mTORC1-dependent Translation of Collapsin Response Mediator Protein-2 Drives Neuroadaptations Underlying Excessive Alcohol Drinking Behaviors

Feng Liu, Rémi Legastelois[#], Sami Ben Hamida[#], and Dorit Ron *

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

Present address:

Rémi Legastelois, INSERM ERi24, Université de Picardie, Amiens, France.

Sami Ben Hamida, Département de Médecine Translationelle et Neurogénétique, Institut de Génétique et de Biologie Moléculaire et Cellulaire, INSERM U-964, CNRS UMR-7104, Université de Strasbourg, Illkirch, France.

* Correspondence should be addressed to Dorit Ron, 675 Nelson Rising Lane, BOX 0663, San Francisco, CA 94143, USA. E-mail: dorit.ron@ucsf.edu

Running title: mTORC1-mediated translation of CRMP-2 and alcohol

30 pages, 5 Figures, 11 Supplementary Figures, Abstract: 152 words, Introduction: 422 words, Article: 3499 words, 64 References.

ABSTRACT

Mammalian target of rapamycin complex 1 (mTORC1) plays an essential role dendritic mRNA translation and participates in mechanisms underlying alcohol drinking and reconsolidation of alcohol-related memories. Here, we report that excessive alcohol consumption increases the translation of downstream targets of mTORC1, including *collapsin response mediator protein-2* (*CRMP-2*), in the nucleus accumbens (NAc) of rodents. We show that alcohol-mediated induction of *CRMP-2* translation is mTORC1-dependent, leading to increased CRMP-2 protein levels. Furthermore, we demonstrate that alcohol intake also blocks glycogen synthase kinase-3β (GSK-3β)-phosphorylation of CRMP-2, which results in elevated binding of CRMP-2 to microtubules and a concomitant increase in microtubules content. Finally, we show that systemic administration of the CRMP-2 inhibitor Lacosamide, or knockdown of *CRMP-2* in the NAc decreases excessive alcohol intake. These results suggest that CRMP-2 in the NAc is a convergent point that receives inputs from two signaling pathways, mTORC1 and GSK-3β, that in turn drives excessive alcohol drinking behaviors.

INTRODUCTION

Addiction is a chronically relapsing disorder, which pathologically usurps mechanisms underlying learning and memory [1, 2]. Long-lasting synaptic plasticity, which underlies the formation of long-term memories, requires new protein synthesis at dendrites [3]. Mammalian target of rapamycin complex 1 (mTORC1) is a complex containing the serine/threonine protein kinase mTOR, and the adaptor protein regulatory-associated protein of mTOR (RAPTOR) [4], which plays an important role in initiating the local translation of synaptic proteins [5, 6]. Specifically, activation of mTORC1 leads to the phosphorylation of its two main downstream effectors, eukaryotic initiation factor 4E binding protein (4E-BP) and ribosomal protein S6 kinase (S6K) [7, 8]. Phosphorylation of 4E-BP and S6K by mTORC1 results in the activation of eukaryotic initiation factor complex, which promotes cap-dependent mRNA translation [9, 10].

Recently, mTORC1 has been shown to contribute to neuroadaptations in several drugs of abuse, including alcohol [11]. Specifically for alcohol, we previously found that excessive alcohol intake leads to the activation of mTORC1 in the nucleus accumbens (NAc) of rodents, and that systemic administration or intra-NAc infusion of the mTORC1 inhibitor, rapamycin [12], reduced excessive alcohol drinking and seeking [13]. More recently we found that mTORC1 participates in the maintenance of alcohol-related memories [14]. As mTORC1 plays an essential role in learning and memory via the translation of mRNAs at dendrites [5, 6], and since psychoactive drugs including alcohol pathologically utilize normal mechanisms underlying learning and memory to cause addiction [2], we hypothesized that mTORC1 mediates neuroadaptations underlying excessive alcohol-drinking behaviors by promoting the translation of synaptic proteins. To test the hypothesis, we selected 8 candidate mRNAs whose translation has been reported to be controlled by mTORC1 [15-19], and whose gene products contribute to plasticity, learning and

memory in the adult brain. Specifically, we focused our studies on: Arc [20], Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [21], the GluA1 subunit of AMPA receptor (GluA1) [22], the GluN1 subunit of NMDA receptor (GluN1) [23], Homer [24], postsynaptic density protein-95 (PSD-95) [25], RACK1 [26], and collapsin response mediator protein-2 (CRMP-2) [27]. Using a rat model of excessive alcohol drinking, we tested the translation of these mRNAs in the NAc, a central component of the reward circuit that is critically involved in the etiology of addiction [2]. We show that a history of excessive alcohol drinking leads to the initiation of the translation of several synaptic proteins, including CRMP-2. We further show that CRMP-2 in the NAc plays a central role in microtubules assembly in response to alcohol and to development and persistence of neuroadaptations underlying excessive alcohol drinking behaviors.

MATERIALS AND METHODS

Information on reagents, materials, animals is detailed in Supplementary Information.

Preparation of solutions

Alcohol solution was prepared from ethyl alcohol solution (190 proof) diluted to 20% (*v/v*) in tap water. Rapamycin (10 mg/kg for rats and 20 mg/kg for mice) [13] was dissolved in DMSO and systemically administered in a volume of 2 ml/kg for both rats and mice. Lacosamide (LCM, 20 mg/kg for rats and 20 or 50 mg/kg for mice) [28] was dissolved in saline solution (0.9%), and systemically administered in a volume of 2 ml/kg for rats and 10 ml/kg for mice.

Collection of brain samples for biochemical analyses

Rats and mice underwent an intermittent access to 20% alcohol two-bottle choice (2BC) drinking paradigm for 2 months. Collection of the NAc was conducted as described previously [29]. See Supplementary Information for detailed procedures.

Polysomal fractionation

Polysome-bound RNA was purified from rat NAc according to a protocol described previously [30]. Fresh rat NAc was snap-frozen in a 1.5 ml Eppendorf tube and pulverized in liquid nitrogen with a pestle. After keeping on dry ice for 5 minutes, the powder of one NAc was resuspended in 1 ml lysis buffer (10 mM Tris pH8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP40, 0.5% sodium deoxycholate, 40 mM dithiothreitol, 400 U/ml Rnasin, 10 mM RVC and 200 µg/ml cycloheximide) followed by 20 times of pipetting to disrupt cells. Two hundred µl of the homogenate was kept as total fraction and subjected to total RNA extraction using TRIzol reagent. The rest of homogenate was centrifuged for 10 seconds at 12,000 g to remove intact nuclei. The supernatant was collected and the ribosomes were further released by adding 2X extraction buffer (200 mM Tris pH7.5, 300 mM NaCl and 200 µg/ml cycloheximide). Samples were kept on ice for 5 minutes and then centrifuged at 12,000 g, 4°C for 5 minutes to remove mitochondria and membranous debris. The resulting supernatant was loaded onto a 15%-45% sucrose gradient and centrifuged in a SW41Ti rotor (Beckman Coulter, Brea, CA) at 38,000 rpm, 4°C for 2 hours. Fractions from the sucrose gradient were collected and further digested with proteinase K (400 µg/ml proteinase K, 10 mM EDTA, 1% SDS) at 37°C for 30 minutes, followed by phenol-chloroform extraction. RNA in the water phase of the polysomal fraction was recovered by ethyl alcohol precipitation.

Reverse transcription-polymerase chain reaction (RT-PCR)

mRNA levels were measured by RT-PCR or quantitative PCR as described previously [31]. Primers information is in Supplementary Information.

Crude synaptosomal fraction and western blot analysis

Protocols for purifying synaptosomal fraction and subsequent western blot analysis are described previously [32]. Detailed procedures are described in Supplementary Information.

Microtubule binding assay and microtubule content assay

Microtubules-related assays were conducted by using microtubule binding protein spin-down assay biochem kit or microtubules/tubulin *in vivo* assay biochem kit from Cytoskeleton (Denver, CO), according to manufacturer's instructions. Detailed procedures are in Supplementary Information.

Generation and infusion of a lentivirus expressing shCRMP-2

The 19 nucleotides short hairpin RNA (shRNA) sequence 5'-GUA AAC UCC UUC CUC GUG U-3' targeting CRMP-2 (Ltv-shCRMP-2) was chosen according to a published sequence [33]. Synthesized DNA oligos containing the above sequence as well as stem loop were annealed and inserted into pLL3.7 vector at HpaI and XhoI sites. The non-related 19 nucleotides sequence 5'-GCG CUU AGC UGU AGG AUU C-3' (Ltv-shCT). The production of lentivirus is described in [34]. Intra-NAc infusion of lentivirus (2×10^7 pg/ml) was conducted as described in [29].

Immunohistochemistry

The protocol of immunohistochemistry for staining GFP in mouse brain sections is described previously [29]. Detailed procedures are described in Supplementary Information.

Intermittent access to 20% alcohol two-bottle choice paradigm

The intermittent-access to 20% alcohol two-bottle choice drinking procedure was conducted as previously described in rats [35] and mice [36].

Effect of Lacosamide on alcohol drinking, voluntary sucrose consumption, locomotion and anxiety-like behavior

These experiments were conducted by using a counterbalanced within-subject design using protocols described previously [29, 37]. See Supplemental Information for detailed procedures.

Voluntary consumption of alcohol and saccharin and spontaneous locomotion after lentiviral infusion

Three weeks after viral infusion, mice received intermittent access to 20% alcohol two-bottle choice paradigm for 3 consecutive weeks as described above. After one week of alcohol withdrawal, mice received two sessions of intermittent access to 0.033% saccharin two-bottle choice drinking paradigm. One day after the last saccharin drinking session, the spontaneous locomotor activity of mice was measured for 60 minutes in activity monitoring chambers (43 cm × 43 cm) with horizontal photo beams (Med Associates, St Albans, VT).

Data analysis

Depending on the experiment, data was analyzed with two-tailed paired or unpaired *t*-test, two-way analysis of variance (ANOVA) or one- or two-way repeated measures- (RM-) ANOVA. Significant main effects and interactions of the ANOVAs were further investigated with the Student-Newman-Keuls *post hoc* test or method of contrast analysis. Statistical significance was set at $p<0.05$.

RESULTS

Alcohol intake promotes the translation of *CRMP-2* mRNA via mTORC1

First, we tested whether excessive drinking of alcohol activates the translation of selected mRNAs whose translation is mTORC1-dependent and are involved in plasticity mechanisms. To do so, rats underwent 8 weeks of repeated cycles of binge drinking of alcohol and withdrawal [38], which leads to excessive amounts of alcohol consumption in rats (5.9 ± 0.4 g/kg/24 hours, n=10). Furthermore, repeated cycles of alcohol intake and abstinence leads to a binge alcohol drinking phenotype in which animals consume large quantities of alcohol in a short time (0.96 ± 0.1 g/kg/30 minutes, n=10) reaching blood alcohol concentration (BAC) that is above 80 mg% [38], which corresponds to BAC values of binge drinking in humans [39]. Rat NAc was dissected immediately after the last binge drinking session (Timeline, Supplementary Figure 1A), and polysomes that contain mRNA undergoing translation were purified (Supplementary Figure 2). As shown in Figure 1, the mRNA levels of *Arc, CaMKIIα, CRMP-2, GluA1*, *Homer2* and *PSD-95*, but not *GluN1* and *RACK1*, were significantly increased in the polysomal fraction of rats that consumed alcohol as compared to rats that drank only water (Figure 1a). The increase in

polysomal mRNA levels of *Arc, CaMKIIα, CRMP-2*, *Homer2* and *PSD-95* mRNAs, but not *GluA1*, were long-lasting, as they were still detected after 24 hours of abstinence (Figure 1b). Alcohol drinking did not produce an increase in the total RNA of these genes (Supplementary Figure 3), indicating that transcriptional activation did not occur in response to alcohol. The level of *TrkB* mRNA, whose translation is not known to be controlled by mTORC1, was also unaltered by alcohol (Figure 1 and Supplementary Figure 3).

mRNAs consisting of a cytidine immediately after the 5' cap, followed by a continuous stretch of 4-14 pyrimidines (5' terminal oligopyrimidine, TOP) are thought to be specifically regulated by mTORC1 [40]. Therefore, we used an *in silico* approach (RefSeq, http://www.ncbi.nlm.nih.gov/refseq/) to examine the transcriptional start site (TSS) of the mRNAs whose translation was activated by alcohol as shown in Figure 1. We found that only *CRMP-2* contains a TOP-like mRNA signature (Supplementary Figure 4); thus, we focused our studies on this gene. First, to confirm that the alcohol-mediated increase in *CRMP-2* translation is indeed mTORC1-dependent, rapamycin or vehicle was systemically administered, and polysomal RNA from rat NAc was isolated 24 hours after the last alcohol drinking session (Timeline, Supplementary Figure 1B). As shown in Figure 1c, the alcohol-mediated increase in polysomal *CRMP-2* mRNA was blocked by rapamycin. In contrast, the mRNA levels of *RACK1* in polysomes were unaltered in the presence or absence of rapamycin (Figure 1d). In addition, alcohol intake did not change the total mRNA levels of either *CRMP-2* or *RACK1* with or without rapamycin treatment (Supplementary Figure 5). Together, these results indicate that alcohol activates the translation of *CRMP-2*, a specific TOP-like mRNA, in an mTORC1-dependent manner.

Binge drinking of alcohol and withdrawal increase CRMP-2 protein levels

Next, we tested whether the alcohol-mediated increase in polysomal *CRMP-2* mRNA corresponds with an upregulation of CRMP-2 protein. As CRMP-2 is located in both soma and synaptic structures [33, 41, 42], we measured the immunoreactivity of the protein in total homogenate as well as in crude synaptosomal fraction of rat NAc. We found that the protein levels of CRMP-2 in total homogenate and synaptic fraction were significantly increased in response to binge drinking of alcohol (Figure 2a-b). In addition, CRMP-2 levels remained elevated after 24 hours of withdrawal (Figure 2c-d). Similarly, alcohol drinking led to an mTORC1-dependent increase in CRMP-2 protein levels in mouse NAc (Supplementary Figure 6). Together, these results suggest that excessive alcohol intake produces an mRNA to protein translation of CRMP-2 in the NAc of both rats and mice.

Excessive drinking of alcohol blocks the phosphorylation of CRMP-2 and increases microtubule content

CRMP-2 is a substrate of glycogen synthase kinase-3β (GSK-3β) [42]. GSK-3β activity is regulated by the serine and threonine kinase AKT [43]. Specifically, AKT phosphorylates GSK-3β at Serine 9, which in turn inactivates the kinase [43]. Excessive alcohol intake activates AKT in the NAc of rodents [44]. Therefore, we hypothesized that the consequences of alcohol-mediated AKT activation are increased phosphorylation of GSK-3β and a subsequent blockade of CRMP-2 phosphorylation at Threonine 514, a site that is specifically targeted by GSK-3β [42]. As shown in Figure 3a-b, the phosphorylation of GSK-3β was significantly elevated in rat NAc after binge drinking of alcohol; and importantly, CRMP-2 phosphorylation was significantly decreased in

total homogenate and in synaptic fraction after a session of excessive alcohol intake (Figure 3c-d) and withdrawal (Supplementary Figure 7).

Phosphorylation of CRMP-2 inhibits its binding to microtubules [27, 45]. Therefore, we tested whether excessive alcohol consumption alters the binding of CRMP-2 to microtubules. To do so, rat NAc was harvested 24 hours after the last drinking session, total homogenates were incubated with pre-assembled microtubules, and microtubules-associated proteins were isolated. As shown in Figure 3e-f, alcohol drinking significantly increased the immunoreactivity of CRMP-2 in the microtubules-enriched pellet fraction, indicating that CRMP-2 binding to microtubules is increased in response to alcohol intake. CRMP-2 promotes microtubule assembly after binding to microtubules [27]. Therefore, we hypothesized that excessive alcohol intake increases microtubule content by promoting its assembly. As shown in Figure 3g-h, alcohol significantly increased the ratio of microtubules-associated tubulin to free tubulin. In contrast, the levels of total tubulin were unaltered by alcohol (Figure 3g, i). Together, these results suggest that excessive alcohol drinking blocks the phosphorylation of CRMP-2 and promotes CRMP-2 binding to microtubules, resulting in microtubule assembly.

Systemic administration of the CRMP-2 inhibitor Lacosamide decreases binge drinking of alcohol in rodents

Microtubules maintain the morphology of neurons and play an important role in structural plasticity [46, 47]. Structural plasticity, which involves the formation, stabilization and elimination of synapses [48], is linked to compulsive drug taking and seeking [49]. We therefore hypothesized that CRMP-2-mediated microtubule assembly participates in the development and/or maintenance of excessive alcohol drinking behaviors. We utilized the CRMP-2 inhibitor,

Lacosamide, which binds with CRMP-2 and inhibits CRMP-2-induced microtubule assembly and neurite growth [50, 51], and tested whether Lacosamide reduces excessive alcohol consumption in rodents (Timeline, Supplementary Figure 1C). As shown in Figure 4, systemic administration of Lacosamide significantly decreased binge drinking of alcohol in rats and mice. Furthermore, systemic administration of Lacosamide did not affect rat sucrose consumption (Figure 4b), suggesting that the drug does not alter general reward. In addition, to rule out putative locomotor or anxiolytic effects of Lacosamide [52], we tested the effect of the drug on spontaneous locomotion and basal anxiety-like behavior. Vehicle and Lacosamide-treated rats did not exhibit significant differences in distance travelled (Supplementary Figure 8A) or time spent in the center of the chamber (Supplementary Figure 8B). Together, these results indicate that Lacosamide decreases binge drinking of alcohol in rodents without affecting the general reward system, basal locomotor activity or anxiety levels.

Knockdown of CRMP-2 in the mouse NAc decreases excessive drinking of alcohol

To test the specific contribution of CRMP-2 in the NAc to alcohol drinking, we generated a lentivirus expressing a short hairpin RNA targeting the *CRMP-2* gene (Ltv-shCRMP-2), and tested whether knockdown of the gene reduces alcohol intake. Infection of Ltv-shCRMP-2 in mouse NAc (Figure 5a) led to a significant decrease in CRMP-2 protein level but not in the immunoreactivity of tubulin, RACK1 or GAPDH (Figure 5b and Supplementary Figure 9A). Furthermore, the knockdown of *CRMP-2* was restricted to the NAc, as no change of CRMP-2 protein level was observed in the neighboring dorsal striatum (Supplementary Figure 9B).

Next, we measured voluntary alcohol intake of mice infected with Ltv-shCRMP-2 or a lentivirus expressing a non-specific short hairpin control sequence (Ltv-shCT) (Timeline,

Supplementary Figure 1D). We found that knockdown of *CRMP-2* in the NAc resulted in a significant decrease in excessive alcohol intake (Figure 5c) and preference (Figure 5d) as compared to Ltv-shCT-infected mice. Water intake was increased after knockdown of *CRMP-2* in the NAc, which is likely a countervailing effect (Figure 5e). Knockdown of *CRMP-2* in mouse NAc did not change the consumption of another rewarding substance, saccharin (Figure 5f), and locomotor activity was unaltered (Supplementary Figure 10). Together, these results indicate that CRMP-2 in the NAc plays an important role in the development of excessive alcohol drinking without altering the general reward system or spontaneous locomotor activity.

DISCUSSION

Here, we show that excessive consumption of alcohol results in the induction of specific mTORC1-dependent mRNA translation in the NAc. One of the identified mRNAs whose translation is triggered by alcohol is the microtubule-binding protein, CRMP-2. We further present data to suggest that the alcohol-mediated inhibition of GSK-3β leads to an increase in CRMP-2 in its hypophosphorylated form. Thus, mTORC1 activation and GSK-3β inhibition in the NAc converge on CRMP-2 resulting in its association with microtubules, which in turn increases microtubule content (Supplementary Figure 11). Importantly, pharmacologic inhibition of CRMP-2 function or specific knockdown of CRMP-2 levels in the NAc attenuates excessive alcohol drinking suggesting that CRMP-2 is an important contributor to neuroadaptations underlying excessive drinking behaviors.

De novo protein synthesis is crucial for the molecular mechanisms underlying long-lasting neuroadaptations in response to drugs of abuse [3, 53], including alcohol [11]. Here, we demonstrate that excessive drinking of alcohol activates the translation of several mTORC1-

dependent candidate mRNAs in the NAc of rats, all of which contribute to synaptic functions. Several of these mTORC1-dependent effectors have been previously linked to various alcohol-related actions. Specifically, Pandey et al. reported that Arc in the amygdala controls alcohol intake in rats [54]; and we previously found that *Arc* mRNA to protein translation is induced in amygdala and cortical regions by retrieval of alcohol-associated memories [14]. Furthermore, the immunoreactivity of GluA1 is increased in the NAc of rodents in response to excessive drinking of alcohol [13], and AMPAR in the dorsomedial striatum (DMS) plays a role in alcohol seeking and drinking [55]. In addition, Easton *et al.* recently reported that CaMKIIα autophosphorylation-deficient mice show reduced alcohol preference compared with wildtype mice [56]. Homer2 protein levels in the NAc have also been shown to be induced by alcohol and contribute to excessive alcohol consumption [57]. Furthermore, PSD-95 knockout mice exhibit hypersensitivity to alcohol intoxication and show decreased voluntary alcohol intake [58]. Our findings therefore put forward the possibility that mTORC1, by simultaneously inducing the translation of a group of synaptic proteins, is a molecular gateway responsible for neuroadaptations induced by alcohol drinking.

We found that the increase in polysomal mRNA of *Arc, CaMKIIα, CRMP-2*, *Homer2* and *PSD-95* is still maintained after 24 hours of alcohol withdrawal. These results are in accordance with our previous findings showing that the activation of mTORC1 is still detected in rodents' NAc after 24 hours of alcohol withdrawal [13]. These findings raise the possibility that the initiation of alcohol-induced mTORC-1-dependent mRNA translation is not directly related to the pharmacological effects of alcohol, but rather are stable changes that depend on the previous history of alcohol exposure and are components of the neuroadaptations that persist through a withdrawal period.

A direct downstream substrate of AKT is GSK-3β [43]. AKT is activated in the NAc in response to excessive alcohol intake [44], and GSK-3β is phosphorylated by AKT after binge drinking of alcohol (as shown herein) and withdrawal [44]. CRMP-2 is a substrate of GSK-3β [42]; and we demonstrate that in the NAc, basal levels of GSK-3β phosphorylation are low, suggesting that GSK-3β is constitutively active. In contrast, CRMP-2 phosphorylation is high in the NAc of water consuming animals, indicating that constitutively active GSK-3β phosphorylates CRMP-2. Importantly, alcohol-mediated GSK-3β phosphorylation (and thus inhibition) is paralleled with a significant reduction in CRMP-2 phosphorylation suggesting that alcohol-mediated inhibition of this kinase has positive biological consequences. It is important to note however that the decrease in CRMP-2 phosphorylation by alcohol may also require the activity of a phosphatase. One possible candidate is protein phosphatase 2A (PP2A), which was reported to dephosphorylate CRMP-2 at Threonine 514 [59]. Another potential candidate is protein phosphatase 1 (PP1), which dephosphorylates CRMP-2 at Threonine 514 when GSK-3β activity is inhibited [60]. Further research is required to test the potential role of phosphatases in CRMP-2 function in response to alcohol.

Microtubule dynamics in dendritic spines is a critical component of structural plasticity [47]. CRMP-2 is a microtubules-associated protein that stabilizes microtubules and promotes neurite outgrowth [27, 61], which further provides the basis for neuronal morphology and structural plasticity [47, 62]. We demonstrate that the functional consequence of alcohol-mediated increase in CRMP-2 protein the blockade of CRMP-2 phosphorylation is the enhanced binding of CRMP-2 to microtubules, leading to the enhancement of microtubule assembly. Thus, our results raise the possibility that alcohol intake changes neuronal morphology through CRMP-2-dependent microtubule dynamics, which may underlie the development and maintenance of alcohol-

drinking behaviors. In support of this possibility, we show that knockdown of CRMP-2 in the NAc results in a robust decrease in excessive alcohol intake and preference, suggesting that CRMP-2 in the NAc is a molecular gateway to control alcohol-drinking behaviors.

Finally, we demonstrate that systemic administration of Lacosamide decreases binge drinking of alcohol in both rats and mice and that this effect is not generalized to other rewarding substances. Lacosamide interacts with CRMP-2 and inhibit CRMP-2-induced microtubule assembly as well as neurite growth [50, 51]. However, Lacosamide has also been shown to increase slow inactivation of voltage-gated sodium channels [63]. Thus, we cannot rule out the possibility that Lacosamide decreases excessive drinking of alcohol, at least in part, through other mechanisms. Nevertheless, Lacosamide, an FDA approved drug used for the treatment of seizures [64], and possibly other CRMP-2-microtuble inhibitors, represent an innovative and promising strategy for the treatment of alcohol-related disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank Eric Zhao, JiHwan Yu and Scott Wegner for technical assistance and Dr. Jacob Beckley for careful review of the manuscript. This research was supported by National Institutes of Health-National Institute on Alcohol Abuse and Alcoholism P50 AA017072 (D.R.) and the State of California (D.R.). The authors have no conflict of interest to declare.

REFERENCES

1. Koob GF, Volkow ND. Neurocircuitry of addiction. *Neuropsychopharmacology* 2010; **35**(1): 217-238.
2. Hyman SE, Malenka RC, Nestler EJ. Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu Rev Neurosci* 2006; **29**: 565-598.
3. Santini E, Huynh TN, Klann E. Mechanisms of translation control underlying long-lasting synaptic plasticity and the consolidation of long-term memory. *Prog Mol Biol Transl Sci* 2014; **122**: 131-167.
4. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011; **12**(1): 21-35.
5. Buffington SA, Huang W, Costa-Mattioli M. Translational control in synaptic plasticity and cognitive dysfunction. *Annu Rev Neurosci* 2014; **37**: 17-38.
6. Jung H, Gkogkas CG, Sonenberg N, Holt CE. Remote control of gene function by local translation. *Cell* 2014; **157**(1): 26-40.
7. Brunn GJ, Hudson CC, Sekulic A, Williams JM, Hosoi H, Houghton PJ *et al.* Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* 1997; **277**(5322): 99-101.
8. Holz MK, Ballif BA, Gygi SP, Blenis J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* 2005; **123**(4): 569-580.
9. Sonenberg N, Hinnebusch AG. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* 2009; **136**(4): 731-745.

10. Aitken CE, Lorsch JR. A mechanistic overview of translation initiation in eukaryotes. *Nat Struct Mol Biol* 2012; **19**(6): 568-576.

11. Neasta J, Barak S, Hamida SB, Ron D. mTOR complex 1: a key player in neuroadaptations induced by drugs of abuse. *J Neurochem* 2014; **130**(2): 172-184.

12. Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov* 2011; **10**(11): 868-880.

13. Neasta J, Ben Hamida S, Yowell Q, Carnicella S, Ron D. Role for mammalian target of rapamycin complex 1 signaling in neuroadaptations underlying alcohol-related disorders. *Proc Natl Acad Sci U S A* 2010; **107**(46): 20093-20098.

14. Barak S, Liu F, Ben Hamida S, Yowell QV, Neasta J, Kharazia V *et al.* Disruption of alcohol-related memories by mTORC1 inhibition prevents relapse. *Nat Neurosci* 2013; **16**(8): 1111-1117.

15. Takei N, Inamura N, Kawamura M, Namba H, Hara K, Yonezawa K *et al.* Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. *J Neurosci* 2004; **24**(44): 9760-9769.

16. Schratt GM, Nigh EA, Chen WG, Hu L, Greenberg ME. BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. *J Neurosci* 2004; **24**(33): 7366-7377.

17. Morita T, Sobue K. Specification of neuronal polarity regulated by local translation of CRMP2 and Tau via the mTOR-p70S6K pathway. *J Biol Chem* 2009; **284**(40): 27734-27745.

18. Loreni F, Iadevaia V, Tino E, Caldarola S, Amaldi F. RACK1 mRNA translation is regulated via a rapamycin-sensitive pathway and coordinated with ribosomal protein synthesis. *FEBS Lett* 2005; **579**(25): 5517-5520.

19. Tang SJ, Reis G, Kang H, Gingras AC, Sonenberg N, Schuman EM. A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. *Proc Natl Acad Sci U S A* 2002; **99**(1): 467-472.

20. Korb E, Finkbeiner S. Arc in synaptic plasticity: from gene to behavior. *Trends Neurosci* 2011; **34**(11): 591-598.

21. Hell JW. CaMKII: claiming center stage in postsynaptic function and organization. *Neuron* 2014; **81**(2): 249-265.

22. Derkach VA, Oh MC, Guire ES, Soderling TR. Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nat Rev Neurosci* 2007; **8**(2): 101-113.

23. Paoletti P, Bellone C, Zhou Q. NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci* 2013; **14**(6): 383-400.

24. Shiraishi-Yamaguchi Y, Furuichi T. The Homer family proteins. *Genome Biol* 2007; **8**(2): 206.

25. Kim E, Sheng M. PDZ domain proteins of synapses. *Nat Rev Neurosci* 2004; **5**(10): 771-781.

26. Adams DR, Ron D, Kiely PA. RACK1, A multifaceted scaffolding protein: Structure and function. *Cell Commun Signal* 2011; **9**: 22.

27. Ip JP, Fu AK, Ip NY. CRMP2: Functional Roles in Neural Development and Therapeutic Potential in Neurological Diseases. *Neuroscientist* 2014. doi: 10.1177/1073858413514278.

28. Beguin C, Potter DN, Carlezon WA, Jr., Stohr T, Cohen BM. Effects of the anticonvulsant lacosamide compared to valproate and lamotrigine on cocaine-enhanced reward in rats. *Brain Res* 2012; **1479**: 44-51.

29. Ben Hamida S, Neasta J, Lasek AW, Kharazia V, Zou M, Carnicella S *et al.* The small g protein h-ras in the mesolimbic system is a molecular gateway to alcohol-seeking and excessive drinking behaviors. *J Neurosci* 2012; **32**(45): 15849-15858.

30. del Prete MJ, Vernal R, Dolznig H, Mullner EW, Garcia-Sanz JA. Isolation of polysome-bound mRNA from solid tissues amenable for RT-PCR and profiling experiments. *RNA* 2007; **13**(3): 414-421.

31. Darcq E, Warnault V, Phamluong K, Besserer GM, Liu F, Ron D. MicroRNA-30a-5p in the prefrontal cortex controls the transition from moderate to excessive alcohol consumption. *Mol Psychiatry* 2014. doi: 10.1038/mp.2014.120.

32. Gibb SL, Hamida SB, Lanfranco MF, Ron D. Ethanol-induced increase in Fyn kinase activity in the dorsomedial striatum is associated with subcellular redistribution of protein tyrosine phosphatase alpha. *J Neurochem* 2011; **119**(4): 879-889.

33. Brittain JM, Piekarz AD, Wang Y, Kondo T, Cummins TR, Khanna R. An atypical role for collapsin response mediator protein 2 (CRMP-2) in neurotransmitter release via interaction with presynaptic voltage-gated calcium channels. *J Biol Chem* 2009; **284**(45): 31375-31390.

34. Lasek AW, Janak PH, He L, Whistler JL, Heberlein U. Downregulation of mu opioid receptor by RNA interference in the ventral tegmental area reduces ethanol consumption in mice. *Genes Brain Behav* 2007; **6**(8): 728-735.

35. Carnicella S, Amamoto R, Ron D. Excessive alcohol consumption is blocked by glial cell line-derived neurotrophic factor. *Alcohol* 2009; **43**(1): 35-43.

36. Warnault V, Darcq E, Levine A, Barak S, Ron D. Chromatin remodeling--a novel strategy to control excessive alcohol drinking. *Transl Psychiatry* 2013; **3**: e231.

37. Ben Hamida S, Darcq E, Wang J, Wu S, Phamluong K, Kharazia V *et al.* Protein tyrosine phosphatase alpha in the dorsomedial striatum promotes excessive ethanol-drinking behaviors. *J Neurosci* 2013; **33**(36): 14369-14378.

38. Carnicella S, Ron D, Barak S. Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. *Alcohol* 2014; **48**(3): 243-252.

39. NIAAA. Council approves definition of binge drinking. *NIAAA Newsletter* 2004; **3**(3).

40. Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS, Sabatini DM. A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature* 2012; **485**(7396): 109-113.

41. Peng J, Kim MJ, Cheng D, Duong DM, Gygi SP, Sheng M. Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. *J Biol Chem* 2004; **279**(20): 21003-21011.

42. Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A, Kaibuchi K. GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* 2005; **120**(1): 137-149.

43. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007; **129**(7): 1261-1274.

44. Neasta J, Ben Hamida S, Yowell QV, Carnicella S, Ron D. AKT signaling pathway in the nucleus accumbens mediates excessive alcohol drinking behaviors. *Biol Psychiatry* 2011; **70**(6): 575-582.

45. Tan M, Ma S, Huang Q, Hu K, Song B, Li M. GSK-3alpha/beta-mediated phosphorylation of CRMP-2 regulates activity-dependent dendritic growth. *J Neurochem* 2013; **125**(5): 685-697.

46. Dent EW, Baas PW. Microtubules in neurons as information carriers. *J Neurochem* 2014; **129**(2): 235-239.

47. Shirao T, Gonzalez-Billault C. Actin filaments and microtubules in dendritic spines. *J Neurochem* 2013; **126**(2): 155-164.

48. Caroni P, Donato F, Muller D. Structural plasticity upon learning: regulation and functions. *Nat Rev Neurosci* 2012; **13**(7): 478-490.

49. Nyberg F. Structural plasticity of the brain to psychostimulant use. *Neuropharmacology* 2014. doi: 10.1016/j.neuropharm.2014.07.004.

50. Wilson SM, Xiong W, Wang Y, Ping X, Head JD, Brittain JM *et al.* Prevention of posttraumatic axon sprouting by blocking collapsin response mediator protein 2-mediated neurite outgrowth and tubulin polymerization. *Neuroscience* 2012; **210**: 451-466.

51. Wilson SM, Khanna R. Specific Binding of Lacosamide to Collapsin Response Mediator Protein 2 (CRMP2) and Direct Impairment of its Canonical Function: Implications for the Therapeutic Potential of Lacosamide. *Mol Neurobiol* 2014. doi: 10.1007/s12035-014-8775-9.

52. Higgins GA, Breysse N, Undzys E, Kuo C, Joharchi N, Derksen DR *et al.* The anti-epileptic drug lacosamide (Vimpat) has anxiolytic property in rodents. *Eur J Pharmacol* 2009; **624**(1-3): 1-9.

53. Kauer JA, Malenka RC. Synaptic plasticity and addiction. *Nat Rev Neurosci* 2007; **8**(11): 844-858.

54. Pandey SC, Zhang H, Ugale R, Prakash A, Xu T, Misra K. Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism. *J Neurosci* 2008; **28**(10): 2589-2600.

55. Wang J, Ben Hamida S, Darcq E, Zhu W, Gibb SL, Lanfranco MF *et al.* Ethanol-mediated facilitation of AMPA receptor function in the dorsomedial striatum: implications for alcohol drinking behavior. *J Neurosci* 2012; **32**(43): 15124-15132.

56. Easton AC, Lucchesi W, Lourdusamy A, Lenz B, Solati J, Golub Y *et al.* alphaCaMKII autophosphorylation controls the establishment of alcohol drinking behavior. *Neuropsychopharmacology* 2013; **38**(9): 1636-1647.

57. Szumlinski KK, Ary AW, Lominac KD. Homers regulate drug-induced neuroplasticity: implications for addiction. *Biochem Pharmacol* 2008; **75**(1): 112-133.

58. Camp MC, Feyder M, Ihne J, Palachick B, Hurd B, Karlsson RM *et al.* A novel role for PSD-95 in mediating ethanol intoxication, drinking and place preference. *Addict Biol* 2011; **16**(3): 428-439.

59. Zhu LQ, Zheng HY, Peng CX, Liu D, Li HL, Wang Q *et al.* Protein phosphatase 2A facilitates axonogenesis by dephosphorylating CRMP2. *J Neurosci* 2010; **30**(10): 3839-3848.

60. Cole AR, Soutar MP, Rembutsu M, van Aalten L, Hastie CJ, McLauchlan H *et al.* Relative resistance of Cdk5-phosphorylated CRMP2 to dephosphorylation. *J Biol Chem* 2008; **283**(26): 18227-18237.

61. Lin PC, Chan PM, Hall C, Manser E. Collapsin response mediator proteins (CRMPs) are a new class of microtubule-associated protein (MAP) that selectively interacts with

assembled microtubules via a taxol-sensitive binding interaction. *J Biol Chem* 2011; **286**(48): 41466-41478.

62. Hoogenraad CC, Akhmanova A. Dendritic spine plasticity: new regulatory roles of dynamic microtubules. *Neuroscientist* 2010; **16**(6): 650-661.

63. Wang Y, Brittain JM, Jarecki BW, Park KD, Wilson SM, Wang B *et al.* In silico docking and electrophysiological characterization of lacosamide binding sites on collapsin response mediator protein-2 identifies a pocket important in modulating sodium channel slow inactivation. *J Biol Chem* 2010; **285**(33): 25296-25307.

64. Perucca E, Yasothan U, Clincke G, Kirkpatrick P. Lacosamide. *Nat Rev Drug Discov* 2008; **7**(12): 973-974.

Figure Legends

Figure 1. Alcohol intake promotes the translation of *CRMP-2* mRNA via mTORC1. (**a**-**d**) Rats experienced 2 months of intermittent access to 20% alcohol two-bottle choice drinking paradigm. Control animals (water, W) underwent the same paradigm but had access to water only. (**a** and **b**) The NAc was removed after 30 minutes binge drinking of alcohol (**a**, binge) or 24 hours after the end of the last drinking session (**b**, withdrawal). Polysomes were isolated and mRNA levels were determined by RT-PCR analysis. The PCR products were separated on 1.5% agarose gels, photographed by Image Lab, and quantified by using ImageJ. Data are expressed as the average ratio of each gene to *GAPDH* ± SEM, and are expressed as percentage of water control. Significance was determined using two-tailed unpaired *t*-test. (**c** and **d**) Three hours before the end of the last 24 hours of alcohol withdrawal, rats were systemically administered with 10 mg/kg of rapamycin or vehicle. The NAc was removed three hours after rapamycin or vehicle treatment and were subjected to polysomal RNA fractionation, and the mRNA levels were determined by RT-qPCR analysis. Data are expressed as the average ratio of *CRMP-2* or *RACK1* to *GAPDH* ± SEM, and expressed as percentage of water plus vehicle. Significance was determined using two-way ANOVA and the method of contrasts. (**a**) *Arc*, $t_{(6)}$=2.805, p=0.031; *CaMKIIα*, $t_{(6)}$=2.475, p=0.048; *CRMP-2*, $t_{(6)}$=2.914, p=0.027; *GluA1*, $t_{(6)}$=2.569, p=0.042; *Homer2*, $t_{(6)}$=3.276, p=0.017; *GluN1*, $t_{(6)}$=1.283, p=0.247; *PSD-95*, $t_{(6)}$=3.209, p=0.018; *RACK1*, $t_{(6)}$=0.902, p=0.401; *TrkB*, $t_{(6)}$=0.903, p=0.401. (**b**) *Arc*, $t_{(6)}$=2.582, p=0.042; *CaMKIIα*, $t_{(6)}$=2.495, p=0.047; *CRMP-2*, $t_{(6)}$=2.485, p=0.048; *GluA1*, $t_{(6)}$=0.497, p=0.637; *Homer2*, $t_{(6)}$=2.512, p=0.046; *GluN1*, $t_{(6)}$=1.971, p=0.096; *PSD-95*, $t_{(6)}$=2.596, p=0.041; *RACK1*, $t_{(6)}$=0.291, p=0.781; *TrkB*, $t_{(6)}$=0.563, p=0.593. (**c**) mRNA levels of *CRMP-2* in the polysomal fraction. Two-way ANOVA showed a significant main effect of alcohol ($F_{(1,16)}$=5.07, p=0.039) and rapamycin ($F_{(1,16)}$=9.087,

p=0.008) but no interaction ($F_{(1,16)}$=1.123, p=0.305); and the method of contrasts detected a significant difference between water and alcohol within the vehicle group (p=0.048) and a significant difference between vehicle and rapamycin within the alcohol group (p=0.029). (**d**) mRNA levels of *RACK1* in the polysomal fraction. Two-way ANOVA showed no main effect of alcohol ($F_{(1,16)}$=0.014, p=0.907) and rapamycin ($F_{(1,16)}$=1.422, p=0.251) and no interaction ($F_{(1,16)}$=0.0692, p=0.796). *p<0.05. (**a**, **b**) n=4 and (**c**, **d**) n=5.

Figure 2. Binge drinking of alcohol and withdrawal increase CRMP-2 protein levels. (**a**-**d**) Rats were trained to drink alcohol as described in Figure 1. The NAc was removed 30 minutes after the beginning (**a** and **b**, binge) or 24 hours after the end of the last drinking session (**c** and **d**, withdrawal) and were subjected to synaptic fractionation. Protein levels were determined by western blot analysis. ImageJ was used for optical density quantification. Data are expressed as the average ratio ± SEM of CRMP-2 or RACK1 to GAPDH, and are expressed as percentage of water control. Significance was determined using two-tailed unpaired *t*-test. (**a**) Protein levels in the total homogenate of binge drinking rats. CRMP-2, $t_{(18)}$=2.537, p=0.02; RACK1, $t_{(10)}$=0.759, p=0.465. (**b**) Protein levels in the synaptic fraction of binge drinking rats. CRMP-2, $t_{(18)}$=3.596, p=0.002; RACK1, $t_{(10)}$=0.223, p=0.828. (**c**) Protein levels in the total homogenate 24 hours after withdrawal. CRMP-2, $t_{(18)}$=4.182, p<0.001; RACK1, $t_{(10)}$=0.335, p=0.729. (**d**) Protein levels in the synaptic fraction 24 hours after withdrawal. CRMP-2, $t_{(18)}$=4.887, p<0.001; RACK1, $t_{(10)}$=0.419, p=0.683. *p<0.05, **p<0.01, ***p<0.001. (**a**-**d**) CRMP-2, n=10; RACK1, n=6.

Figure 3. Binge drinking of alcohol blocks the phosphorylation of CRMP-2 and increases microtubule content. (**a**-**i**) Rats were trained to drink alcohol as described in Figure 1. (**a**-**d**) The

NAc was removed 30 minutes after the beginning of the last drinking session (binge). Phosphorylation of GSK-3β and CRMP-2 was determined by western blot analysis. Quantification was conducted as in Figure 2. Data are expressed as the average ratio of phospho-GSK-3β to total GSK-3β or phospho-CRMP-2 to total CRMP-2 ± SEM, and are expressed as percentage of water control. Significance was determined using two-tailed unpaired *t*-test. (**a**) [S^{9}]GSK-3β phosphorylation in total homogenate, $t_{(10)}$=3.141, p=0.011. (**b**) [S^{9}]GSK-3β phosphorylation in the synaptic fraction, $t_{(10)}$=3.574, p=0.005. (**c**) [T^{514}]CRMP-2 phosphorylation in total homogenate, $t_{(14)}$=3.119, p=0.007. (**d**) [T^{514}]CRMP-2 phosphorylation in the synaptic fraction, $t_{(14)}$=3.18, p=0.008. (**e-i**) The NAc were removed 24 hours after the end of the last drinking session (withdrawal). (**e** and **f**) Microtubule binding assay. (**e**) CRMP-2 and tubulin levels in the pellet fraction were determined by western blot analysis. (**f**) Optical density quantification of Microtubules-bound CRMP-2 is expressed as the ratio of CRMP-2 (pellet) to tubulin (pellet) ± SEM. $t_{(11)}$=2.271, p=0.044. (**g-i**) Microtubule content assay. Tubulin and GAPDH levels in every fraction were detected by western blot analysis. Optical density quantification of the microtubule content (**h**) and total tubulin (**i**) are expressed as the ratio of tubulin (pellet) to tubulin (supernatant) ± SEM. $t_{(14)}$=2.42, p=0.029, and tubulin (total) to GAPDH (total), $t_{(14)}$=1.207, p=0.248, respectively. *p<0.05, **p<0.01. (**a**, **b**) n=6, (**c**, **d**) n=8, (**e**, **f**) n=6-7 and (**g-i**) n=8.

Figure 4. Systemic administration of the CRMP-2 inhibitor Lacosamide decreases binge drinking of alcohol in rodents. (**a**) Rats were subjected to an intermittent-access to 20% alcohol two-bottle choice drinking procedure for 2 months. Vehicle (Veh) or Lacosamide (LCM, 20 mg/kg) was systemically administered 90 minutes before the beginning of a drinking session.

Alcohol intake was measured 2 hours after vehicle or Lacosamide treatment. Data are presented as mean ± SEM. Significance was determined using two-tailed paired *t*-test, $t_{(8)}$=4.176, p=0.003. (**b**) Rats were subjected to an intermittent-access to sucrose solution two-bottle choice drinking procedure for 2 weeks. Vehicle (Veh) or Lacosamide (LCM, 20 mg/kg) was systemically administered 90 minutes before the beginning of a drinking session. Sucrose intake was measured 2 hours after vehicle or Lacosamide treatment. Data are presented as mean ± SEM. Significance was determined using two-tailed paired *t*-test, $t_{(9)}$=-0.059, p=0.954. (**c**-**e**) Mice experienced 2 months of intermittent access to 20% alcohol two-bottle choice drinking paradigm. Vehicle (Veh) or Lacosamide (LCM, 20 mg/kg or 50 mg/kg) was systemically administered 90 minutes before the beginning of a drinking session. Alcohol intake (**c**), alcohol preference (**d**) and water intake (**e**) were measured at the end of a 4 hours drinking session. Data are presented as mean ± SEM. Significance was determined using one-way RM-ANOVA and *post hoc* Student–Newman–Keuls test. (**c**) Alcohol intake. One-way RM-ANOVA showed a significant main effect of Lacosamide ($F_{(2,20)}$=9.842, p=0.001), and *post hoc* Student–Newman–Keuls test, q=6.081, p=0.001. (**d**) Alcohol preference is expressed as the ratio of alcohol intake to total fluid intake. One-way RM-ANOVA showed a significant main effect of Lacosamide ($F_{(2,20)}$=8.715, p=0.002), and *post hoc* Student–Newman–Keuls test, q=5.453, p=0.003. (**e**) Water intake. One-way RM-ANOVA showed a significant main effect of Lacosamide ($F_{(2,20)}$=4.029, p=0.034), and *post hoc* Student–Newman–Keuls test, q=3.627, p=0.047. *p<0.05, **p<0.01, ***p<0.001. (**a**, **b**) n=9-10 and (**c**-**e**) n=11.

Figure 5. Knockdown of CRMP-2 in the mouse NAc decreases excessive drinking of alcohol. (**a**-**f**) A lentivirus expressing non-specific control shRNA (Ltv-shCT) (2×10^7 pg/ml) or shRNA

targeting mouse CRMP-2 (Ltv-shCRMP-2) (2×10^7 pg/ml) was infused bilaterally into the mouse NAc (1.2 µl/side). After three weeks of recovery, mice underwent an intermittent-access to 20% alcohol two-bottle choice drinking procedure for 8 sessions. The NAc was dissected at the end of behavioral experiment and used for immunohistochemistry (**a**), and western blot analysis (**b**). Alcohol intake (**c**), alcohol preference (**d**), water intake (**e**) and saccharin intake (**f**) were measured after each 24-hours drinking session and expressed as an average of every 2 drinking sessions. Data are expressed as mean ± SEM. Significance was determined using two-tailed unpaired *t*-test (**b** and **f**) or two-way RM-ANOVA (**c**-**e**). (**a**) Ltv-shCRMP-2 infects NAc neurons. Slices were stained with anti-GFP antibodies. Left panel (5X) depicts the specificity of the site of virus infection. Scale bar, 500 µm. Middle image (40X) depicts Ltv-shCRMP-2 infection of neurons. Scale bar, 25 µm. Right panel (63X) shows a representative neurite from an infected neuron. Scale bar, 5 µm. (**b**) Ltv-shCRMP-2 infection decreases CRMP-2 expression in the NAc. Left, the protein levels of CRMP-2, tubulin, RACK1 and GAPDH were determined by western blot analysis. Right, the histogram shows the ratio of CRMP-2 level to GAPDH level in the NAc, $t_{(6)}$=6.414, p=0.0007. (**c**) Alcohol intake. Two-way RM-ANOVA showed a significant main effect of virus infusion ($F_{(1,20)}$=6.883, p=0.016), a significant effect of session ($F_{(3,60)}$=51.502, p<0.001) and no interaction between virus infusion and session ($F_{(3,60)}$=1.969, p=0.128). (**d**) Alcohol preference is expressed as the ratio of alcohol intake to total fluid intake. Two-way RM-ANOVA showed a significant main effect of virus infusion ($F_{(1,20)}$=8.585, p=0.008), a significant effect of session ($F_{(3,60)}$=29.666, p<0.001) and no interaction between virus infusion and session ($F_{(3,60)}$=1.344, p=0.269). (**e**) Water intake. Two-way RM-ANOVA showed a significant main effect of virus infusion ($F_{(1,20)}$=6.155, p=0.022), no effect of session ($F_{(3,60)}$=2.175, p=0.100) and no interaction between virus infusion and session ($F_{(3,60)}$=1.265, p=0.294). (**f**) One week after the

alcohol drinking test, the same mice were subjected to an intermittent-access to saccharin two-bottle choice drinking procedure for 2 sessions. Saccharin intake was measured after each session and expressed as an average of the two sessions, $t_{(19)}$=0.530, p=0.602. *p<0.05, **p<0.01, ***p<0.001. (**b**) n=4 and (**c**-**f**) n=11.