Supporting Information

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SI Materials and Methods

Materials. The following antibodies were purchased from Cell Signaling Technology: phospho-S6K Thr389 (no. 9234), phospho-4E-BP Thr37/46 (no. 2855), total S6K (no. 2708), and total 4E-BP2 [no. 2845; 4E-BP2 is the main 4E-BP isoform in the brain (1, 2)]. GAPDH (sc-25778) and pan Homer (sc-8921) antibodies were purchased from Santa Cruz Biotechnology. GluR1 antibody (no. 06-306) was from Upstate. EDTA-free Complete Mini Protease Inhibitors Mixture was purchased from Roche, and Phosphatase Inhibitors Mixtures 1 and 2 were purchased from Sigma-Aldrich. BCA Protein Assay kit was purchased from Pierce, NuPAGE Bis-Tris precasted gels were from Invitrogen, and nitrocellulose membrane was purchased from Millipore. Enhanced Chemiluminescence Plus was purchased from GE Healthcare and BioMax MR Film was purchased from Kodak. Alcohol was purchased from Gold Shield Chemical and DMSO from Sigma-Aldrich. Rapamycin (R-5000) was purchased from LC Laboratories.

Animals. Male C57BL/6J and DBA/2J mice were obtained from Jackson Laboratories (9–15 wk), and male Long-Evans rats (300–350 g) were purchased from Harlan. Mice and rats were individually housed and maintained in a temperature- and humidity-controlled room under a 12-h light:dark cycle (lights on at 0700 hours) 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).

Preparation of Solutions. 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 i.p. administration or in 5% DMSO in PBS solution for intra-NAc infusions. The vehicles used for control treatments are 100% DMSO and 5% DMSO in PBS solution for i.p. injection and intra-NAc infusion, respectively.

Western Blot Analysis. Mice were killed by cervical dislocation. Rats were anesthetized by isoflurane and were killed by decapitation. Brains were rapidly removed and placed on an ice-cold platform. The NAc was immediately homogenized in radio-immunoprecipitation assay buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, EDTA 1 mM, 1% (vol/vol) Nonidet P-40, 0.5% (weight/vol) sodium deoxycholate, and 0.1% (weight/vol) SDS, and protease and phosphatase inhibitors. Protein concentration was determined using BCA assay, and an equal amount of samples (40 μg) was denatured with Laemmli buffer, boiled for 10 min and resolved on a 4% to 12% SDS/PAGE, and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h with 5% (wt/vol) nonfat milk in Tris-buffered saline solution/0.1% (vol/vol) Tween-20 (TBS-T) and then incubated overnight at 4 °C in the same blocking solution including the appropriate antibody. After extensive washing with TBS-T, bound primary antibodies were detected with HRP-conjugated secondary antibody and visualized by Enhanced Chemiluminescence Plus. After measuring the level of phosphoproteins, membranes were then stripped for 30 min at 50 °C in buffer containing 100 mM 2-β-mercaptoethanol, 2% (weight/vol) SDS, 62.5 mM Tris-HCl, pH 6.7, followed by extensive washing in TBS-T before reblocking and reprobing with the appropriate

total antibody. The optical density of the relevant immunoreactive band was quantified by using the NIH Image 1.63 program. The level of GluR1 and Homer were normalized to the level of GAPDH. The values of the phosphoprotein signal were normalized to the signal of the total protein in the same sample. Results were expressed as a percentage of the control.

Mouse Experiments. *Systemic administration of alcohol.* Mice were habituated to the i.p. administration procedure by being injected daily with saline solution (0.9% sodium chloride; Hospira) for 3 d. One day later, mice were systemically treated (i.p.) with alcohol [2.5 g/kg or 2 g/kg, 20% (vol/vol)] or saline solution.
Measurement of alcohol-induced locomotor sensitization. Locomotor activity assessment was carried out in 43-cm × 43-cm locomotor activity chambers (Med Associates). Horizontal locomotor activity was detected via infrared beam breaks. Before the beginning of the experiment, DBA/2J mice experienced three habituation sessions. No injection was given during the first habituation session, and daily injection (i.p.) of saline solution was given on days 2 and 3. Immediately after saline solution injection on the third day, mice were placed in the center of the activity chamber, and locomotor activity was measured and used as the baseline. Following habituation sessions, mice were assigned to sensitized or control groups (n = 31–33 per group) in a counterbalanced manner according to the baseline locomotor activity. Animals were administered saline solution or alcohol (2 g/kg, i.p.) once daily for 11 d. On locomotor test days (days 1, 4, 7, and 11), mice were taken from their home cage and brought to the testing room at least 30 min before the beginning of the session to habituate them to the room. Mice were then placed in the locomotor activity chambers for 30 min and locomotor activity was recorded (pretest session). Animals were then administered saline solution or alcohol (2 g/kg) and put back into the locomotor chambers and locomotor activity was monitored for an additional 15 min (test session). At the end of the session, mice were returned to their home cages. On days when locomotor activity was not recorded (days 2, 3, 5, 6, 8, 9, and 10), mice were brought to the test room for 30 min, administered saline solution or alcohol, and returned to their home cages. On the challenge day (day 11) alcohol-pretreated (sensitized) and saline solution-pretreated (control) animals were subdivided into four challenge groups: group 1, control + vehicle; group 2, control + rapamycin; group 3, sensitized + vehicle; and group 4, sensitized + rapamycin. Vehicle or rapamycin (10 mg/kg) was administered (i.p.) 3 h before alcohol administration (2 g/kg, i.p.) to all mice. The distance traveled by each animal in the locomotor activity chambers was recorded 5 min before and for 15 min after alcohol administration. Pretest and test sessions lasted 30 min and 15 min respectively, and data were collected in 1-min bins.
Measurement of rapamycin-induced CCP and conditioned place aversion. The place conditioning boxes (Columbus Instrument) consist of two distinct compartments that differ in color and floor texture. On day 1 (preconditioning test), the initial preference was assessed. DBA/2J mice were placed in the central compartment and were allowed to freely explore the apparatus for 15 min, and the time spent in each compartment was recorded. Animals that spent more than 65% of the time in either one of the compartments during the preconditioning baseline test were excluded from the study. This screening allowed the use an unbiased design in which the two compartments were equally preferred before conditioning as indicated by the group average (unbiased apparatus) and to pseudorandomly assigned the compartment paired with rapa-

mycin (unbiased assignment procedure). The next day, the conditioning training started with one conditioning trial per day for 6 d (days 2–7). On days 2, 4, and 6, mice were administered (i.p.) vehicle 3 h before being confined to the unpaired compartment for 15 min. On days 3, 5, and 7 mice received rapamycin (10 mg/kg, i.p.) 3 h before being confined to the rapamycin-paired compartment for 15 min. On day 8 (postconditioning phase), mice had free access to both compartments, and time spent in each compartment was measured.

Measurement of alcohol-induced conditioned place preference. Apparatus, habituation, preconditioning, and unbiased assignments were conducted as described above. Conditioning training started with one conditioning trial per day for 6 d (days 2–7). Mice were administered (i.p.) saline solution and confined immediately to one of the compartments for 5 min (unpaired compartment). The next day, mice were administered saline solution (saline group) or alcohol (1.8 g/kg, 20% vol/vol solution; alcohol group) and were confined to the other compartment (drug-paired compartment). This schedule was repeated twice more (i.e., three alcohol-conditioning tests). On day 8, one half of each group was administered (i.p.) rapamycin (10 mg/kg; saline/rapa, alcohol/rapa), and the other half with vehicle (saline/veh, alcohol/veh). Three hours later, animals were allowed to explore the entire apparatus for 15 min (postconditioning test). CPP score was calculated as time spent in the drug-paired compartment on the test day minus time spent in the same compartment on the preconditioning day.

BAC measurements. Trunk blood was collected in heparinized capillary tubes at the end of the 4-h limited-access 20% alcohol-drinking session. Serum was extracted with 3.4% trichloroacetic acid followed by a 5-min centrifugation at $420 \times g$ and assayed for alcohol content using the NAD^+-NADH enzyme spectrophotometric method (3, 4). BACs were determined using a standard calibration curve. An average value of 97 ± 9 mg% (21 ± 2 mM) was obtained ($n = 8$).

Intermittent limited-access 20% alcohol drinking paradigm in mice. C57BL/6J mice were acclimated on a reverse dark cycle for 1 wk (lights off from 1000 h to 2200 h). After acclimatization, mice were given access to alcohol using an intermittent limited-access procedure. Mice had access to a solution of 20% alcohol in water (vol/vol) for 4 h every other day, with access beginning at 1200 h. A water bottle was always available except during the 4-h alcohol access sessions. The amount of alcohol consumed (g/kg of body weight/4 h) was calculated as the difference in volume at the beginning and at the end of the limited-access session. This paradigm leads to a high level of alcohol intake (7 ± 2 g/kg/4 h) resulting in BACs of 97 ± 9 mg%. Drinking volumes were corrected for spillage by placing a bottle containing alcohol solution in an empty cage during the session. Spillage was never more than 0.1 mL (<7% of total fluid intake).

Experiment 1: Effect of rapamycin on alcohol consumption. After 3 wk of alcohol consumption leading to a stable alcohol intake of 7 ± 2 g/kg/4 h, mice were systemically (i.p) administered vehicle or rapamycin at the indicated dose (volume, 2 mL/kg) 3 h before the beginning of the alcohol-drinking session. Alcohol intake was monitored 4 h later.

Experiment 2: Effect of rapamycin on water consumption. After 2 wk of access to water only, mice were systemically (i.p) administered vehicle or rapamycin (10 mg/kg) 3 h before the beginning of the water-drinking session starting at 1200 h, and water intake was measured 4 h later.

Quinine taste test. C57BL/6J mice were given a 20-h concurrent access to one bottle of quinine (3 or 300 μM) and one bottle of water for 2 wk. Water was always available ad libitum. The position of the quinine bottle was altered daily. One week after the beginning of the experiment, mice were administered (i.p.) vehicle rapamycin (10 mg/kg) 3 h before the beginning of the quinine-drinking session. Water and quinine intakes were mea-

sured 20 h later. Drinking volumes were corrected for spillage. All subjects received vehicle or rapamycin treatment in a counterbalanced manner (one injection per week). Quinine preference was calculated according to the following formula: [quinine intake / (quinine intake + water intake)] × 100.

Motor coordination in a Rotarod test. Experiment 1: Effect of rapamycin treatment on motor coordination. Drug-naive C57BL/6 mice were first trained to remain on a Rotarod (Accurotor; AccuScan Instruments) rotating at 10 rpm for 180 s. At the end of the training, animals were randomly divided into two groups and then systemically administered (i.p.) vehicle or rapamycin (10 mg/kg). Three hours later, mice were tested every 15 min for 90 min and the latency to fall was recorded for each trial.

Experiment 2: Effect of rapamycin treatment on alcohol-induced ataxia. Training and treatments were conducted as described earlier. Three hours after vehicle or rapamycin treatment, mice were first tested to ensure they could stay on the Rotarod for 180 s and then treated (i.p.) with 1.5 g/kg of alcohol. Mice were retested every 15 min for 120 min and the latency to fall from the rod was recorded in each trial.

Rat Experiments. Intermittent-access 20% alcohol two-bottle choice drinking paradigm. Intermittent access of 20% alcohol was similar to the paradigm described previously (5). Briefly, animals were given 24-h concurrent access to one bottle of 20% (vol/vol) alcohol in tap water and one bottle of water. Drinking sessions started at 1200 h on Monday, Wednesday, and Friday, with 24 or 48 h (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 as a result of the experimental manipulations during the test sessions. The spillage was never greater than 0.5 mL (<2.5% of the total fluid intake). The water and alcohol bottles were weighed 30 min and 24 h after the beginning of the session. The time point of 30 min was selected because we previously observed that rats drink approximately 25% of the total alcohol consumed during the first 30 min of the 24-h session (5). For Western blot analyses, rats experienced at least 3 mo (38 sessions) of the intermittent-access 20% alcohol two-bottle choice drinking paradigm.

Surgery. Surgical procedures began after 19 sessions of the two-bottle choice drinking paradigm described earlier, when rats reached a stable baseline of alcohol consumption of 5 to 6 g/kg/24 h (Fig. S3A). Stereotaxic surgeries were conducted under isoflurane anesthesia (Baxter). Rats were positioned in a stereotaxic frame (David Kopf Instruments) and bilateral guide cannulae (C235G-2.0, 26-gauge; 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) (6). 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 d of recovery, the intermittent-access 20% alcohol procedure was resumed and microinfusions started when alcohol drinking returned to a stable baseline.

Intra-NAc infusions of rapamycin. Rats were microinjected with vehicle or rapamycin (0.005, 0.5, 5, or 50 ng) 3 h before the beginning of the 24-h alcohol-drinking session. A total of 1 μL of rapamycin or vehicle was infused over a period of 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 h after the beginning of the session. All subjects received each dose of rapamycin 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. High levels of voluntary alcohol consumption were obtained in an intermittent-access 20% alcohol 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 for 30 min in operant self-administration chambers (Med Associates) under a Fixed Ratio 3 (FR3) schedule. Experiments started after 6 wk of alcohol self-administration upon acquisition of a stable baseline of responding. Rats were systemically (i.p) administered vehicle or 10 mg/kg of rapamycin 3 h before the beginning of the operant self-administration session. The same rats were used to test the effect of the rapamycin (10 mg/kg, 2 mL/kg, 3 h before the session) in extinction. Rats continued to maintain a baseline level of responding for alcohol for 3 wk. Animals were then tested in a single extinction trial in which animals could press the lever for 30 min without receiving access to alcohol.

All studies were performed with a "within-subjects" design in which rats received both treatments in counterbalanced order. All trials were separated by at least 1 wk of standard alcohol self-administration, allowing the lever-press responding for alcohol to return to baseline.

Operant sucrose self-administration. The operant sucrose self-administration procedure was performed as previously described (7) with minor modifications. 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 schedule 5 d per week during 30-min sessions. Experiments started when the rats reached a stable level of presses. Rats were systemically administered (i.p.) vehicle or rapamycin (10 mg/kg). All injections were given 3 h 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.

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

Histology. Rats implanted with cannulae were killed by i.p. injection of pentobarbital and perfused transcardically with 4% paraformaldehyde. Coronal sections of the forebrain were stained with thionin to allow visualization of probe tracks in the NAc (Fig. S3B). Subjects with injection cannulae located in the NAc were included in the study.

Data analysis. Unless otherwise stated, biochemical data were analyzed with a two-tailed 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 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 ± SEM.

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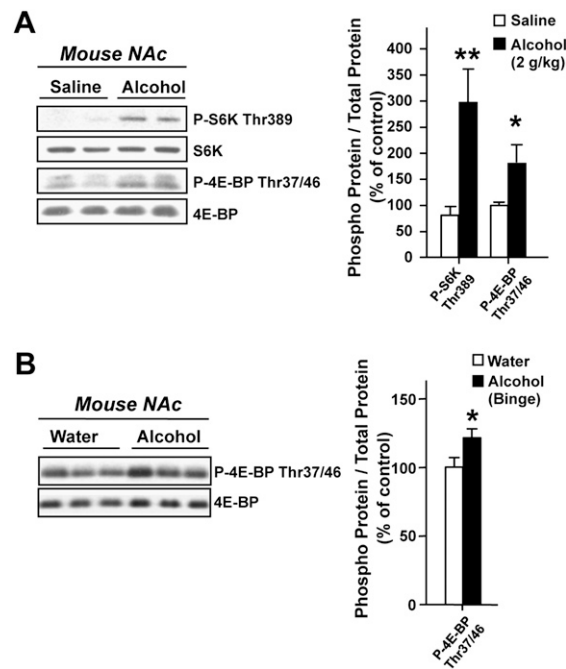
Fig. S1. Exposure to alcohol activates mTORC1-mediated signaling pathway in the NAc of mice. (*A*) DBA/2J mice were systemically administered (i.p.) 2 g/kg of alcohol or saline solution and the NAc was removed 30 min later. The levels of S6K and 4E-BP phosphorylation were determined by Western blot analyses (*n* = 8 per group). Data are presented as mean ± SEM and expressed as percentage of control; *$P < 0.05$ and **$P < 0.01$, two-tailed unpaired *t* test. (*B*) C57BL/6J mice had access to a 20% solution of alcohol for 4 h every other day for 3 wk. After the tenth 4-h alcohol-drinking session, the NAc was removed; *n* = 7 per group; *$P < 0.05$, two-tailed unpaired *t* test.
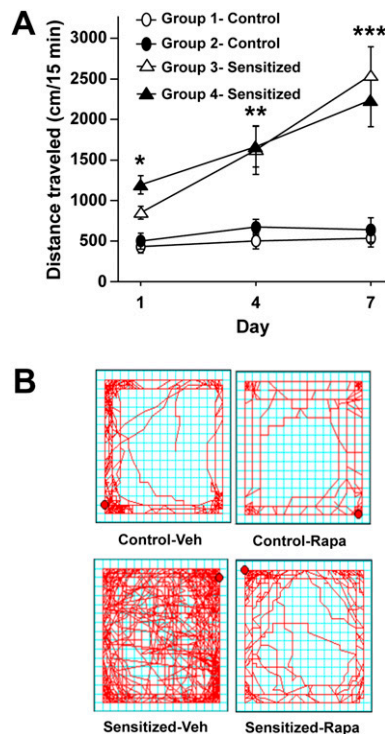
Fig. S2. Systemic administration of rapamycin reduces the expression of alcohol-induced locomotor sensitization. (*A*) DBA/2J mice were administered saline solution alone (groups 1 and 2) or with 2 g/kg of alcohol (groups 3 and 4) daily. On days 1, 4, and 7, locomotor activity was recorded for 15 min following the alcohol injection; *n* = 15–17 per group; data presented as mean ± SEM. Two-way ANOVA with repeated measures (Newman-Keuls post hoc test) showed an interaction between Groups and Day [$F_{(3,120)}$ = 23.34, $P < 0.001$], *$P < 0.05$ (group 4 vs. groups 1 and 2 on day 1), **$P < 0.01$ (groups 3 and 4 vs. groups 1 and 2 on day 4), and ***$P < 0.001$ (groups 3 and 4 vs. groups 1 and 2 on day 7). (*B*) On day 11, mice were treated (i.p.) with vehicle or rapamycin (10 mg/kg). Three hours later, all mice received alcohol (2 g/kg, i.p.) and locomotor activity was monitored. The panel shows a representative example of the total distance traveled by each group of mice in the locomotor activity chambers during the 15 min following alcohol administration.
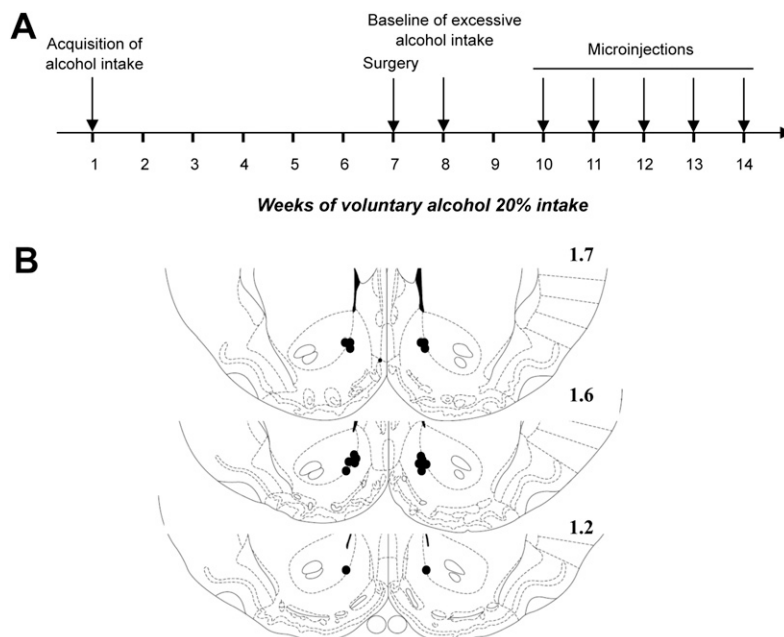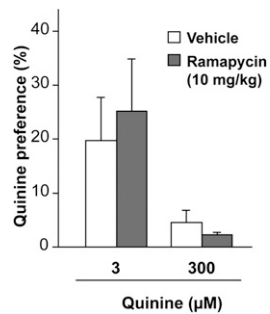
Fig. S3. Schematic representation of the experimental design of rapamycin intra-NAc infusions in rats. (*A*) Voluntary alcohol drinking was performed using a two-bottle choice intermittent drinking paradigm. Animals had a 24-h concurrent access to two bottles, one with 20% alcohol and one with water, starting at 1200 h 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 h) were selected and stereotaxic surgery to implant guide cannulae in the NAc was performed. After 3 d of recovery, rats returned to the intermittent-access 20% alcohol procedure and intra-NAc infusions began several sessions later when rats maintained a stable baseline of 5–6 g/kg/24 h of alcohol consumption. Infusions were conducted once per week on Monday. (*B*) Schematic drawings of coronal sections of the 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. [This article was published in *The Rat Brain in Stereotaxic Coordinates*, 4th Ed, Paxinos G, Watson C, Copyright Academic Press (1998).]
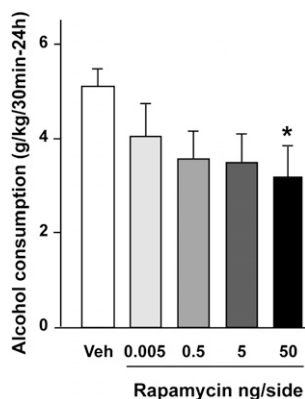
Fig. S4. Intra-NAc infusion of rapamycin reduces voluntary alcohol consumption in rats during the 30 min to 24 h period of the drinking session. Vehicle (Veh) or rapamycin (0.005, 0.5, 5, and 50 ng/side) were infused into the NAc 3 h before the beginning of the drinking session in rats trained to consume 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-h period of the drinking session. One-way ANOVA with repeated measures showed significant effects of treatment [$F_{(4,32)}$ = 2.77, $P < 0.05$]; n = 9 per group. Data are represented as mean ± SEM; *$P < 0.05$ vs. vehicle (Newman-Keuls post hoc test).

Fig. S5. Systemic administration of rapamycin dose-dependently decreases alcohol intake without altering water consumption in mice. (*A*) Schematic representation of the experimental procedure of rapamycin treatment in mice. C57BL/6J mice were trained to drink alcohol in a limited-access 20% alcohol session every other day (Monday, Wednesday, and Friday) for 3 wk. After acquisition of a stable baseline of alcohol drinking, mice were habituated to the i.p. injection procedure to reduce effects of the stress of handling and receiving injections. On the test day, mice were randomly assigned into vehicle or rapamycin groups and systemically administered (i.p.) vehicle or rapamycin 3 h before the beginning of the limited-access 20% alcohol session. Three hours before the substitution of water with a 20% solution of alcohol, C57BL/6J mice were systemically administered (i.p.) vehicle (Veh) or 1 or 5 mg/kg rapamycin (*B*), 10 mg/kg rapamycin (*C*), or 20 mg/kg rapamycin (*D*). (*E*) Mice were systemically administered (i.p.) vehicle (Veh) or 10 mg/kg of rapamycin 3 h before the beginning of a 4-h water-drinking session (*n* = 8–12 per group). Data presented as mean ± SEM (*B–E*); one-way ANOVA (Newman-Keuls post hoc test). (*B*) [$F_{(2,27)}$ = 4.15, $P < 0.05$], *$P < 0.05$; (*C*) [$F_{(1,20)}$ = 20.31, $P < 0.001$], *** $P < 0.001$; (*D*) [$F_{(1,17)}$ = 9.53, $P < 0.01$], **$P < 0.01$ vs. vehicle.

Fig. S6. Rapamycin does not induce place aversion in mice. (*A*) Schematic representation of the place conditioning procedure associated with rapamycin treatment in mice. (*B*) After a preconditioning test (day 1), DBA/2J mice were administered (i.p.) vehicle (Veh) or rapamycin (10 mg/kg) on alternate days 3 h before the beginning of a 15-min conditioning test (day 2–7). On day 8, a 15-min postconditioning test was run in the same manner as the initial preconditioning test (*n* = 8–9 per group). Data presented as mean ± SEM.
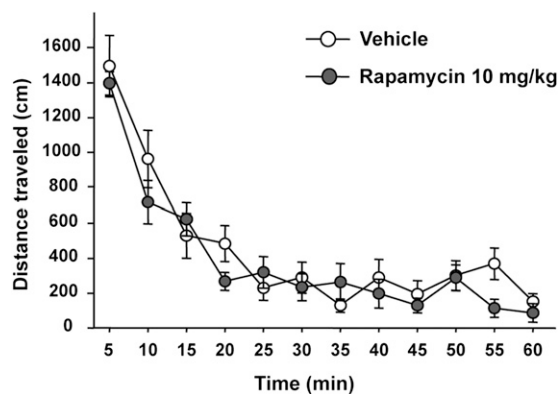
Fig. S7. Systemic administration of rapamycin dose not alter aversive response to quinine in mice. C57BL/6J mice had daily access to one bottle of water and one bottle of a quinine solution (3 or 300 μM) for 20 h during 2 wk. Three hours before the seventh 20-h quinine-drinking session, mice were treated (i.p.) with vehicle or 10 mg/kg of rapamycin. Quinine and water intake were measured at the end of the 20-h drinking session (n = 8–9 per group). Data presented as mean ± SEM.
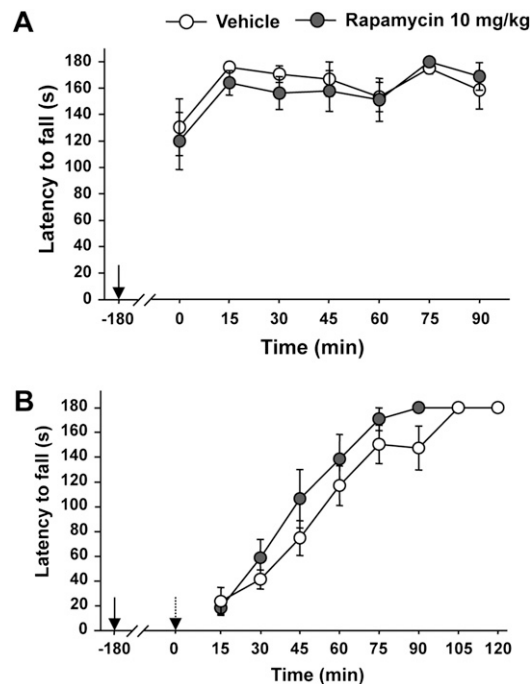
Fig. S8. Systemic administration of rapamycin does not alter sensorimotor coordination in mice in absence and in presence of alcohol. (*A*) C57BL/6J mice were systemically administered (i.p.) vehicle or rapamycin (10 mg/kg) 3 h before the beginning of a Rotarod test. The trials were conducted every 15 min for 90 min. (*B*) Three hours before i.p. administration of alcohol (1.5 g/kg), mice were systemically administered (i.p.) rapamycin (10 mg/kg) or vehicle. Then, mice were placed back on the rod every 15 min for 2 h. Solid and dashed arrows indicate the time points of injection of rapamycin and alcohol, respectively (n = 8–9 per group). Data presented as mean ± SEM.

Fig. S9. Systemic administration of rapamycin does not alter locomotor activity in rats. Animals were systemically administered (i.p.) vehicle or rapamycin (10 mg/kg) 3 h before the beginning of a 1-h session of motor activity recording (n = 8–9 per group). Data presented as mean ± SEM.