**Supplementary information**

**Supplemental Material and methods**

Rabbit anti-SGK1 (#ab43606), rabbit anti-pSer422SGK1 (#ab55281) and mouse anti-GFP (#ab290) antibodies were purchased from Abcam (Cambridge, MA). Rabbit anti-Rictor (#2114), rabbit anti-pAKT Ser473 (#4058), rabbit anti-AKT (#9272), and rabbit anti-pGSK3β (#9323) antibodies were purchased from Cell signaling technology (Danvers, MA). Rabbit anti-GAPDH (#sc-25778) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-rabbit horseradish peroxidase (HRP) and donkey anti-mouse HRP conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Chicken anti-GFP (#A10262), donkey anti-mouse IgG AlexaFluor 564, donkey anti-chicken AlexaFluor 488 antibodies, and NuPAGE Bis-Tris precast gels were purchased from Life Technologies (Grand Island, NY). Mouse anti-NeuN (#MAB377), mouse anti-GSK3β (#05-412) antibodies and nitrocellulose membranes were obtained from Millipore (Billerica, MA). EDTA-free complete mini Protease Inhibitor Cocktails were from Roche (Indianapolis, IN). Mouse anti-actin antibodies (#A2228) and phosphatase inhibitor Cocktails 2 and 3 were from Sigma Aldrich (St. Louis, MO). Enhance Chemiluminescence (ECL) was from GE Healthcare (Buckinghamshire, UK). G-actin/F-actin assay kit was from Cytoskeleton Inc. (Denver, CO). Pierce bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific (Rockford, IL). The HIV-1 p24 antigen ELISA kit for determining the titer of lentivirus was purchased from ZeptoMetrix Corporation (Buffalo, NY). Ethyl alcohol (190 proof) was purchased from VWR ([Radnor, PA](https://www.google.com/search?biw=1280&bih=907&q=Radnor+Pennsylvania&stick=H4sIAAAAAAAAAOPgE-LUz9U3sMiKT7JU4gIxjQqMk4uytbSyk63084vSE_MyqxJLMvPzUDhWGamJKYWliUUlqUXFAA3oA2VFAAAA&sa=X&sqi=2&ved=0ahUKEwjzwPr758rLAhUJ32MKHVk8CEMQmxMImgEoATAR)). A-443654 was purchased from Medchem Express LLC (Monmouth Junction, NJ). Other common reagents were from Sigma Aldrich or ThermoFisher Scientific (Waltham, MA).

**Animals**

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were 8-10 weeks old at the beginning of the experiment. Mice were individually housed in a temperature- and humidity- controlled room under a reversed 12 h light/dark cycle (lights on at 10 PM), with food and water available *ad libitum*. All animal procedures were approved by the University of California San Francisco Institutional Animal Care and Use Committee (IACUC) and conducted in agreement with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

**Preparation of solutions**

Alcohol solution was prepared from ethyl alcohol solution (190 proof) diluted to 20% (v/v) in tap water. Saccharin solution (0.01%) was prepared in tap water. A-443654 (1 g/l) was prepared in 0.1% DMSO in PBS. This formulation of DMSO in PBS constituted the vehicle solution.

**Westernblot analysis**

Tissue was homogenized in ice-cold radio immunoprecipitation assay (RIPA) buffer (in mM: 50 Tris-HCL, 5 EDTA, 120 NaCl, and 1% NP-40, 0.1% deoxycholate, 0.5% SDS, proteases and phosphatases inhibitors). Samples were homogenized using a sonic dismembrator. Protein content was determined using BCA™ protein assay kit and 20-40 g of tissue homogenate was loaded for separation by SDS-PAGE. Following separation by SDS-PAGE, tissue homogenates were transferred onto nitrocellulose membrane at 300 mA for 2 hours. Membranes were blocked with 5% milk-PBS with 0.1% Tween 20 at room temperature (RT) for 30 minutes and then probed with primary antibodies overnight at 4ºC (anti-pAKT 1/1000, anti-AKT 1/1000, anti-pGSK3β 1/1000, anti-GSK3β 1/500, anti-pSGK1 1/1000, anti-SGK1 1/2000, anti-GAPDH 1/2000, anti-Rictor 1:2000, anti-actin 1/5000). Membranes were washed and probed with HRP-conjugated secondary antibodies for one hour at RT. Membrane were developed using the ECL reagent and band intensities were quantified using ImageJ software (NIH).

**F-actin/G-actin assay**

F-actin/G-actin assay was performed using the G-actin/F-actin *in vivo* assay biochem kit (Cytoskeleton Inc.) as previously described in (Laguesse *et al*, 2017) Specifically, DMS and DLS punches were homogenized in 250 l ice-cold LAS02 buffer with protease and phosphatases inhibitors cocktails and centrifuged (350g) for 5 minutes at 4ºC to remove cellular debris. Protein concentrations were determined in the supernatant using BCA™ protein assay kit. Supernatant was centrifuged at 15,000g for 30 minutes at 4ºC. The resultant supernatant contained soluble actin (G-actin). The insoluble F-actin in pellet was then re-suspended and incubated on ice for 1 hour in 250 l F-actin depolymerization buffer, with gently mixing every 15 minutes. Samples were centrifuged at 15,000g for 30 minutes at 4ºC and this supernatant was used to measure F-actin content by western blot. Twenty l of the G-actin fraction and 40 l of the F-actin fractions were loaded onto an SDS PAGE gel and analyzed by western blot.

**Immunohistochemistry**

Mice were euthanized by transcardial perfusion with 0.01M PBS followed by 4% paraformaldehyde (PFA) in phosphate buffer, pH 7.4. Brains were removed, fixed in 4% PFA overnight at 4ºC, and then cryopreserved in 30% sucrose for 3 days. Brains were then rapidly frozen and coronally sectioned into 50 m sections using a Leica CM3050 cryostat (Leica Biosystems, Richmond, IL). Free-floating sections containing the infection site in the DMS were selected. Coronal sections were blocked with 5% normal donkey serum in PBS for 1 hour and were then incubated for 24 hours at 4°C on an orbital shaker with antibodies for either a neuronal marker (anti-NeuN antibody, 1:500) in combination with the anti-GFP antibody (1:5000), diluted in PBS plus 3% BSA and 0.05% Triton X-100. Sections were then washed 3 times, for 5 minutes each, in PBS followed by incubation for 4 hours with the following secondary antibodies: Alexa Fluor 488-labeled donkey anti-rabbit and Alexa Fluor 594-labeled donkey anti-mouse (both at 1:500). After staining, the sections were washed 3 times, for 5 minutes each, in PBS, and mounted with Vectashield mounting medium. Images were acquired using a Zeiss LSM 510 META laser confocal microscope (Zeiss MicroImaging, Jena, Germany).

**Construction, preparation, characterization and intra-DMS infusion of a lentivirus expressing shRNA targeting Rictor**

Synthesized DNA oligonucleotides containing the above sequences were annealed and inserted into pLL3.7 vector (Addgene, Cambridge, MA) at HpaI and XhoI sites. Plasmids DNA were prepared using a Plasmid Maxi Kit (Qiagen, Venlo, Netherlands)**.** All constructs were verified by sequencing. The efficiency of Rictor shRNA plasmid in knocking down the protein was verified in Neuroblastoma cells by western blot analysis (data not shown). The production of lentivirus was conducted as described in (Lasek *et al*, 2007). Briefly, HEK lentiX cells (Takara Bio inc, Mountain View, CA) were transfected with the lentiviral packaging vectors psPAX2 and pMD2.G, together with the pLL3.7 shRictor or pLL3.7 SCR using lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA) in OPTIMEM medium (Sigma Aldrich, St. Louis, MO). Six hours after transfection, medium was changed to DMEM-FBS 10%. Sixty hours after transfection, supernatant containing the viral particles was collected, filtered into 0.22 m filters and purified by ultracentrifugation at 26,000g for 90 minutes at 4ºC. Pellet containing the virus was re-suspended in sterile PBS, aliquoted and stored at -80 ºC until use. Virus titer was determined using the HIV-1 p24 antigen ELISA kit (ZeptoMetrix Corporation, Buffalo, NY).

Mice were continuously anesthetized using isoflurane (Baxter,Deerfield, IL) and bilateral microinfusions were made using stainless steel injectors (33 gauges, Hamilton) into the DMS (anterioposterior +1.6mm and +1.1mm, mediolateral 1.25 mm and dorsoventral +3.0 mm; all coordinates are from bregma). Ltv-SCR or ltv-shRictor (1 l/side with 2 sites of infusion per hemisphere) was infused at an injection rate of 0.2 l/min and a titer of 1×108 pg/ml. After each infusion, the injectors were left in place for an additional 10 minutes to allow diffusion of the virus. After each infusion, the injectors were left in place for an additional 10 minutes to allow diffusion of the virus. Mice recovered for 4 weeks before experiments were initiated. A cohort of naïve animals was used to verify virus expression by immunohistochemistry and efficacy of shRNA-mediated knockdown of Rictor was confirmed by western blot analysis. At the end of the behavioral studies, one mm thick coronal sections were collected using a brain dissection block and examined for virus expression by GFP visualization using a fluorescent microscope.

**Morphological analysis**

Morphology analysis was conducted as described previously (Laguesse *et al*, 2017). Mice underwent the IA20%-2BC paradigm for 4 weeks. Afterwards, mice were divided into groups with similar baseline alcohol consumption and ltv-SCR or ltv-shRictor (1x105 pg/ml) was infused into the DMS. One week after surgery, mice underwent 4 additional weeks of IA20%-2BC (8 weeks total). Alcohol intake values on the last week of drinking were 17.85±1.05 g/kg/24h (ltv-SCR) and 17.93±0.79 g/kg/24h (ltv-shRictor). Four hours after the beginning of the last drinking session, mice were perfused and tissue sectioned coronally at 100 m. Images of overall dendritic branches and the soma of GFP stained DMS neurons were acquired with a 20x objective with a z interval of 3 m (30-35 images per cell). Images were reconstructed in 2D and GFP neurons were traced using Neurolucida software (MBF Biosciences, Williston, VT). Dendritic branches were quantified using Sholl analysis (Sholl, 1953), with the center of all concentric spheres defined as the center of the soma. Starting radius was 10 m and end radius was 150 m from the center of the soma with an interval of 10 m between radii. For dendritic spines analysis, images were acquired with a 100x oil immersion lens. Individual neurons were chosen for spine analysis based on the following criteria: (i) There was minimal or no overlap with other labeled cells. (ii) At least three primary dendrites needed to be visible for the cell to be used for analysis. (iii) Only distal dendrites (3rd or 4th order) of at least 25 m long in focus plane were analyzed (Laguesse *et al*, 2017; Lee *et al*, 2006). Image z-stacks of between 15 and 20 images were acquired at a z separation of 0.3 m. Images of spines were deconvoluted by AutoQuant X3 (Media Cybernetics, Rockville, MD) and morphological properties were analyzed by using FIJI software (NIH) as described in (Laguesse *et al*, 2017). Protrusions from dendrites were classified into 4 types based on length and neck and head morphology (Hering and Sheng, 2001). Filopodia were defined as long filamentous protrusions >2 m in length that lacked a discernable head. Stubby protuberances were defined as protrusions <1 m in length and with a head width > 0.3 m that did not appear to have a neck. Mushroom-shaped spines were defined as dendritic protrusions < 2 m in length, and characterized by a short neck and large spine head (head width > 0.5 m). Thin spines were defined as protrusions < 2 m in length that had elongated spine necks with small heads (head width < 0.5 m). For each of the 5 animals examined in each group, at least 12 neurons were analyzed, with at least 2 dendrites analyzed per neuron (Laguesse *et al*, 2017; Wang *et al*, 2015).

**Voluntary consumption of alcohol**

The IA20%–2BC procedure was conducted as previously described in (Laguesse *et al*, 2016). Specifically, mice were given 24 hours 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 hours (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). Mice that drink more than 14 g/kg/24 h, respectively, were considered excessive alcohol drinkers and included in the study (70 percent of the animals).

For the biochemical experiments testing mTORC2 activation and F/G actin content, mice underwent IA20%-2BC for 8 weeks (24 drinking sessions). Mice reaching an average level of alcohol intake higher than 14g/kg/24h on sessions 17 to 23, (i.e. last two weeks of IA20%-2BC) were selected for the study. Control mice had access to water only. Mice were sacrificed by rapid cervical dislocation and striatal tissue dissected 4-h after the beginning of the last drinking session. At this time point, mice reach a blood alcohol concentration greater than 80 mg% (Neasta *et al*, 2010), which meets the criteria of the NIAAA for binge drinking in humans (NIAAA, 2004).

For experiments testing the consequences of Rictor knockdown on spine morphology, mice underwent the IA20%-2BC paradigm for 4 weeks. Afterwards, mice were divided into groups based upon similar alcohol intake values, and low titer ltv-SCR or ltv-shRictor (1x105 pg/ml) was infused into the DMS. One week after surgery, mice underwent 4 additional weeks of IA20%-2BC (8 weeks total). Alcohol intake values on the last week of drinking were 17.85±1.05 g/kg/24h (ltv-SCR) and 17.93±0.79 g/kg/24h (ltv-shRictor).

For experiments testing the consequences of Rictor knockdown on alcohol intake, mice were bilateral infused with ltv-SCR or ltv-shRictor in the DMS. One month following surgery, animals were subjected to the IA20%-2BC paradigm for two weeks during which fluid (alcohol and water) intake was measured 4 and 24 hours after the beginning of each drinking sessions. Drinking values were corrected for spillage.

**Locomotion paradigm**

At the end of the alcohol drinking experiments, mice infected with ltv-Rictor or ltv-SCR had access to water only for one week and were tested for spontaneous locomotor activity. To do so, mice were individually placed in the center of an open-field (43 cm x 43 cm) equipped with horizontal photo beams (Med Associates, St Albans, VT). Horizontal locomotor activity by the mice was recorded as the number of photo beam breaks for 30 min.

**Saccharine intake**

At the end of the locomotion experiment, mice infected with ltv-shRictor or ltv-SCR in the DMS underwent 2 weeks of intermittent access to a solution of 0.01% saccharine or water (IA0.01%sac-2BC) during which fluid (saccharin and water) intake was measured 4 and 24 hours after the beginning of each drinking sessions.

**Cannula implantation and microinfusion of the mTORC2 activator A-443654**

Mice were continuously anesthetized using isoflurane (Baxter, Deerfiels, IL). Bilateral guide cannula (Plastic One, Roanoke, VA) were implanted in the DMS (anterioposterior +1.3mm, mediolateral ±1.25mm and dorsoventral +2.5 mm; all coordinates are from bregma) and secured with dental adhesive (C&B Metabond, Parkell Inc, Edgewood, NY) and cement (Ortho-Jet, Lang Dental, Wheeling, IL). Animals were allowed to recover for 1 week before IA20%2BC. Two weeks after surgery, animals were handled for drug microinfusions for a minimum of 3 days. Mice subsequently received microinjections of either vehicle (0.1% DMSO in PBS) or A-443654 (Han *et al*, 2007) (1g/l; 1l/side) 15-min prior to an alcohol-drinking session. Drugs were injected with bilateral infusion needles (Plastics One, Roanoke, VA) that projected 0.5 mm past the end of the guide cannulae, at a rate of 0.5ul/min. Each mouse received each microinjected treatment (vehicle and or A-443654) twice on different test session, in a counter-balanced manner. For each animal, the average of the two drinking sessions was used for each treatment. Microinjections were conducted on Wednesday and Friday of the second and third week of IA20%2BC. Fluid intake was measured 1 and 2 hours after the beginning of the test session. One week following the completion of the alcohol drinking study, mice underwent IA0.01%sac-2BC for 2 weeks. Saccharin intake is less variable than alcohol and each mouse received each treatment (vehicle and A-443654) once on different test session, in a counter-balanced manner. Microinjections and testing were conducted as described above, with saccharin drinking testing carried out on Wednesday and Friday of the second week of IA0.01%sac-2BC. After completion of the behavioral experiments, brains were harvested and post-fixed in 4% PFA for one week. Fixed brains were then rapidly frozen and coronally sectioned at 50 m using a Leica CM3050 cryostat (Leica Biosystems, Richmond, IL). Slices were stained with cresyl violet before visualization by light microscopy.

**Supplemental tables**

**Table S1.** Individual alcohol-drinking data of mice used for biochemical experiments

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Figure | Brain Region | Animal number | Mouse Number | Last session (g/kg/24hr) | Binge session (g/kg/4hr) |
| 1B | DMS | N=7 | 1  2  5  7  5  6  7  Mean ± s.e.m. | 20.1  15.6  14.33  14.55  14.01  14.28  14.53  15.34 ± 0.81 | 6.0  4.4  5.14  3.96  2.5  3.60  2.30  3.98 ± 0.51 |
| 1C | DLS | N=8 | 1  2  3  4  5  6  7  8  Mean ± s.e.m. | 22.31  18.05  18.80  16.16  16.18  15.21  10.23  12.73  16.21 ± 1.31 | 10.32  7.04  6.88  6.11  4.79  5.48  4.81  6.67  6.51 ± 0.63 |
| 2A | DMS | N=9 | 1  2  3  4  5  6  7  8  9  Mean ± s.e.m. | 8.92  11.30  17.71  16.91  12.75  14.44  18.9  16.57  17.31  14.98 ± 1.12 | 4.76  3.57  3.54  5.43  3.48  3.47  3.15  5.14  5.97  4.28 ± 0.35 |
| 2B | DLS | N=5 | 1  2  3  4  5  Mean ± s.e.m. | 22.5  12.4  16.5  14  20.1  17.1 ± 1.87 | 7.5  4.9  5.2  6.4  6  6 ± 0.46 |
| 2C | DMS | N=9 | 1  2  3  4  5  6  7  8  9  Mean ± s.e.m. | 22.5  12.4  16.5  14.0  12.75  14.44  18.9  16.57  17.31  16.16 ± 1.07 | 7.5  4.89  5.16  6.42  3.48  3.47  3.15  5.14  5.97  5.02 ± 0.49 |
| 2D | DLS | N=9 | 1  2  3  4  5  6  7  8  9  Mean ± s.e.m. | 22.5  12.4  16.5  14  20.1  12.75  14.44  18.9  16.57  16.46 ± 1.16 | 7.5  4.9  5.2  6.4  6  3.48  3.47  3.15  5.14  5.03 ± 0.49 |

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

**Table S2.** Individual alcohol-drinking data of mice used for dendritic morphology analysis

|  |  |  |  |
| --- | --- | --- | --- |
| Animal number | SCR | Session 19-21  (Before Ltv-SCR infusion) | Session 32-34  (After Ltv-SCR infusion) |
| N=6 | 1  2  3  4  5  6  Mean± s.e.m. | 21.79  15.08  14  15.47  17.76  15.97  16.67 ± 1.14 | 15.38  22.01  16.52  18.28  19.39  15.55  17.85 ± 1.05 |
|  | shRictor |  |  |
| N=6 | 1  2  3  4  5  6  Mean± s.e.m. | 15.06  15.63  15.35  18.7  16.11  18.79  16.60 ± 0.69 | 16.83  17.63  15.79  21.38  17.35  18.62  17.93 ± 0.79 |

Individual alcohol drinking data of mice used for dendritic morphology analysis. Alcohol intake is expressed as mean ±s.e.m

**Supplemental figure legends**

**Figure S1: Rictor knockdown in the DMS does not alter saccharin intake or locomotor activity.** Experimental timeline is depicted in Fig. 5A.(**A**) Saccharin intake (ml/kg/4h) across drinking sessions (left, no main effect of virus F(1,13)=0.07, *p*=0.79), and averaged saccharin on sessions 1 to 6 (right, *t*(13)=0.26, *p*=0.79). (**B**) Water intake on sessions (s1-6) in the IA0.01% sac-2BC paradigm *t*(13)=0.05, *p*=0.96. (**C**) Locomotor activity as measured by the number of beam breaks across a 30-min open-field test (left, F(1,60)=2.83, *p*=0.118), and as average beam break across the total duration of the test (right, *t*(12)=1.68, *p*=0.118). Data are expressed as mean ± S.E.M. n=7-8 per treatment.

**Figure S2:** **mTORC2 activation in the DMS does not influence saccharin intake.** Experimental timeline is depicted in Fig. 5D.(**A**) Schematic drawings of coronal sections of the mouse brain depicting the placement of bilateral infusion sites in the DMS. (**B**) saccharin intake (ml/kg/1hr) in the IA0.01%sac-2BC paradigm following intra-DMS infusion of vehicle or A-443654. *t*(7)=0.37, *p*=0.721. (**C**) water intake (ml/kg/1hr) in the IA0.01%sac-2BC paradigm following intra-DMS infusion of vehicle or A-443654. *t*(7)=1.19, *p*=0.274. n=9 per treatment.

**References**

Han EK, Leverson JD, McGonigal T, Shah OJ, Woods KW, Hunter T*, et al* (2007). Akt inhibitor A-443654 induces rapid Akt Ser-473 phosphorylation independent of mTORC1 inhibition. *Oncogene* **26**(38): 5655-5661.

Hering H, Sheng M (2001). Dendritic spines: structure, dynamics and regulation. *Nat Rev Neurosci* **2**(12): 880-888.

Laguesse S, Morisot N, Phamluong K, Ron D (2016). Region specific activation of the AKT and mTORC1 pathway in response to excessive alcohol intake in rodents. *Addict Biol*.

Laguesse S, Morisot N, Shin JH, Liu F, Adrover MF, Sakhai SA*, et al* (2017). Prosapip1-Dependent Synaptic Adaptations in the Nucleus Accumbens Drive Alcohol Intake, Seeking, and Reward. *Neuron* **96**(1): 145-159 e148.

Lasek AW, Janak PH, He L, Whistler JL, Heberlein U (2007). Downregulation of mu opioid receptor by RNA interference in the ventral tegmental area reduces ethanol consumption in mice. *Genes, brain, and behavior* **6**(8): 728-735.

Lee KW, Kim Y, Kim AM, Helmin K, Nairn AC, Greengard P (2006). Cocaine-induced dendritic spine formation in D1 and D2 dopamine receptor-containing medium spiny neurons in nucleus accumbens. *Proc Natl Acad Sci U S A* **103**(9): 3399-3404.

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**(46): 20093-20098.

NIAAA (2004). NIAAA council approves definition of binge drinking. *NIAAA Newsletter, Winter 2004, Number 3*. Office of Research Translation and Communications, NIAAA, NIH, DHHS: Bethesda, MD. Vol 3, p 3.

Sholl DA (1953). Dendritic organization in the neurons of the visual and motor cortices of the cat. *J Anat* **87**(4): 387-406.

Wang J, Cheng Y, Wang X, Roltsch Hellard E, Ma T, Gil H*, et al* (2015). Alcohol Elicits Functional and Structural Plasticity Selectively in Dopamine D1 Receptor-Expressing Neurons of the Dorsomedial Striatum. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **35**(33): 11634-11643.