Online supplemental information

Reagents

Anti-AKT (#9272S), anti-phospho-AKT (ser473) (# 4058S), anti-S6 (#2217S) and anti-phospho-S6 (ser235-236) (#2211S) were obtained from Cell Signaling Technology (Danvers, MA). Anti-GAPDH (sc-25778) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-rabbit horseradish peroxidase (HRP) was purchased from the Jackson Immunoresearch laboratories (West Grove, PA). Anti-NeuN (#MAB377) and nitrocellulose membranes were purchased from Millipore. EDTA-free complete mini Protease Inhibitor tabs were purchased from Roche (Indianapolis, IN). Phosphatase Inhibitor Cocktails 2 and 3 were from Sigma Aldrich (St. Louis, MO). NuPAGE Bis-Tris precast gels, donkey anti-rabbit IgG AlexaFluor 594 (A21207), and donkey anti-mouse AlexaFluor 488 (#A21202) were purchased from Life Technologies (Grand Island, NY). Enhance Chemiluminescence (ECL) plus was from GE Healthcare (Buckinghamshire, UK). Pierce Bicinchoninic Acid (BCA) protein assay kit was obtained from Thermo Scientific (Rockford, IL). Ethyl alcohol (190 proof) was purchased from VWR (Randor, PA).

Two-bottle choice drinking paradigm

Intermittent access to 20% alcohol

IA20%-2BC procedure was conducted as previously described in rats (Carnicella et al., 2014) and mice (Warnault et al., 2013). Specifically, mice were given 24 hrs of concurrent access to one bottle of 20% alcohol (v/v) in tap water and one bottle of water. The drinking session started at 12:00PM 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. The water and alcohol bottles were **weighed** at the beginning and at the end of each alcohol drinking session. Rats and mice were weighted once a week. Length of drinking paradigm was 7-8 weeks (21 drinking sessions).

Continuous access to 10% alcohol

Rats underwent a 3 weeks (21 drinking sessions) continuous access to two bottles (CA10%-2BC); one containing a 10% alcohol solution (v/v) in water and the other containing tap water. The bottles were weighted every day at 12:00PM. Rats were **weighed** once a week. Rats consumed on average 1.5 g/kg/24h and were considered moderate alcohol drinkers.

Intermittent access to 1% sucrose

The procedure was similar to the IA-2BC paradigm described above, except that rats were given a solution of 1% sucrose instead of 20% alcohol. Because of a strong preference for sucrose over water, the maximum amount of sucrose available during a 24h-drinking session was capped at 20 ml. This volume of sucrose closely matches the maximum amount of alcohol consumed by excessive drinkers on the final 24h-drinking session.

Immunochemistry (IHC)

The procedure was conducted as previously described (Barak et al., 2013). Thirty min after the beginning of the last 20% alcohol IA-2BC drinking session ("binge" time point), rats were deeply anesthetized with Euthasol (Virbac, Fort Worth, TX, USA) and perfused with 1% PBS, followed by 4% paraformaldehyde (PFA) in phosphate buffer, pH 7.4. Brains were removed and fixed in the same fixative overnight, then transferred to 30% sucrose and stored at 4°C until the brain sank to the bottom of the tube. Frozen 50µm thick coronal sections were cut on a cryostat

(Leica CM3050 cryostat (Leica Biosystems)) and collected into 24-well dishes. Free-floating sections containing the striatum (Bregma +1.20;+1.00), medial prefrontal cortex (mPFC, bregma +3.20;+2.70) and the orbitofrontal cortex (OFC, bregma +3.70;+3.20) were selected and blocked with 5% normal donkey serum in PBS for 1-hr and then incubated for 24 hrs at 4°C on an orbital shaker with antibodies against the neuronal marker (anti-NeuN antibody, 1:500) and anti-phospho-S6 antibodies (anti-pS6 1:500), diluted in PBS plus 3% bovine serum albumin (BSA) and 0.05% Triton X-100. The sections were then washed 3 times for 5 min each in PBS followed by incubation for 4 hrs with the secondary antibodies Alexa Fluor 488-labeled donkey anti-rabbit and Alexa Fluor 594-labeled donkey anti-mouse (both at 1:500). After staining, sections were washed 3 times for 5 min each in PBS, and mounted in Vectashield mounting medium. Images were acquired on the Zeiss LSM510 Meta confocal microscope with the 20X Plan-Apochromat objective. Quantification was completed using the cell counter plugin in ImageJ software (NIH).

Western blot analysis

Procedure was conducted as previously described (Gibb et al., 2011; Neasta et al., 2010). Specifically, tissue homogenates (30µg per sample) were separated on a 4%-12% SDS-PAGE gel and were transferred onto nitrocellulose membrane at 30V for 2 hrs. Blots were blocked with 5% milk-PBS with 0.1% Tween 20 at RT and then probed with the appropriate primary antibodies (anti-pS6 1:2000, anti-S6 1:2000, anti-pAKT 1:1000, anti-AKT 1:1000) overnight at 4ºC. Membranes were then washed and probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for one hr at RT. Membrane were developed using the ECL and band intensities were quantified using ImageJ software (NIH).

Barak S, Liu F, Ben Hamida S, Yowell QV, Neasta J, Kharazia V, Janak PH, Ron D (2013) Disruption of alcohol-related memories by mTORC1 inhibition prevents relapse. Nat Neurosci 16:1111-1117.

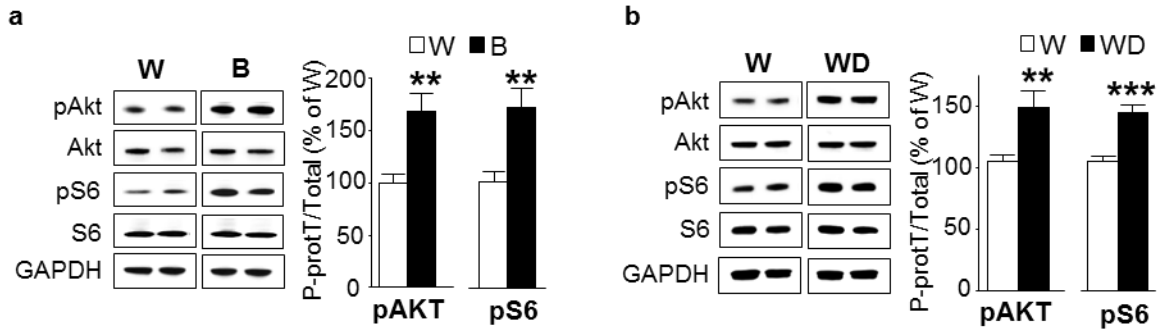
Carnicella S, Ron D, Barak S (2014) Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. Alcohol 48:243-252.

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

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

Warnault V, Darcq E, Levine A, Barak S, Ron D (2013) Chromatin remodeling — a novel strategy to control excessive alcohol drinking. Translational Psychiatry 3:e231.

Supplementary figure



Supplementary Figure 1. Excessive alcohol intake activates AKT/mTORC1 signaling in the mouse NAc.

After 7-8 weeks of IA20%-2BC, the NAc of mice were removed 4 hrs after the beginning of the last drinking session (Binge, B, see Fig.1) (a) or 24 hrs after the end of the last drinking session (Withdrawal, WD) (b). Phosphorylation of AKT and S6 was determined by western blot

analysis. ImageJ was used for optical density quantification. Data are expressed as the average ratio +/- S.E.M of phospho-AKT to AKT and phospho-S6 to S6 and are expressed as percentage of water (W) controls. Significance was determined using two-tailed unpaired *t*-test. (a) Phosphorylation level of AKT and S6 in the NAc of alcohol binge drinking mice (B) pAKT ***p*<0.01**; pS6 ***p*<0.01** n=8 alcohol, 7 water (b) Phosphorylation level of AKT and S6 in the NAc of mice after 24 hrs of withdrawal (WD) pAKT ***p*<0.01**; pS6 ***p*<0.001**. n=8 per group.**p<0.01; ***p<0.001.