mTORC1 and its downstream effector CRMP2 drive reinstatement of alcohol reward seeking

Sami Ben Hamida[1#], Sophie Laguesse[1#], Nadege Morisot[1#], Anthony L Berger[1], Jong-Hyun Park[2,3], Khanhky Phuamluong[1], Ki Duk Park[2,3], Dorit Ron[1]*

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

[2]Convergence Research Center for Diagnosis, Treatment and Care System of Dementia, Korea Institute of Science and Technology (KIST), Seoul 02792, Republic of Korea.

[3]Division of Bio-Medical Science &Technology, KIST School, Korea University of Science and Technology, Seoul 02792, Republic of Korea.

[#]Present address: SBH: Douglas Research Center, McGill University, Montreal, Canada

[#]Present address: SL: GIGA-Neurosciences, University of Liege, Belgium

[#]Present address: NM: Brains On-line LLC, South San Francisco, CA, United States

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

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Abstract

Alcohol use disorder (AUD) 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 (NAc) 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 NAc. 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 FDA approved drugs, rapamycin and lacosamide, for the treatment of AUD.

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., 2017; Heilig and Egli, 2006)). One of the most troubling aspects of alcohol use disorder (AUD) is the relapse that may occur after several years of abstinence (Moos and Moos, 2006). Thus, the high rate of relapse characterizing AUD represents the major challenge for the treatment of the disorder. Hence, a better understanding of the mechanisms underlying relapse is of great merit and one strategy to prevent relapses in alcohol addicts could be the attenuation of reward-related memories

Drugs of abuse are thought to usurp the mechanisms underlying learning and memory processes (Russo et al., 2010; Torregrossa et al., 2011). The mammalian target of rapamycin complex 1 (mTORC1) is a multiprotein complex centered on the serine and threonine kinase mTOR required for numerous forms of long-lasting synaptic plasticity, learning, and memory (Lipton and Sahin, 2014). mTORC1 phosphorylates the p70 ribosomal S6 Kinase (S6K), which in turn phosphorylates the ribosomal protein S6, as well as initiation factor 4E binding protein (4E-BP) (Lipton and 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 and Sabatini, 2017). In the central nervous system (CNS), the 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 and Sahin). Accumulating evidence reveals 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 (Barak et al., 2013; Beckley et al., 2016; Laguesse et al., 2017a; Liu et al., 2017; Neasta et al., 2010). Specifically, we previously reported that excessive alcohol consumption activates mTORC1 in the nucleus accumbens (NAc) of rodents (Beckley et al., 2016; Laguesse et al., 2017a; Neasta et al., 2010), 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 (Laguesse et al., 2017b; Liu et al., 2017). Finally, we reported that retrieval of memories associated with an alcohol experience increases mTORC1 activity in cortical and amygdalar regions, and that systemic or intra-CeA mTORC1 inhibition disrupts the reconsolidation of alcohol-related memories (Barak et al., 2013). 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 stress (Sanchis-Segura and 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, to identify the molecular mechanisms that underlie the behavior.

Materials and Methods

Detailed information regarding reagents and preparation of solutions can be found in Supporting Information.

Subjects

Male DBA/2J mice (Jackson Laboratory) were 8 weeks old at the beginning of the experiment and group-housed (4/cages) in a temperature- and humidity-controlled colony room (22±2 °C, relative humidity: 50–60%) under normal 12-h 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 (IACUC) and were conducted in agreement with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

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 the Supporting Information. 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 min (lacosamide experiment) later, saline- 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 min. The timing between pre-treatment and reinstatement testing was chosen based on previous studies (Liu et al., 2017; Neasta et al., 2010).

Effect of lacosamide on spontaneous locomotor activity

New Cohort of animals were used to assess the effect of lacosamide on spontaneous locomotor activity. The experiment was conducted in the CPP apparatus and was detected by infrared photobeams. Lacosamide (10 mg/kg, i.p.) or vehicle was administered intraperitoneally 90 min prior placing mouse in the CPP apparatus. Spontaneous locomotor activities were monitored for 30 min.

Western blot analysis

Western blot analysis was conducted as described in (Laguesse et al., 2017a). Further information can be found in the Supporting Information.

Immunohistochemistry

Immunohistochemistry was conducted as described previously (Ben Hamida et al., 2012). Further information can be found in the Supporting Information.

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. The accepted value for significance is $p<0.05$.

Results

Alcohol priming 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 Fig. 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 g/kg 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 to the group conditioned with saline ($p<0.01$)), however, the rewarding effects

of (0.9 g/kg) dose of alcohol was significantly lower than those induced by (1.8 g/kg) dose (p<0.05)). Importantly, acute administration of (1.8 g/kg) of alcohol, but not the priming dose (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 Fig. 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 vs. 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 to saline-treated animals (p's<0.0001). During the *Extinction phase,* the acquired alcohol CPP was extinguished throughout four days with saline injection prior to counterbalanced confinement to the unpaired and alcohol-paired compartment. As shown in Fig. 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 Fig. 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 min 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 mTORC1 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 (Beckley et al., 2016; Laguesse et al., 2017a; Neasta et al., 2010). 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 Fig. 1a. One hour following the end of the reinstatement test (Fig. 2a), mTORC1 activity in the NAc, DLS and DMS (Fig. 2b) were determined by analyzing the phosphorylation levels of S6 (Buffington et al., 2014). As shown in Fig. 2c, one hour after reinstatement, mTORC1 activity was significantly increased in the NAc of Alc/Alc mice compared to 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, r2 = 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; 2-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 (Corbit et al., 2016; Janak and Chaudhri, 2010). 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 Fig. 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 to 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 mTORC1 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 Fig. 1a. Next, on the reinstatement test day, animals received an i.p. administration of rapamycin three 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 min (Fig. 4a). As expected, a robust CPP was observed in animals conditioned with alcohol compared to those treated with saline. Specifically, as shown in Fig. 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 to saline-treated animals (p's <0.001). The acquired alcohol CPP was then extinguished during the extinction phase. As shown in Fig. 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 Fig. 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 the systemic administration of rapamycin efficiently prevents the reinstatement of alcohol seeking induced by priming injections of alcohol.

Alcohol priming-induced reinstatement of alcohol place preference increases CRMP2 levels in the NAc

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 and 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 (Laguesse et al., 2017b; Liu et al., 2017 Beckley, 2016 #102; Neasta et al., 2010). 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; $p_{Conditioning}$ =0.23, $p_{Priming}$ = 0.53 and $p_{interaction}$=0.044), PSD-95

(Fig. 5b; $p_{Conditioning}$ = 0.03, $p_{Priming}$ = 0.16 and $p_{interaction=0.29}$), GluA1 (Fig. 5c; $pc_{onditioning}$ = 0.63, $p_{Priming}$ = 0.34 and $p_{interaction}$=0.56), Arc (Fig. 5d; $p_{Conditioning}$ = 0.87, $p_{Priming}$ = 0.98 and $p_{interaction=}$0.78) and Prosapip1 (Fig. 5e; $p_{Conditioning}$ = 0.14, $p_{Priming}$ = 0.81 and $p_{interaction}$=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 Fig. 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; $p_{Conditioning}$ = 0.29, $p_{Priming}$ = 0.57 and $p_{interaction=0.83}$). 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

CRMP2 is a microtubule-binding protein implicated in the regulation of dendritic morphology (Ip et al., 2014; Quach et al., 2015). Abnormal CRMP2 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 knock-down of CRMP2 in the NAc decrease excessive alcohol consumption 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 Fig. 1a, and on the *reinstatement test* day, animals were i.p. administered with saline or lacosamide (Beyreuther et al., 2007), a specific inhibitor of CRMP2 that prevents CRMP2-induced microtubule assembly (Wilson and Khanna, 2015; Wilson et al., 2012). Ninety minutes later, animals were injected with a priming dose of alcohol or saline (Fig. 6a) and tested for alcohol reinstatement. As shown in Fig. 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 to saline-treated animals (p's p<0.001). The acquired alcohol CPP was then extinguished during the extinction phase. As shown in Fig. 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 to 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 Fig. 6c, systemic administration

of 10 mg/kg of lacosamide did not alter mice locomotion ($t_{(8)}$=0.69, p=0.51) . 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 and 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 (Bossert et al., 2013; Epstein et al., 2006). Here, in order to examine the involvement of mTORC1 in the relapse of alcohol seeking, we used a CPP paradigm widely used to explore the association between the rewarding effects of a drug and specific environmental cues (Napier et al., 2013; Tzschentke, 2007). We confirmed previous results (Al-Hasani et al., 2013; Bhutada et al., 2012; Roger-Sanchez et al., 2012) 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 and Barak, 2016) including the NAc (Salgado and Kaplitt, 2015). We found that mTORC1 is activated in the NAc but not in the other striatal regions, the DLS and DMS in response to reinstatement of alcohol seeking induced by priming administration of alcohol. 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 core of mice following alcohol priming-induced reinstatement of alcohol CPP. Together, data indicate that alcohol exposure induces distinct neuroadaptations in different NAc 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 and Barak, 2016). These findings are also in line with previous studies showing that the NAc shell is critical for cue- (Richard and Fields, 2016) and context- (Perry and 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. 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 an 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 did not have prior exposure 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 and Bevins, 2000). Another difference between these studies is the choice of reactivation parameters (pharmacologically priming herein vs. 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 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 (Laguesse et al., 2017b; Liu et al., 2017), and that a single alcohol drinking session activates 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; Nagai et al., 2017; Quach et al., 2015), and 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 (Liu et al., 2017). Moreover, we showed 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 a 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 (Lamprecht, 2014; Shirao and Gonzalez-Billault, 2013). Interestingly, an increased dendritic spine diameter 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. 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 also note that lacosamide blockade repetitive firing of neuronal cells by interfering with sodium channel slow inactivation processes (Rogawski et al., 2015). Thus, we cannot exclude the possibility that this drug abolishes alcohol reinstatement, at least in part, through CRMP-2-independent mechanisms.*

Commentaire [s.1]: Blocks?

In summary, our work shows that reactivation of alcohol-associated memory using alcohol priming in a CPP task recruits the mTORC1/CRMP2 signaling and that pharmacological 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.

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Figures legends

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, 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 ± 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), DBA/2J mice were administered (i.p.) by alcohol (0.9 or 1.8 g/kg) or saline solution and were then confined in the drug- or non-drug-paired compartment. One day after the sixth session, the post-acquisition test was conducted for 15 min. (**e**) CPP score on the *post-acquisition* (Post-acq.), *post-extinction* (Post-ext.) and *reinstatement* (Reinst.) tests. For each test day, mice were placed in the central neutral area and allowed to explore both compartments of the apparatus for 15 min. In the *reinstatement test* day, mice previously conditioned with saline- (Sal) or alcohol- (Alc, 1.8g/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 ±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 vs. all other groups. (**d**) **p<0.01 and ***p<0.001 vs. saline-conditioned mice; $p<0.05 Alc 0.9 vs Alc 1.8. (**e**) ###p<0.001 vs. saline-conditioned mice; ***p<0.001 vs. all other groups.

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 Fig. 1. DLS, DMS and NAc of mice were dissected 60 min after the end of the *reinstatement test*. (**b**) Schematic drawing of a coronal section of the mouse brain showing the sectioned DLS, DMS 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**), DLS (**e**) and DLS (**f**). ImageJ was used for optical density quantification. Data are expressed as the mean ratio ± 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 vs. all other groups.

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 Fig. 1. Mice were euthanized 30 min 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 100µm. *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 vs. all other groups.

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 Fig. 1. On day 14, mice were pre-treated with vehicle (Veh) or rapamycin (Rapa, 10mg/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 ± 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 vs. saline-conditioned mice and *p<0.05 vs. all other groups.

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 Fig. 2 **c-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 ± SEM of protein to GAPDH and are expressed as percentage of the control sal/sal group. (**a-g**) n=4, **p<0.01 vs. all other groups.

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 Fig.1. On day 14, mice were pre-treated with vehicle (Veh) or lacosamide (LCM, 10 mg/kg, i.p.). Ninety min 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 ± 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 min prior to the beginning of the locomotion test. Data are represented as mean percentage ± SEM of spontaneous locomotor activity measured during 30 min. (**b**) Veh/Sal n=11, LCM/Sal n=10, Veh/Alc n=12, LCM/Alc n=14, (**c**) n=5. (**b**) $^{###}$p<0.001 vs. saline-conditioned mice and *p<0.05 vs. Veh/Alc group.