Only data from rats with cannulae located within the CeA were included in the analysis (**Supplementary Fig. 4).**

Statistical analyses. Data from western blot and immunohistochemistry were analyzed using unpaired *t* tests. Operant self-administration, alcohol consumption and place preference data were analyzed using two-way ANOVA with repeated measures. Fisher least significant difference (LSD) *post-hoc* analysis was used where indicated. Correlation was analyzed by linear regression, and the effect size (R^2 value) was calculated. No statistical test was run to determine sample size a priori. The sample sizes we chose are similar to those used in previous publications[21,49].

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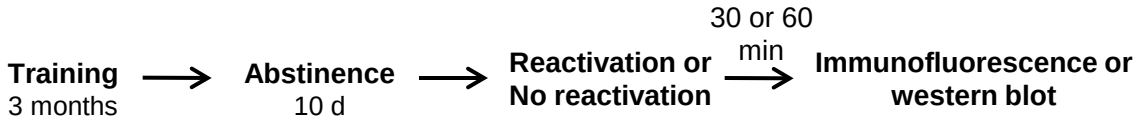
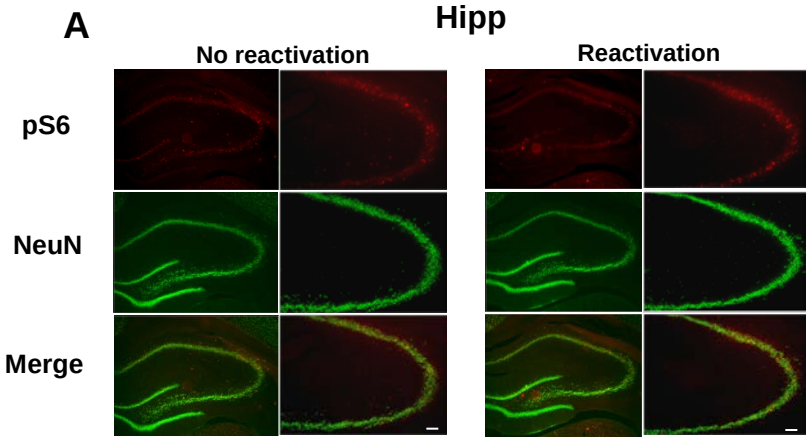
Supplementary information

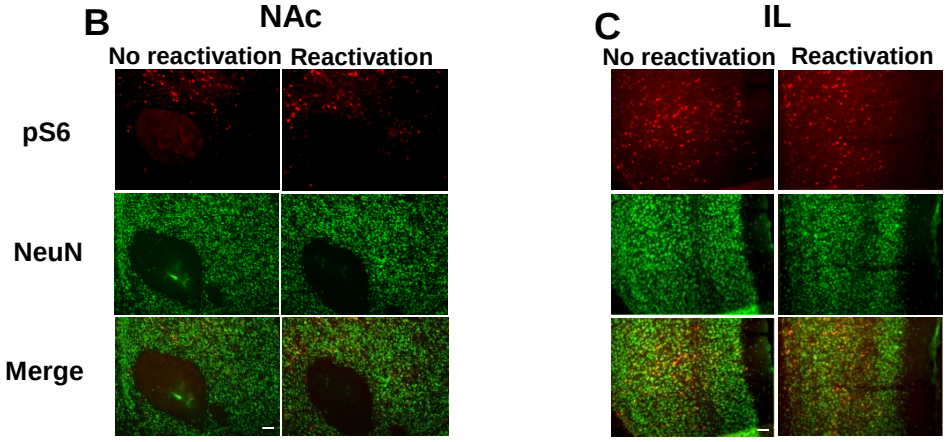
Supplemental Table 1. Lever presses (Mean ± SEM) during the 5-min reactivation session in the operant chambers. Vehicle, rapamycin or anisomycin was administered immediately after the reactivation session. Differences are not significant, p's>0.05.

Experiment		Vehicle	Rapamycin/anisomycin
Systemic rapamycin (20 mg/kg)	Immunohistochemistry	19.00 ± 5.53	-
	Western blot I	22.25 ± 5.68	-
	Western blot II	19.50 ± 3.29	20.75 ± 2.30
	Test/reacquisition	18.00 ± 2.71	16.42 ± 3.07
	Sucrose	8.07 ± 2.30	10.71 ± 2.13
Intra-CeA	Rapamycin (50 µg/side)	18.13 ± 3.46	20.38 ± 4.01
	Anisomycin (50 µg/side)	19.33 ± 2.88	17.29 ± 3.54

Barak et al., Supplemental Fig. 1

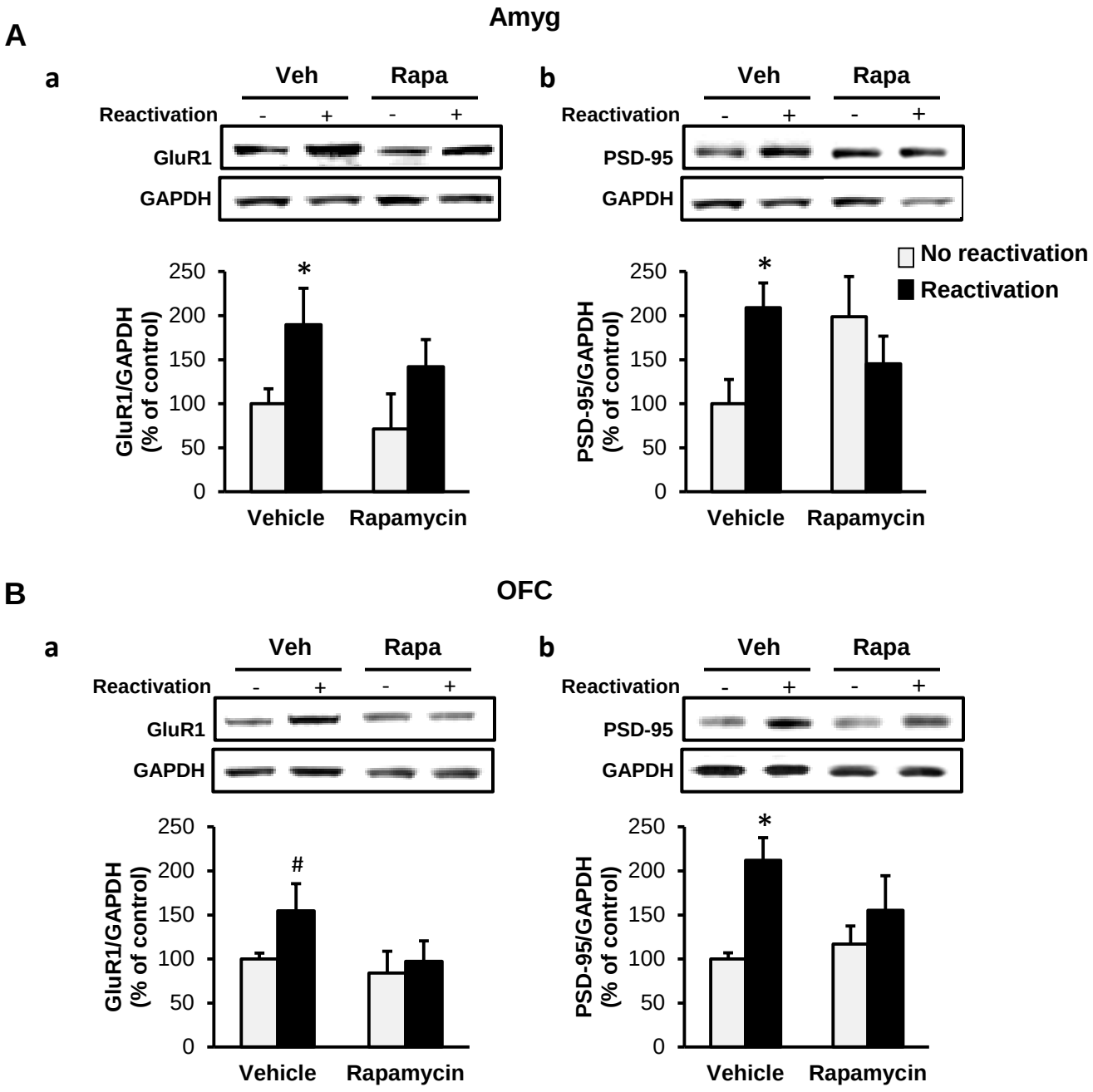






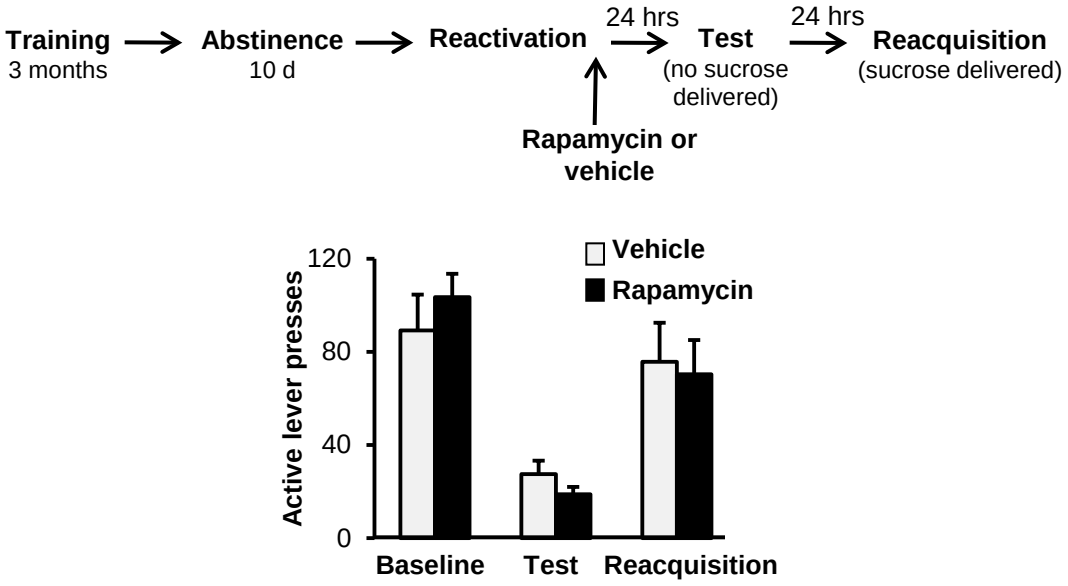
Supplemental Figure 1. The mTORC1 signaling pathway is not activated in the hippocampus, nucleus accumbens and infralimbic cortex following reactivation of alcohol-associated memories. A-C. *Immunohistochemical staining of S6 phosphorylation*. A-C. Shown is dual-channel immunofluorescence images of phosphoS6 (pS6, red), NeuN (a marker for neurons, green), and overlay (yellow), the dorsal hippocampus (A); the nucleus accumbens (NAc; B); and the infralimbic region of the medial prefrontal cortex (IL; C). Images are representative of results from 4 rats (3-4 sections/region/rat). Scale bar: 100 µm**.**

Barak et al., Supplemental Fig. 2



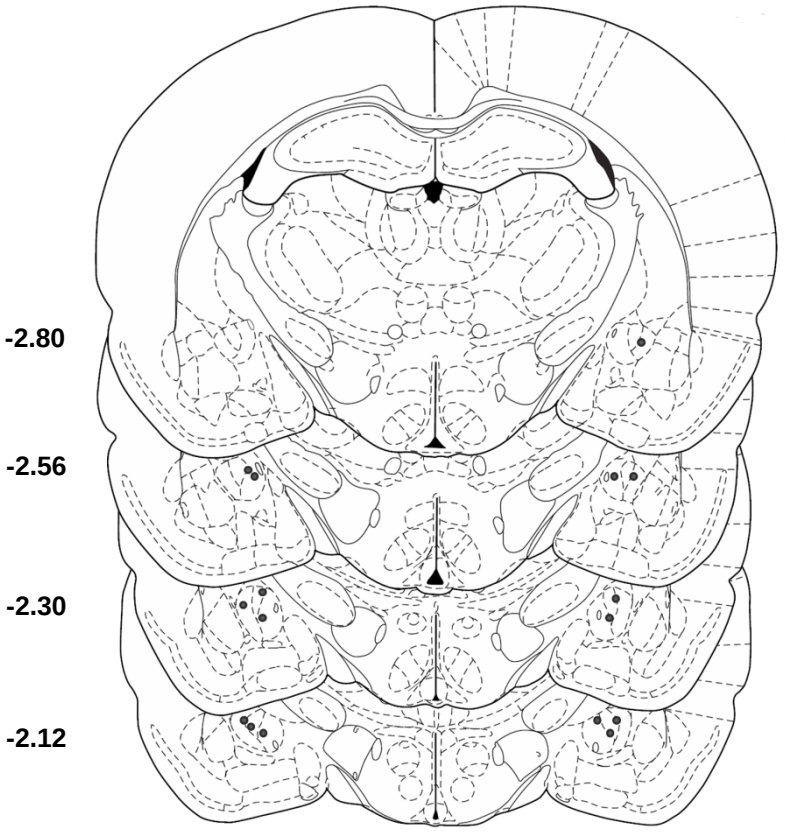
Supplemental Figure 2. Reactivation of alcohol-associated memories increases levels of synaptic proteins. *Immunoblotting of GluR1 (Aa, Ba) and PSD-95 (Ab, Bb) in the amygdala (Amyg; A) and OFC (B), 60 min after reactivation of alcohol-associated memory.* The levels of GluR1 and PSD-95 determined by western blot analysis and normalized to GAPDH. Rapamycin (20 mg/kg, i.p) was administered immediately after memory reactivation. Data are mean ± SEM and expressed as percentage of control. Aa, Ba, Bb, Two-way ANOVA; non-significant Reactivation X Treatment interaction; Ab, Reactivation X Treatment interaction [F(1, 11=4.54, p=0.05] post hoc comparisons *p<0.05 #p=0.07; n=3-4 per group).

Barak et al., Supplemental Fig. 3



Supplemental Figure 3. Inhibition of mTORC1 after reactivation of sucrose-associated memories does not affect relapse measured as instrumental responding for sucrose. Effects of rapamycin (20 mg/kg ,i.p.) given immediately after memory reactivation using presentation of context as well as a sucrose solution prime, on lever presses during test and reacquisition. Data are mean ± SEM of active lever presses before abstinence (baseline), and during retention test and reacquisition stages. (Two-way ANOVA; Stage X Treatment interaction [$F(2,26)=1.65$, $p=0.21$], $n=14$).

Barak et al., Supplemental Fig. 4



Supplementary Figure 4. Schematic representation of the cannulae placement in the central nucleus of the amygdala in coronal sections[3]. The locations of the cannulae tips are represented by black circles. Numbers indicate the distance relative to Bregma, in mm.

Barak et al., Supplemental Fig. 5

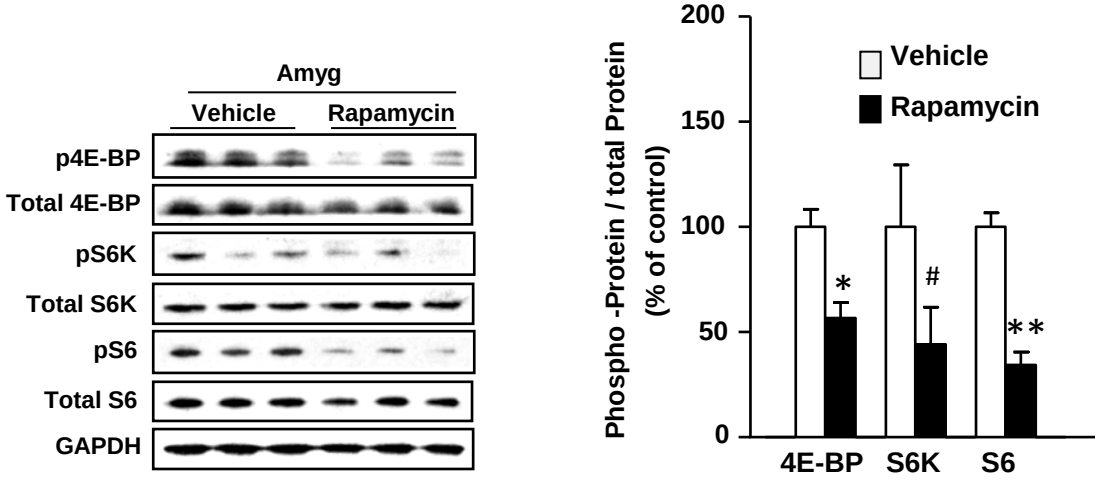
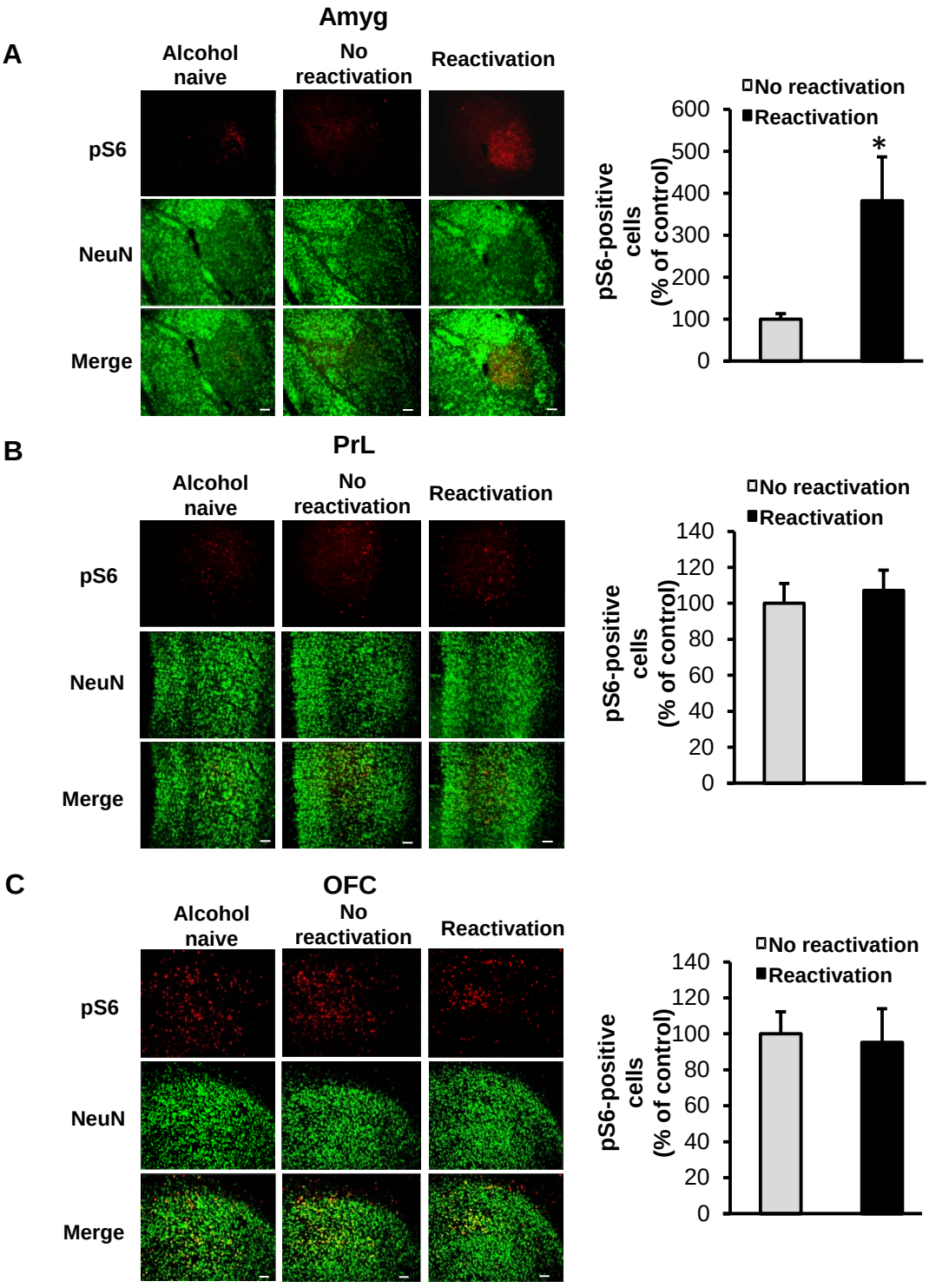


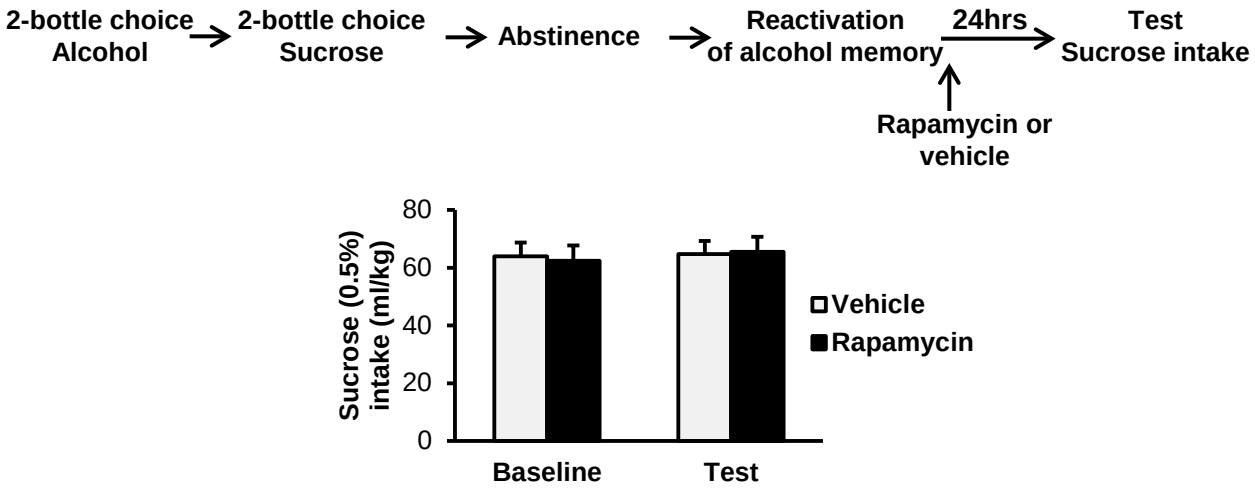
Figure 5. Infusion of rapamycin into the central nucleus of the amygdala (CeA) locally inhibits mTORC1 signaling. Rapamycin (50 µg/side) was infused unilaterally into rats' CeA; the other side was infused with vehicle. Three hrs later, Immunoreactivity of 4E-BP, S6K and S6 phosphorylation was determined by western blot analysis, normalized to the total protein level. Data are expressed as percentage of vehicle. (t's(4)>2.65, #p=0.06; *p<0.05, **p<0.01, n=3).

Barak et al., Supplemental Fig. 6



Supplemental Figure 6. The mTORC1 signaling pathway is activated in the CeA but not in the PrL or OFC following reactivation with of alcohol-associated memories odor-taste cue. A-C. Shown is dual-channel immunofluorescence images of phosphoS6 (pS6, red), NeuN (a marker for neurons, green), and overlay (yellow), the CeA (A), the PrL (B) and the OFC (C) of alcohol-naïve and alcohol-experienced rats that underwent memory reactivation by a brief exposure to the odor-taste of alcohol in the home cage (alcohol-naïve and reactivation, respectively), and of alcohol-experienced control rats that did not have a memory reactivation session (no reactivation). Images are representative of results from 4 rats (3-4 sections/region/rat). Scale bar, 100 µm. Quantification of the immunohistochemical staining was conducted by blind counting of pS6-positive cells normalized by the total area, in 3 slices per brain region from each rat. Data are mean ± SEM ($t(6)>2.67$; *$p<0.05$, $n=4$).

Barak et al., Supplemental Fig. 7



Supplemental Figure 7. Inhibition of mTORC1 after reactivation of alcohol-associated memories does not affect non-reactivated memories. Effects of rapamycin given after reactivation of alcohol-associated memories on reinstatement of sucrose intake in a 2-bottle choice procedure. Data are presented as mean ± SEM of sucrose (0.5% solution) intake (ml/kg/24 hrs) during a 24 hrs 2-bottle choice session, in rapamycin- or vehicle-treated rats before abstinence (baseline) and 24 hrs after reactivation (test; two-way ANOVA; no significant Stage X Treatment interaction $F(1,14)=0.63$, $p=0.84$; $n=8$).