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ORIGINAL ARTICLE

mTORC1-dependent translation of collapsin response mediator protein-2 drives neuroadaptations underlying excessive alcohol-drinking behaviors

F Liu¹, S Laguesse, R Legastelois², N Morisot, S Ben Hamida³ and D Ron

Mammalian target of rapamycin complex 1 (mTORC1) has an essential role in 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-3 β (GSK-3 β)-phosphorylation of CRMP-2, which results in elevated binding of CRMP-2 to microtubules and a concomitant increase in microtubule 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-3 β , that in turn drives excessive alcohol-drinking behaviors.

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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 translation initiation factor 4E binding protein (4E-BP) and ribosomal protein S6 kinase (S6K).^{7,8} Phosphorylation of 4E-BP reduces its binding with the eukaryotic translation initiation factor 4E (eIF4E), leading to the disinhibition of eIF4E; together with the phosphorylation-dependent S6K activation, dissociation of eIF4E from phosphorylated 4E-BP enables the formation of eukaryotic initiation factor complex, which promotes cap-dependent mRNA translation.^{9,10}

Recently, mTORC1 has been shown to contribute to neuroadaptations of several drugs of abuse, including alcohol.¹¹ Specifically for alcohol, we 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 reduces excessive alcohol drinking and seeking.¹³ We further showed that mTORC1 participates in the maintenance of alcohol-related memories.¹⁴ As mTORC1 has an essential role in learning and memory

via the translation of mRNAs at dendrites,^{5,6} and as psychoactive drugs including alcohol pathologically utilize normal mechanisms underlying learning and memory to cause addiction,² we hypothesized that mTORC1 mediates neuroadaptations underlying alcohol-drinking behaviors by promoting the translation of synaptic proteins. To test the hypothesis, we selected eight 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 Ca²⁺/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 RACK1²⁶ and collapsin response mediator protein-2 (CRMP-2).²⁷ Using a rat model of excessive alcohol drinking, we measured 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 has a central role in microtubules assembly in response to alcohol exposure and to the development and persistence of neuroadaptations underlying excessive alcohol-drinking behaviors.

MATERIALS AND METHODS

Further details regarding reagents and procedures can be found in the Supplementary Information section.

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Animals

Male Long-Evans rats (8–9 week old) were obtained from Harlan (Indianapolis, IN, USA). Rats were individually housed and kept in a temperature- and humidity-controlled environment under a 12-hour light/dark cycle (lights on at 07:00 AM) with free access to food and tap water. Male C57BL/6J mice (8–9 week old) were obtained from the Jackson Laboratory (Farmington, CT, USA) and were housed in a temperature- and humidity-controlled room under a reversed 12 h light/dark cycle (lights on at 1000 hrs), with food and water available *ad libitum*. All animal procedures in this report were approved by the Gallo Center and the University of California San Francisco (UCSF) Institutional Animal Care and Use Committee and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals (National Research Council, Gallo Center) and the Association for Assessment and Accreditation of Laboratory Animal Care (UCSF).

Preparation of solutions

Alcohol solution was prepared from ethyl alcohol solution (190 proof) diluted to 10% or 20% (v/v) in tap water. Rapamycin (10 mg kg⁻¹ for rats and 20 mg kg⁻¹ for mice)¹³ was dissolved in dimethyl sulfoxide and systemically (intraperitoneally) administered in a volume of 2 ml kg⁻¹ for both rats and mice. Lacosamide (20 mg kg⁻¹ for rats and 20 or 50 mg kg⁻¹ for mice)²⁸ was dissolved in saline solution (0.9%), and administered intraperitoneally in a volume of 2 ml kg⁻¹ for rats and 10 ml kg⁻¹ for mice.

Polysomal fractionation

Collection of the NAc was conducted as described previously.²⁹ 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 min, the powder of one NAc was resuspended in 1 ml lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 1% NP40, 0.5% sodium deoxycholate, 40 mM dithiothreitol, 400 U ml⁻¹ Rnasin, 10 mM RVC and 200 µg ml⁻¹ cycloheximide) followed by 20 times of pipetting to disrupt cells. In total, 200 µl of the homogenate was subjected to total RNA extraction using TRIzol reagent. The rest of the homogenate was centrifuged for 10 s at 12 000 g to remove intact nuclei. The supernatant was collected and the ribosomes were further released by adding 2 extraction buffer (200 mM Tris pH 7.5, 300 mM NaCl and 200 µg ml⁻¹ cycloheximide). Samples were kept on ice for 5 min and then centrifuged at 12 000 g, 4 °C for 5 min 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, USA) at 38 000 rpm, 4 °C for 2 h. Fractions from the sucrose gradient were collected and further digested with a solution containing (400 µg ml⁻¹ proteinase K, 10 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate) at 37 °C for 30 min, followed by phenol–chloroform extraction. RNA in the water phase of the polysomal fraction was recovered by ethyl alcohol precipitation.

Reverse transcription-PCR

mRNA levels were measured by reverse transcription-PCR or quantitative PCR as previously described.³¹ Primer sequences are specified in the Supplementary Information section.

Crude synaptosomal fraction and western blot analysis

Purification of the synaptosomal fraction and western blot analysis are described in.³² Detailed procedures are also 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, USA), according to manufacturer's instructions. Detailed procedures are described in Supplementary Information.

Generation and usage of a lentivirus expressing shCRMP-2

The 19 nucleotides short hairpin RNA sequence 5'-GUAACUCCUCCUC GUGU-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'-GCGCUUAGCUGUAGGAUUC-3' (Ltv-shCT) was used as control. The production of lentivirus is described in.³⁴ Intra-NAc infusion of lentivirus (2 × 10⁷ pg ml⁻¹) was conducted as described in.²⁹

Immunohistochemistry

Immunohistochemistry protocol is described in.²⁹ Detailed procedures are also described in Supplementary Information.

Behavioral experiments

Alcohol-drinking paradigms. Continuous access to 10% and intermittent access to 20% alcohol two-bottle choice (CA10%-2BC, IA20%-2BC) drinking procedures were conducted as previously described in rats³⁵ and mice.³⁶

Effect of lacosamide on alcohol-drinking, voluntary sucrose consumption, locomotion and anxiety-like behavior. Experiments were conducted by using a counterbalanced within-subject design using protocols described previously.^{29,37}

Behavioral experiments following lentiviral infusion. Three weeks after viral infusion, mice were subjected to IA20%-2BC paradigm for three consecutive weeks as described above. After 1 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, spontaneous locomotor activity was measured for 60 min in activity monitoring chambers (43 cm × 43 cm) with horizontal photo beams (Med Associates, St Albans, VT, USA).

Data analysis

Depending on the experiment, data were analyzed using appropriate statistical test, including two-tailed paired or unpaired *t*-test, two-way analysis of variance (ANOVA) or one- or two-way repeated measures ANOVA is detailed in the Figure Legends. Significant main effects and interactions of the ANOVAs were further investigated with the Student–Newman–Keuls *post hoc* test or method of contrast analysis. Data are expressed as mean ± s.e.m. Statistical significance was set at *P* < 0.05. D'Agostino–Pearson normality test and *F*-test/Levene test were used to verify the normal distribution of variables and the homogeneity of variance, respectively. The excessive drinking of alcohol animals included for biochemical and behavioral experiments were chosen based on the criteria described previously.^{36,38} No randomization method was used to allocate animals in each group. No blinding analysis was performed for the animal allocation. Samples were collected from each animal brain. The exact number of samples indicates biological replicates and are indicated in each Figure Legend. No statistical analysis was used to determine sample size *a priori*. The sample sizes we chose here are similar to those used in previous publications.^{13,39}

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 excessive alcohol intake and withdrawal.³⁸ This paradigm results in a binge alcohol-drinking phenotype in which animals consume large quantities of alcohol in a short time reaching blood alcohol concentration that is above 80 mg%,³⁸ which corresponds to blood alcohol concentration values of binge drinking in humans.⁴⁰ Rat NAc was dissected immediately after the last binge-drinking session (Timeline, Figure 1a), and polysomes that contain mRNA undergoing translation were purified (Supplementary Figure 1). 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 with rats that drank only water (Figure 1b and Table 1). The increase in polysomal mRNA levels of *Arc*, *CaMKIIα*,

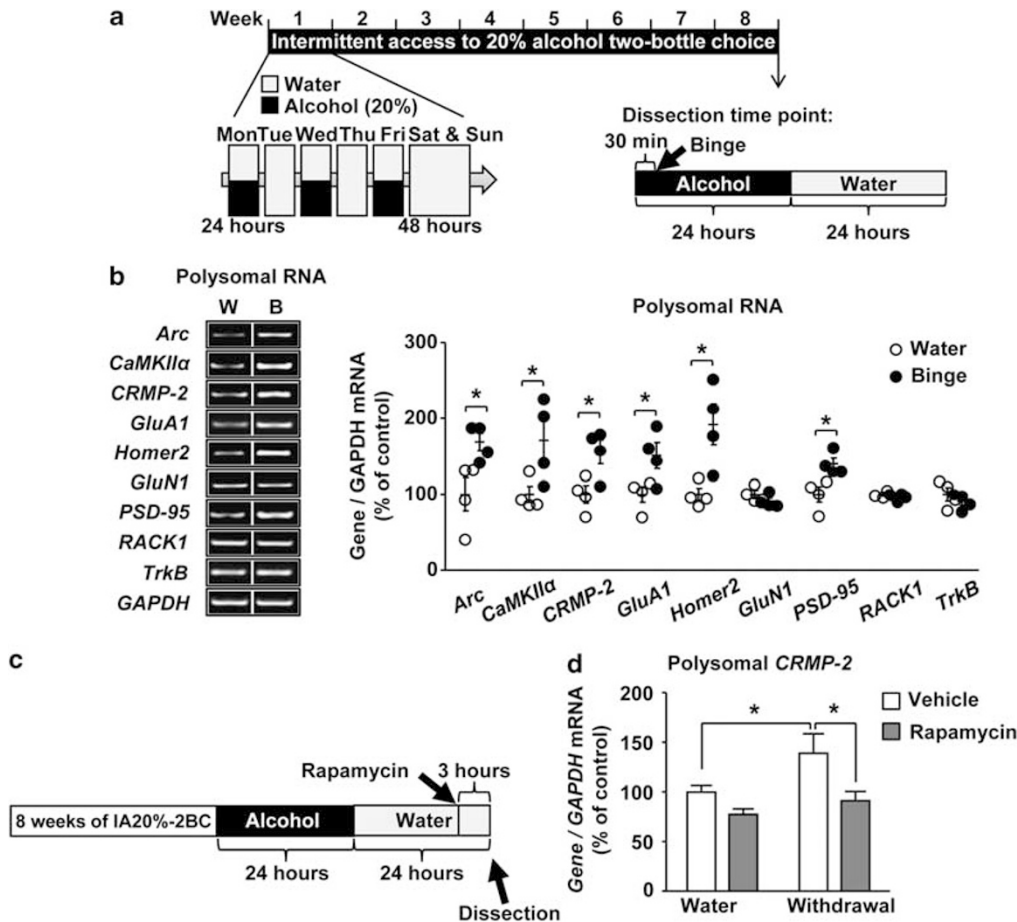


Figure 1. Alcohol intake promotes the translation of *CRMP-2* mRNA via mTORC1. (a) Timeline of experiment depicted in (b). (b) Rats were subjected to IA20%-2BC-drinking procedure for 2 months. The NAC was removed at the end of the last 30 min binge alcohol-drinking session. Control animals underwent the same paradigm but had access to water only (W). 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. The vertical lines indicate that the samples were from the same gel but were not run in adjacent lanes. Data are expressed as the average ratio of each gene to *GAPDH* \pm s.e.m., and are expressed as percentage of water control. Significance was determined using two-tailed unpaired *t*-test. *Arc*, $t_{(6)}=2.805$, $P=0.031$; *CaMKIIa*, $t_{(6)}=2.475$, $P=0.048$; *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*, $t_{(6)}=0.903$, $P=0.401$. (c) Timeline of experiment depicted in (d). Three hours before the end of the last 24 h of alcohol withdrawal, rats were systemically administered with 10 mg kg⁻¹ of rapamycin or vehicle. (d) The NAC was removed 3 h after rapamycin or vehicle treatment and was subjected to polysomal RNA fractionation. mRNA levels were measured by RT-qPCR analysis. Data are expressed as the average ratio of *CRMP-2* to *GAPDH* \pm s.e.m., and expressed as percentage of water plus vehicle. Significance was determined using two-way ANOVA and the method of contrasts. 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 ($P=0.029$). (b) $n=4$, (d) $n=5$ for each group. * $P < 0.05$.

CRMP-2, *Homer2* and *PSD-95* mRNAs, but not *GluA1*, were still detected after 24 h of abstinence (Supplementary Figure 2A and Table 1). Alcohol drinking did not produce an increase in the total RNA of any of the genes (Supplementary Figure 2B and C and Table 1), 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 1b and Supplementary Figure 2).

mRNAs consisting a 5' terminal oligopyrimidine structure are thought to be regulated by mTORC1.⁴¹ Therefore, we used an *in silico* approach (RefSeq, <http://www.ncbi.nlm.nih.gov/refseq/>) to examine the transcriptional start site of the mRNAs whose translation was activated by alcohol. Among the candidate mRNAs listed in Figure 1b, we observed that only *CRMP-2* contains a terminal oligopyrimidine-like mRNA signature (Supplementary Figure 3). As *CRMP-2* mRNA was previously identified as a terminal oligopyrimidine mRNA,¹⁷ we focused our studies on this gene.

First, we confirmed that *CRMP-2* mRNA is increased in the polysomal fraction in response to alcohol intake and withdrawal by quantitative reverse transcription-PCR (Supplementary Figure 4 and Table 1). The total mRNA levels of *CRMP-2* were unaltered in response to alcohol intake, and neither were the total and polysomal levels of *RACK1* (Supplementary Figure 4). Next, 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 h after the last alcohol-drinking session (timeline, Figure 1c). As shown in Figure 1d, the alcohol-mediated increase in polysomal *CRMP-2* mRNA was blocked by rapamycin. In contrast, the polysomal mRNA levels of *RACK1* or total mRNA levels of *CRMP-2* and *RACK1* were unaltered in the presence or absence of rapamycin (Supplementary Figure, Table 1). Together, these results suggest that alcohol activates the translation of *CRMP-2*,

Table 1. Individual alcohol-drinking data in rodents used for biochemical experiments

<i>Animals drinking data</i>				
Figure 1b and Supplementary Figure 2B	<i>Drinking paradigm</i> IA20%-2BC binge rat	<i>Rat number</i>	<i>Last drinking session (g kg⁻¹ 24 h⁻¹)</i>	
			<i>Binge (g kg⁻¹ 30 min⁻¹)</i>	
	Animal numbers N = 4	1 2 3 4	6.10 7.23 6.31 6.07	0.89 1.89 0.72 1.46
		Mean ± s.e.m.	6.43 ± 0.27	1.24 ± 0.27
Figure 1d and Supplementary Figure 5	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session (g kg⁻¹ 24 h⁻¹)</i>	
			<i>Vehicle group</i>	<i>Rapamycin group</i>
	Animal numbers N = 5	1 2 3 4 5	4.75 5.20 4.11 5.89 3.62	4.92 4.32 4.30 5.76 5.24
		Mean ± s.e.m.	4.71 ± 0.40	4.91 ± 0.28
Figures 2b and c	<i>Drinking paradigm</i> IA20%-2BC binge rat	<i>Rat number</i>	<i>Last drinking session (g kg⁻¹ 24 h⁻¹)</i>	
			<i>Binge (g kg⁻¹ 30 min⁻¹)</i>	
	Animal numbers N = 10	1 2 3 4 5 6 7 8 9 10	5.65 7.24 8.56 5.79 6.65 4.38 5.60 5.67 7.43 5.17	0.85 1.46 1.16 1.54 0.83 0.69 0.68 1.54 1.52 1.84
		Mean ± s.e.m.	6.21 ± 0.39	1.21 ± 0.13
Figures 2d and e	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session (g kg⁻¹ 24 h⁻¹)</i>	
	Animal numbers N = 10	1 2 3 4 5 6 7 8 9 10	5.00 4.51 5.58 6.51 4.59 7.22 4.45 3.90 4.70 3.47	
		Mean ± s.e.m.	4.99 ± 0.36	
Figures 3a and b	<i>Drinking paradigm</i> IA20%-2BC binge rat	<i>Rat number</i>	<i>Last drinking session (g kg⁻¹ 24 h⁻¹)</i>	
			<i>Binge (g kg⁻¹ 30 min⁻¹)</i>	
	Animal numbers N = 6	1 2 3 4 5 6	4.73 4.58 5.67 7.43 5.17 4.87	1.32 1.00 1.54 1.52 1.84 1.43
		Mean ± s.e.m.	5.41 ± 0.43	1.44 ± 0.11
Figures 3c and d	<i>Drinking paradigm</i> IA20%-2BC binge rat	<i>Rat number</i>	<i>Last drinking session (g kg⁻¹ 24 h⁻¹)</i>	
			<i>Binge (g kg⁻¹ 30 min⁻¹)</i>	
	Animal numbers N = 8	1 2 3 4 5 6 7 8	4.26 4.01 4.73 4.58 5.67 7.43 5.17 4.87	0.90 1.43 1.32 1.00 1.54 1.52 1.84 1.43
		Mean ± s.e.m.	5.09 ± 0.38	1.37 ± 0.11

Table 1. (Continued)

<i>Animals drinking data</i>				
Figure 3f and Supplementary Figure 9B	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)	
	Animal numbers N = 6	1	3.50	
		2	5.30	
		3	5.00	
		4	6.07	
		5	5.96	
		6	4.19	
		Mean ± s.e.m.	5.00 ± 0.41	
Figures 3h and i	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)	
	Animal numbers N = 8	1	3.07	
		2	6.78	
		3	4.84	
		4	3.03	
		5	6.37	
		6	3.12	
		7	6.10	
		8	3.84	
		Mean ± s.e.m.	4.64 ± 0.56	
Supplementary Figure 2A (except CRMP-2), 2C	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)	
	Animal numbers N = 4	1	5.84	
		2	6.15	
		3	6.93	
		4	6.47	
		Mean ± s.e.m.	6.35 ± 0.23	
Supplementary Figure 2A CRMP-2	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)	
	Animal numbers N = 7	1	5.28	
		2	3.10	
		3	3.43	
		4	5.84	
		5	6.15	
		6	6.93	
		7	6.47	
		Mean ± s.e.m.	5.23 ± 0.66	
Supplementary Figure 4D-E	<i>Drinking paradigm</i> IA20%-2BC binge rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)	<i>Binge</i> (g kg ⁻¹ 30 min ⁻¹)
	Animal numbers N = 6	1	6.05	1.01
		2	9.69	1.40
		3	3.54	0.93
		4	6.10	0.89
		5	7.23	1.89
		6	6.31	0.72
		Mean ± s.e.m.	6.49 ± 0.81	1.14 ± 0.18
Supplementary Figure 4F-G	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)	
	Animal numbers N = 6	1	5.28	
		2	3.10	
		3	3.43	
		4	5.84	
		5	6.15	
		6	6.93	
		Mean ± s.e.m.	5.12 ± 0.63	
Supplementary Figure 6A-B	<i>Drinking paradigm</i> IA20%-2BC binge rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)	<i>Binge</i> (g kg ⁻¹ 30 min ⁻¹)
	Animal numbers N = 6	1	5.65	0.85
		2	7.24	1.46
		3	8.56	1.16
		4	5.79	1.54
		5	6.65	0.83
		6	4.38	0.69
		Mean ± s.e.m.	6.38 ± 0.59	1.09 ± 0.14

Table 1. (Continued)

<i>Animals drinking data</i>			
Supplementary Figure 6C-D	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)
	Animal numbers N = 6	1 2 3 4 5 6	5.00 4.51 5.58 6.51 4.59 7.22
		Mean ± s.e.m.	6.57 ± 0.45
	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)
	Animal numbers N = 4	1 2 3 4	3.29 7.18 2.16 3.25
		Mean ± s.e.m.	3.97 ± 1.10
Supplementary Figure 7A	<i>Drinking paradigm</i> IA20%-2BC 1 week-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)
	Animal numbers N = 4	1 2 3 4	5.20 3.45 2.79 5.24
		Mean ± s.e.m.	4.17 ± 0.62
Supplementary Figure 7B-C	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)
	Animal numbers N = 3	1 2 3	5.00 4.51 5.58
		Mean ± s.e.m.	5.03 ± 0.31
Supplementary Figure 7D	<i>Drinking paradigm</i> C10%-2BC rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)
	Animal numbers N = 4	1 2 3 4	2.97 0.84 0.34 2.89
		Mean ± s.e.m.	1.76 ± 0.53
Supplementary Figure 8B-C	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal mouse	<i>Mouse Number</i>	<i>Last drinking session</i> (g kg ⁻¹ 24 h ⁻¹)
			<i>Vehicle group</i> <i>Rapamycin group</i>
	Animal numbers N = 5	1 2 3 4 5	11.11 10.38 13.00 11.50 11.56 9.41 12.03 10.43 8.18 10.37
		Mean ± s.e.m.	11.18 ± 0.81 10.42 ± 0.33
Supplementary Figure 9C	<i>Drinking paradigm</i> IA20%-2BC 24 h-withdrawal rat	<i>Rat number</i>	<i>Last drinking session</i> (g kg 24 h ⁻¹)
	Animal numbers N = 7	1 2 3 4 5 6 7	3.50 3.10 5.30 5.00 6.07 5.96 4.19
		Mean ± s.e.m.	4.73 ± 0.44

Individual alcohol drinking data in rodents used for biochemical experiments. Alcohol intake is expressed as mean ± s.e.m.

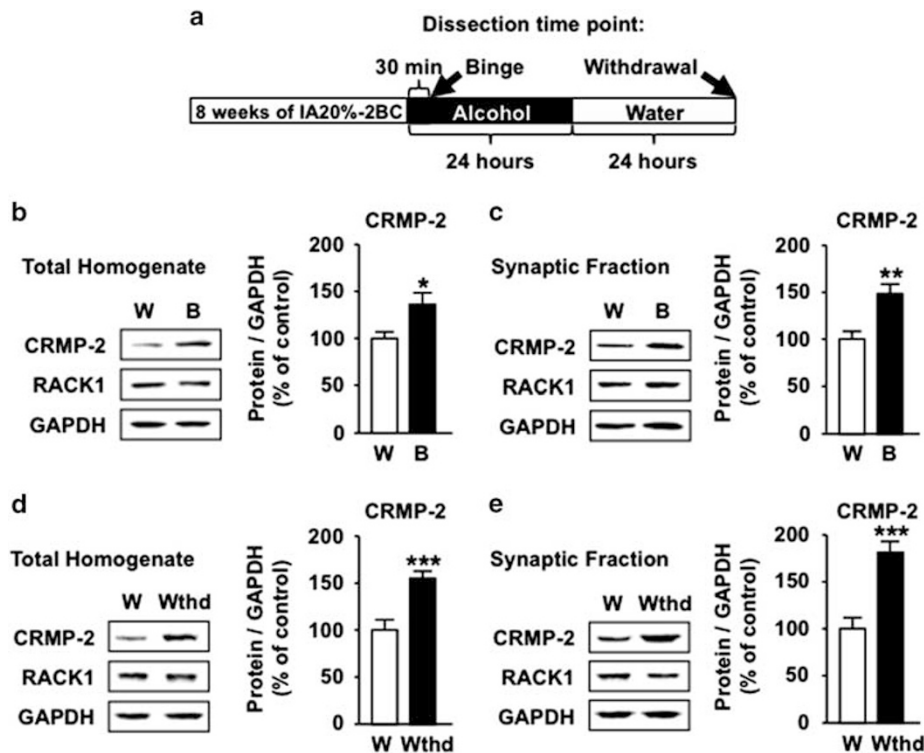


Figure 2. Binge drinking of alcohol and withdrawal increase CRMP-2 protein levels. (a) Timeline of experiment depicted in (b–e). The NAC was removed 30 min after the beginning (b and c, binge, B) or 24 h after the end of the last drinking session (d–e, withdrawal). Protein levels were determined by western blot analysis. ImageJ was used for optical density quantification. Data are expressed as the average ratio \pm s.e.m. of CRMP-2 or RACK1 to GAPDH, and are expressed as percentage of water (W) control. Significance was determined using two-tailed unpaired *t*-test. (b) Protein levels in the total homogenate of binge-drinking rats. CRMP-2, $t_{(18)} = 2.537$, $P = 0.02$. (c) Protein levels in the synaptic fraction of binge drinking rats. CRMP-2, $t_{(18)} = 3.596$, $P = 0.002$. (d) Protein levels in the total homogenate 24 h after withdrawal. CRMP-2, $t_{(18)} = 4.182$, $P < 0.001$. (e) Protein levels in the synaptic fraction 24 h after withdrawal. CRMP-2, $t_{(18)} = 4.887$, $P < 0.001$. $n = 10$ for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

a terminal oligopyrimidine-like mRNA, in an mTORC1-dependent manner.

Binge drinking of 20% alcohol and acute withdrawal increase CRMP-2 protein levels in the NAC

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,^{33,42,43} we measured the immunoreactivity of the protein in total homogenate as well as in crude synaptosomal fraction of rat NAC, which were dissected either at the end of last binge-drinking session or after 24 h of abstinence (timeline, Figure 2a). We found that the protein levels of CRMP-2, but not RACK1, were significantly increased in total homogenate and synaptic fraction in response to binge drinking of alcohol (Figures 2b and c and Supplementary Figure 6A and B, Table 1). CRMP-2 levels remained elevated after 24 h of withdrawal (Figures 2d and e and Supplementary Figure 6C and D, Table 1), but returned to basal levels after 1 week of withdrawal (Supplementary Figure 7A, Table 1). We did not detect alterations in CRMP-2 levels in response to alcohol intake in the motor cortex and cerebellum (Supplementary Figure 7B and C, Table 1). Furthermore, CRMP-2 levels in the NAC were unchanged in response to consumption of moderate amounts of 10% alcohol for 21 consecutive days (Supplementary Figure 7D, Table 1). Finally, similarly to the results in rats, excessive alcohol drinking led to an mTORC1-dependent increase in CRMP-2 protein levels in mouse NAC (Supplementary Figure 8, Table 1). Together, these results

suggest that repeated cycles of binge drinking and withdrawal promotes the translation of *CRMP-2* mRNA and that the change is specifically localized to the NAC.

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

CRMP-2 is a substrate of glycogen synthase kinase-3 β (GSK-3 β).⁴³ GSK-3 β activity is regulated by the serine and threonine kinase AKT.⁴⁴ Specifically, AKT phosphorylates GSK-3 β at Serine 9, which in turn inactivates the kinase.⁴⁴ Excessive alcohol intake activates AKT in the NAC of rodents.⁴⁵ Therefore, we hypothesized that the consequences of alcohol-mediated AKT activation are increased phosphorylation of GSK-3 β and a subsequent blockade of CRMP-2 phosphorylation at Threonine 514, a site that is specifically targeted by GSK-3 β .⁴³ As shown in Figures 3a and b, the phosphorylation of GSK-3 β 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 (Figures 3c and d, Table 1) and withdrawal (Supplementary Figure 9, Table 1).

Phosphorylation of CRMP-2 inhibits its binding to microtubules.^{27,46} Therefore, we tested whether excessive alcohol consumption alters the binding of CRMP-2 to microtubules. To do so, rat NAC was harvested 24 h after the last drinking session, total homogenates were incubated with pre-assembled microtubules, and microtubules-associated proteins were isolated. As shown in Figures 3e and f, alcohol drinking significantly increased the immunoreactivity of CRMP-2 in the microtubules-enriched

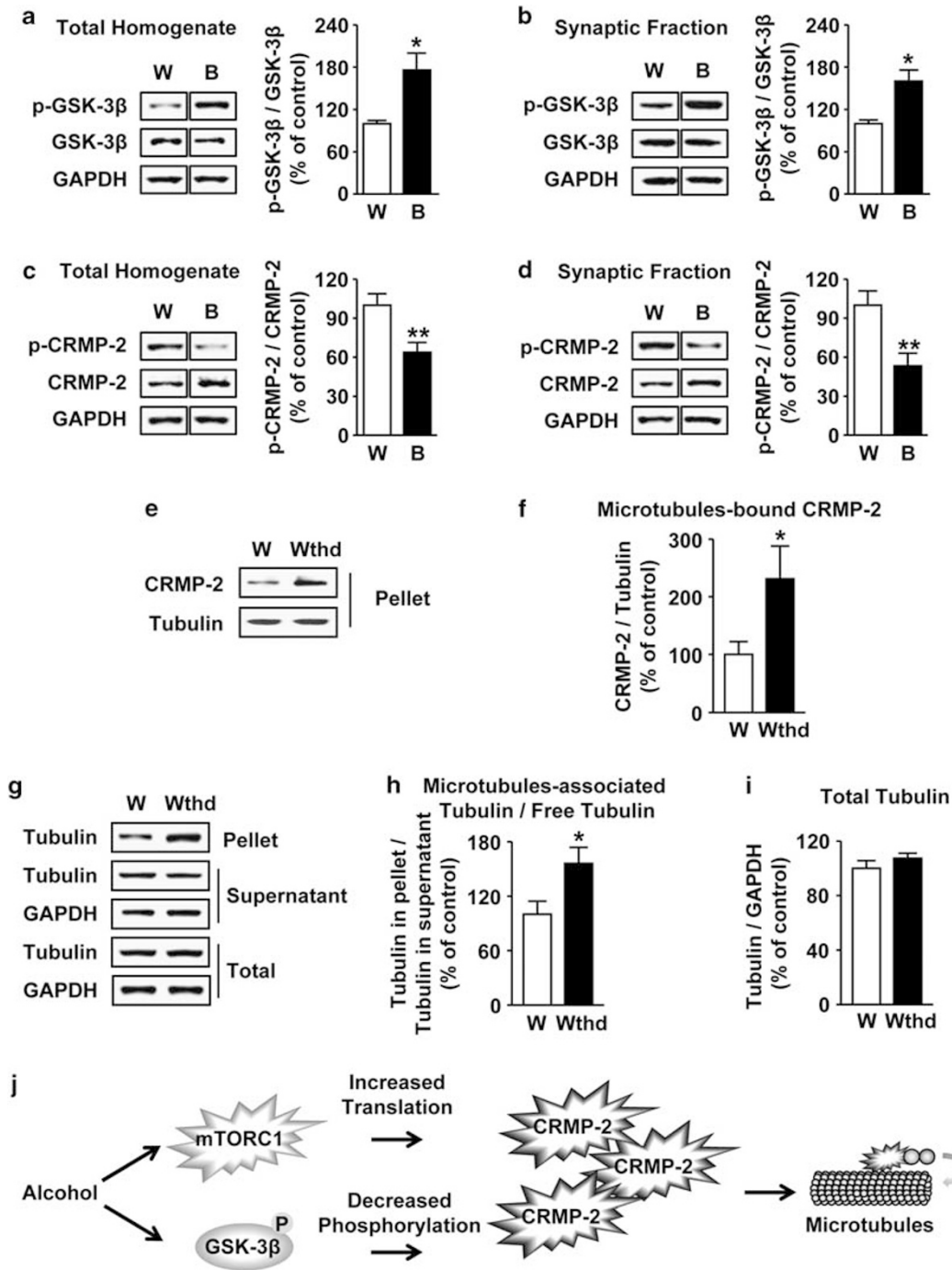


Figure 3. Binge drinking of alcohol blocks the phosphorylation of CRMP-2 and increases microtubule content. (a–i) Rats were trained to drink alcohol as described in Figures 1a. (a–d) The NAc was removed 30 min after the beginning of the last drinking session (binge, B). Phosphorylation of GSK-3β and CRMP-2 was determined by western blot analysis. Quantification was conducted as in Figure 2. The vertical lines indicate that separates groups were from the same gel but were not run in adjacent lines. Data are expressed as the average ratio of phospho-GSK-3β to total GSK-3β or phospho-CRMP-2 to total CRMP-2 ± s.e.m., and are expressed as percentage of water (W) control. Significance was determined using two-tailed unpaired *t*-test. (a) [³²P]GSK-3β phosphorylation in total homogenate, $t_{(10)} = 3.141$, $P = 0.011$. (b) [³²P]GSK-3β phosphorylation in the synaptic fraction, $t_{(10)} = 3.574$, $P = 0.005$. (c) [³²P]CRMP-2 phosphorylation in total homogenate, $t_{(14)} = 3.119$, $P = 0.008$. (d) [³²P]CRMP-2 phosphorylation in the synaptic fraction, $t_{(14)} = 3.18$, $P = 0.007$. (e–i) The NAc were removed 24 h 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) ± s.e.m. $t_{(11)} = 2.271$, $P = 0.044$. (g–i) Microtubule content assay. Tubulin and GAPDH levels in 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) ± s.e.m. $t_{(14)} = 2.42$, $P = 0.029$, and tubulin (total) to GAPDH (total), $t_{(14)} = 1.207$, $P = 0.248$, respectively. (j) Diagram of the proposed signaling cascade. Excessive drinking of alcohol leads to newly synthesized CRMP-2 in a hypophosphorylated state through the activation of mTORC1 and the inhibition of GSK-3β that in turn promotes microtubule assembly. (a, b) $n = 6$, (c–d, h–i) $n = 8$ for each group; (f) $n = 7$ water, $n = 6$ withdrawal. * $p < 0.05$, ** $p < 0.01$.

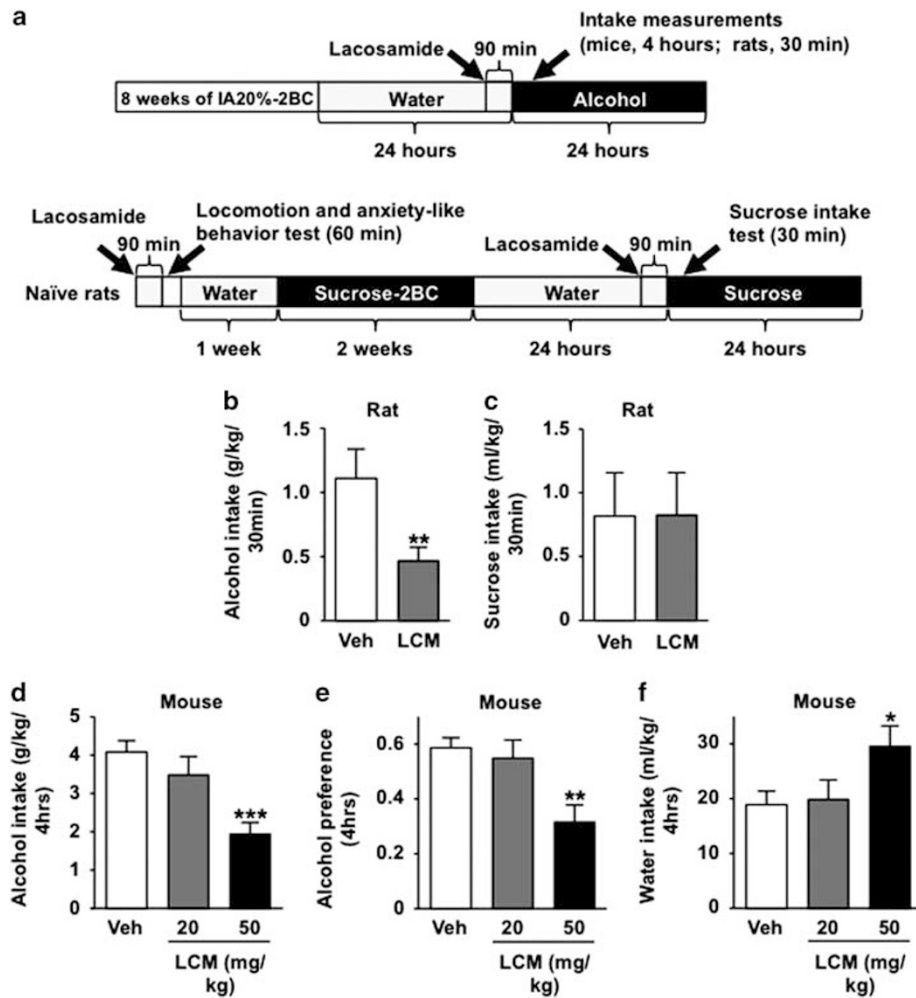


Figure 4. Systemic administration of the CRMP-2 inhibitor lacosamide decreases binge drinking of alcohol in rodents. **(a)** Timeline of experiments depicted in **(b–f)**. **(b)** Rats were subjected to IA20%-2BC for 2 months. Vehicle (Veh) or lacosamide (LCM, 20 mg kg⁻¹) was systemically administered 90 min before the beginning of a drinking session. Alcohol intake was measured 30 min after the beginning of a drinking session. Data are presented as mean ± s.e.m. Significance was determined using two-tailed paired *t*-test, $t_{(8)} = 4.176$, $P = 0.003$. **(c)** 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⁻¹) was systemically administered 90 min before the beginning of a drinking session. Sucrose intake was measured 30 min after the beginning of a drinking session. Data are presented as mean ± s.e.m. Significance was determined using two-tailed paired *t*-test, $t_{(9)} = -0.059$, $P = 0.954$. **(d–f)** Mice experienced 2 months of IA20%-2BC-drinking paradigm. Vehicle (Veh) or lacosamide (LCM, 20 or 50 mg kg⁻¹) were systemically administered 90 min before the beginning of a drinking session. Alcohol intake **(d)**, alcohol preference **(e)** and water intake **(f)** were measured at the end of a 4 h drinking session. Data are presented as mean ± s.e.m. Significance was determined using one-way RM-ANOVA and *post hoc* Student–Newman–Keuls test. **(d)** 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, $q = 6.081$, $P = 0.001$. **(e)** Alcohol preference. 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, $q = 5.453$, $P = 0.003$. **(f)** 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, $q = 3.627$, $P = 0.047$. **(b)** $n = 9$, **(c)** $n = 10$, **(d–f)** $n = 11$ for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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 tested whether excessive alcohol intake increases microtubule content by promoting its assembly. As shown in Figures 3g and h, alcohol significantly increased the ratio of microtubules-associated tubulin to free tubulin. In contrast, the levels of total tubulin were unaltered by alcohol (Figures 3g and i, Table 1). Together, these findings suggest that excessive alcohol drinking blocks the phosphorylation of CRMP-2 and promotes newly synthesized, hypophosphorylated, CRMP-2 binding to microtubules, resulting in microtubule assembly (Figure 3j).

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

Microtubules maintain the morphology of neurons and have an important role in structural plasticity.^{47,48} Structural plasticity, which involves the formation, stabilization and elimination of synapses,⁴⁹ is linked to compulsive drug taking and seeking.⁵⁰ 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,^{51,52} and tested whether lacosamide reduces excessive alcohol

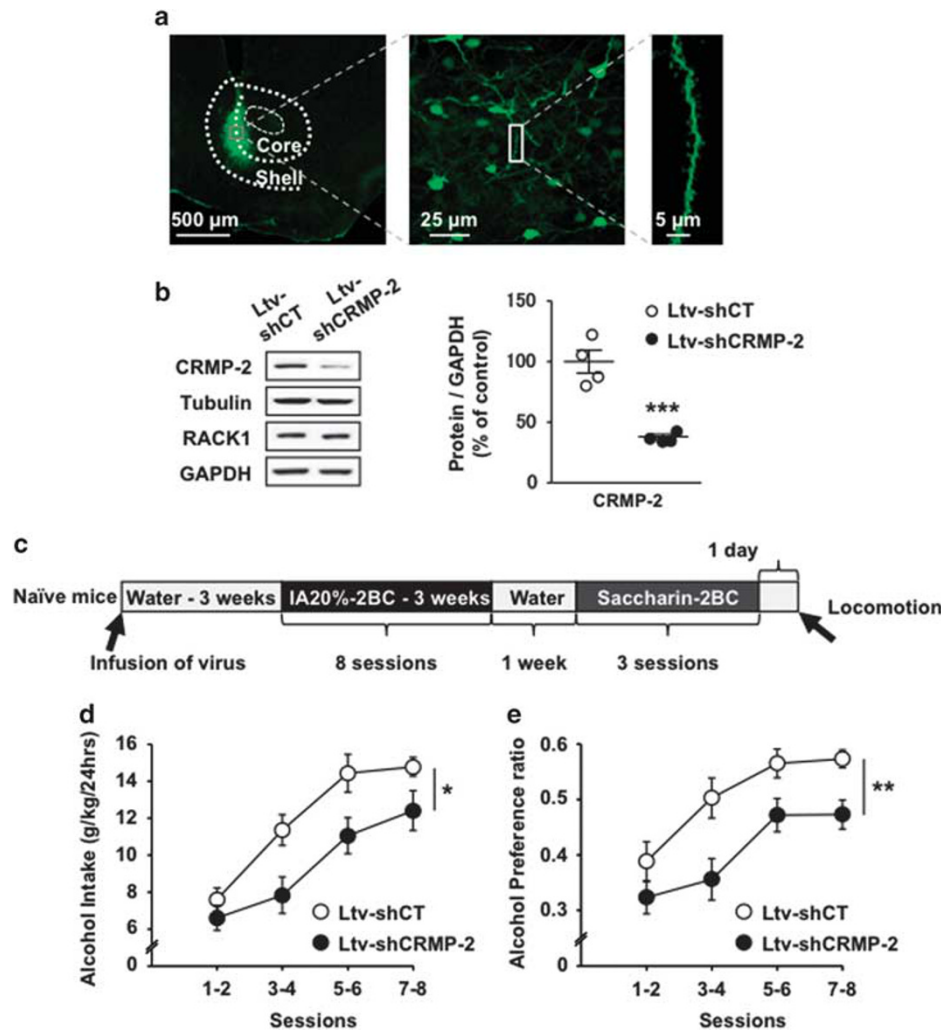


Figure 5. Knockdown of CRMP-2 in the mouse NAc decreases excessive drinking of alcohol. (a–e) A lentivirus expressing non-specific control shRNA (Ltv-shCT) (2×10^7 pg ml⁻¹) or shRNA-targeting mouse CRMP-2 (Ltv-shCRMP-2) (2×10^7 pg ml⁻¹) was infused bilaterally into the mouse NAc (1.2 μl per side). After 3 weeks of recovery, mice underwent IA20%-2BC drinking procedure for eight sessions. The NAc was dissected at the end of behavioral experiment and used for immunohistochemistry (a) and western blot analysis (b). (a) Ltv-shCRMP-2 infects NAc neurons. Slices were stained with anti-GFP antibodies. Left panel ($\times 5$) depicts the specificity of the site of virus infection. Scale bar, 500 μm . Middle image ($\times 40$) depicts Ltv-shCRMP-2 infection of neurons. Scale bar, 25 μm . Right panel ($\times 63$) 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, histogram depicts the ratio of CRMP-2 to GAPDH levels. Significance was determined using two-tailed unpaired *t*-test, $t_{(6)} = 6.414$, $P = 0.0007$. (c) Timeline of experiments depicted in (d) and (e). Alcohol intake (d) and alcohol preference (e) were measured after each 24 h drinking session and expressed as an average of every two drinking sessions. (d) 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 ($F_{(3,60)} = 1.969$, $P = 0.128$). (e) 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 ($F_{(3,60)} = 1.344$, $P = 0.269$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (b) $n = 4$, (d and e) $n = 11$ for each group.

consumption in rodents (timeline, Figure 4a). As shown in Figure 4b (rats) and 4D-F (mice), systemic administration of lacosamide significantly decreased binge drinking of alcohol, alcohol preference, but did not reduce the consumption of water. Systemic administration of lacosamide did not affect rat sucrose consumption (Figure 4c), suggesting that the drug does not alter general reward. In addition, to rule out putative locomotor or anxiolytic effects of lacosamide,⁵³ we tested the effect of the drug on spontaneous locomotion and basal anxiety-like behavior (Supplementary Figure 10). Vehicle and lacosamide-treated rats did not exhibit significant differences in distance traveled

(Supplementary Figure 10) or time spent in the center of the chamber (Supplementary Figure 10). 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 11A). Furthermore, the knockdown of CRMP-2 was restricted to the NAc, as no change in CRMP-2 protein level was observed in the neighboring dorsal striatum (Supplementary Figure 11B).

Next, we measured the levels of voluntary alcohol intake of mice infected with Ltv-shCRMP-2 or a lentivirus expressing a non-specific short hairpin control sequence (Ltv-shCT) (Timeline, Figure 5c). We found that knockdown of CRMP-2 in the NAc resulted in a significant decrease in excessive alcohol intake (Figure 5d) and preference (Figure 5e) as compared with Ltv-shCT-infected mice. Water intake was increased after knockdown of CRMP-2 in the NAc, which is likely a countervailing effect (Supplementary Figure 12A). Knockdown of CRMP-2 in mouse NAc did not change the consumption of another rewarding substance, saccharin (Supplementary Figure 12B), and locomotor activity was also unaltered (Supplementary Figure 12C). Together, these results indicate that CRMP-2 in the NAc has 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-3 β leads to an increase in CRMP-2 in its hypophosphorylated form. Thus, mTORC1 activation and GSK-3 β inhibition in the NAc converge on CRMP-2 resulting in its association with microtubules, which in turn increases microtubule content (Supplementary Figure 13). 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,^{3,54} 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.*⁵⁵ reported that Arc in the amygdala controls alcohol intake in rats; 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 has a role in alcohol seeking and drinking.⁵⁶ In addition, Easton *et al.*⁵⁷ recently reported that CaMKII α autophosphorylation-deficient mice show reduced alcohol preference compared with wild-type mice. Homer2 protein levels in the NAc have also been shown to be induced by alcohol and to contribute to excessive alcohol consumption.⁵⁸ Furthermore, PSD-95 knockout mice exhibit hypersensitivity to alcohol intoxication and show decreased voluntary alcohol intake.⁵⁹ 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*, *CaMKII α* , *CRMP-2*, *Homer2* and *PSD-95* was still maintained after 24 h 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 h of alcohol withdrawal.¹³ These findings raise the possibility that the initiation of alcohol-induced mTORC1-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 short withdrawal period. We further observed that CRMP-2 levels returned to baseline levels after 1 week of withdrawal. It is plausible that downstream effectors of CRMP-2 are participating in the neuroadaptations that are observed during prolonged period of abstinence from alcohol drinking. Importantly, the increase in CRMP-2 levels were dependent on the concentration of alcohol as the increases were detected in response to a history of drinking of intermittent access to 20% alcohol, a paradigm that mimics excessive drinking of alcohol in humans,³⁸ but not after continuous access to 10% alcohol that results in moderate levels of drinking. These results are likely to reflect the notion that the mechanisms underlying moderate and excessive drinking of alcohol are different.⁶⁰ Finally, alcohol-mediated increase in CRMP-2 expression is brain region specific as CRMP-2 levels were elevated in the NAc but not in the motor cortex or cerebellum, possibly because both regions are not directly related to the reward pathway.

CRMP-2 is a substrate of GSK-3 β ,⁴³ which is a direct downstream substrate of AKT⁴⁴ (Supplementary Figure 13). Phosphorylation of GSK-3 β inhibits its enzymatic activity⁴⁴ and we show GSK-3 β is phosphorylated by AKT after binge drinking of alcohol (as shown herein) and withdrawal.⁴⁵ We found that in the NAc, basal levels of GSK-3 β phosphorylation are low, suggesting that GSK-3 β is constitutively active. In contrast, CRMP-2 phosphorylation is high in the NAc of water-consuming animals, indicating that constitutively active GSK-3 β phosphorylates CRMP-2. Importantly, alcohol-mediated GSK-3 β 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, which was reported to dephosphorylate CRMP-2 at Threonine 514.⁶¹ Another potential candidate is protein phosphatase 1, which dephosphorylates CRMP-2 at Threonine 514 when GSK-3 β activity is inhibited.⁶² 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.⁴⁸ CRMP-2 is a microtubules-associated protein that stabilizes microtubules and promotes neurite outgrowth,^{27,63} which further provides the basis for neuronal morphology and structural plasticity.^{48,64,65} Our results suggest that the functional consequences of alcohol-mediated increases in CRMP-2 protein levels and 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 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. The relationship between alcohol exposure and structural plasticity in the striatum has been recently reported. Specifically, DePoy *et al.*⁶⁶ showed that chronic intermittent alcohol vapor exposure results in neuronal dendritic hypertrophy

in the dorsolateral striatum, and we recently found that excessive drinking of alcohol leads to changes in dendrite morphology in dopamine D1 receptor neurons in the dorsomedial striatum of mice.⁶⁷ It would therefore be of interest to determine whether CRMP-2-mediated changes in microtubule content contribute to these or others synaptic morphology changes induced by alcohol.

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.^{51,52} However, lacosamide has also been shown to increase slow inactivation of voltage-gated sodium channels.⁶⁸ Thus, we cannot rule out the possibility that lacosamide decreases excessive drinking of alcohol, at least in part, through other mechanisms. Unfortunately, we could not examine alcohol induction of microtubule assembly after knockdown of CRMP-2 or lacosamide administration, as we could not differentiate between changes in microtubule assembly that are due to the treatment *per se* or that are due to the reduction in alcohol intake. Nevertheless, lacosamide, an FDA-approved drug used for the treatment of seizures.⁶⁹ Thus, this drug and possibly other CRMP-2-microtubule inhibitors represent a promising strategy for the treatment of alcohol-related disorders.

The raw data can be found at <https://portal.g-node.org/data/>. Readers can access the data by creating an account and searching for "Liu".

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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