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Authors

Hamida, Sami Ben
Laguesse, Sophie
Morisot, Nadege
[et al.](#)

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Mammalian target of rapamycin complex 1 and its downstream effector collapsin response mediator protein-2 drive reinstatement of alcohol reward seeking

Sami Ben Hamida¹, Sophie Laguesse¹, Nadege Morisot¹, Jong-Hyun Park^{2,3}, Khanhky Phuamluong¹, Anthony L. Berger¹, Ki Duk Park^{2,3}, Dorit Ron¹

¹Department of Neurology, University of California, San Francisco, San Francisco, CA, 94143, USA

²Convergence Research Center for Diagnosis, Treatment and Care System of Dementia, Korea Institute of Science and Technology (KIST), Republic of Korea

³Division of Bio-Medical Science & Technology, KIST School, Korea University of Science and Technology, Republic of Korea

Abstract

Alcohol use disorder is a chronic relapsing disease. Maintaining abstinence represents a major challenge for alcohol-dependent patients. Yet the molecular underpinnings of alcohol relapse remain poorly understood. In the present study, we investigated the potential role of the mammalian target of rapamycin complex 1 (mTORC1) in relapse to alcohol-seeking behavior by using the reinstatement of a previously extinguished alcohol conditioned place preference (CPP) response as a surrogate relapse paradigm. We found that mTORC1 is activated in the nucleus accumbens shell following alcohol priming-induced reinstatement of alcohol place preference. We further report that the selective mTORC1 inhibitor, rapamycin, abolishes reinstatement of alcohol place preference. Activation of mTORC1 initiates the translation of synaptic proteins, and we observed that reinstatement of alcohol CPP is associated with increased protein levels of one of mTORC1's downstream targets, collapsin response mediator protein-2 (CRMP2), in the nucleus accumbens. Importantly, the level of mTORC1 activation and CRMP2 expression positively correlate with the CPP score during reinstatement. Finally, we found that systemic administration of the CRMP2 inhibitor, lacosamide, attenuates alcohol priming-induced reinstatement of CPP. Together, our results reveal that mTORC1 and its downstream target, CRMP2, contribute to mechanisms underlying reinstatement of alcohol reward seeking. Our results could have important implications for the treatment of relapse to alcohol use and position the Food and Drug Administration approved drugs, rapamycin and lacosamide, for the treatment of alcohol use disorder.

Corresponding author: Dorit Ron, 675 Nelson Rising Lane, BOX 0663, San Francisco, CA 94143, dorit.ron@ucsf.edu3.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Data S1. Supporting Information

INTRODUCTION

Harmful alcohol use continues to be a major worldwide concern with severe socioeconomic consequences (WHO 2014). The etiology of alcohol dependence remains poorly understood, and only a few treatments are available (for reviews, see Akbar *et al.* 2018 #133; Heilig & Egli 2006 #73). One of the most troubling aspects of alcohol use disorder (AUD) is relapse to alcohol use that may occur even after several years of abstinence (Moos & Moos 2006). Thus, the high rate of relapse characterizing AUD represents a major challenge, and hence, a better understanding of the mechanisms underlying relapse is of great merit. One strategy to prevent relapse in alcohol addicts could be the attenuation of reward-related memories. Drugs of abuse are thought to usurp normal mechanisms underlying learning and memory processes (Russo *et al.* 2010; Torregrossa *et al.* 2011). The mammalian target of rapamycin complex 1 (mTORC1) is required for numerous forms of long-lasting synaptic plasticity, learning and memory (Lipton & Sahin 2014). mTORC1 is a multiprotein complex including the serine and threonine kinase mTOR (Lipton & Sahin 2014). mTORC1 phosphorylates the p70 ribosomal S6 Kinase, which in turn phosphorylates the ribosomal protein S6, as well as the initiation factor 4E binding protein (Lipton & Sahin 2014). These phosphorylation events promote the assembly of the translation initiation complex resulting in the translation of a subset of mRNA to proteins (Saxton & Sabatini 2017). In the central nervous system, activation of mTORC1 triggers the local dendritic translation of synaptic proteins, inducing structural and functional neuroadaptations that in turn participate in synaptic plasticity learning and memory (Buffington *et al.* 2014; Lipton & Sahin 2014). Accumulating evidence suggest that mTORC1 signaling also plays a central role in the molecular mechanisms underlying addiction (Neasta *et al.* 2014), and we have generated data implicating mTORC1 in processes that drive alcohol drinking behaviors (Neasta *et al.* 2010; Barak *et al.* 2013; Beckley *et al.* 2016; Liu *et al.* 2017; Laguesse *et al.* 2017a). Specifically, we previously reported that excessive alcohol consumption activates mTORC1 in the nucleus accumbens (NAc) of rodents (Neasta *et al.* 2010; Beckley *et al.* 2016; Laguesse *et al.* 2017a), resulting in the translation of synaptic proteins such as the collapsin response mediator protein-2 (CRMP2) (Liu *et al.* 2017) and Prosap2-interacting protein 1 (Prosapip1) (Laguesse *et al.* 2017b). We further have shown that inhibiting the function of these mTORC1 targets attenuates excessive alcohol consumption (Liu *et al.* 2017; Laguesse *et al.* 2017b). We also reported that retrieval of memories associated with an alcohol experience increases mTORC1 activity in cortical and amygdalar regions and that systemic or intra-CeA administration of the selective mTORC1 inhibitor, rapamycin, disrupts the reconsolidation of alcohol-related memories (Barak *et al.* 2013). Finally, in line with the potential important role of mTORC1 in mechanisms underlying AUD, genetic variants in mTOR signaling-related genes predict heavy alcohol consumption in humans (Meyers *et al.* 2015).

The reinstatement of drug-seeking behavior in rodents refers to the renewal of a behavior previously reinforced by drugs, by stimuli such as priming, cues, context or by stress (Sanchis-Segura & Spanagel 2006). Wang *et al.* previously reported that exposure to cocaine-related cues increased mTORC1 activity in the NAc core, but not shell and that specific inhibition of mTORC1 in the NAc core, using rapamycin, reduced reinstatement of

cocaine-seeking triggered by cocaine cues (Wang *et al.* 2010). Furthermore, James *et al.* reported that intra-NAc shell infusion of rapamycin, during operant self-administration training attenuated reinstatement of cocaine seeking (James *et al.* 2014).

Here, we sought to determine whether mTORC1 signaling also drives the reinstatement of alcohol-seeking behavior, and if so, we aimed to identify the molecular mechanisms that underlie the behavior.

MATERIALS AND METHODS

Detailed information regarding reagents and preparation of solutions can be found in Data S1

Subjects

Male DBA/2 J mice (Jackson Laboratory, Bar Harbor, Maine, US) were 8 weeks old at the beginning of the experiment and were group-housed (4/cages) in a temperature- and humidity-controlled colony room ($22 \pm 2^\circ\text{C}$, relative humidity: 50–60%) under normal 12-hour light/dark cycle (lights on at 07:00 AM) with food and water available ad libitum. Mice were weighed prior to drug administration. All animal procedures were approved by the University of California San Francisco Institutional Animal Care and Use Committee and were conducted in agreement with the Association for Assessment and Accreditation of Laboratory Animal Care.

Conditioned place preference procedures

Acquisition, extinction and reinstatement of alcohol-induced conditioned place preference—The protocol used to acquire alcohol CPP was performed accordingly to (Neasta *et al.* 2010). Further information can be found in Data S1. The CPP score was calculated as time spent in the drug-paired compartment on the *post-acquisition*, *post-extinction* or *reinstatement test* days minus time spent in the same compartment on the *pre-acquisition day*.

Rapamycin or lacosamide treatment—Different groups of mice were used to test the effect of treatment with saline and rapamycin or lacosamide on reinstatement of alcohol seeking. Mice conditioned with saline or alcohol were pseudo-randomly divided in two groups with a similar CPP scores on the *post-acquisition* and *post-extinction tests*. On day 14, half of each conditioned group of the mice was treated (i.p.) with vehicle (sal/veh and alc/veh groups), and the other half with rapamycin (10 mg/kg; sal/rapa and alc/rapa groups) or lacosamide (10 mg/kg; sal/LCM and alc/LCM groups). Three hours (rapamycin experiment) or 90 minutes (lacosamide experiment) later, saline-conditioned and alcohol-conditioned mice received a priming injection of saline or alcohol (0.9 g/kg), and immediately after mice underwent the *reinstatement* test for 15 minutes. The timing between pre-treatment and reinstatement testing was chosen based on previous studies (Neasta *et al.* 2010; Liu *et al.* 2017). Effect of lacosamide on spontaneous locomotor activity

A separate cohort of animals was used to assess the effect of lacosamide on spontaneous locomotor activity. Locomotor activity assessment was conducted in the CPP apparatus and

was detected by infrared photobeams. Lacosamide (10 mg/kg, i.p.) or vehicle was administered 90 minutes prior to placing mice in the CPP apparatus. Spontaneous locomotor activity was monitored for 30 minutes.

Western blot analysis

Western blot analysis was conducted as described in Laguesse *et al.* (2017a). Further information can be found in Data S1.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted as described previously (Ben Hamida *et al.* 2012). Further information can be found in Data S1.

Data analysis

Biochemical, IHC and CPP results were analyzed using one-way or a two-way analysis of variance (ANOVA). The Newman–Keuls *post hoc* tests and the method of contrast are used for individual group comparisons. Correlations between CPP scores and either IHC or biochemical data were analyzed using Pearson’s correlation tests. Statistical significance was set at $P < 0.05$.

RESULTS

Alcohol priming dose induces reinstatement of conditioned place preference to alcohol

To test whether mTORC1 plays a role in the reinstatement of alcohol reward, we used the Pavlovian-based CPP procedure in which animals develop an association between the rewarding action of a drug and specific environmental cues (Tzschentke 2007). This paradigm is used to study the reinforcing effect of drugs and motivated drug-seeking behaviors (Napier *et al.* 2013). The experimental timeline is illustrated in Figure 1a. For the *acquisition* phase, a none-hypnotic dose of alcohol (1.8 g/kg), which has previously been shown to induce robust alcohol CPP (Neasta *et al.* 2010), was used. For the *reinstatement test*, we used 0.9 g/kg of alcohol as a priming dose. Both doses of alcohol (0.9 and 1.8 g/kg) produced place preference (Fig. 1d; one-way ANOVA showed a significant effect of the alcohol doses ($F_{(2, 28)} = 16.13, P < 0.001$), and Newman–Keuls *post hoc* test showed a significant increase in the CPP score in alcohol treated groups compared with the group conditioned with saline ($P < 0.01$)); however, the rewarding effects of 0.9 g/kg the dose of alcohol was significantly lower than those induced by the 1.8 g/kg dose ($P < 0.05$). Acute administration of 1.8 g/kg of alcohol, but not the priming dose of 0.9 g/kg, produced a robust activation of mTORC1 in the NAc, as shown by the increased phosphorylation levels of its downstream target S6 (Fig. 1b–c; One-way ANOVA showed a significant main effect of alcohol doses ($F_{(2, 14)} = 16.23, P = 0.0004$), and Newman–Keuls *post hoc* test showed a significant increase in S6 phosphorylation levels only after injection of alcohol 1.8 g/kg ($P < 0.001$). Importantly, data shown in Figure 1e demonstrate that priming injection of alcohol reinstated alcohol place preference in mice conditioned with alcohol. Specifically, on the *Post-acquisition test* (Fig. 1e left panel), animals spent significantly more time in alcohol-paired compartment versus the saline-paired compartment. Two-way ANOVA showed a significant main effect of Conditioning ($F_{(1, 30)} = 39.69, P < 0.0001$), no effect of Groups ($F_{(1, 30)} =$

0.21, $P = 0.6$) and no interaction between the two factors ($F_{(1, 30)} = 0.25$, $P = 0.62$). Following analysis using method of contrast indicated that alcohol-conditioned animals exhibited a significantly higher CPP compared with saline-treated animals ($P < 0.0001$). During the *Extinction phase*, the acquired alcohol CPP was extinguished throughout 4 days with saline injection prior to counterbalanced confinement to the unpaired and alcohol-paired compartment. As shown in Figure 1e (middle panel), on the *Post-extinction test*, no significant difference was observed in the CPP scores between mice conditioned with saline and those conditioned with alcohol. Two-way ANOVA showed no significant effects of Conditioning ($F_{(1, 30)} = 1.313$, $P = 0.26$), Groups ($F_{(1, 30)} = 0.001$, $P = 0.96$) and interaction between the two factors ($F_{(1, 30)} = 0.09$, $P = 0.76$). Finally, as shown in Figure 1e (right panel), on the *reinstatement test day*, all mice received a priming injection of 0.9 g/kg of alcohol or saline, and CPP scores were determined after 15 minutes of free ambulation in the CPP apparatus. Two-way ANOVA showed a significant main effect of Conditioning ($F_{(1, 30)} = 15.11$, $P < 0.001$), a significant main effect of Priming ($F_{(1, 30)} = 16.77$, $P < 0.001$) and a significant interaction between the two factors ($F_{(1, 30)} = 4.507$, $P = 0.04$). Newman–Keuls *post hoc* tests detected a significant difference between the alcohol-conditioned and alcohol-primed mice (Alc/Alc group) and all the other groups ($P < 0.001$).

Alcohol priming-induced reinstatement of alcohol place preference activates mammalian target of rapamycin complex 1 in the nucleus accumbens shell

The NAc plays a major role in context–reward associations and guiding motivated behaviors (Everitt 2014), and we previously reported that excessive alcohol drinking activates mTORC1 in the NAc (Neasta *et al.* 2010; Beckley *et al.* 2016; Laguesse *et al.* 2017a). In addition, reinstatement of cocaine seeking increases mTORC1 activity in the NAc (Wang *et al.* 2010). Therefore, we hypothesized that reactivation of previously acquired memories by alcohol priming activates mTORC1 signaling in the NAc. To this end, an independent cohort of mice underwent the same paradigm as described in Figure 1 a. One hour following the end of the reinstatement test (Fig. 2a), mTORC1 activity in the NAc, dorsolateral striatum (DLS) and dorsomedial striatum (DMS) (Fig. 2b) were determined by analyzing the phosphorylation levels of S6 (Buffington *et al.* 2014). As shown in Figure 2c, 1 hour after reinstatement, mTORC1 activity was significantly increased in the NAc of Alc/Alc mice compared with all other groups. Two-way ANOVA showed no significant effect of Conditioning ($F_{(1, 12)} = 3.56$, $P = 0.083$), no significant effect of Priming ($F_{(1, 30)} = 3.73$, $P = 0.077$) and a significant interaction between the two factors ($F_{(1, 12)} = 6.1542$, $P = 0.029$). Newman–Keuls *post hoc* tests detected a significant difference between Alc/Alc group and all the other groups ($P < 0.05$). Interestingly, the reinstatement scores were positively correlated with the observed increased phosphorylation of S6 (Fig. 2d; Pearson correlation, $r^2 = 0.249$, $P = 0.05$). In contrast, no change in mTORC1 activity following alcohol conditioning or priming was observed in the dorsal striatum (Fig. 2e–f). Specifically, S6 phosphorylation was unaltered by priming injections of alcohol in the DMS [Fig. 2e; two-way ANOVA showed no significant effects of Conditioning ($F_{(1, 12)} = 0.015$, $P = 0.904$), Priming ($F_{(1, 12)} = 0.4221$, $P = 0.528$) and interaction between the two factors ($F_{(1, 12)} = 0.3792$, $P = 0.549$)], or in the DLS (Fig. 2f; two-way ANOVA showed no significant effects of Conditioning ($F_{(1, 12)} = 0.0541$, $P = 0.82$), Priming ($F_{(1, 12)} = 0.03$, $P = 0.864$) and interaction between the two factors ($F_{(1, 12)} = 0.488$, $P = 0.498$). Together, these data suggest

that mTORC1 is specifically activated in the NAc following priming-induced reinstatement of alcohol seeking.

The NAc is divided into the core and shell subregions, which are known to play distinct roles in motivation processes and modulation of alcohol seeking by context (Janak & Chaudhri 2010; Corbit *et al.* 2016). We previously showed that intake of large quantities of alcohol activates mTORC1 specifically in the NAc shell but not the core of rodents (Beckley *et al.* 2016; Laguesse *et al.* 2017a). In order to determine whether mTORC1 is differentially regulated in the NAc core and shell by reinstatement of alcohol seeking, we analyzed the immunoreactivity of phospho-S6 in the two NAc regions 30 minutes after the end of reinstatement test (Fig. 3a–b). As shown in Figure 3c–d, reinstatement of the alcohol-induced CPP promoted the activation of mTORC1 specifically in the NAc shell. Two-way ANOVA showed a significant main effect of Conditioning ($F_{(1, 30)} = 6.68, P = 0.014$), a significant main effect of Priming ($F_{(1, 30)} = 8.229, P = 0.007$) and a significant interaction between the two factors ($F_{(1, 30)} = 15.28, P < 0.001$). Newman–Keuls *post hoc* test detected a significant increase of phospho-S6 in Alc/Alc group compared with all other groups ($P < 0.001$). In addition, a positive correlation was observed between phospho-S6 labeling and the reinstatement scores (Fig. 3d, Pearson regression, $r^2 = 0.2, P = 0.0089$). However, no change in phospho-S6 immunoreactivity was observed in the NAc core (Fig. 3e, two-way ANOVA showed no effects of Conditioning ($F_{(1, 30)} = 0.098, P = 0.755$), Priming ($F_{(1, 30)} = 0.068, P = 0.796$) and interaction between the two factors ($F_{(1, 30)} = 0.0005, P = 0.982$)). These results suggest that priming-induced reinstatement of alcohol seeking activates mTORC1 specifically in the NAc shell.

Inhibition of mammalian target of rapamycin complex 1 by systemic administration of rapamycin prevents priming-induced reinstatement of alcohol seeking

Next, we tested whether inhibition of mTORC1 is sufficient to suppress alcohol priming induced-reinstatement of alcohol place preference by using the selective mTORC1 inhibitor, rapamycin (Dowling *et al.* 2010). To do so, mice underwent the acquisition and extinction of alcohol CPP according to the schedule shown in Figure 1a. Next, on the reinstatement test day, animals received an i.p. administration of rapamycin 3 hours before the alcohol priming treatment (saline or alcohol) (Fig. 4a). Immediately after the alcohol prime administration, mice were placed in the CPP apparatus and were given free access to the whole apparatus for 15 minutes (Fig. 4a). As expected, a robust CPP was observed in animals conditioned with alcohol compared with those treated with saline. Specifically, as shown in Figure 4b (left panel), in the *Post-acquisition test*, two-way ANOVA showed a significant main effect of alcohol Conditioning ($F_{(1, 48)} = 43.57, P < 0.0001$), no effect of Group ($F_{(1, 48)} = 0.47, P = 0.49$) and no interaction between the two factors ($F_{(1, 48)} = 0.23, P = 0.63$). Following analysis using method of contrast indicated that alcohol-conditioned animals exhibited a significantly higher CPP compared with saline-treated animals ($P < 0.001$). The acquired alcohol CPP was then extinguished during the extinction phase. As shown in Figure 4b (middle panel), in the *Post-extinction test*, all groups showed similar levels of place preference to the target compartment. Two-way ANOVA showed no significant effects of Conditioning ($F_{(1, 48)} = 0.01, P = 0.9$), Group ($F_{(1, 48)} = 0.18, P = 0.66$) and interaction between the two factors ($F_{(1, 48)} = 0.13, P = 0.71$). Finally, as shown in Figure 4b

(right panel), in the *reinstatement test* day, treatment with rapamycin abolished the alcohol priming-induced reinstatement of alcohol place preference. Two-way ANOVA showed a significant main effect of Treatment ($F_{(1, 48)} = 5.5, P = 0.023$), a significant main effect of Priming ($F_{(1, 48)} = 4.25, P = 0.045$) and significant interaction between the two factors ($F_{(1, 48)} = 4.1, P = 0.049$). Newman–Keuls *post hoc* test showed a significant difference between vehicle and rapamycin treatment in alcohol-primed mice ($P < 0.05$). Together, our results reveal that systemic administration of rapamycin efficiently prevents the reinstatement of alcohol seeking induced by a priming injection of alcohol.

Alcohol priming-induced reinstatement of alcohol place preference increases collapsin response mediator protein-2 levels in the nucleus accumbens

We next set out to identify a possible downstream target of mTORC1, which contributes to the reinstatement of alcohol place preference. mTORC1 has been shown to promote the translation of a subset of synaptic proteins (Buffington *et al.* 2014; Lipton & Sahin 2014), and we generated data suggesting that excessive alcohol drinking initiates the translation of Arc, GluA1, Homer, PSD-95, Prosapip1 and CRMP2 in the NAc of rodents (Neasta *et al.* 2010; Beckley *et al.* 2016; Liu *et al.* 2017; Laguesse *et al.* 2017b). Thus, we measured the protein level of these synaptic proteins in the NAc of mice that underwent the previously described acquisition, extinction and reinstatement of alcohol CPP paradigm (Fig. 1a). We observed no change in the protein levels of Homer (Fig. 5a; $p_{\text{Conditioning}} = 0.23, p_{\text{Priming}} = 0.53$ and $p_{\text{interaction}} = 0.044$), PSD-95 (Fig. 5b; $p_{\text{Conditioning}} = 0.03, p_{\text{Priming}} = 0.16$ and $p_{\text{interaction}} = 0.29$), GluA1 (Fig. 5c; $p_{\text{conditioning}} = 0.63, p_{\text{Priming}} = 0.34$ and $p_{\text{interaction}} = 0.56$), Arc (Fig. 5d; $p_{\text{Conditioning}} = 0.87, p_{\text{Priming}} = 0.98$ and $p_{\text{interaction}} = 0.78$) and Prosapip1 (Fig. 5e; $p_{\text{Conditioning}} = 0.14, p_{\text{Priming}} = 0.81$ and $p_{\text{interaction}} = 0.8$) in the NAc after reinstatement of CPP. In contrast, CRMP2 protein levels were significantly increased in the NAc of mice in response to alcohol prime-induced reinstatement of alcohol place preference (Fig. 5f). Two-way ANOVA showed a significant main effect of Conditioning ($F_{(1, 12)} = 11.03, P = 0.006$), no significant effect of Priming ($F_{(1, 12)} = 2.16, P = 0.16$) and significant interaction between the two factors ($F_{(1, 12)} = 9.04, P = 0.01$). Newman–Keuls *post hoc* tests detected a significant difference between the Alc/Alc group and all the other groups ($P < 0.01$). As shown in Figure 5g, a positive correlation was observed between CRMP2 levels and the reinstatement scores (Pearson regression, $r^2 = 0.4, P = 0.0091$). In contrast, we observed no change in CRMP2 levels in the DMS after CPP reinstatement (Fig. 5h; $p_{\text{Conditioning}} = 0.29, p_{\text{Priming}} = 0.57$ and $p_{\text{interaction}} = 0.83$). Together, these results suggest that reinstatement of alcohol CPP increases the protein levels of the mTORC1 downstream target, CRMP2, specifically in the NAc.

Systemic administration of lacosamide reduces alcohol priming-induced reinstatement of alcohol place preference

Collapsin response mediator protein-2 is a microtubule-binding protein implicated in the regulation of dendritic morphology (Ip *et al.* 2014; Quach *et al.* 2015). Abnormal CRMP2 function has been associated with the development of several neurological disorders (Ip *et al.* 2014; Quach *et al.* 2015). We previously found that excessive alcohol drinking increases CRMP2 translation in the NAc (Liu *et al.* 2017). We further found that pharmacological inhibition of CRMP2 as well as shRNA-mediated knockdown of CRMP2 in the NAc

decrease excessive alcohol consumption (Liu *et al.* 2017). As CRMP2 protein levels are elevated as a result of alcohol-induced reinstatement of CPP, we hypothesized that CRMP2 plays a role in mechanisms underlying reinstatement of alcohol CPP. To test this possibility, mice underwent the acquisition and extinction of alcohol CPP protocol as shown in Figure 1a, and on the *reinstatement test* day, animals were i.p. administered with saline or 10 mg/kg of lacosamide (Beyreuther *et al.* 2007), a specific inhibitor of CRMP2 that acts in part by preventing CRMP2 binding to microtubules (Wilson *et al.* 2012; Wilson & Khanna 2015). Ninety minutes later, animals were injected with a priming dose of alcohol or saline (Fig. 6a) and tested for alcohol reinstatement. As shown in Figure 6b (left panel), in the *Post-acquisition test*, two-way ANOVA showed a significant main effect of Conditioning ($F_{(1, 42)} = 49.71, P < 0.0001$), no effect of Groups ($F_{(1, 42)} = 0.06, P = 0.81$) and no interaction between the two factors ($F_{(1, 42)} = 0.09, P = 0.76$). Following analysis using method of contrast indicated that alcohol-conditioned animals exhibited a significantly higher CPP scores compared with saline-treated animals ($P < 0.001$). The acquired alcohol CPP was then extinguished during the extinction phase. As shown in Figure 6b (middle panel), in the *Post-extinction test*, no significant difference was observed in the CPP scores between mice conditioned with saline and those conditioned with alcohol. Two-way ANOVA showed no effects of Conditioning ($F_{(1, 42)} = 2.85, P = 0.09$), Groups ($F_{(1, 42)} = 0.13, P = 0.72$) and interaction between the two factors ($F_{(1, 42)} = 0.07, P = 0.78$). Importantly, lacosamide treatment significantly reduced reinstatement of CPP after subsequent alcohol exposure compared with vehicle treated group (Fig. 6b (right panel)). Two-way ANOVA showed significant main effects of Priming ($F_{(1, 42)} = 17.84, P < 0.001$), significant main effects of Treatment ($F_{(1, 42)} = 4.1, P = 0.049$) but no significant interaction between both factors ($F_{(1, 42)} = 2.95, P = 0.093$). Following analysis using method of contrast revealed a significant difference between vehicle and lacosamide treatment in alcohol-primed mice ($P < 0.05$). Finally, as shown in Figure 6c, systemic administration of 10 mg/kg of lacosamide did not alter mice locomotion ($t_{(8)} = 0.69, P = 0.51$), and we previously showed that even a higher dose of lacosamide (20 mg/kg) does not alter spontaneous locomotor activity in open field test (Liu *et al.* 2017). Together, these data suggest that inhibition of CRMP2 function prior to alcohol priming is sufficient to reduce the re-emergence of alcohol-seeking behavior. It also indicates that CRMP2 is critical for the recall of previously extinguished alcohol place preference.

DISCUSSION

Here, we present data to suggest that reinstatement of alcohol reward memory activates mTORC1 in the NAc shell resulting in the translation of its downstream targets CRMP2. Our data further reveal that pharmacologic inhibition of either mTORC1 or CRMP2 with the FDA approved drugs rapamycin (Dowling *et al.* 2010) and lacosamide (Wilson & Khanna 2015), respectively, attenuates alcohol-reward associated memories.

Reinstatement of reward-seeking behaviors classically refers to the renewal of an extinguished drug-seeking or drug-taking behavior in response to exposure to the drug itself, drug-associated cue or context, or acute stress (Epstein *et al.* 2006; Bossert *et al.* 2013). Here, in order to examine the involvement of mTORC1 in the relapse to alcohol seeking, we used a CPP paradigm widely used to explore the association between the rewarding effects

of a drug and specific environmental cues (Tzschentke 2007; Napier *et al.* 2013). We confirmed previous results (Bhutada *et al.* 2012; Roger-Sanchez *et al.* 2012; Al-Hasani *et al.* 2013) indicating that an extinguished alcohol-induced CPP could be reinstated by the non-contingent administration of a low priming dose of alcohol. The level of the reinstated response is considered as an index of level of motivation for environmental contexts previously paired with drug treatments (Bossert *et al.* 2013), and the alcohol prime is likely to enable reinstatement of alcohol place preference by facilitating the retrieval of a complex association between context (alcohol-associated compartment) and the rewarding properties of alcohol.

The induction, expression, extinction and reinstatement of alcohol seeking are thought to depend on drug-induced neuroadaptations in several brain areas (Al-Hasani *et al.* 2013) indicating that an extinguished alcohol-induced CPP could be reinstated by the non-contingent administration of a low priming dose of alcohol. The level of the reinstated response is considered as an index of level of motivation for environmental contexts previously paired with drug treatments (Bossert *et al.* 2013), and the alcohol prime is likely to enable reinstatement of alcohol place preference by facilitating the retrieval of a complex association between context (alcohol-associated compartment) and the rewarding properties of alcohol.

The induction, expression, extinction and reinstatement of alcohol seeking are thought to depend on drug-induced neuroadaptations in several brain areas that regulate drug rewarding and motivation effects (Ron & Barak 2016) including the NAc (Salgado & Kaplitt 2015). Interestingly, the priming dose of alcohol used in this study 0.9 g/kg produces a small but detectable CPP, but a single systemic administration of 0.9 g/kg alcohol did not affect mTORC1 activity. It is highly plausible that repeated systemic administration of alcohol sensitizes mTORC1 signaling pathway, leading to an increased activity of mTORC1. It is also highly plausible that mTORC1 is activated during the retrieval of alcohol-associated memories. This result is in line with our previous work showing that reactivation of alcohol-associated memories using context, alcohol odor and taste activates mTORC1 in several brain regions in rats, resulting in relapse to alcohol seeking and drinking (Barak *et al.* 2013). Our data further show that there is a tight correlation between the level of mTORC1 activation in the NAc and the CPP score. Furthermore, we observed that mTORC1 is activated in the NAc shell but not in the Nac core, the DLS or DMS of mice following alcohol priming-induced reinstatement of alcohol CPP. Together, data indicate that alcohol exposure induces distinct neuroadaptations in different striatal regions. These findings are in line with previous data showing that different signaling cascades are induced by alcohol in discrete brain regions and subpopulations of neurons (Ron & Barak 2016). These findings are also in line with previous studies showing that the NAc shell is critical for cue- (Richard & Fields 2016) and context- (Perry & McNally 2013) induced reinstatement of extinguished drug-seeking behavior. Additionally, context-induced reinstatement of operant responding for a beer solution was shown to be correlated with increased expression of c-Fos in the ventral part of the NAc shell (Hamlin *et al.* 2007).

Importantly, we found that the systemic administration of rapamycin abolished reinstatement of alcohol CPP. This result could not be due to a nonspecific effects of

rapamycin such as alteration in locomotor activity or motor coordination as both behaviors are intact in response to a single systemic or subchronic administration of the drug (Blundell *et al.* 2008; Cleary *et al.* 2008; Neasta *et al.* 2010; Hadamitzky *et al.* 2018).

We previously reported that alcohol-related memories could be reactivated after exposure to sensory properties of alcohol itself (odor and taste), which specifically activates mTORC1 in select amygdalar and cortical regions in rats tested in operant conditioning paradigms (Barak *et al.* 2013). Furthermore, we (Barak *et al.* 2013) and others (Lin *et al.* 2014) demonstrated that rapamycin administration immediately following memory reactivation disrupts the alcohol consolidated memories. Interestingly, the retrieval of alcohol-associated memories during the reconsolidation window is not driven by mTORC1 pathway in the NAc (Barak *et al.* 2013). There are a few but critical differences in the methods used herein and in Barak *et al.* (2013). The main one is that Barak *et al.* assessed the effects of cues/context-induced recall of alcohol-associated memories in rats that underwent long-term exposure to alcohol in operant self-administration task whereas the mice in the current study were not exposed to alcohol prior to the acquisition of alcohol place preference phase. Moreover, unlike the CPP paradigm, the operant self-administration is an appetitive learning task where animals escalate their voluntary alcohol intake driven by progressive increase of the motivation for the drug. This possibility is consistent with several lines of evidence suggesting that neurobiological mechanisms underlying CPP and self-administration are distinct (Bardo & Bevins 2000). Another difference between the two studies is the choice of reactivation parameters [pharmacologically priming herein versus context and odor-taste cue in (Barak *et al.* 2013)]. Therefore, although the activation of mTORC1 in specific brain regions probably depends on the length of alcohol exposure and the behavioral paradigm, it is clear that the mTORC1 in the mesocorticolimbic system is critical for relapse of alcohol-seeking behaviors.

We previously showed that long-term excessive alcohol consumption induces the translation of GluA1, Arc, CamKII, PSD-95, CRMP2, Prosapip1 and Homer in the NAc (Liu *et al.* 2017; Laguesse *et al.* 2017b) and that a single alcohol drinking session initiates the translation of Homer and GluA1 but not PSD-95 (Beckley *et al.* 2016). Here, we report that the reinstatement of alcohol CPP is associated with increased protein levels CRMP2 (Liu *et al.* 2017) but not Homer, PSD-95, GluA1, Arc and Prosapip1 in the NAc. These data suggest that although mTORC1 is activated in the NAc in response to long-term excessive drinking of alcohol as well as a single administration of a subthreshold dose of alcohol, the molecular transducers of mTORC1 are not the same in both situations.

CRMP2 is a microtubule-binding protein that regulates microtubule assembly (Ip *et al.* 2014; Quach *et al.* 2015; Nagai *et al.* 2017). We previously reported that excessive alcohol consumption promotes the mTORC1-dependent translation of CRMP2 in the NAc, resulting in increased microtubule assembly (Liu *et al.* 2017). We further showed that downregulation of CRMP2 levels greatly reduced alcohol consumption and that disrupting CRMP2 function using lacosamide also reduced alcohol drinking (Liu *et al.* 2017). Although we herein measured the protein levels rather than the direct translation of CRMP2 mRNA, it is likely that the increase in the protein levels of CRMP2 by a priming dose of alcohol is the result of the mTORC1-dependent translation of the mRNA. Microtubules infiltrations into dendritic

spines have been correlated with spine enlargement and synaptic strengthening, which are considered to be the basis of memory formation (Shirao & Gonzalez-Billault 2013; Lamprecht 2014). Interestingly, an increased dendritic spine diameter in the Nac core was found in response to context-induced reinstatement of drug seeking (Stankeviciute *et al.* 2014). It is therefore tempting to speculate that reinstatement of alcohol-seeking is mediated by CRMP2-dependent structural plasticity in the NAC.

We show that systemic administration of the CRMP2 inhibitor, lacosamide, attenuates alcohol priming-induced reinstatement of CPP. This effect is not related to a nonspecific effect of lacosamide because spontaneous locomotion [Fig. 6c and (Liu *et al.* 2017)] and basal anxiety-like behavior (Liu *et al.* 2017) are preserved in animals treated with this drug. Thus, our study also suggests that lacosamide initially developed for the treatment of epilepsy (Stohr *et al.* 2007) could represent a promising new strategy for the treatment of relapse of alcohol-seeking behavior. It is important to note however, that lacosamide was shown to block neuronal firing by interfering with sodium channel slow inactivation processes (Rogawski *et al.* 2015). Thus, we cannot exclude the possibility that lacosamide abolishes alcohol reinstatement, at least in part, through CRMP2-independent mechanisms.

In summary, our work shows that reactivation of alcohol-associated memories using alcohol priming in a CPP task recruits the mTORC1/CRMP2 signaling and that pharmacological inhibition of this pathway disrupts priming-induced reinstatement of alcohol place preference. Our results provide a molecular mechanism of how re-exposure to alcohol after abstinence leads to reactivation of alcohol-related memories. Importantly, our study also provides new evidence to suggest that targeting mTORC1 signaling could be an effective relapse-prevention strategy in alcoholics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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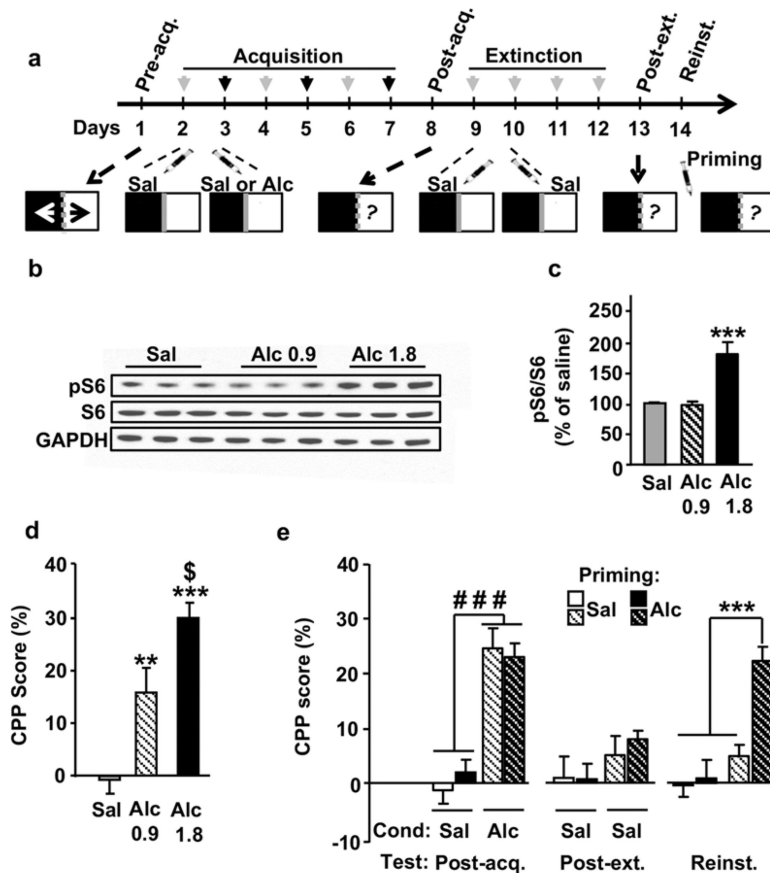


Figure 1. Alcohol priming induces reinstatement of alcohol place preference. (a) **Experimental timeline depicting the acquisition, extinction and reinstatement of alcohol-induced CPP.** (b–c) Mice were systemically administered with saline or alcohol (0.9 or 1.8 g/kg). One hour after the i.p. injection, the NAc were dissected and phosphorylation levels of S6 were determined by western blot analysis. ImageJ was used for optical density quantification. Data are expressed as the mean ratio \pm SEM of phospho-S6 to S6 and are expressed as percentage of the saline control. (d) CPP score on the *post-acquisition* test. During the conditioning phase (6 d), Mice were administered (i.p.) by alcohol (0.9 or 1.8 g/kg) or saline solution and were then confined in the drug-paired or non-drug-paired compartment. One day after the sixth session, the post-acquisition test was conducted for 15 minutes. (e) CPP score of the *post-acquisition* (Post-acq.), *post-extinction* (Post-ext.) and *reinstatement* (Reinst.) tests. In each test day, mice were placed in the central neutral area and allowed to explore both compartments of the apparatus for 15 minutes. In the *reinstatement* test day, mice previously conditioned with saline- (Sal) or alcohol- (Alc, 1.8 g/kg) received a priming injection of alcohol (0.9 g/kg, i.p.) or saline immediately prior to the beginning of the test session. Data are represented as mean percentage \pm SEM of time spent in the drug-paired compartment during the post-acquisition, post-extinction and reinstatement tests minus time spent in the same compartment during the pre-acquisition session. (b–c) $n = 5$, (d) $n = 10–11$, (e) $n = 13–18$. (c) *** $P < 0.001$ versus all other groups. (d) ** $P < 0.01$ and *** $P < 0.001$ versus saline-conditioned mice; \$ $P < 0.05$ Alc 0.9 versus Alc 1.8. (e) ### $P < 0.001$ versus

saline-conditioned mice; *** $P < 0.001$ versus all other groups. CPP, conditioned place preference

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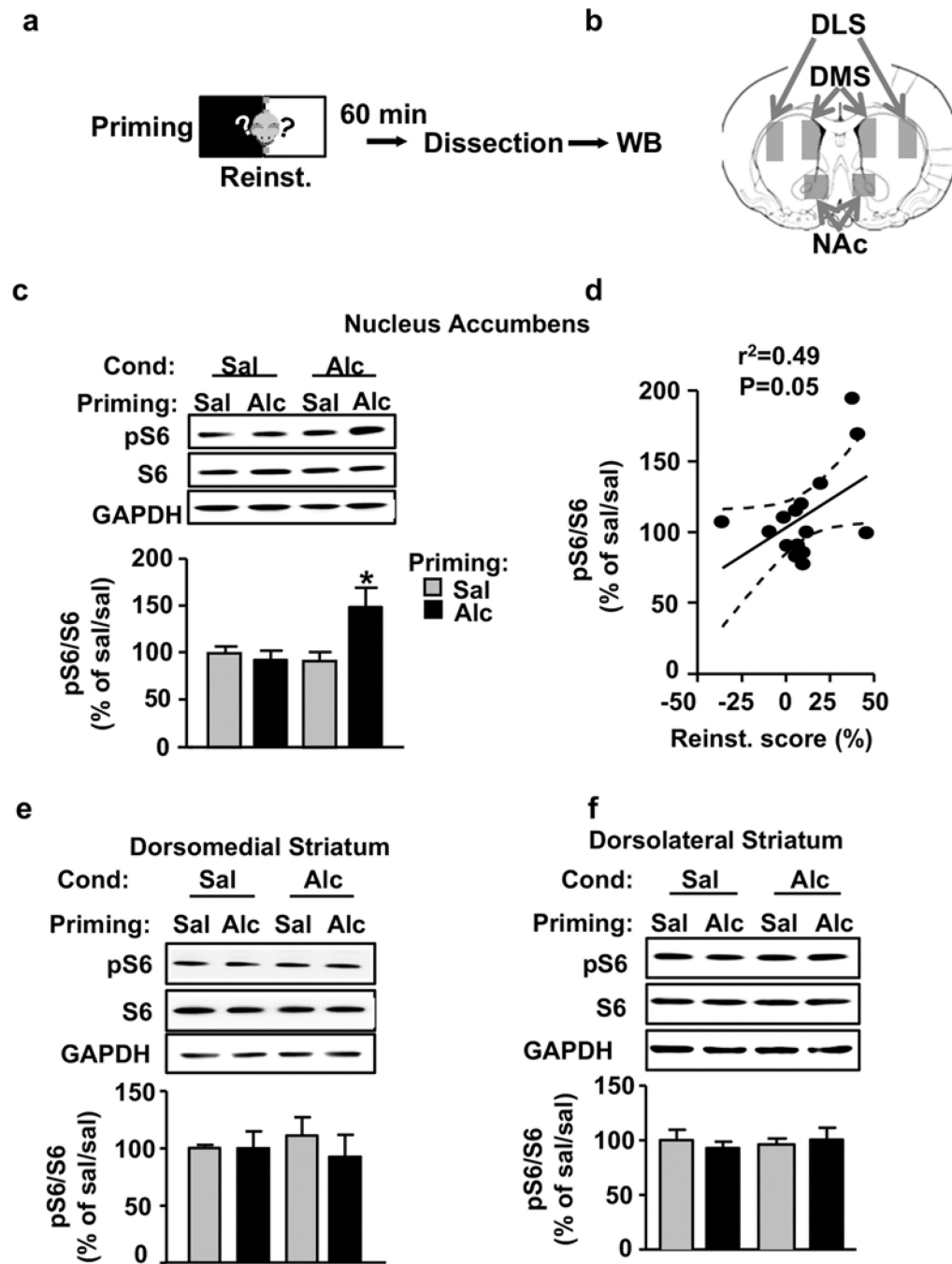


Figure 2. mTORC1 is activated in the nucleus accumbens during reinstatement of alcohol place preference.

(a) Mice underwent acquisition, extinction and reinstatement of alcohol place preference as depicted in Figure 1. DMS, DLS and NAc of mice were dissected 60 minutes after the end of the *reinstatement test*. (b) Schematic drawing of a coronal section of the mouse brain showing the sectioned DMS, DLS and NAc at bregma DV = +1.10/+0.70. (c–f) Phosphorylation level of S6 by saline- (Sal) or alcohol- (Alc) in conditioned and primed mice was determined by western blot analysis in the NAc (c), DMS (e) and DLS (f). ImageJ was used for optical density quantification. Data are expressed as the mean ratio \pm SEM of

phospho-S6 to S6 and are expressed as percentage of the control sal/sal group. (d) Scatter plot showing the relationship between CPP score on the *reinstatement test* and phospho-S6 in the NAc. Centerline is the linear regression and dashed lines are the 95% confidence interval. $n = 4$, $*P < 0.05$ versus all other groups. CPP, conditioned place preference; DLS, dorsolateral striatum; DMS, dorsomedial striatum; mTORC1, mammalian target of rapamycin complex 1; NAc, nucleus accumbens

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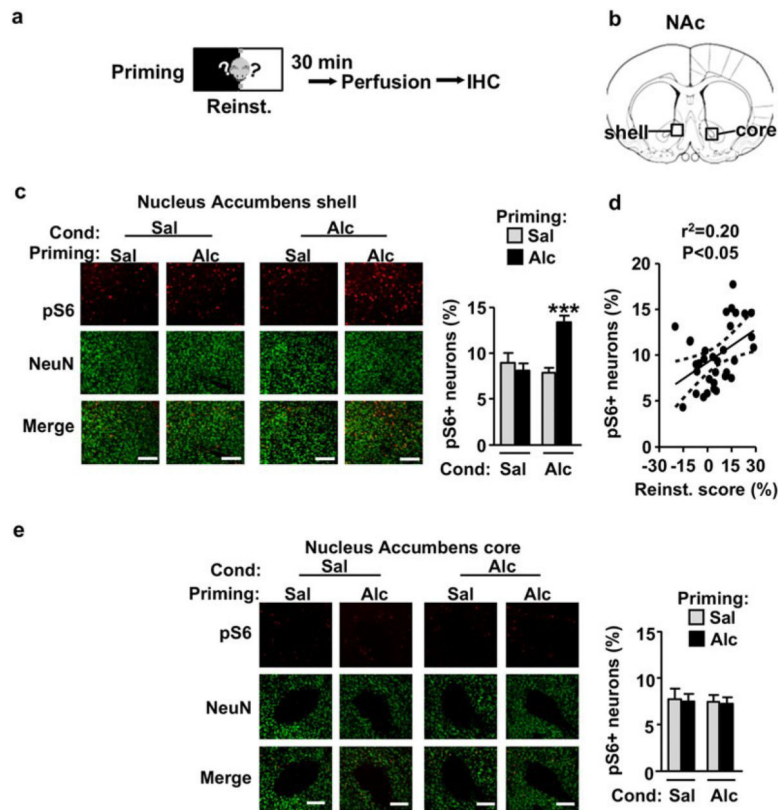


Figure 3. mTORC1 activation following priming-induced reinstatement of alcohol-CPP is restricted to the NAc shell.

(a) Mice underwent acquisition, extinction and reinstatement of alcohol place preference as depicted in Figure 1. Mice were euthanized 30 minutes after the end of the *reinstatement test*. (b) Schematic drawing of a coronal section of the mouse brain showing the shell and core portions of the NAc. (c–e) Phospho-S6 levels in the NAc shell (c) and core (e) (bregma DV = +1.10/+0.70) by saline- (Sal) or alcohol- (Alc) conditioned and primed mice were determined by immunohistochemistry. *Left panels*, Representative images of mouse NAc shell (c) and core (e) labeled with phospho-S6 in red and NeuN in green. Scale bar 100 μ m. *Right panels*, Phospho-S6 labeled neurons are expressed as percentage of NeuN positive cells. (d) Scatter plot showing the relationship between CPP score on the reinstatement test and phospho-S6+ neurons in the NAc shell. Centerline is the linear regression and dashed lines are the 95% confidence interval. Sal/Sal $n = 7$, Sal/Alc $n = 9$, Alc/Sal $n = 7$, Alc/Alc $n = 10$. *** $P < 0.001$ versus all other groups. CPP, conditioned place preference; mTORC1, mammalian target of rapamycin complex 1; NAc, nucleus accumbens

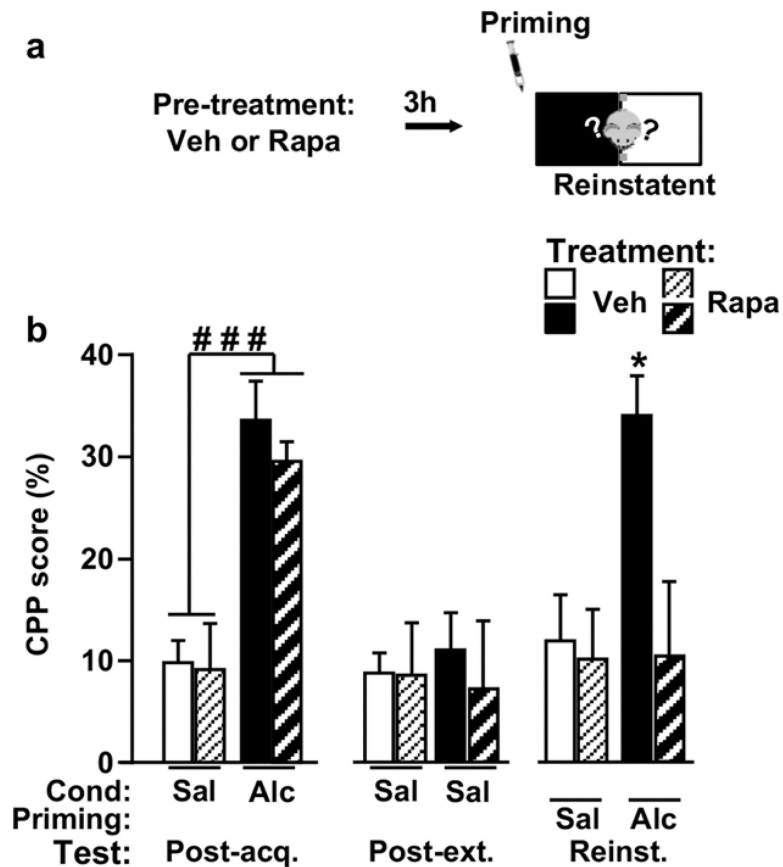


Figure 4. Systemic administration with the mTORC1 inhibitor, rapamycin, blocks priming-induced reinstatement of alcohol place preference.

(a) Mice underwent acquisition and extinction of alcohol place preference as depicted in Figure 1. On day 14, mice were pre-treated with vehicle (Veh) or rapamycin (Rapa, 10 mg/kg, i.p.). Three hours later, mice received a priming injection of saline or alcohol (0.9 g/kg, i.p.) and underwent the *reinstatement test*. (b) CPP scores on the *post-acquisition* (Post-acq.), *post-extinction* (Post-ext.) and *reinstatement* (Reinst.) tests. Data are represented as mean percentage \pm SEM of time spent in the drug-paired compartment during the tests minus time spent in the same compartment on the pre-acquisition session. Veh/Sal $n = 13$, Rapa/Sal $n = 14$, Veh/Alc $n = 13$, Rapa/Alc $n = 13$. $###P < 0.001$ versus saline-conditioned mice and $*P < 0.05$ versus all other groups. CPP, conditioned place preference; mTORC1, mammalian target of rapamycin complex 1

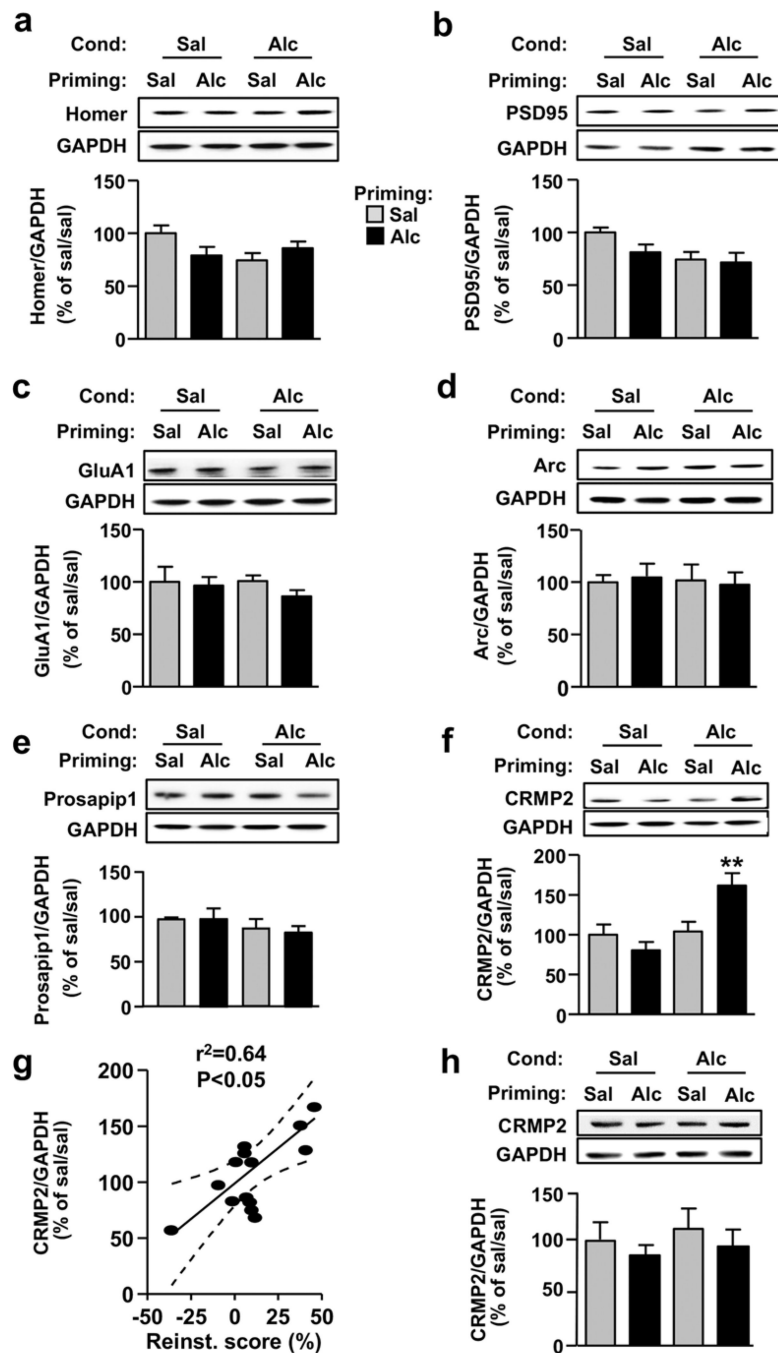


Figure 5. CRMP2 levels are increased in the NAC following priming-induced reinstatement of alcohol place preference. Homer (a), PSD95 (b), GluA1 (c), Arc (d), Prosapip1 (e) and CRMP2 (f) levels in the NAC of saline- (Sal) or alcohol- (Alc) conditioned and primed mice was determined by western blot analysis. NAc samples are identical to the tissue samples used in Figure 2c–d. (g) Scatter plot showing the relationship between CPP score on the reinstatement test and CRMP2 levels in the NAc. Centerline is the linear regression and dashed lines are the 95% confidence interval. (h) CRMP2 levels in the DMS were determined by western blot analysis. ImageJ was used for optical density quantification. Data are represented as the mean ratio \pm

SEM of protein to GAPDH and are expressed as percentage of the control sal/sal group. $n = 4$, $**P < 0.01$ versus all other groups. CPP, conditioned place preference; CRMP2, collapsin response mediator protein-2; DMS, dorsomedial striatum; NAc, nucleus accumbens

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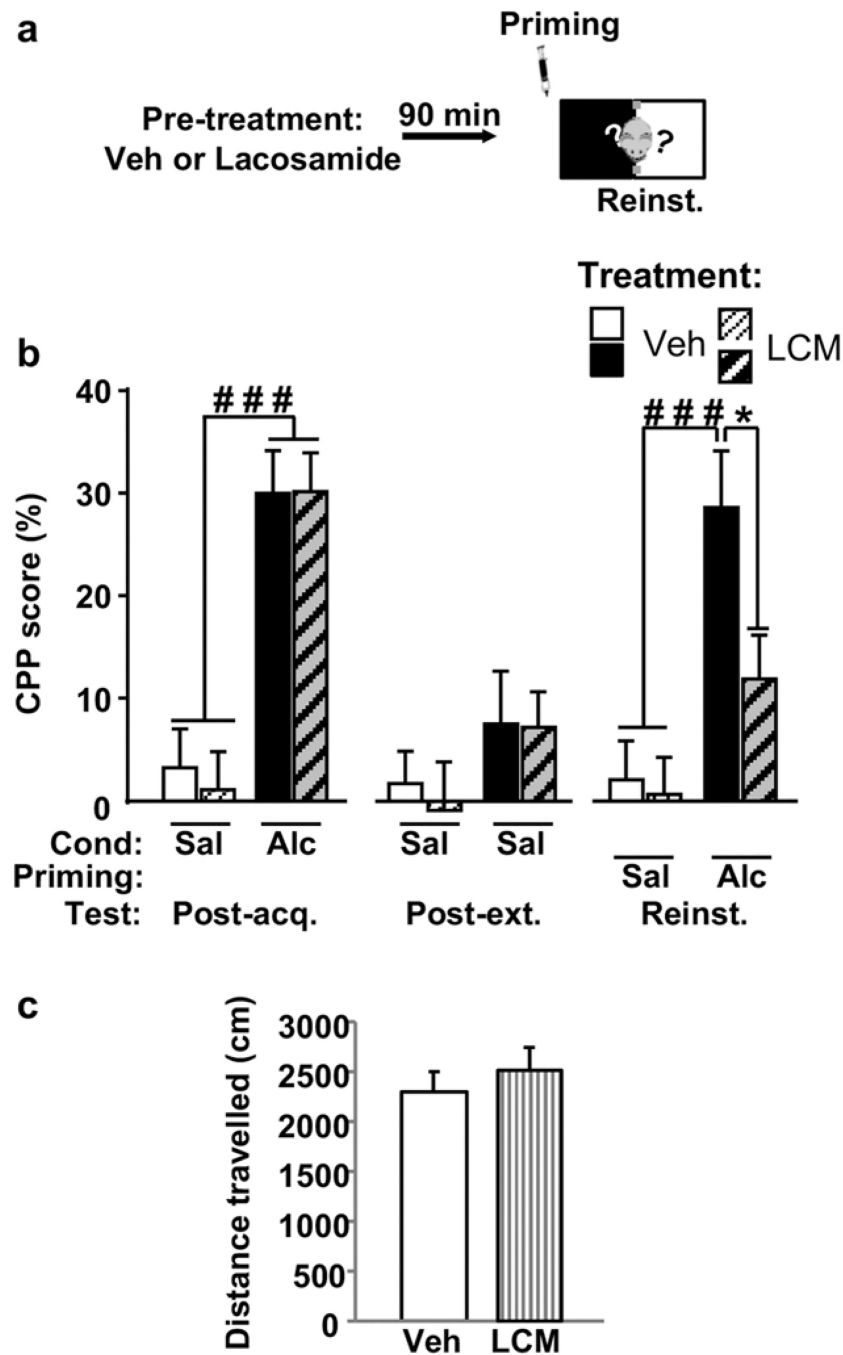


Figure 6. Systemic administration of the CRMP2 inhibitor lacosamide prevents reinstatement of alcohol place preference and does not affect spontaneous locomotor activity.

(a) Mice underwent acquisition and extinction of alcohol place preference as depicted in Figure 1. On day 14, mice were pre-treated with vehicle (Veh) or lacosamide (LCM, 10 mg/kg, i.p.). Ninety minutes later, mice received a priming injection of saline or alcohol (0.9 g/kg, i.p.) and underwent the *reinstatement test*. (b) CPP score on the *post-acquisition* (Post-acq.), *post-extinction* (Post-ext.) and *reinstatement* (Reinst.) tests. Data are represented as mean percentage \pm SEM of time spent in the drug-paired compartment during the tests minus time spent in the same compartment on the pre-acquisition session. (c) Mice were

injected with either Veh or LCM (10 mg/kg, i.p.) 90 minutes prior to the beginning of the locomotion test. Data are represented as mean percentage \pm SEM of spontaneous locomotor activity measured during 30 minutes. (b) Veh/Sal $n = 11$, LCM/Sal $n = 10$, Veh/Alc $n = 12$, LCM/Alc $n = 14$, (c) $n = 5$. (b) $###P < 0.001$ versus saline- conditioned mice and $*P < 0.05$ versus Veh/Alc group. CPP, conditioned place preference

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