

## Supplemental Information

### Methods & Materials

**Materials.** Anti-phospho-AKT Ser473 (#4058), phospho-GSK3 $\alpha/\beta$  Ser21/9 (#9323), total AKT (#9272), and total ERK1/2 (#9102) antibodies are from Cell Signaling Technology (Danvers, MA). Anti-phospho-AKT Thr308 (sc-16646-R), and phospho ERK1/2 Tyr204/187 (sc-7976-R) antibodies are from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-total GSK-3 $\alpha/\beta$  antibody (#05-412) is from Millipore (Billerica, MA). Phosphatase Inhibitors Cocktails 1 and 2 and wortmannin are from Sigma Aldrich (St. Louis, MO) and triciribine from Calbiochem (San Diego, CA). EDTA-free complete mini Protease Inhibitor Cocktails are from Roche (Indianapolis, IN). NuPAGE® Bis-Tris precast gels are from Invitrogen (Carlsbad, CA) and nitrocellulose membrane is from Millipore. Enhanced Chemiluminescence (ECL) plus is from GE Healthcare (Piscataway, NJ).

**Preparation of solutions.** Alcohol solution was prepared from ethyl alcohol absolute anhydrous (190 proof) diluted to 20% alcohol (vol/vol) in tap water. Wortmannin was dissolved in 25% dimethyl sulfoxide (DMSO) in phosphate buffered saline (PBS) and triciribine was dissolved in 10% DMSO in PBS. The vehicles used for control treatments were 25% DMSO or 10% DMSO in PBS for wortmannin and triciribine, respectively.

**Systemic administration of alcohol.** Mice were habituated to the i.p. administration procedure by being injected daily with saline (0.9% sodium chloride, Hospira, Lake Forest, IL) for 3 days.

One day later, mice were systemically treated (i.p.) with alcohol (2 g/kg, 20% (vol/vol)) or saline and killed 15 min later.

**Intra-NAc infusion of wortmannin in mice.** Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and placed in a stereotactic apparatus (David Kopf Instruments, Tujunga, CA). Their skull was leveled, small holes were drilled and 1  $\mu$ l (0.2  $\mu$ l/min) of wortmannin (0.4  $\mu$ g/side) or vehicle was injected using a Hamilton syringe bilaterally into the NAc (+1.7 AP,  $\pm$ 0.8 ML and -4.35 DV relative to Bregma). One hr later, animals were systemically administered with alcohol (2 g/kg, i.p.) or saline solution, and the NAc was removed after 15 min.

**Intermittent-access 20% alcohol 2-bottle choice drinking paradigm.** Animals were given 24-hr concurrent access to one bottle of 20% (vol/vol) alcohol in tap water and one bottle of water. Drinking sessions started at 12:00 pm on Monday, Wednesday and Friday, with 24 or 48 hr (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  $\leq$ 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 drinking session. To test the effect of intra-NAc infusion of wortmannin and triciribine on alcohol consumption, cannulae were implanted into the NAc after 19 sessions of the 2-bottle choice drinking paradigm when rats reached a stable baseline of alcohol consumption. After 4 days of recovery, the intermittent-access 20% alcohol procedure was

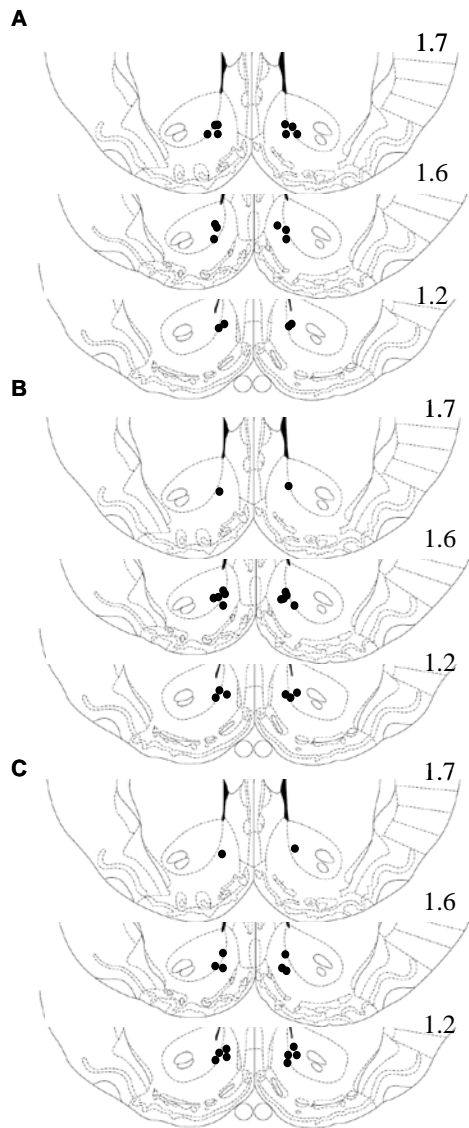
resumed and microinfusions started when alcohol drinking returned to a stable baseline. All subjects received each dose of wortmannin and triciribine in a counterbalanced manner, with one microinjection per week. The water and alcohol bottles were weighed 30 min, and 24 hrs after the beginning of the session.

**Surgery.** Procedures began after 19 sessions of the 2-bottle choice drinking paradigm described above, when rats reached a stable baseline of alcohol consumption of 5-6 g/kg/24 hrs. Stereotaxic surgeries were conducted under isoflurane anesthesia (Baxter Health Care Corporation, Deerfield, IL). Rats were positioned in a stereotaxic frame (David Kopf Instruments) and bilateral guide cannulae (C235G-2.0, 26 ga, Plastics One, Roanoke, VA) 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 (1). 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 4 days of recovery, the intermittent-access 20% alcohol procedure was resumed and microinfusions started when alcohol drinking returned to a stable baseline.

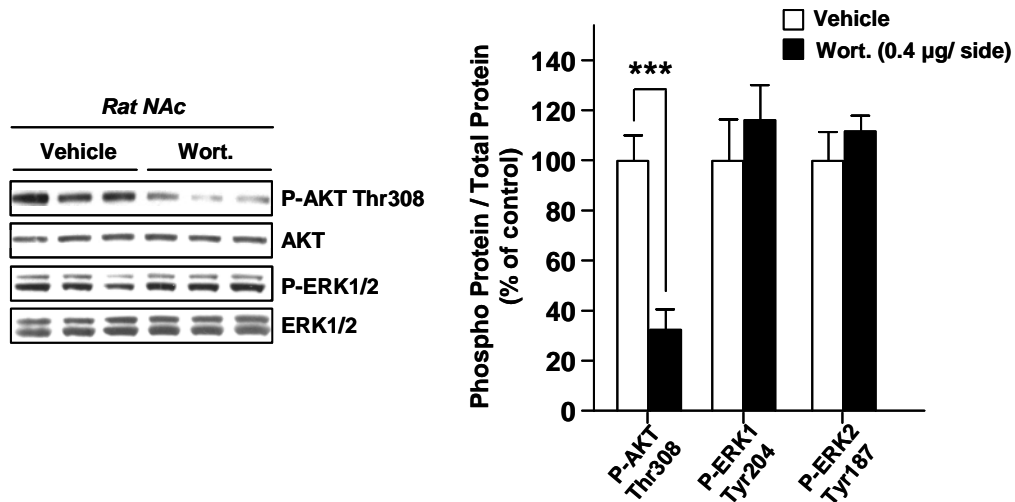
**Operant self-administration of alcohol.** High levels of voluntary alcohol consumption were obtained in an intermittent-access 20% alcohol (vol/vol) two-bottle-choice drinking paradigm as described above. After achieving a stable baseline of consumption, rats were trained to self-administer a 20% alcohol solution in operant self-administration chambers (Med Associates, St Albans, VT). 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 ratio 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. In a first experiment, all subjects received vehicle or wortmannin in a counterbalanced manner, with one microinfusion per week. One week later, the same animals were used to test the effect of the triciribine.

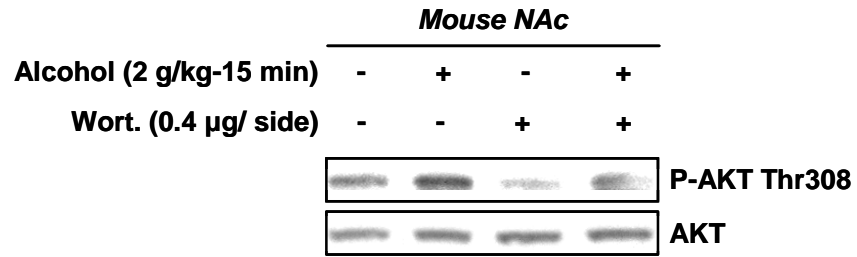
**Operant self-administration of sucrose.** Rats were trained to self-administer a solution of 1.5% of sucrose. Briefly, rats were initially trained under FR1 by using 0.1 ml of an 8% sucrose solution (weight/vol) as the reinforcer. The FR schedule was then progressively increased to FR3, and sucrose 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. In a first experiment, all subjects received vehicle or wortmannin in a counterbalanced manner, with one microinfusion per week. Two weeks later, the same animals were used to test the effect of the triciribine.



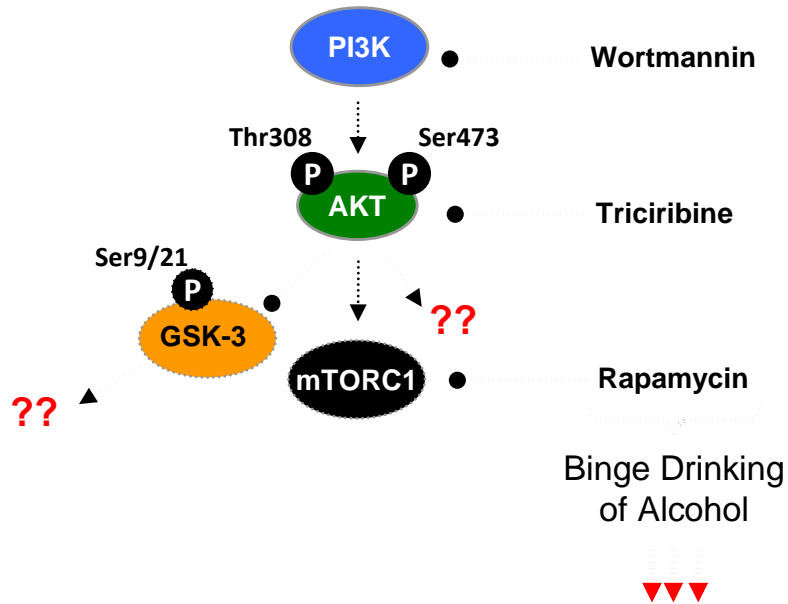
**Figure S1.** Schematic drawings of coronal sections of the rat brain showing the placement of bilateral injection sites in the nucleus accumbens (NAc) (reprinted with permission from Paxinos and Watson (1)). **(A)** Injection sites corresponding to Figures 3 and 4. **(B)** Injection sites corresponding to Figures 5 and 6. **(C)** Injection sites corresponding to Figure 7. 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.



**Figure S2.** Intra-nucleus accumbens (NAc) infusion of wortmannin in rats inhibits AKT without affecting extracellular signal-regulated kinase (ERK). Vehicle or wortmannin (Wort, 0.4 µg/ side) were infused into the NAc of rats. One hour later, the NAc were collected and the levels of AKT and ERK phosphorylation were determined by western blot analyses.  $n = 5$  per group. Data are presented as mean  $\pm$  SEM and expressed as percentage of control. \*\*\* $p < 0.001$ , one-tailed unpaired  $t$ -test.



**Figure S3.** Intra-nucleus accumbens (NAc) infusion of wortmannin in mice inhibits alcohol-induced AKT phosphorylation. Vehicle or wortmannin (Wort, 0.4 µg/ side) were infused into the NAc of mice. One hour later, mice were systemically administered (i.p.) with 2 g/kg of alcohol or saline solution, and the NAc was removed after 15 min. The level of AKT phosphorylation on threonine 308 was determined by western blot analysis. Data are representative of 2 experiments.



**Figure S4.** Excessive voluntary drinking of alcohol results in the activation of the PI3K/AKT and mTORC1 signaling pathways as well as to the inhibition of GSK-3 within the nucleus accumbens (NAc). Recurring cycles of excessive alcohol consumption and withdrawal periods result in the activation of PI3K within the NAc which in turn leads to AKT activation. AKT kinase plays a pivotal role by triggering the activation of mTORC1 signaling and at the same time by shutting down GSK-3 signaling. Intra-NAc inhibition of PI3K, AKT and mTORC1 using wortmannin, triciribine and rapamycin respectively, decreases binge drinking of alcohol in rodents.

## Reference

1. Paxinos G, Watson C (1998): *The Rat Brain in Stereotaxic Coordinates. 4th Edition.* San Diego, CA: Academic Press.