Role for mammalian target of rapamycin complex 1 signaling in neuroadaptations underlying alcohol-related disorders.

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The Mammalian Target of Rapamycin:

A New Target for Alcohol Use Disorders

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Abstract

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that has in recent years been implicated in long-lasting forms of synaptic plasticity, the basis of learning and memory. Aberrant synaptic plasticity plays a major role in neuroadaptations underlying addiction to drugs of abuse and alcohol. Here we show that alcohol consumption results in the activation of the mTOR signaling pathway in the nucleus accumbens (NAc), a brain region involved in reward. Importantly, systemic or intra-NAc administration of the mTOR inhibitor rapamycin, selectively attenuates alcohol-drinking and -seeking behaviors. Together, our results put forward mTOR within the reward pathway as a novel contributor to molecular mechanisms underlying alcohol-drinking behaviors, and as a new target to treat alcohol use disorders.

The serine-threonine kinase mTOR signals through two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (*1-2*), and is a focal point in cell signaling integrating and responding to extracellular stimuli such as nutrients, stress, hormones or mitogens (*1, 3*). mTORC1, unlike mTORC2, is sensitive to the acute treatment of the inhibitor rapamycin (*2*). mTORC1 is best characterized for its ability to control the translation initiation of a subset of mRNAs via the phosphorylation and activation of the p70 ribosomal S6 kinase (S6K), and the phosphorylation of the eukaryotic translation initiation factor-4E binding protein (4E-BP) (*1, 3-4*).

In the central nervous system, long-lasting forms of synaptic plasticity and memory depend on new protein synthesis (*4-5*), specifically at dendrites (*6*) where mTORC1 and its downstream effectors S6K and 4E-BP are localized (*7-8*). The mTORC1-mediated local protein translation is critical for stabilizing plastic changes occurring at synapses (*9-10*). In addition, the mTORC1 pathway controls soma and dendritic morphology (*11*), long-term potentiation (LTP) and depression (LTD) (*4, 12*). Finally, the mTORC1 signaling pathway has been shown to contribute to memory formation, consolidation and reconsolidation (*4, 13*), as well as memory deficits (*14*).

Drug and alcohol addiction is a disorder that shares striking molecular similarities with the neural mechanisms underlying learning and memory (*15*), particularly in the mesocorticolimbic circuit where plasticity induced by drugs of abuse is well documented (*15-17*). Addiction is a chronically relapsing psychiatric disease characterized by compulsion to seek and consume drugs, loss of control and emergence of a negative emotional state when access to drug is withdrawn (*17*). Despite its massive health and

socioeconomic impact on the world population (*18-19*), molecular targets and pharmacotherapies for the treatment of drug and alcohol addiction are still limited.

Since mTORC1 plays a major role in neural plasticity, we hypothesized that this complex contributes to neuroadaptations within the mesolimbic system that result in excessive drug intake. We therefore tested the contribution of mTORC1 to alcohol consumption using rodent models of alcohol-drinking behaviors. First, we examined whether acute administration of alcohol alters the mTORC1-mediated signaling pathway in the NAc, a brain region within the mesolimbic system that is a critical component of the circuitry underlying reward and addiction (*16, 20*). To do so, mice were systemically (intraperitoneally, i.p.) administered with a non-hypnotic dose of alcohol (2.5 g/kg), and the phosphorylation levels of the mTORC1 substrates, S6K and 4E-BP, were measured in the NAc 30 min after treatment. We found that acute *in vivo* exposure of mice to alcohol significantly increased the phosphorylation level of both proteins (Fig. 1A) indicating that alcohol exposure triggers the activation of the mTORC1 signaling pathway in the NAc.

Next, we assessed whether the mTORC1 pathway was activated in the NAc in rodent models of excessive drinking of alcohol. Recurring alcohol consumption and withdrawal periods trigger seeking and excessive drinking-behaviors (*19, 21*), and a similar paradigm was used in mice and rats resulting in voluntary alcohol consumption that generated blood alcohol concentrations which correspond to human binge-drinking as defined by the National Institute on Alcohol Abuse and Alcoholism (*22-23*). Mice and rats were therefore trained to drink large quantities of alcohol (*23*), and activation of the mTORC1 signaling pathway in the NAc was determined. We found that binge-drinking of alcohol results in the activation of the mTORC1-mediated signaling cascade as

reflected by a higher phosphorylation-state of 4E-BP in the NAc of mice (fig. S1), and rats (Fig. 1B), as well as the phosphorylation of S6K in rats (Fig. 1B). Interestingly, the phosphorylation levels of 4E-BP and S6K proteins in the rat NAc were also enhanced after 24 hrs of alcohol withdrawal (Fig. 1C), suggesting a long-lasting activation of the mTORC1 signaling pathway following repeated exposure to alcohol. We therefore hypothesized that activation of mTORC1 signaling within the NAc is necessary for the maintenance of neuroadaptations contributing to excessive alcohol intake.

To test this possibility, we used the mTORC1 inhibitor rapamycin, an FDA-approved drug used for the prevention of host-rejection of organ transplants (*24*). Rapamycin was administered to binge-drinking mice 3 hrs before the beginning of an alcohol-intake session (fig. S2). We found that systemic administration of rapamycin dose-dependently reduced excessive alcohol drinking (Fig. 2A and figs. S3A-C) without affecting water intake (Fig. 2B and fig. S3D). Acute administration of a high dose (40 mg/kg) of rapamycin does not alter locomotor activity in mice (*13*), and we observed that rapamycin did not affect mice motor coordination in the absence or presence of alcohol (fig. S4). Thus, it is unlikely that the reduction in alcohol consumption induced by rapamycin was due to a nonspecific effect of the drug such as general decrease in fluid intake or an alteration in locomotor activity. Next, we tested for the contribution of mTORC1 activity within the mesolimbic system to excessive voluntary alcohol intake in rats (fig. S5). We found that intra-NAc infusion of rapamycin 3 hrs before the beginning of the session significantly decreased alcohol (Fig. 3A) but not water (Fig. 3B) intake over a period of 24-hr access. Notably, we also observed that inhibiting mTORC1 in the NAc was effective in reducing both binge drinking occurring during the first 30 min of

the session (Fig. 3C), as well as alcohol intake during the rest of the drinking period (i.e. 30 min-24 hrs) (fig. S6). These data show that in the NAc, mTORC1 is implicated not only in the first bout of excessive alcohol intake but also in the sustained consumption of alcohol.

To gain insight into the behavioral processes underlying the decrease in alcohol intake by rapamycin, we examined the effect of systemically administered rapamycin on the motivation of rats to consume alcohol. For this purpose, rats with a history of excessive alcohol consumption were trained to self-administer alcohol in an operant procedure on a fixed-ratio 3 schedule in which 3 lever-presses result in the delivery of 0.1ml of a 20% alcohol solution (*25*). Consistent with our previous results, systemic rapamycin treatment significantly reduced operant responding for alcohol (Fig. 4A), which was accompanied by a large decrease (more than 50%) in the amount of alcohol consumed by rats during the 30-min session (Fig. 4B). Because mTORC1 plays a key role in learning and memory (*4*), the reduction in operant self-administration in response to rapamycin could be a consequence of deficits in memory processes associated with lever pressing. However, rapamycin-treated rats were still able to discriminate between the lever delivering alcohol and the inactive lever suggesting an absence of such memory deficits (Fig. 4A). Moreover, the effect of rapamycin on alcohol self-administration cannot be attributed to a nonspecific alteration in locomotion since acute administration of rapamycin to rats did not affect their locomotor activity in an open-field (fig. S7). Importantly, we observed that rapamycin reduced the high rate of lever presses occurring at the beginning of the self-administration session (Fig. 4C), suggesting that rapamycin decreased the motivation to seek alcohol. To test this possibility, instrumental

performance was analyzed during extinction where alcohol was not delivered upon lever-pressing. As shown in Fig. 4D, rapamycin administration decreased alcohol-seeking in rats as reflected by a reduction of the number of presses on the alcohol lever during an extinction session. Finally, to determine if the effect of rapamycin is specific to alcohol or could be generalized to other reinforcing substances, we examined the ability of rapamycin to modulate the self-administration of sucrose, a non-drug reinforcer. We observed that systemic rapamycin administration did not affect lever-press responding for sucrose (Fig. 4E). This result further confirms that the decrease in alcohol self-administration induced by rapamycin was not due to a general locomotor or memory deficit. It also indicates that the effect of rapamycin on operant self-administration is selective for alcohol and does not reflect a general attenuation of motivation to obtain a reward. Taken together, our data show that in rats with a history of excessive alcohol consumption, rapamycin selectively decreases alcohol intake by reducing their motivation to specifically seek alcohol.

Overall, the present work shows that mTORC1 positively regulates alcohol-seeking and excessive alcohol consumption. The molecular mechanism by which mTORC1 contributes to neuroadaptations that underlie alcohol-drinking and -seeking behaviors remains to be determined. Nevertheless, we found that both mTORC1 substrates S6K and 4E-BP are phosphorylated after 24 hrs of alcohol deprivation in the NAc of rats with a history of high level of alcohol intake. Since both of these proteins are part of the mTORC1-mediated translation machinery (*1, 3*), the kinase is likely to regulate alcohol intake, at least in part, via the translation of proteins whose expression is

necessary for the maintenance of mechanisms underlying alcohol drinking behaviors in the mesolimbic system.

In summary, we present data to suggest that alcohol exposure induces a long-lasting activation of the mTORC1 signaling pathway in the NAc that contributes to mechanisms underlying excessive alcohol drinking. Our results further document that the FDA-approved drug rapamycin decreases excessive alcohol intake as well as the motivation to seek alcohol in pre-clinical rodent models of alcohol abuse. Moreover, this effect of rapamycin is specific for alcohol and does not reflect a non-selective inhibition of the reward and motivational system, which is a critical point from a therapeutic perspective (*26*). Importantly, we found that rapamycin attenuates "binge-like" alcohol drinking behavior that is likely to result from a decrease in the motivation to seek alcohol. This last finding is of particular interest because binge-drinking is an increasingly prevalent problem during adolescence and young adulthood and is a strong predictor of future alcohol-related problems (*27-28*). Our findings are also particularly relevant for the treatment of alcoholism where the pattern of drug consumption is often characterized by successive cycles of daily episodes of binge drinking and withdrawal (*21*). In addition, lapse and relapse to alcohol drinking, which result in excessive alcohol consumption, are fundamental problems in alcoholism treatment. Our data therefore put forward the possibility that targeting the mTORC1 signaling cascade as an innovative and valuable strategy for the treatment of alcohol use and abuse disorders.

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Figure legends:

Figure 1: Alcohol exposure activates the mTORC1 signaling pathway in the NAc of rodents. (**A)** Mice were systemically treated i.p. with 2.5g/kg of alcohol or saline, and the NAc was removed 30 min later. (**B)** and (**C)** Rats experienced at least 3 months of intermittent-access 20% alcohol two-bottle-choice drinking sessions (*23*). Control rats had access to only water for the duration of the experiments. (**B)** After the last 24 hrs of alcohol deprivation, rats had access to a 20% solution of alcohol for 30 min leading to an average intake of 1.16 ± 0.06 g/kg, and the NAc were immediately removed. (**C)** The NAc were removed after the last 24 hrs of alcohol deprivation session. The level of S6K and 4E-BP phosphorylation was determined by western-blot analysis as described in supporting online material. Optical density quantification of S6K and 4E-BP phosphorylation is expressed as the ratio of the phosphorylated protein to the total protein. (**A**), n=6 per group. (**B**) and (**C**), n=9 per group. Data are represented as mean ± S.E.M. and expressed as percentage of control. Unpaired t-test, *$p<0.05$, **$p<0.01$.

Figure 2: Systemic administration of rapamycin in mice dose-dependently reduces alcohol intake. (**A**) Mice had access to a 20% solution of alcohol for 4 hrs every other day for 3 weeks (*23*). Three hrs before the tenth 4 hr-alcohol-drinking session, mice were treated with vehicle (Veh), or 1, 5, 10 or 20 mg/kg of rapamycin. Alcohol intake was measured at the end of the 4 hr-drinking session. (**B**) After 2 weeks without access to alcohol, mice were systemically administrated with 10 mg/kg of rapamycin or vehicle 3 hrs before the beginning of a water-drinking session. Water intake was measured 4 hrs

later. (**A**), n=26 for the vehicle group and n=11-12 for the rapamycin groups. (**B**), n=11-12 per group. Data are represented as mean ± S.E.M. and expressed as percentage of vehicle. One-way ANOVA (Newman-Keuls post-hoc test), *p<0.05, **p<0.01, ***p<0.001 compared to the vehicle group and #p<0.05 (1 mg/kg vs. 20 mg/kg).

Figure 3: Intra-NAc infusion of rapamycin reduces alcohol drinking in rats. Vehicle (Veh) or 0.005, 0.5, 5 or 50 ng/side of rapamycin were infused into the NAc 3 hrs before the beginning of the 24-hr alcohol-drinking session in rats trained to consume a large amount of a 20% solution of alcohol in a two-bottle choice paradigm. Alcohol (**A**) and water (**B**) intake were measured at the end of the 24-hr session. (**C**) Alcohol intake after the first 30 min of the session was also measured. Alcohol and water consumptions are expressed in grams per kilogram (g/kg) of body weight. (**A-C**), n=9 per group. Data are represented as mean ± S.E.M. One-way ANOVA with repeated measures followed by a Newman-Keuls post-hoc test *p<0.05, **p<0.01 compared to vehicle.

Figure 4: Systemic administration of rapamycin selectively decreases operant alcohol self-administration-related behaviors. (**A-D**) Rats with a history of high level of alcohol consumption were trained to self-administer a solution of 20% alcohol as described in supporting online material. Three hrs before the beginning of a 30-min session, rats were administered (i.p.) with 10 mg/kg of rapamycin or vehicle (Veh). (**A)** Number of lever presses during the 30-min operant alcohol self-administration session. (**B)** Amount of alcohol consumed during the session. (**C**) Cumulative mean presses in bins of 2 min, indicative of the rate of presses for alcohol during the session**.** (**D)** Number of presses on

the alcohol lever during a 30-min extinction session. (**E)** Three hrs before the beginning of a 30-min session, rats were systemically treated with 10 mg/kg of rapamycin or vehicle (Veh) and the number of presses on the sucrose lever in rats trained to self-administer a solution of 1.5% sucrose was recorded. (**A-E**), n=7 per group. Data are represented as mean ± S.E.M. (**A**), **$p<0.01$ compared to vehicle. (**B**) and (**D**), *$p<0.05$ compared to vehicle. (**C**), all p's<0.05 compared to vehicle for all time intervals. For detailed statistical analyses, see the full figure legend in the supporting online material.

Fig. 1

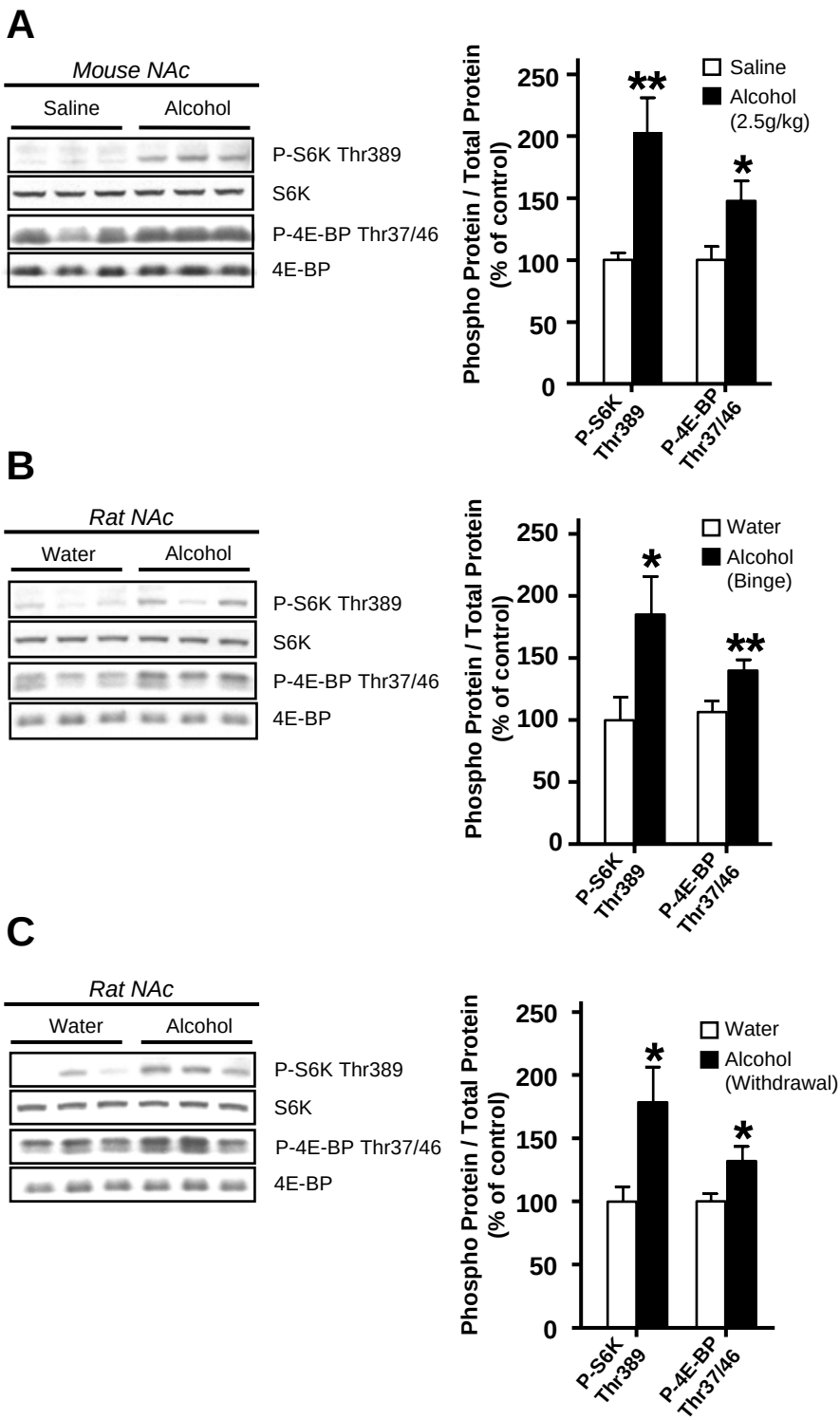

A
Mouse NAc
Saline
Alcohol
P-S6K Thr389
S6K
P-4E-BP Thr37/46
4E-BP
Phospho Protein / Total Protein (% of control)
Saline
Alcohol (2.5g/kg)
P-S6K Thr389
P-4E-BP Thr37/46
B
Rat NAc
Water
Alcohol
P-S6K Thr389
S6K
P-4E-BP Thr37/46
4E-BP
Phospho Protein / Total Protein (% of control)
Water
Alcohol (Binge)
P-S6K Thr389
P-4E-BP Thr37/46
C
Rat NAc
Water
Alcohol
P-S6K Thr389
S6K
P-4E-BP Thr37/46
4E-BP
Phospho Protein / Total Protein (% of control)
Water
Alcohol (Withdrawal)
P-S6K Thr389
P-4E-BP Thr37/46

Fig. 2

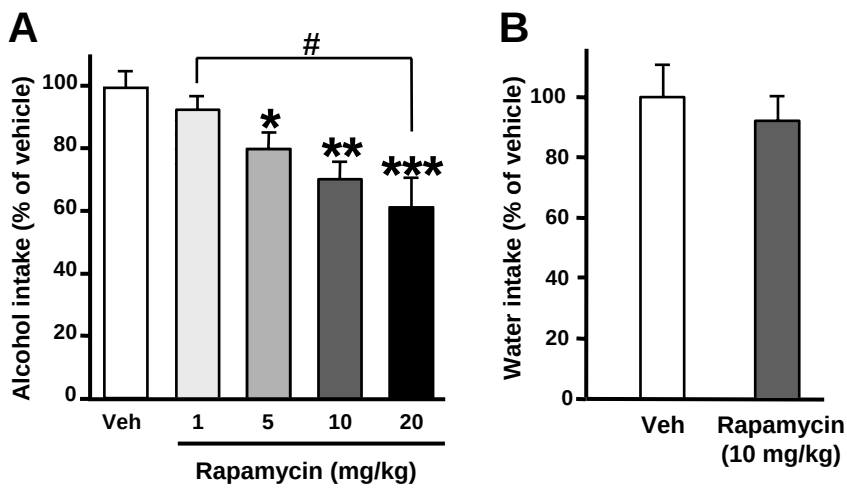

A
B
#
*
**
***
Alcohol intake (% of vehicle)
Water intake (% of vehicle)
100
80
60
40
20
0
Veh
1
5
10
20
Rapamycin (mg/kg)
Rapamycin
(10 mg/kg)

Fig. 3

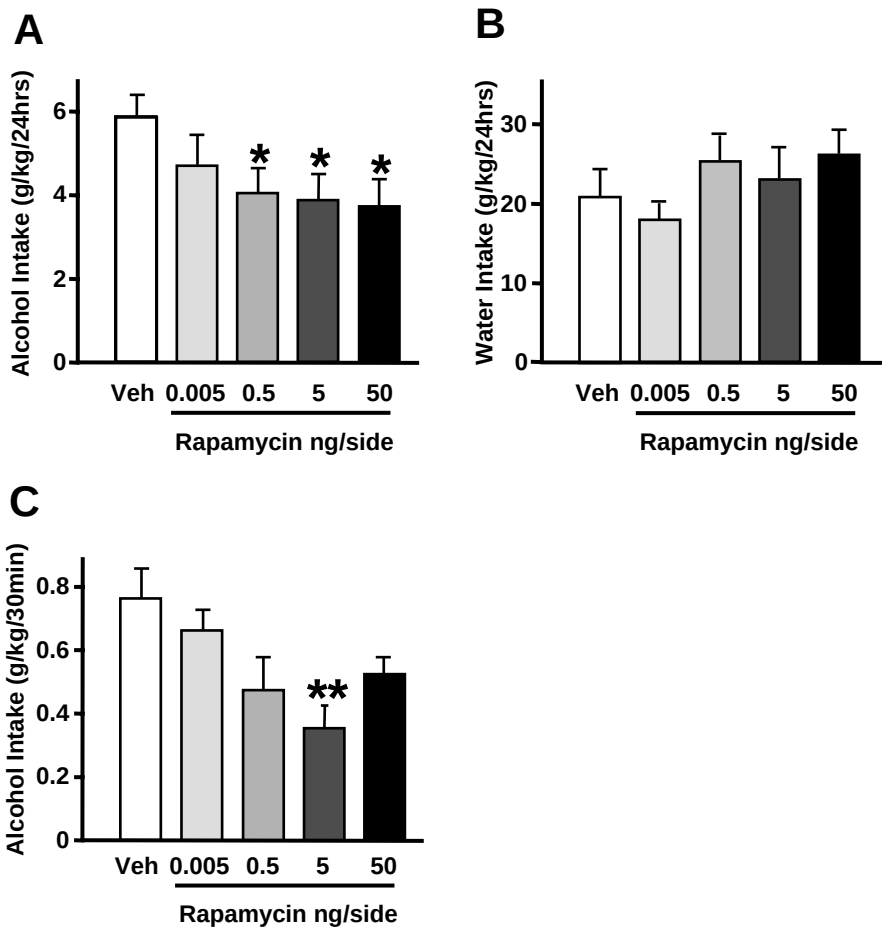
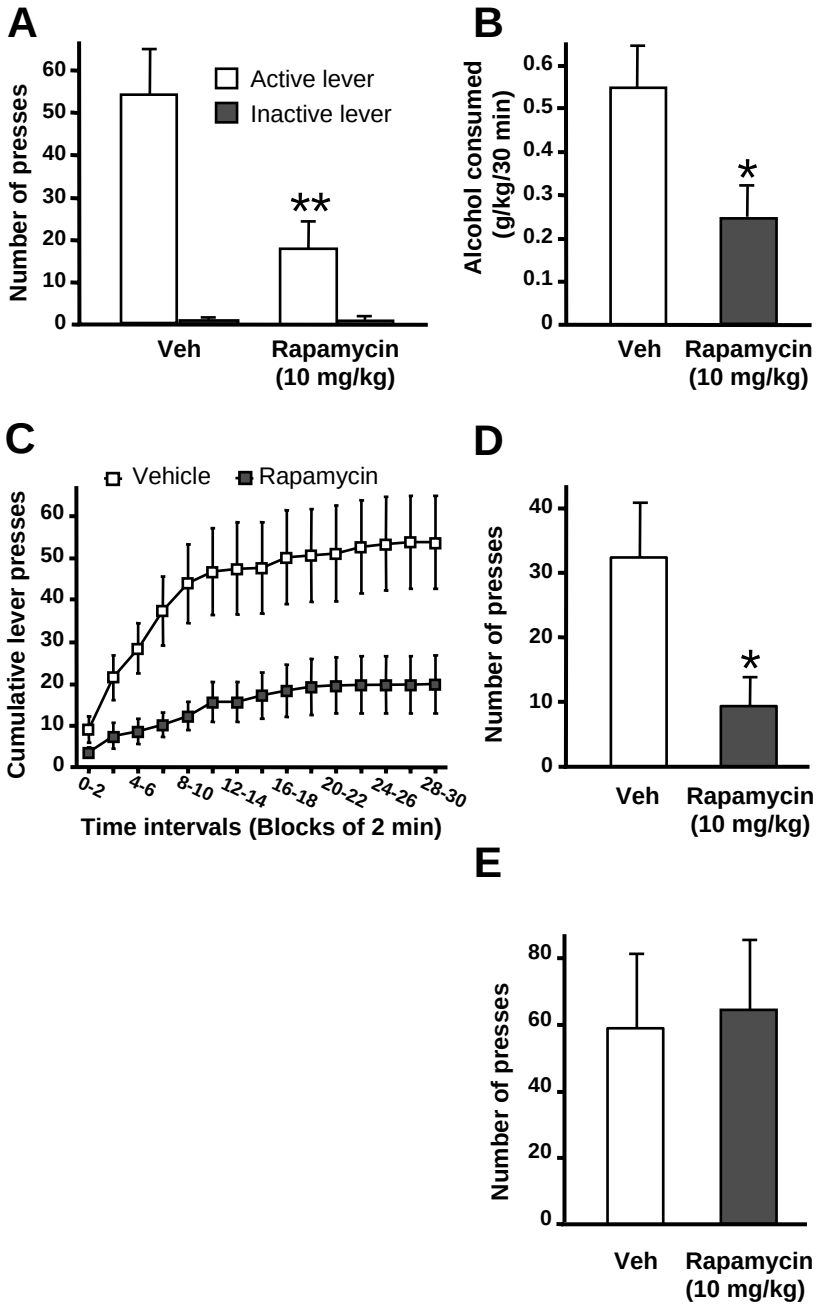

A
Alcohol Intake (g/kg/24hrs)
0
2
4
6
*
*
*
Veh
0.005
0.5
5
50
Rapamycin ng/side
B
Water Intake (g/kg/24hrs)
0
10
20
30
Veh
0.005
0.5
5
50
Rapamycin ng/side
C
Alcohol Intake (g/kg/30min)
0
0.2
0.4
0.6
0.8
**
Veh
0.005
0.5
5
50
Rapamycin ng/side

Fig. 4


A
Number of presses
Active lever
Inactive lever
**
Veh
Rapamycin (10 mg/kg)
B
Alcohol consumed (g/kg/30 min)
*
Veh
Rapamycin (10 mg/kg)
C
Vehicle
Rapamycin
Cumulative lever presses
0-2
4-6
8-10
12-14
16-18
20-22
24-26
28-30
Time intervals (Blocks of 2 min)
D
Number of presses
*
Veh
Rapamycin (10 mg/kg)
E
Number of presses
Veh
Rapamycin (10 mg/kg)

Supporting Online Material for

The Mammalian Target of Rapamycin:

A New Target for Alcohol Use Disorders

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-Supporting text: detailed Fig.4 legend including statistics

-References for materials and methods

-Supplemental figures S1-8

MATERIALS AND METHODS

Materials

The following antibodies were purchased form Cell Signaling Technology: Phospho-S6K Thr389 (#9234), Phospho-4E-BP Thr37/46 (#2855), total S6K (#2708), total 4E-BP2 (#2845, 4E-BP2 is the main 4E-BP isoform in the brain (*1-2*)). Secondary anti-rabbit IgG-horseradish peroxidase (HRP) conjugated antibody (sc-2313) was purchased from Santa Cruz Biotechnology. EDTA-free Complete mini Protease inhibitors Cocktail were purchased from Roche, and the Phosphatase Inhibitors Cocktails 1 and 2 were purchased from Sigma Aldrich. BCA™ Protein Assay kit was purchased from Pierce, NuPAGE® Bis-Tris precast gels were from Invitrogen, nitrocellulose membrane was purchased from Millipore. Enhanced Chemiluminescence (ECL) plus was purchased from GE Healthcare, and BioMax MR Film was purchased from Kodak. Alcohol was purchased from Gold Shield Chemical Company, dimethyl sulfoxide (DMSO) from Sigma-Aldrich. Rapamycin (# R-5000) was purchased from LC-laboratories.

Animals

Male C57BL/6J mice were obtained from Jackson Laboratories (9 to 15 weeks old at the start of each experiment) and male Long-Evans rats (300–350 g at the beginning of the experiments) were purchased from Harlan. Mice and rats were individually housed and maintained in a temperature and humidity-controlled room under a 12 hr light: dark cycle (lights on at 07.00 am) with food and water available *ad libitum*, unless stated otherwise.

All animal procedures in this report were approved by the Gallo Center Institutional Animal Care and Use Committee and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

Treatments

Alcohol solution was prepared from ethyl alcohol absolute anhydrous (190 proof) diluted to 20% alcohol (vol/vol) in tap water. Rapamycin was dissolved in 100% DMSO for systemic intraperitoneal (i.p.) administration or in 5% DMSO in phosphate buffered saline (PBS) for intra-NAc infusions. The vehicles used for control treatments are 100% DMSO and 5% DMSO in PBS for i.p. injection and intra-NAc infusion respectively.

Systemic administration of alcohol in mice

Mice were habituated to the i.p. administration procedure by being injected daily with saline (0.9 % sodium chloride, Hospira) for 3 days. One day later, mice were i.p. injected with alcohol (2.5g/kg, 20% (vol/vol)) or saline. Mice were killed by cervical dislocation 30 min later and their brains were rapidly removed and placed on an ice-cold platform. The NAc were collected for western-blot analysis.

Western-blot analysis

After removal, the NAc was immediately homogenized in a RadioImmuno Precipitation Assay (RIPA) buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, EDTA 1mM, 1% (vol/vol) NP-40, 0.5% (weight/vol) sodium deoxycholate and 0.1% (weight/vol) SDS, protease and phosphatase inhibitors. Protein concentration was determined using

BCA™ assay, and an equal amount of samples (40µg) was denatured with Laemmli buffer, boiled for 10 min and resolved on a 4-12% SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were blocked for 1 hr with 5% (weight/vol) non-fat milk in Tris Buffer Saline/0.1% (vol/vol) Tween 20 (TBS-T) and then incubated overnight at 4ºC in the same blocking solution supplemented with the appropriate anti-phospho-specific antibody. After extensive washing with TBS-T, bound primary antibodies were detected with HRP-conjugated secondary antibody and visualized by ECL plus. Membranes were then striped for 30 min at 50ºC in buffer containing 100mM 2-Mercaptoethanol, 2% (weight/vol) SDS, 62.5 mM Tris-HCl pH 6.7, followed by extensive washing in TBS-T before re-blocking and re-probing with the appropriate total antibody. The optical density of the relevant immunoreactive band was quantified using NIH Image 1.63 program. For quantitative purposes, the optical density values of the phospho-protein bands were normalized to the total protein bands in the same sample and expressed as a percentage of the control.

Intermittent-limited-access 20% alcohol drinking paradigm in mice

Mice were acclimated on a reverse dark cycle for 1week (lights off from 10:00 am to 10:00 pm). Mice were individually housed and were allowed continuous access to tap water. Body weights were recorded weekly throughout the study. After acclimatization, mice were given access to alcohol using a limited-intermittent access procedure. Mice were given a solution of 20% alcohol in water (vol/vol) for 4 hrs every other day, with access beginning at 12:00 pm. A water bottle was always available when alcohol was not given, except during the 4-hr access sessions. Bottles were weighed immediately before

and after the 4 hr-access period. The amount of alcohol consumed (g/kg of body weight/4 hrs) was calculated as the difference in volume from the beginning and the end of the limited-access session. Drinking volumes were corrected for spillage using bottles containing alcohol solution placed in an empty cage during the limited access session. This paradigm leads to a high level of alcohol intake (5-9 g/kg/4 hrs) in a restricted period of time resulting in blood alcohol concentrations (BECs) higher than 80 mg% (see below).

Experiment 1: effect of rapamycin on alcohol drinking

After 3 weeks of alcohol consumption leading to a stable alcohol intake of 5-9 g/kg/4 hrs, mice were randomly separated into rapamycin or vehicle-treated groups. Mice were injected i.p. with vehicle or rapamycin (1, 5, 10 and 20 mg/kg) 3 hrs before the beginning of the alcohol-drinking session in a volume of 2 ml/kg (fig. S2). Alcohol intake was monitored 4 hrs later as described above.

Experiment 2: effect of rapamycin on water drinking

After 2 weeks without access to alcohol, mice were randomly separated into rapamycin or vehicle-treated groups. Then, mice were injected i.p. with rapamycin (10 mg/kg) or vehicle 3 hrs before the beginning of the water-drinking session starting at 12:00 pm. Water intake was measured 4 hrs later.

BEC measurements in mice

Trunk blood was collected in heparinized capillary tubes at the end of the 4-hr limited-access 20% alcohol drinking session. Serum was extracted with 3.4% trichloroacetic acid followed by a 5 min centrifugation at 420 g and assayed for alcohol content using the

NAD^+-NADH enzyme spectrophotometric method (*3-4*). The BECs were determined using a standard calibration curve. An average value of 97 ± 9 mg% (21 ± 2 mM) was obtained (n=8).

Measure of the smotor coordination in mice using the rotarod test

Experiment 1: Effect of rapamycin treatment on motor coordination

Mice were first trained to remain on a rotarod (Accurotor Rotarod; AccuScan Instruments, Inc) rotating at 10 RPM for 180 sec. At the end of the training, animals were randomly divided in 2 groups and then injected i.p. with rapamycin (10 mg/kg) or vehicle. Three hrs later, mice were tested every 15 min for 90 min and latency to fall was recorded for each trial.

Experiment 2: Effect of rapamycin treatment on alcohol-induced ataxia

Mice were first trained to remain on a rotarod rotating at 10 RPM for 180 sec. The following day, animals were randomly divided in 2 groups and then injected i.p. with rapamycin (10 mg/kg) or vehicle. Three hours later, each mouse was first tested to ensure it could stay on the rotarod for 180 sec and then was injected i.p. with 1.5 g/kg of alcohol. Each mouse was retested every 15 min for 120 min. The latency to fall from the rod in each trial was recorded.

Intermittent-access 20% alcohol two-bottle-choice drinking paradigm in rats

Intermittent-access of 20% alcohol was similar to the paradigm described previously (*5*). Briefly, animals were given 24-hr concurrent access to one bottle of 20% (vol/vol) alcohol in tap water and one bottle of water. Drinking session started at 12:00 pm on

Monday, Wednesday and Friday, with 24 or 48 hrs (weekend) alcohol-deprivation periods between the alcohol-drinking sessions. The placement (left or right) of each solution was alternated between each session to control for side preference. A bottle containing water in a cage without rats was used to evaluate the spillage due to the experimental manipulations during the test sessions. The spillage was always ≤0.5 ml (<2.5% of the total fluid intake). The water and alcohol bottles were weighed 30 min, and 24 hrs after the beginning of the session. The time point of 30 min was selected because we previously found that along the 24-hr free-access alcohol session, rats drink around 25% of the total alcohol consumed during the first 30 min of the session (*5*). This pattern of consumption results in BECs higher than 80 mg% (*5*) that corresponds to binge-drinking as defined by the National Institute on Alcohol Abuse and Alcoholism (*6*).

For western-blots analyses of rat NAc, rats experienced at least 3 months (38 sessions) of intermittent-access 20% alcohol two-bottle-choice drinking paradigm.

Surgery

Surgery procedures began after 19 sessions of the two-bottle-choice drinking paradigm as described above, when rats reached a stable baseline of alcohol consumption of 5-6 g/kg/24 hrs (fig. S5). Stereotaxic surgeries were conducted under isoflurane anesthesia (Baxter Health Care Corporation). Rats were positioned in a stereotaxic frame (David Kopf Instruments) and bilateral guide cannulae (C235G-2.0, 26 ga, Plastics One) were aimed at the NAc at the following coordinates (1.6 mm posterior to bregma, 1 mm mediolateral, 5.9 mm ventral to the skull surface (*7*). Cannulae were secured with stainless-steel screws and dental acrylic; stylets were inserted into the guide cannulae to

maintain injector site clear of debris. After 3 days of recovery, intermittent-access 20% alcohol procedure was resumed and microinfusions started when alcohol drinking returned to a stable baseline.

Intra-NAc infusion of rapamycin

Rats were microinjected with rapamycin (0.005, 0.5, 5 or 50 ng) or vehicle 3 hrs before the beginning of the 24-hr alcohol-drinking session. A total of 1 µl of rapamycin or vehicle was infused over 2 min into the NAc of gently restrained rats via injection cannulae extending 1 mm beyond the guide cannula tip. Injection cannulae were left in place for an additional 1 min. After infusion, stylets were replaced in the guide cannulae and the animal was put back in the home cage. Alcohol and water intake were calculated 30 min and 24 hrs after the beginning of the session.

All subjects received each rapamycin dose in a counterbalanced manner, with one microinjection per week. Two alcohol-drinking sessions without treatment were carried out between microinjections to allow alcohol intake to return to the baseline level.

Operant alcohol self-administration after a history of high voluntary alcohol consumption

The procedure was adapted from (*8*). High levels of voluntary alcohol consumption were obtained in an intermittent-access 20% alcohol (vol/vol) two-bottle-choice drinking paradigm as described above. After achieving a stable baseline of consumption, rats were trained to self-administer a 20% alcohol solution in operant self-administration chambers (Med Associates). The chambers contain two levers: an active lever, for which presses

result in delivery of 0.1 ml of the alcohol solution, and an inactive lever, for which presses are counted but no programmed events occur. The levers are retractable and they extend into the chamber at the initiation of each session and retract at the end of the session. After 2 to 3 nights in the chambers to allow acquisition of a lever-press response for alcohol under a fixed ration 1 (FR1), operant sessions were conducted 5 days per week with the schedule requirement increased to FR3 and the length of session shortened from 60 to 30 min over the first 2 weeks.

Experiments started after 6 weeks of alcohol self-administration upon acquisition of a stable baseline of responding. Rats were injected i.p. 3 hrs before the beginning of the operant self-administration session with 10 mg/kg of rapamycin or vehicle. The study was performed twice with a "within subjects" design in which rats received both treatments in counterbalanced order, with a week without treatment between the experiments to allow the lever-press responding for alcohol to return to baseline. Three weeks after the end of this experiment, the same rats were used to test the effect of the rapamycin (10 mg/kg, 3 hrs before the session) on extinction of alcohol self-administration. Animals were probed with a single extinction trial during which animals could press the lever for 30 min without receiving access to alcohol (active lever responding activated the pump on an FR3 schedule, but no alcohol was delivered). All subjects received rapamycin or the vehicle in a counterbalanced manner. The extinction trials were separated by 2 weeks of standard alcohol self-administration as described above allowing the lever-press responding for alcohol to return to baseline between treatments.

Operant sucrose self-administration in rats

The operant sucrose self-administration procedure was performed as previously described (*8*) with minor modifications. Briefly, rats were initially trained under FR1 by using 0.1 ml of an 8% sucrose solution (weight/vol) as the reinforcer. The FR schedule was then progressively increased to FR3, and sucrose concentration was progressively decreased to 1.5%. Animals were trained under this final schedule 5 days per week during 30-min sessions. Experiments started when the rats reached a stable level of presses. Rats were injected i.p. with rapamycin (10 mg/kg) or vehicle. All injections were given 3 hrs before the operant session. The study was performed twice, with a "within subjects" design in which rats received both treatments in counterbalanced order. Five sucrose-drinking sessions without any injection were carried out before counterbalancing the treatment.

Rat locomotor activity procedure

Rats were first habituated to handling and injection procedures with injections of saline (i.p.) once a day for 2 days. Testing was conducted using 40 × 40 cm open-field locomotor activity-monitoring chambers equipped with horizontal photobeams (Med Associates). Horizontal locomotor activity was monitored during 1-hr sessions over 3 consecutive days. On day 1, rats were placed in the activity chamber for 1 hr for habituation. On day 2, animals were injected i.p. with saline 3 hrs before a session of 1 hr. On day 3, animals were divided into 2 groups with equal locomotor activities according to the data from day 2. Animals received a single injection of rapamycin (10 mg/kg) or vehicle 3 hrs before the 1 hr recording of spontaneous activity. At the end of each session, animals were returned to the home cage. Data were measured as the distance traveled in centimeters.

Histology

Rats implanted with cannulae were sacrificed by systemic administration of pentobarbital and were perfused transcardically with 4% paraformaldehyde. Coronal sections of the forebrain were stained with thionin to allow visualization of probe tracks in the NAc (fig. S8). Subjects with injection cannulae located in the NAc were included in the study.

Data analysis

Biochemical data were analyzed with an unpaired t-test. Rat two-bottle-choice and operant self-administration experiments were conducted in a within-subject design whereas mice limited-access drinking experiments were conducted in a between-subject design. Depending on the experiment, data were analyzed with one-way ANOVA or with one-way and two-way ANOVA with repeated measures. Significant main effects and interactions of the ANOVAs were further investigated with the Student-Newman-Keuls test or the method of contrasts. Statistical significance was set at $p < 0.05$. Data are presented as mean ± S.E.M.

SUPPORTING TEXT: detailed figure legend 4 including statistical analysis

Figure 4: Systemic administration of rapamycin selectively decreases operant alcohol self-administration-related behaviors. (**A-D**) Rats with a history of high alcohol consumption were trained to self-administer a solution of 20% alcohol as described in supporting online material. Three hrs before the beginning of a 30-min session, rats were injected i.p. with 10 mg/kg of rapamycin or vehicle (Veh). (**A)** Number of lever presses during the 30 min operant alcohol self-administration session. (**B)** Amount of alcohol consumed during the session. (**C**) Cumulative mean presses in bins of 2 min, indicative of the rate of presses for alcohol during the session**.** (**D)** Number of presses on the alcohol lever during a 30-min extinction session. (**E)** Three hrs before the beginning of a 30-min session, rats were systemically injected with 10 mg/kg of rapamycin or vehicle (Veh) and the number of presses on the sucrose lever in rats trained to self-administer a solution of 1.5% sucrose was recorded. (**A-E**), n=7 per group. Data are represented as mean ± S.E.M. (**A),** two-way ANOVA with repeated-measures revealed a significant main effect of lever and treatment and an interaction between both factors, Newman-Keuls post-hoc test, **$p<0.01$ compared to vehicle. (**B**) and (**D**) one-way ANOVA with repeated measures, *$p<0.05$ compared to vehicle. (**C**), two-way ANOVA with repeated measures showed a significant main effect of time and treatment. Subsequent analysis using the method of contrasts detected a significant difference between vehicle and rapamycin for all time intervals (all p's<0.05).

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Fig. S1

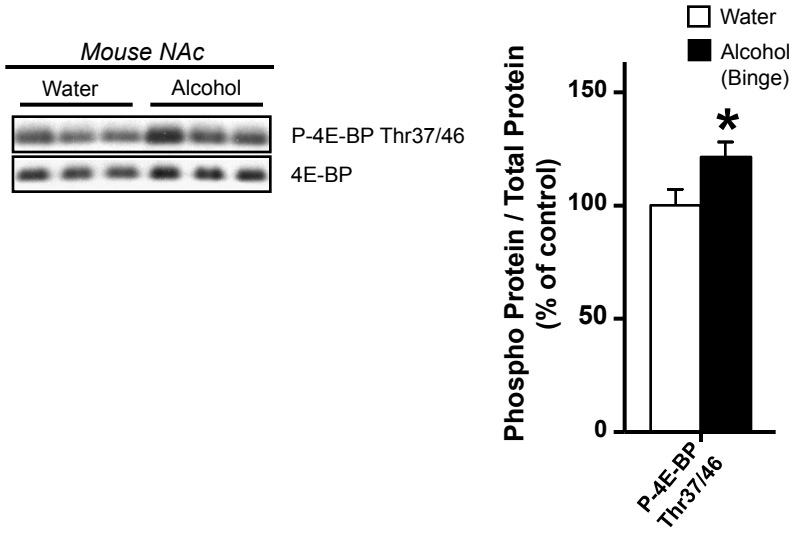

Figure S1: The level of 4E-BP phosphorylation in mice NAc is increased after binge-drinking of alcohol. Mice had access to a 20% solution of alcohol for 4 hrs every other day during 3 weeks. Control mice had access to only water for the duration of the experiments. After the tenth 4hr-alcohol-drinking session, the NAc were removed and 4E-BP phosphorylation was determined by western-blot analysis as described in supporting online material. Optical density quantification of 4E-BP phosphorylation is expressed as the ratio of the phosphorylated protein to the total protein. The level of S6K phosphorylation was too low to be accurately quantified. n=7 per group. Data are represented as mean ± S.E.M. and expressed as percentage of control. *$p<0.05$, unpaired t-test.

Fig. S2

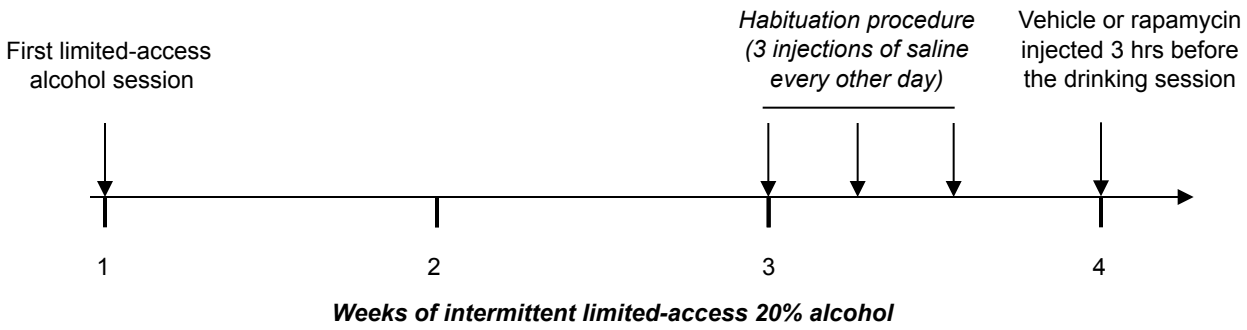

Figure S2: Schematic representation of the experimental procedure of rapamycin treatment in mice. Animals were trained to drink alcohol in a 4-hr limited-access 20% alcohol session every other day (Monday, Wednesday, and Friday) for 3 weeks. After acquisition of a stable baseline of alcohol drinking, mice were habituated to the systemic injection procedure to reduce effects due to the stress of handling and receiving injections. On the test day, mice were randomly assigned into vehicle or rapamycin-groups and administered with rapamycin or vehicle (i.p.) 3 hrs before the beginning of the 4-hr limited-access 20% alcohol session.

Fig. S3

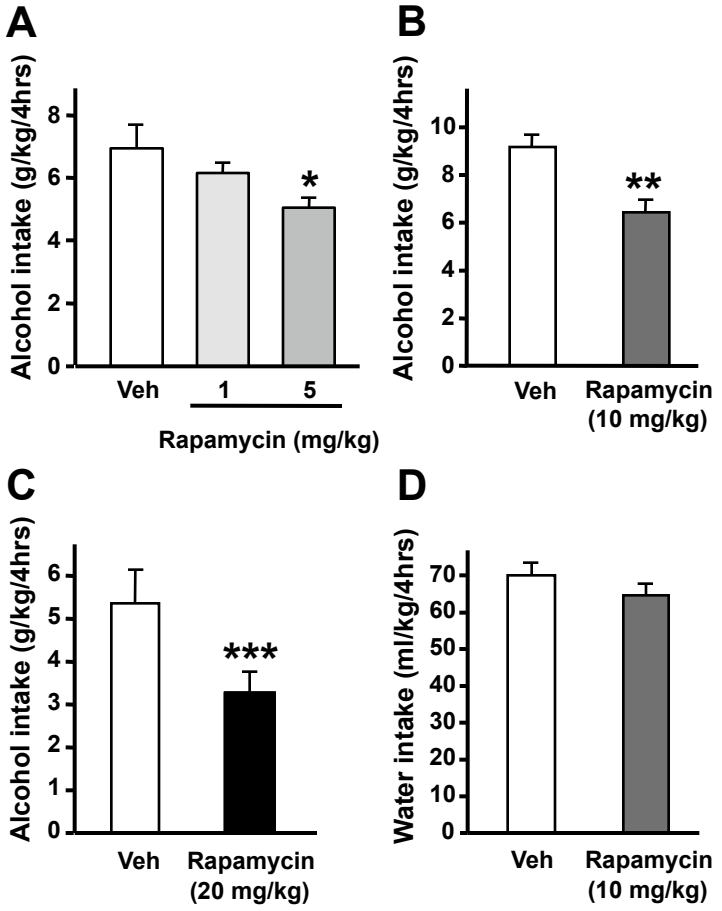

Figure S3: Systemic administration of rapamycin dose-dependently decreases alcohol intake without altering water consumption in mice. (**A-C**), Three hrs prior to the substitution of water with a 20% solution of alcohol, mice were injected i.p. with vehicle (Veh) or 1 and 5 mg/kg rapamycin (**A**), 10 mg/kg rapamycin (**B**) and 20 mg/kg rapamycin (**C**). (**D)** Mice were treated (i.p). with 10 mg/kg of rapamycin 3 hrs before the beginning of a 4-hr water drinking session. n=8-12 per group. Data are represented as mean ± S.E.M. One-way ANOVA (Newman-Keuls post-hoc test). *p<0.05, **p<0.01, ***p<0.001 compared to vehicle.

Fig. S4

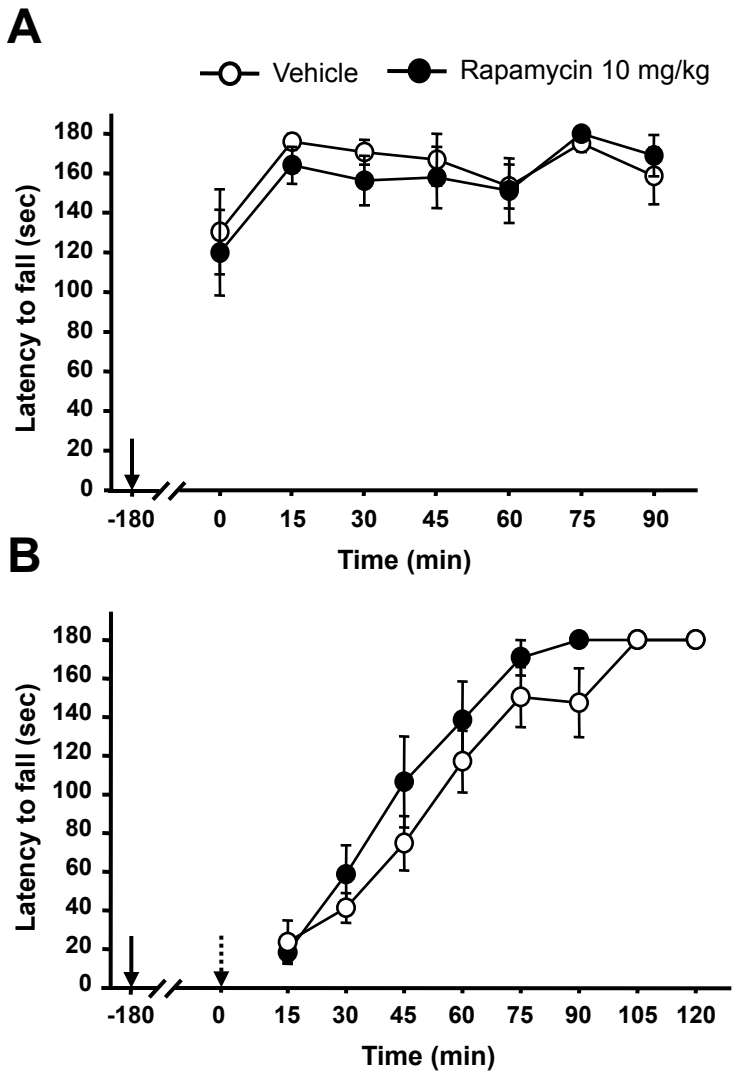

Figure S4: Systemic administration of rapamycin does not alter sensorimotor coordination in mice in absence and in presence of alcohol. (**A)** Animals were injected i.p. with rapamycin (10 mg/kg) or vehicle 3 hrs before the beginning of a rotarod test. The trials were conducted every 15 min for 90 min. (**B**) Three hours before systemic administration of alcohol (1.5 g/kg), rapamycin (10 mg/kg) or vehicle were administered (i.p.). Mice were then placed back on the rod every 15 min for 2 hrs. Solid and dashed arrows indicate the time points of injection of rapamycin and alcohol respectively. n=8-9 per group. Data are represented as mean ± S.E.M.

Fig. S5

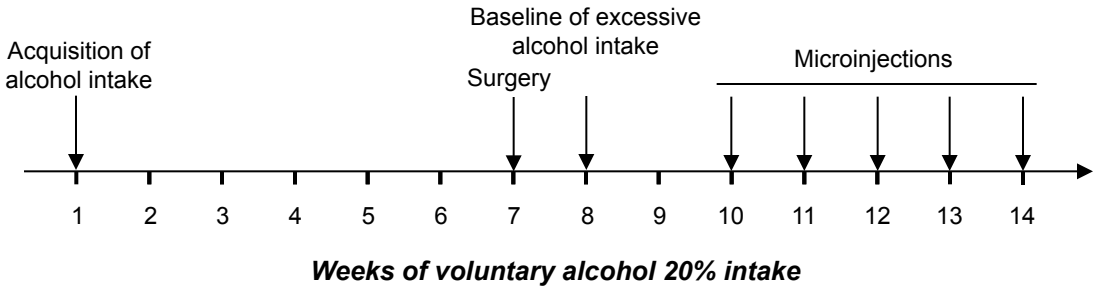

Figure S5: Schematic representation of the experimental design of intra-Nac infusions of rapamycin in rats. Voluntary alcohol drinking was performed using a two-bottle choice intermittent drinking paradigm. Animals had a 24-hr concurrent access to two bottles, one with 20% alcohol and one with water, starting at 12:00 p.m. on Monday, Wednesday, and Friday. After the rats reached a baseline of voluntary alcohol drinking, animals with a high level of consumption (5-6 g/kg/24 hrs) were selected and proceeded to perform stereotaxic surgery to implant guide cannulae in the NAc (see fig. S8). After 3 days of recovery, rats returned to the intermittent-access 20% alcohol procedure and microinjections began several sessions later when rats maintained a stable baseline of 5-6 g/kg/24 hrs of alcohol consumption. Microinjections were conducted once per week on Monday.

Fig. S6

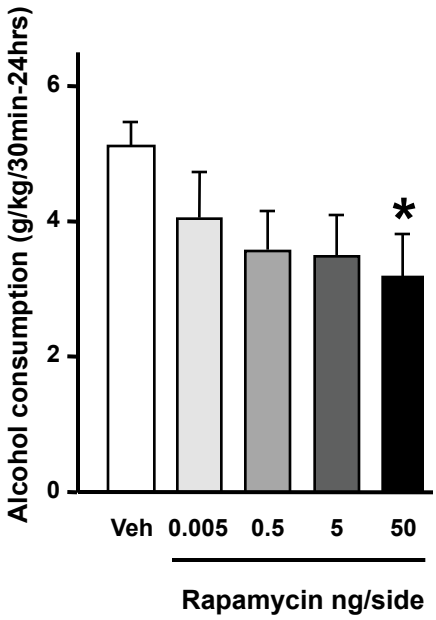

Figure S6: Intra-NAc infusion of rapamycin reduces voluntary alcohol consumption in rats during the 30 min to 24 hrs period of the drinking session. Rapamycin (0.005, 0.5, 5 and 50 ng/side) or vehicle (Veh) were infused into the NAc 3 hrs before the beginning of the drinking session in rats trained to consume a high amount of a 20% solution of alcohol in a two-bottle choice paradigm. The bar graph represents alcohol intake during the 30 min to 24 hrs period of the drinking session. n=9 per group. Data are represented as mean ± S.E.M. One-way ANOVA with repeated measures (Newman-Keuls post-hoc test), *$p<0.05$ compared to vehicle.

Fig. S7

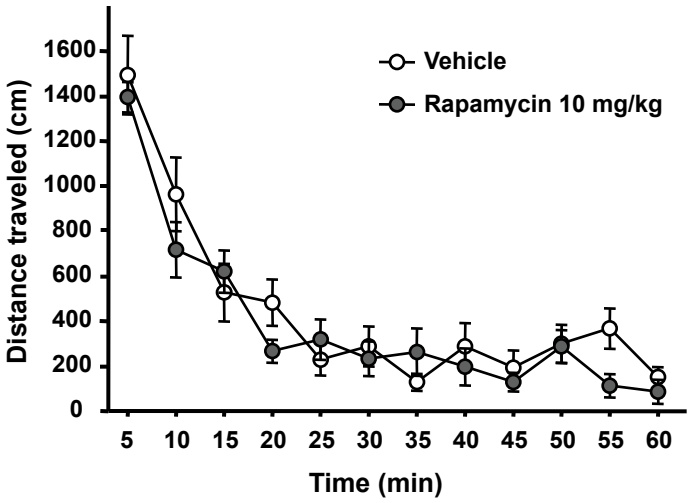

Figure S7: Systemic administration of rapamycin does not alter locomotor activity in rats. Animals were injected i.p. with rapamycin (10 mg/kg) or vehicle 3 hrs before the beginning of a 1 hr session of recorded motor activity. n=8-9 per group. Data are represented as mean ± S.E.M.

Fig. S8

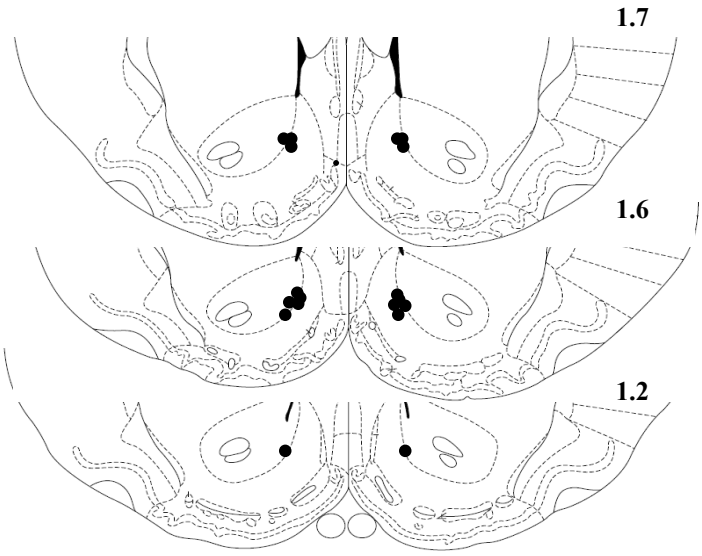

Figure S8: Schematic drawings of coronal sections of rat brain showing the placement of bilateral injection sites in the NAc. Only data from animals in which the histologically reconstructed sites of infusions were localized in the NAc were included in the analysis of each experiment. The drawings are taken from the Paxinos and Watson brain atlas (*7*).