mTORC1 IN THE ORBITOFRONTAL CORTEX PROMOTES HABITUAL ALCOHOL SEEKING

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ABSTRACT

The mechanistic target of rapamycin complex 1 (mTORC1) plays an important role in dendritic translation and in learning and memory. We previously showed that heavy alcohol use activates mTORC1 in the orbitofrontal cortex (OFC) of rodents (1). Here, we set out to determine the consequences of alcohol-dependent mTORC1 activation in the OFC. We found that inhibition of mTORC1 activity attenuates alcohol seeking and restores sensitivity to outcome devaluation in rats that habitually seek alcohol. In contrast, habitual responding for sucrose was unaltered by mTORC1 inhibition, suggesting that mTORC1's role in habitual behavior is specific to alcohol. We further show that inhibition of GluN2B in the OFC attenuates alcohol-dependent mTORC1 activation, alcohol seeking and habitual responding for alcohol. Together, these data suggest that the GluN2B/mTORC1 axis in the OFC drives alcohol seeking and habit.

INTRODUCTION

mTORC1 is a multiprotein complex that contains the serine/threonine protein kinase mTOR, the regulatory associated protein of TOR (Raptor), and other enzymes and adaptor proteins (2). Upon activation, mTORC1 phosphorylates eIF4E-binding protein (4E-BP) and the ribosomal protein S6 kinase (S6K), which in turn phosphorylates its substrate, S6 (3). These phosphorylation events lead to the translation of a subset of mRNAs to proteins (2, 3). In the CNS, mTORC1 is responsible for the local dendritic translation of synaptic proteins (4, 5). As such, mTORC1 plays an important role in synaptic plasticity, and learning and memory (6).

Increasing lines of evidence in rodents suggest that mTORC1 is a key player in mechanisms underlying alcohol use disorder (AUD) (7). For instance, excessive alcohol intake and reinstatement of alcohol place preference activate mTORC1 in the rodent nucleus accumbens (NAc) (1, 8-10), resulting in the translation of synaptic proteins, which in turn produce synaptic and structural adaptations that drive and maintain heavy alcohol use and relapse (1, 8, 9, 11, 12). Repeated cycles of voluntary binge drinking of alcohol and withdrawal also produces a robust and sustained activation of mTORC1 in the OFC of rodents (1); however, the behavioral consequences of this activation are unknown.

The OFC integrates sensory and reward information (13) and is an essential player in decision making (13-15), in stimulus-outcome association (16-18), in updating the value of predicted outcomes (15, 19), in goal-directed behaviors (16, 20, 21), and in compulsive behavior (22, 23).

Exposure to drugs of abuse impairs the performance of OFC-dependent behavioral tasks (24), and aberrant neuroadaptations induced by drugs of abuse in the OFC contribute to drug-seeking and compulsive drug taking (24-26). For example, withdrawal from alcohol vapor

exposure produces morphological and cellular alterations in rodents (27, 28). Inactivation of the OFC enhances alcohol consumption in alcohol-dependent mice (29), and chronic alcohol use alters the protein landscape in the primate OFC (30). In humans, degraded OFC white matter and reduced neuronal density are associated with alcohol-dependence (31, 32), and presentation of alcohol-associated cues to alcohol-dependent subjects elicits OFC activation (33-35).

Here, using rats as a model system, we set out to examine whether mTORC1 in the OFC contributes to the mechanisms underlying AUD.

METHODS

Reagents

Anti-cFos (1:500), anti-phospho-S6 (S235/236, 1:500), anti-S6 (1:1000), Alexa Fluor 488-labeled anti-phospho-S6 (S235/236, 1:250), anti-phospho-4E-BP (T37/46, 1:500), anti-4E-BP (1:1000), anti-Raptor (1:1000), anti-phospho-CaMKII (T286, 1:1000), anti-phospho-S6K (T389, 1:500) and anti-S6K (1:500) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-CaMKII (1:500) and anti-GAPDH (1:5000) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Actin (1:10,000) antibodies, phosphatase Inhibitor Cocktails 2 and 3, Dimethyl sulfoxide (DMSO), D-sucrose and NMDA were from Sigma Aldrich (St. Louis, MO). Nitrocellulose membranes and anti-NeuN antibodies were purchased from EMD Millipore (Billerica, MA, USA). Enhanced Chemiluminescence (ECL) was purchased from GE Healthcare (Pittsburg, PA, USA). The secondary antibodies Alexa Fluor 488-labeled donkey anti-rabbit, Alexa Fluor 594-labeled donkey anti-rabbit and Alexa Fluor 594-labeled donkey anti-mouse, Prolong Gold mounting media and NuPAGE® Bis-Tris pre-casted gels were purchased from LifeTechnologies (Carlsbad, CA, USA). Anti-GFP antibodies were purchased from Abcam (Cambridge, MA, USA). Donkey anti-rabbit horseradish peroxidase (HRP), and donkey anti-mouse horseradish peroxidase (HRP) were purchased from Jackson ImmunoResearch (West Grove, PA). EDTA-free complete mini Protease Inhibitor Cocktails were purchased from Roche (Indianapolis, IN). NuPAGE Bis-Tris precast gels and Phosphate buffered saline (PBS) were purchased from Life Technologies (Grand Island, NY). Pierce Bicinchoninic Acid (BCA) protein assay kit was obtained from Thermo Scientific (Rockford, IL), and ProSignal Blotting Film were purchased from Genesee Scientific (El Cajon, CA). Ethyl alcohol (190 proof) was purchased from

VWR (Radnor, PA). Rapamycin was purchased from LC laboratories (Woburn, MA). Ro25-6981 was purchased from Tocris Bioscience (Bristol, UK).

Subjects

Male Long Evans rats (Harlan, Indianapolis, IN) were one (*ex vivo* experiment) or two months (*in vivo* experiments) old at their arrival. Animals were single-housed in a temperature- and humidity-controlled colony room (22±2 °C, relative humidity: 50–60%) under a normal 12h light/dark cycle (lights on at 7:00AM) with food and water available ad libitum. Rats were given a week of habituation to the housing conditions before the beginning of the experiments. All animal procedures were approved by the University of California San Francisco Institutional Animal Care and Use Committee (IACUC) and were conducted in agreement with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Preparation of solutions

Alcohol was diluted to 20% (v/v) and sucrose to 1-8% (w/v) in tap water. Rapamycin (50ng/µl) was dissolved in PBS. Ro25-6981 (10µg/µl) and NMDA (25µM) were dissolved in 0.1% DMSO in PBS.

Intermittent access to 20% alcohol using two bottle choice and drug infusion

Rats underwent intermittent access to 20% alcohol in a 2-bottle choice (IA-20%2BC) paradigm for 7 weeks as described in (36). Specifically, rats were given 24 hours of concurrent access to one bottle of 20% alcohol (v/v) in tap water and one bottle of water. Control rats had access to water only. Drinking sessions started at 12:00pm on Monday, Wednesday and Friday, with 24- or 48

hours (weekend) of alcohol-deprivation periods in which rats consumed only water. The placement (left or right) of the water or alcohol solution was alternated between each session to control for side preference. Water and alcohol bottles were weighed at the beginning and at the end of each alcohol drinking session. Rats were weighed once a week. Rats drinking more than 3.5 g/kg/24h on the last week of IA-20%2BC were selected for the study, approximately 70-80% of the cohort.

Stereotaxic Surgery

Following stable baseline of alcohol drinking, rats underwent stereotaxic surgery. Specifically, rats were continuously anesthetized using isoflurane (Baxter) and bilateral guide cannula (Plastic One) were implanted in the ventrolateral OFC (anterioposterior +3.5mm, mediolateral ±2mm and dorsoventral +3.9mm; all coordinates are from bregma), secured with screws (Plastic One) and dental cement (Ortho-Jet, Lang Dental).

Alcohol seeking and drug infusion

First, rats underwent IA-20%2BC paradigm for 7 weeks, as described above. Rats drinking more than 3.5 g/kg/24h on the last week of IA-20%2BC were selected for alcohol self-administration training in an operant self-administration (OSA) paradigm (37) with modification adapted from (21, 38, 39). Training was conducted in operant chambers (Med-Associates; Georgia, VT) using a fixed ratio 3 (FR3) (i.e. three active lever presses result in the delivery of one alcohol reward) during daily 30 minutes-session (Mon-Fri) as previously described (37). An inactive lever was also extended during the operant sessions but had no programmed consequence. Following stable baseline of alcohol responding, rats underwent stereotaxic surgery for cannula placement in the OFC, as described above. After a one-week recovery period, operant self-administration procedure

was resumed and handling for drug microinfusions began. Rats received infusion of either vehicle or rapamycin (50ng/µl/side) 3 hours or R025-6981 (10µg/µl/side) 15 minutes before a single 30 minutes-extinction session. Drugs were injected with bilateral infusion needles (Plastics One) that projected 0.5 mm past the end of the guide cannula, at a rate of 0.5µl/minute. Drug testing was performed using a "within-subject" design in which rats received both treatments in counterbalanced order.

Upon establishing a baseline of IA-20%2BC, a separate cohort of animals received an infusion of R025-6981 (10 µg/µl/side) or vehicle 15 minutes prior to the end of the last withdrawal session. The OFC was harvested at the end of the 24-hrs withdrawal session and were processed for western blot analysis.

Habitual and goal-directed alcohol seeking and drug infusion

After 7 weeks of IA-20%2BC, high-drinking rats (alcohol intake >3.5 g/kg/24h) were trained to self-administer 20% alcohol in an operant self-administration (OSA) paradigm (37) with modification adapted from (21, 38, 39). Self-administration training was initiated under FR1 for 3 consecutive overnight sessions. Afterwards, the sessions lasted for an hour. Only one lever was available during the operant sessions. Rats were pseudo-randomly assigned to 2 groups and were subjected to a random interval (RI), or a random ratio (RR) schedule of reinforcement which biases responding towards habitual or goal directed actions, respectively (16).

RI training: During RI training, each reward was delivered following one lever press occurring after variable reward delivery time intervals (16). Three sessions were under RI-5 (i.e. inter-reward time was 5 seconds on average, ranging from 1 to 10 seconds), followed by three RI-15 sessions (i.e. inter-reward time was 15 seconds on average ranging from 10 to 30 seconds). Reinforcement

was then shifted to RI-30 (i.e. inter-reward time was 30 seconds on average ranging from 10 to 50s) for at least 20 sessions.

RR training: During RR training, reward was delivered following a variable number of lever responses (16). Six sessions under RR-2 (1 reward delivery following on average 2 lever presses with number of presses ranging from 1 to 3) followed by RR-3 (1 reward delivery following on average 3 lever presses with number of presses ranging from 1 to 5) for at least 20 sessions.

Training data e.g. lever presses and alcohol consumed are shown in **Supplemental Fig. 3**. Only rats that self-administered alcohol more than 0.3g/kg/hour were included in the study.

Following stable RI-30 or RR-3 responding, rats underwent alcohol devaluation testing as described in (38) with modifications. Rats were given home-cage 2BC access to 1% sucrose on non-devalued (ND) days or 20% alcohol on devalued (D) days for 30 minutes (**Fig. 2a**). The amount of alcohol and sucrose consumed on ND and D day, respectively, are reported in **Table 1**. On each day, immediately after home-cage pre-feeding, rats underwent a 10-minutes extinction test during which unrewarded lever presses were recorded. A "within-subject" design was used in which ND or D sessions were in a counterbalanced order, with 2 standard retraining sessions between the tests. Rats that did not acquire the behavior and were pressing less than 5 lever presses per session on ND day were excluded from analysis. In order to visualize individual lever pressing, we normalized ND and D lever presses, which minimizes the effects of individual variance in total lever pressing and has been used extensively to study outcome devaluation (15, 16, 21). Normalized lever pressing was calculated as ND or D lever presses/total number of lever presses, respectively. Normalizing devaluation lever presses produces a distribution of data, where 50% is equivalent to equal lever pressing between ND and D (16). Devaluation index was calculated as the ratio of lever presses on ND day–lever presses on D day/total number of lever presses. At the

end of the training period and upon acquiring the behavior (RR, goal directed behavior and RI, habitual behavior), rats underwent stereotaxic surgery to bilaterally implant a guide cannula in the ventrolateral OFC, as described above. One week later, RI-30 or RR-3 training was resumed for 2 weeks after which rats received microinjections of either vehicle or rapamycin (50ng/µl/side) 3 hours or R025-6981 (5µg/µl/side) 15 minutes prior an alcohol devaluation testing.

Habitual sucrose seeking

RI30 training was similar to the procedure described for alcohol except that 8% sucrose was initially used and progressively reduced to 1% across training sessions. Only ten RI30 sessions (2 weeks) were conducted prior to the initial outcome devaluation test. Rats were then implanted with a guide cannula in the OFC as described above. After one-week recovery period, sucrose self-administration was resumed for two weeks, after which rats received microinjections of vehicle or drug in a counter-balanced manner, prior to the devaluation test. Sensitivity to changes in sucrose value was tested using a sucrose devaluation procedure which was similar to alcohol devaluation except that rats had 2BC access to 20% alcohol on ND day and 1% sucrose on D day (**Fig. 3a and Timeline Fig. 3b**). The amount of alcohol and sucrose consumed on ND and D day, respectively, are reported in **Table 1**. RI-trained animals displayed insensitivity to sucrose devaluation (**Fig. 3c-d, vehicle**), revealing a rapid shift toward habitual sucrose responding as previously described (38).

Histology

For verification of cannula placement, brains were post-fixed in 4% PFA for one week, then rapidly frozen and sectioned into 50μm coronal slices using a Leica CM3050 cryostat (Leica

Biosystems). Slices were stained using cresyl violet and examined for cannula placement using a bright-field microscope. All rats had correct guide cannula placement and were included in the study. For verification of virus expression, brains were sectioned in 1mm-thick coronal section using a brain mold, and GFP fluorescence was visualized using a fluorescent microscope. One rat had no virus expression and was excluded from the study.

Ex vivo NMDA treatment

Naïve rats were euthanized by isoflurane and decapitated. Brains were removed and sectioned into 300 μm-thick slices at 4° C in a solution of aCSF containing (in mM) 200 sucrose, 1.9 KCl, 1.4 NaH2PO4, 0.5 CaCl2, 6 MgCl2, 10 glucose, 25 NaHCO3, 4 ascorbic acid; 310-330 mOsm. Immediately after sectioning, OFC-containing slices were recovered in aCSF (in mM: 125 NaCl, 2.5 KCl, 1.4 NaH2PO4, 25 NaHCO3, 2 CaCl2, 1.3 MgCl2, 25 glucose, 0.4 ascorbic acid) heated at 32° C for 30 minutes and then moved to an aCSF solution containing 1 μM TTX at room temperature for 30 minutes (40). The OFC was dissected and incubated for 30 minutes in aCSF containing 1 μM TTX at room temperature. OFC sections were then treated for 3 minutes with vehicle (0.1% DMSO) or NMDA (25μM) in TTX-containing aCSF , and western blot analysis was conducted as described below.

Western blot analysis

Tissue were homogenized in ice-cold radio immunoprecipitation assay (RIPA) buffer (in mM: 50 Tris-Cl, 5 EDTA, 120 NaCl, 1% NP-40, 0.1% deoxycholate, 0.5% SDS, protease and phosphatase inhibitors), using a sonic dismembrator. Protein content was determined using BCA kit. Tissue homogenates (30 μg per sample) were resolved on NuPAGE 10% Bis-Tris gels at 100 V for 2

hours and transferred onto nitrocellulose membranes at 30V for 2 hours. Blots were blocked with 5% milk-phosphate-buffered saline with 0.1% tween-20 at room temperature and then probed with primary antibodies overnight at 4°C. Membranes were washed and probed with HRP-conjugated secondary antibodies for 2 hours at room temperature, and bands were visualized using ECL. Band intensities were quantified by ImageJ (National Institutes of Health, MD, USA).

Immunohistochemistry

Immunohistochemistry was conducted as described previously (1). Rats were euthanized with Euthasol (Virbac Corp., Fort Worth, TX) and transcardially perfused 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 50μm sections using a Leica CM3050 cryostat (Leica Biosystems). Free-floating sections containing the infection site in the OFC were selected. Coronal sections were blocked with 5% normal donkey serum in PBS for 1 hour and then incubated for 24 hours at 4°C on an orbital shaker with anti-NeuN and anti-GFP antibodies diluted in PBS plus 3% BSA and 0.05% Triton X-100. The 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 in Vectashield mounting medium. Images were acquired on the Zeiss LSM510 Meta confocal microscope (Zeiss MicroImaging, Jena, Germany) with the 20X Plan-Apochromat objective. Quantification was completed using the cell counter plugin in ImageJ software (NIH).

Data analysis

All data are expressed as the mean ± SEM. GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot and analyze the data. D'Agostino–Pearson normality test and *F*-test/Levene test were used to verify the normal distribution of variables and the homogeneity of variance, respectively. The sample sizes were based on previous publications (8, 41). Data between two groups were compared using two-tailed paired or unpaired *t*-test. Data from multiple groups against one group were compared using a two-way repeated-measures analysis of variance followed when appropriate by a Tukey multiple comparisons test or a Sidak's multiple comparisons test. Statistical significance was set at $p<0.05$.

RESULTS

mTORC1 in the OFC contributes to alcohol seeking

We first examined whether activation of mTORC1 in the OFC contributes to the maintenance of heavy alcohol use. Rats that underwent 7 weeks of IA-20%2BC received bilateral intra-OFC infusion of vehicle or the mTORC1 inhibitor, rapamycin (50ng/μl), 3 hours prior to a drinking session (8). As shown in **Supplementary Fig. 1,** intra-OFC administration of rapamycin did not impact voluntary intake of alcohol. Next, rats that underwent IA-20%2BC for 7 weeks were trained to self-administer 20% alcohol in an OSA paradigm (42). Upon establishing a baseline of alcohol lever presses, rats received bilateral infusion of vehicle or rapamycin (50ng/μl) into the OFC 3 hours prior to an OSA session, and the number of lever presses and alcohol consumption were measured (8). As shown in **Fig. 1a-c**, inhibition of mTORC1 in the OFC failed to alter the number of lever presses (**Fig.1a-b**), and alcohol consumed (**Fig. 1c**). Inactive lever pressing, and the latency to the first or last press were also unaltered (**Supplementary Fig. 2a-c**). Next, we determined whether mTORC1 plays a role in alcohol seeking by testing whether rapamycin alters lever presses during an extinction session. Intra-OFC administration of rapamycin suppressed both the number (**Fig.1d-e**), and the rate (**Fig. 1f**) of rats' responding on the lever that was previously associated with alcohol. The reduction in alcohol seeking was not due to alterations in locomotion, as inter-response intervals were similar in vehicle and rapamycin-treated rats (**Supplementary Fig. 2d**). Together, these data suggest that mTORC1 in the OFC does not contribute to alcohol drinking *per se,* but does promote alcohol seeking.

mTORC1 in the OFC participates in habitual responding for alcohol

Reward seeking can be driven by goal-directed or habitual compulsive behaviors (43-45). We next examined whether mTORC1 in the OFC plays a role in goal-directed or habitual alcohol seeking using a satiety outcome devaluation procedure (**Fig. 2a**). Rats that first underwent 7 weeks of IA-20%2BC were then trained to lever press for 20% alcohol using either an RR or RI schedule of reinforcement, which biases lever responding toward goal-directed (RR) or habitual (RI) actions (16, 21, 38) (**Supplementary Fig. 3a-b**). RR and RI trained rats showed a similar number of presses throughout the training (**Supplementary Fig. 3a**), however alcohol consumption was slightly higher during RI training, presumably due to fewer lever presses being required to obtain the same volume of alcohol, compared with RR training. Rats trained to self-administer alcohol in a goal-directed manner are sensitive to outcome devaluation, while habitually-trained rats are not; animals who habitually press a lever to obtain a reward will continue to press that lever, even when the relative value of the reward is reduced due to satiety (16, 21, 38, 46). Thus, we conducted a devaluation procedure to differentiate between goal-directed and habitual responding, (**Fig. 2a**). On the devalued (D) day, rats had 30 minutes of home-cage access to 20% alcohol, the reward previously associated with lever responding, and on the non-devalued (ND) day, rats had 30 minutes of home-cage access to 1% sucrose, a reward not previously associated with lever responding (**Fig. 2a**). Rats then underwent a 10-minute extinction test, during which lever presses were recorded (**Fig. 2a**). A timeline for the experiments is outlined in **Fig. 2d**. In order to minimize the effects of individual variability in overall lever pressing, and to graphically represent the distribution of lever presses between ND and D, we normalized lever-pressing data such that 50% indicates identical lever pressing on both days (15, 16, 21). As shown in **Fig. 2b-c,** rats trained on an RR schedule exhibit a decrease in responding following alcohol devaluation, while RI-trained

rats remain insensitive to devaluation. These data shows that RI OSA training can produce habitual responding for alcohol in rats.

Next, to test whether mTORC1 in the OFC participates in goal-directed or habitual alcohol seeking, we infused rapamycin (50ng/μl) or vehicle bilaterally, 3 hours prior to a devaluation session, and examined lever presses in RR- and RI-trained groups. Rapamycin administration did not alter home cage alcohol intake prior to the extinction session in either group (**Supplementary Fig. 4**). Surprisingly, intra-OFC administration of rapamycin did not impact responding in RR-trained (goal-directed) cohorts, as lever presses were reduced in rats that were pre-fed with alcohol and treated with either vehicle or rapamycin (**Fig. 2e-f**). In contrast, inhibition of mTORC1 in the OFC reduced responding in the RI-trained (habitual) rats in the devalued condition (**Fig.2g-h**). Critically, the reduction in responding in the rapamycin-treated RI-trained rats paralleled the responding of RR-trained animals (**Fig.2e-h**). These data suggest that mTORC1 inhibition in the OFC restores goal-directed alcohol seeking in animals trained to habitually self-administer alcohol.

To determine whether mTORC1 in the OFC plays a role in habitual responding to a natural reward, animals were trained to self-administer sucrose under an RI schedule of reinforcement, and lever presses were measured in rats treated with rapamycin or vehicle during an extinction session in animals that were first offered sucrose (devalued) or alcohol (non-devalued) in their home cage **(Timeline, Fig. 3a-b)**.. Rapamycin administration did not alter home cage sucrose intake prior to the extinction session (**Supplementary Fig. 5**) As shown in **Fig. 3c-d**, habitual responding for sucrose was similar in the vehicle- and rapamycin-treated animals. Together, these data suggest that mTORC1's contribution to habitual behavior is not generalized to all rewarding substances.

Alcohol-dependent mTORC1 activation in the OFC requires GluN2B

Next, we set out to elucidate the mechanism by which long-term heavy alcohol use activates mTORC1 in the OFC. Glutamatergic inputs from cortical and subcortical structures project to the OFC (47, 48). Extracellular glutamate content is elevated in cortical regions of humans and rats during alcohol withdrawal (49). N-methyl D-Aspartate receptors (NMDAR) are targets of alcohol (50). For example, GluN2B-containing NMDARs are activated by alcohol in the hippocampus and dorsal striatum (50). Stimulation of GluN2B activates the guanine nucleotide releasing factor 1 (GRF1), the activator of H-Ras (51). Since H-Ras is upstream of mTORC1 (52), we hypothesized that alcohol-dependent stimulation of GluN2B activates mTORC1 in the OFC. We first tested whether NMDAR stimulation activates mTORC1. To do so, OFC slices were briefly treated with NMDA (25μM), and the activation of CaMKII and mTORC1 were tested by measuring the phosphorylation of mTORC1 downstream targets, S6K and S6. We found that *ex vivo* treatment of the OFC with NMDA led to increased phosphorylation, and thus activation, of CaMKII, a marker of NMDAR activation (**Fig. 4a-b, Supplementary Fig. 6**), as well as of mTORC1 (**Fig. 4a-b, Supplementary Fig. 6**). These data suggest that NMDAR stimulation activates mTORC1 in the OFC.

We previously showed that mTORC1 is activated in the OFC in response to a binge drinking session which persists for at least 24 hours into alcohol abstinence (1). Thus, we next tested whether GluN2B is required for alcohol-dependent mTORC1 activation. To do so, rats that underwent 7-weeks of IA-20%2BC paradigm or that drank water only for the same duration of time, received bilateral intra-OFC infusion of the selective GluN2B antagonist Ro 25-6981 (53) (10μg/ul) or vehicle, 15 minutes prior to the end of the last alcohol-deprivation session, and mTORC1 activation was examined by measuring the phosphorylation of mTORC1 downstream

targets, 4-EBP and S6. In vehicle-treated rats, alcohol produced a robust activation of CaMKII (**Fig. 4c,f, Supplementary Fig. 7**) and mTORC1, as measured by S6 (**Fig. 4d,g, Supplementary Fig. 7**), and 4-EBP phosphorylation (**Fig. 4e,h, Supplementary Fig. 7**). In contrast, R025-6981-treated rats exhibited a marked reduction in both CaMKII and mTORC1 activation during alcohol withdrawal **Fig. 4c-h**. However, GluN2B protein levels in the OFC were unchanged by alcohol (**Supplementary Fig. 8).** Together our data suggest that GluN2B mediates mTORC1 activation following alcohol consumption.

GluN2B in the OFC is required for alcohol seeking and habit

We reasoned that, if GluN2B is upstream of mTORC1, then GluN2B should also participate in habitual alcohol seeking. First, we examined whether inhibition of GluN2B alters alcohol seeking during extinction. We found that intra-OFC administration of Ro25-6981 (10μg/μl) 15 minutes prior to an extinction session suppressed alcohol seeking, as indicated by a reduction in cumulative (**Fig. 5a**) and active lever presses (**Fig. 5b**), and the rate (**Fig. 5c**) of lever pressing. Importantly, intra-OFC administration of Ro25-6981 (5μg/μl) reverted habitual responding for alcohol to goal directed responding in rats trained under the RI (habitual) schedule of reinforcement (**Fig.5d-e**). In contrast, intra-OFC infusion of R025-6981, did not impact alcohol consumption in a 2BC test during devalued days (**Supplementary Fig. 9**). Finally, as shown in **Supplementary Fig. 10,** inhibition of GluN2B in the OFC did not change locomotion. Together, these data indicate that the recruitment of GluN2B signaling by alcohol is required for alcohol seeking and habit.

DISCUSSION

Using operant training protocols that bias alcohol seeking towards habitual or goal-directed behavior, we present data implicating mTORC1 in the OFC in mechanisms underlying the development and/or maintenance of habitual behavior associated with alcohol use. In addition, we show that GluN2B in the OFC is required for alcohol-dependent mTORC1 activation and function. Together, our data suggest that the GluN2B/mTORC1 axis acts as a molecular switch that converts the OFC from driving goal directed to habitual alcohol use.

We found that inhibition of mTORC1 in the OFC attenuates lever pressing during extinction, suggesting that mTORC1 regulates alcohol seeking. The OFC has been implicated in updating reward anticipation based on the value of an expected outcome (17, 54), and O'Doherty et al. showed that the lateral OFC (lOFC) is activated during reward anticipation in humans (55). Thus, it is plausible that one of the roles of mTORC1 in the OFC is the strengthening of alcohol reward anticipation, thus promoting alcohol seeking.

We further show that blockade of mTORC1 activity in the OFC converts habitual alcohol responding to a more goal directed behavior. Habitual behavior is thought to rely on the strengthening of the dorsolateral striatum (DLS) over dorsomedial striatum (DMS), a brain region which participates in goal-directed behaviors (38, 56, 57). Renteria and colleagues showed that alcohol vapor exposure generates habitual behavior via the disruption of OFC to DMS circuit (39). Thus, it is plausible that mTORC1 activation is strengthening OFC projections to the DLS, and/or is weakening OFC to DMS projections. One caveat to consider, however, is that we measured habitual vs. goal directed responding for alcohol, whereas Renteria et al. measured responding for food. It is plausible that Renteria et al. tested whether alcohol alters decision making in general, while the current study is focused on habitual responding specifically for alcohol. It would be of

interest to determine the normal role of mTORC1 in the OFC, and whether mTORC1 suppresses other decision-making tasks.

We previously showed that mTORC1 activation is detected in the lOFC (1), which was targeted in the current study. However, the structure of the OFC is complex. The medial OFC (mOFC) and lOFC are each part of anatomically and functionally distinct corticostriatal circuits in humans (58) and rodents (59). The mOFC has been reported to play a larger role in determining, updating, and storing the relative reward values of stimuli and actions (60). The lOFC, on the other hand, is proposed to be more involved in encoding the relationship between stimuli/actions and outcomes (60), reward anticipation and goal directed behavior (16). As mTORC1's activation is localized to the lOFC, more research is required to determine if mTORC1 contributes to other lOFC-related behaviors.

The OFC has been associated with compulsive behavior (22), and recently Pascoli et al. reported that OFC neurons projecting to the dorsal striatum are associated with compulsive lever pressing in response to optogenetic activation of dopaminergic ventral tegmental area neurons (23). Compulsive drug use is associated with maladaptive habitual responding (57, 61), and we and others, provided animal data to support the notion that alcohol consumption can become compulsive (44, 62, 63). Thus, it is possible that mTORC1 signaling in the OFC is driving both habitual and compulsive alcohol seeking.

Interestingly, mTORC1 inhibition in the OFC does not alter habitual responding for sucrose, indicating that mTORC1 plays a specific role in the formation of habits in response to alcohol exposure and not natural rewards. The difference between the consequences of mTORC1 inhibition on habitual alcohol vs. sucrose seeking is striking, but it is important to note that, unlike alcohol, voluntary sucrose intake does not activate mTORC1 in the OFC (1).

Curiously, Corbit et al. showed that RR-trained rats exhibit habitual alcohol self-administration after 8 weeks of training on an RR3 schedule of reinforcement (38). In the current study, rats undergoing RR training demonstrated persistent goal-directed alcohol seeking behaviors. The difference between the two studies is most likely to be due to the length of RR training (long in Corbit et al. (38) vs. short herein). It is possible that further training would drive our RR-trained goal-directed rats to exhibit more habitual phenotypes. Further research would be required to compare the models more accurately.

We previously reported that repeated cycles of binge alcohol drinking and withdrawal activates mTORC1 in the OFC (1), and we demonstrate herein that mTORC1 activation in the OFC depends, at least in part, on GluN2B suggesting that GluN2B is activated in response to alcohol use. The NMDAR is a target of alcohol (50), and alcohol enhances GluN2B phosphorylation in brain regions such as the cerebellum, the DMS and the hippocampus (50). GluN2B phosphorylation enhances the activity of the channel (69). Thus, it is plausible that alcohol-dependent GluN2B phosphorylation in the OFC enhances GluN2B function thereby enabling mTORC1 activation. Alternatively, the hyperactivity of GluN2B could be a compensatory homeostatic plasticity resulting from the inhibition of NMDAR by alcohol (50). In contrast to our findings that alcohol does not alter the expression of GluN2B in the OFC, Nimitvilai and colleagues showed that chronic exposure to alcohol vapor decreases in the expression of GluN2B (28). One important difference between our study and Nimitvilai et al. as well as additional reports on the interaction between the OFC and alcohol discussed herein, relates to the use of contingent vs. non-contingent alcohol administration. Specifically, prior to the OSA, rats were subjected to 7 weeks of IA-20%2BC. The IA-20%2BC paradigm models "problem drinkers" e.g. human subjects that suffer from AUD phenotypes such as binge drinking, compulsive drinking

and craving (64). In our case, rats achieve blood alcohol concentrations (BAC) of 80 mg/dl (65) a BAC equivalent to binge drinking humans (66). In contrast, the majority of other reports described herein involved passive exposure of alcohol vapor, which achieves BAC of 150-250 mg/dl, and leads to physical dependence on alcohol (67). Humans who are dependent on alcohol show profoundly degraded white matter and reduced neuronal density in the OFC (31, 32), possibly an indication of alcohol-induced damage to this structure. Thus, it is likely that cycles of binge drinking do not damage the OFC which may be the case in animal models of alcohol dependence. As only a small percentage of alcohol users exhibit physical dependence (68), we believe that our findings have important implications for the understanding of the mechanisms underlying problem drinking and AUD.

What could be the mechanism by which mTORC1 contributes to alcohol seeking and habit? Activation of mTORC1 triggers the translation of a subset of mRNAs to protein in the cell body and in dendrites (3, 70). We previously showed that long-term excessive alcohol intake and reinstatement of alcohol place preference initiate the translation of several synaptic proteins in the NAc (11, 12, 36). We also found that reconsolidation of alcohol reward memories increases the immunoreactivity of mTORC1 targets in the OFC (41). Thus, it is possible that the consequences of mTORC1 activation in the OFC is the translation of synaptic proteins.

We show here that GluN2B activation in the OFC is sufficient to influence the formation and/or maintenance of alcohol seeking and habit. In line with the possibility that GluN2B activation contributes to the development of habit, DePoy et al. showed that habitual behavior, developed upon subchronic cocaine treatment during adolescence (71), is inhibited by the treatment of animals with the GluN2B inhibitor ifenprodil (71). DePoy et al. further showed that cocaine exposure during adolescence produces structural changes in the OFC which were

attenuated by ifenprodil (71). While our data suggest that GluN2B in the OFC participates in behavioral inflexibility, Brigman et al. reported that GluN2B in the OFC plays a role in choice learning (72), It is plausible that alcohol-dependent neuroadaptations tilt the function of GluN2B from choice learning, e.g. a goal directed behavior, to habitual responding.

Gremel et al. showed that the endocannabinoid/CB1 receptor system in in OFC to DLS circuit promotes habit formation by weakening the OFC to DMS circuitry required for goal directed behavior (21). Interestingly, cannabinoid signaling has been shown to activate mTORC1 in hippocampal neurons (73). Furthermore, THC-dependent impairment of novel object recognition was reversed by the mTORC1 inhibitor, rapamycin (73). Thus, it is possible that endocannabinoids and mTORC1 are part of the same signaling cascade that gates goal-directed behaviors, thereby promoting habit. Finally, Gourley and colleagues reported that the brain-derived neurotrophic factor (BDNF) in the OFC participates in goal-directed behaviors (18, 74, 75). BDNF and mTORC1 play opposing roles in AUD (76). For example, BDNF keeps alcohol intake in moderation, and mTORC1 is associated with excessive alcohol use (76). As breakdown of the BDNF signaling cascade results in compulsive alcohol use (62). It is tempting to speculate that the balance between BDNF and mTORC1 signaling in the OFC determines whether alcohol use is kept in moderation or becomes habitual.

ACKNOWLEDGEMENTS AND DISCLOSURES

This research was supported by the National Institute of Alcohol Abuse and Alcoholism, P50 AA017072 (D.R.) and R01 AA027474 (D.R.).

The authors do not have a financial or non-financial competing interests.

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FIGURE LEGENDS

Figure 1. Inhibition of mTORC1 in the OFC reduces alcohol seeking

(a-c) Intra-OFC infusion of rapamycin does not alter self-administration of alcohol. Rats underwent 7 weeks of IA-20%2BC, and were then trained to self-administer 20% alcohol using a FR3 schedule. Vehicle (white) or rapamycin (50ng/µl, red) was infused bilaterally in the OFC 3 hours before a 30 min self-administration session. **(a)** RM ANOVA of cumulative lever presses did not identify a significant main treatment effect ($F_{1,12}$=0.26, p>0.05). **(b)** Number of active lever presses (t_6=1.40, p>0.05) and **(c)** amount of alcohol consumed (t_6=1.51, p>0.05) did not differ between treatment groups. **(d-f) Intra-OFC infusion of rapamycin inhibits lever presses during extinction.** Vehicle (white) or rapamycin (50ng/µl, red) was infused in the OFC 3 hours before a 30 min extinction session, and responses on the previously active lever were recorded. (**d**). RM ANOVA revealed significant main treatment effect ($F_{1,16}$=4.40, p=0.05) on cumulative lever presses. Number (**e**) and rate (per 5 min) (**f**) of lever presses were reduced in the rapamycin-treated animals. Significance was determined using two-tailed paired t-test ((**e**) t_8=3.31, p<0.05 and (**f**) t_8=3.30, p<0.05). Data are presented as individual values and mean±SEM. **(a-c)** n=7, **(d-e)** n=9. *p<0.05.

Figure 2. Inhibition of mTORC1 in the OFC attenuates habitual responding for alcohol

Rats were trained to self-administer 20% alcohol using RR (green) or RI (red) schedule of reinforcement. **(a) Alcohol devaluation test.** On test day, rats were pre-fed with 20% alcohol for 30 minutes in their home cage during devalued day (D) or with 1% sucrose in the non-devalued day (ND) and normalized lever pressing (percent of total presses on D or ND, see methods) was

used to evaluate goal-directed or habitual responding during a 10-minuted extinction session. **(b-c) RR trained animals are goal directed whereas RI trained animals show habitual behavior.** RR trained rats (green) press less during devalued day as compared to RI trained animals (red). Two-way ANOVA with Sidak post hoc analysis indicates a significant decrease in normalized lever pressing on D vs ND in RR-trained rats (p=0.0018), but not in RI-trained rats (p=0.8014) (Main effect of devaluation: F (1, 20) = 9.391, p=0.0061; reinforcement schedule X devaluation: F (1, 20) = 4.736, p=0.0417). **(c)** Devaluation index was calculated as the ratio of ND-D/ND+D. RR trained rats show a higher devaluation index as compared to the RI trained rats. Two-tailed unpaired t-test shows a significant difference in devaluation index (t_{16}=3.266, p=0.0049). **(d) Timeline of experiment.** Rats underwent 7 weeks of IA20%-2BC. Rats were the trained to operant self-administer 20% alcohol during 3 overnight FR1 sessions and pseudo-randomly assigned to 2 groups. One group was subjected to a progressive RI reinforcement schedule, while the other was assigned a RR schedule. Following stable RI-30 or RR-3 responding, rats underwent alcohol devaluation testing depicted in **a**. Upon establishing the behavior, rats underwent stereotaxic surgery to bilaterally implant a guide cannula. One week later, RI-30 or RR-3 training was resumed for 2 weeks, after which rats received microinjections of vehicle or drug in a counter-balanced manner, prior to the devaluation test. **(e-f) Intra-OFC infusion of rapamycin does not alter goal-directed alcohol seeking.** Rats that were trained using an RR (green) schedule of reinforcement received an intra-OFC infusion of vehicle (hatched green) or rapamycin (50ng/μl) (green) 3 hours before the alcohol devaluation procedure as in (**a-c**), and the number of lever presses were measured during extinction. **(e)** Total lever press-based normalization indicates significant decreases in lever pressing between ND and D days in both vehicle-treated (p=0.0085) and rapamycin-treated (p=0.0117) RR-trained rats (Main effect of devaluation: F(1,40)=17.65,

p=0.0001) **(f)** Devaluation index. No significant difference was detected (two-tailed paired t-test, t_{13}=1.135, p=0.2768)**. (g-h) Intra-OFC infusion of rapamycin reduces habitual lever presses.** Rats that were trained using an RI schedule of reinforcement (red) received an intra-OFC infusion of vehicle (hatched red) or rapamycin (50ng/μl) (red) 3 hours before the alcohol devaluation procedure as in (**a-c**), and the number of lever presses were measured during extinction. **(g)** Total lever press-based normalization indicates significant decreases upon intra-OFC infusion of rapamycin- (p=0.0005), but not vehicle (p=0.6381) in RI trained rats on D compared to ND days (Main effect of devaluation: F(1,32)=12.38, p=0.0013; treatment X devaluation: F(1,32)=5.327, p=0.0276) **(h)** Devaluation index. Two-tailed paired t-test shows a significant difference in devaluation index (t_{16}=3.557, p=0.0026). Data are presented as mean±SEM. *p<0.05, **p<.01 ***p<0.001. **(b-c)** n=8-10, **(e-f)** n=8-11, **(g-h)** n=8-9.

Figure 3. Habitual sucrose responding in rats with a history of excessive alcohol drinking is not affected by mTORC1 inhibition in the OFC

(a) Sucrose devaluation test. Rats were trained to respond for sucrose in an operant self-administration paradigm under a random interval (RI) procedure. Habitual behavior was probed in an identical way to the alcohol-trained cohorts with the exception that sucrose pre-feeding was considered the devaluing context and alcohol was the non-devalued substance. **(b) Timeline of experiment.** Rats underwent 7 weeks of IA20%-2BC, and were then trained to operant self-administer initially 8% sucrose. Sucrose concentration was progressively reduced to 1% during 3 overnight FR1 sessions. Rats were subjected to a progressive RI reinforcement schedule. Following stable RI-30 responding, rats underwent alcohol devaluation testing depicted in **a**. Upon establishing the behavior, rats underwent stereotaxic surgery to bilaterally implant a guide cannula.

One week later, RI-30 training was resumed for 2 weeks, after which rats received microinjections of vehicle or drug in a counter-balanced manner, prior to the devaluation test. **(c)** Two-way RM ANOVA failed to show a significant effect of repeated testing (Main effect of devaluation: $F(1,26)=1.779$, $p=0.1939$; treatment X devaluation: $F(1,26)=0.6074$, $p=0.4428$) on lever presses between devalued and non-devalued days when habitually responding animals were pretreated with either vehicle or rapamycin. **(d)** Devaluation index. No significant difference was detected ($t14=0.96$, $p=0.36$). Significance between treatment groups was determined using ANOVAs and two-tailed paired t-tests. n=14.

Figure 4. GluN2B is required for mTORC1 activation by alcohol.

(a-b) Stimulation of NMDA receptors activates mTORC1 in the OFC. Vehicle (0.1% DMSO) or NMDA (25μM) was applied to OFC slices of naïve rats for 3 minutes. Phosphorylation of CaMKII and S6K and S6 were determined by western blot analysis. Total levels of the proteins were also measured as well, as actin which was used as a loading control. **(a)** Representative blots of CaMKII (left), S6K (middle), and S6 (right) phosphorylation in OFC slices treated with vehicle or NMDA. **(b)** ImageJ was used for optical density quantification. Data are expressed as the average ratio +/-S.E.M of phospho-CaMKII to CaMKII, phospho-S6K to S6K and phospho-S6 to S6, and are expressed as percentage of vehicle. Significance was determined using two-tailed unpaired *t*-test. (CaMKII: $t_{12}=5.61$, $p<0.001$; S6K: $t_{12}=4.77$, $p<0.001$; S6: $t_{12}=4.51$, $p<0.001$). **(c-h) Alcohol-dependent mTORC1 activation is attenuated by the GluN2B inhibitor, Ro25-6981.** Rats underwent 7 weeks of IA-20%2BC resulting in an average of alcohol intake of 4.37 g/kg/24 hours (**Table 2**). Rats received bilateral intra-OFC infusions of vehicle (saline) or Ro25-6981 (Ro25, 10μg/μl) 15 minutes prior to the end of the 24-hour alcohol deprivation session. The OFC

was harvested 24 hours after the last 24 hours of alcohol drinking session. Water only consuming rats were used as controls. (**c**-**e**) Representative images of CaMKII (**c**), S6 (**d**) and 4E-BP (**e**) phosphorylation in water vs. alcohol-exposed animals that were pre-treated with vehicle or Ro25-6981. (**f**-**h**) ImageJ was used for optical density quantification. Data are expressed as the average ratio +/- S.E.M of phospho-CaMKII to CaMKII (**f**), phospho-S6 to S6 (**g**), and phospho-4E-BP to 4E-BP (**h**), and are expressed as percentage of water + vehicle group. Two-way RM ANOVA showed a significant effect of alcohol on pCaMKII ($F_{1,20}$=24.66, p<0.0001), pS6 ($F_{1,20}$=21.92, p=0.0001), and p4E-BP ($F_{1,20}$=48.23, p<0.0001). In addition, two-way RM ANOVAs indicated a main effect of drug treatment on pCaMKII ($F_{1,20}$=48.51, p<0.0001), pS6 ($F_{1,20}$=4.58, p<0.05), and p4E-BP ($F_{1,20}$=8.49, p<0.01), as well as treatment x alcohol withdrawal interaction on pCaMKII ($F_{1,20}$=18.57, p<0.01), pS6 ($F_{1,20}$=7.559, p<0.05), and p4E-BP ($F_{1,20}$=13.22, p<0.01). Tukey's multiple comparison post hoc analysis revealed that alcohol increased pCaMKII (p<0.001), pS6 (p<0.001), and p4E-BP (p<0.001) in vehicle-treated animals and that pretreatment with R025-6981 reduced the phosphorylation of pCaMKII (p<0.0001), S6 (p<0.05), and 4E-BP (p<0.001). (**a-b**) n = 7 per group, (**c-h**) *p<0.05, ***p<0.001. n = 6 per group.

Figure 5. GluN2B in the OFC promotes alcohol seeking and habitual alcohol responding

(**a-c**) **Intra-OFC administration of Ro25-6981 decreases alcohol seeking.** Rats underwent 7 weeks of IA-20%2BC, and were then trained to self-administer 20% alcohol. Vehicle (white) or Ro25-6981 (Ro25, 10μg/μl, blue) was infused bilaterally in the OFC 15 minutes prior to a 30 minutes self-administration session. **(a)** Cumulative lever presses. Two-way RM ANOVA revealed a significant main treatment effect ($F_{1,12}$=10.89, p<0.05). **(b)** Number and **(c)** rate (per 5 minutes) of lever press. Significance was determined using two-tailed paired t-test (both t_6=2.62,

$p<0.05$). **(d-e) Intra-OFC administration of Ro25-6981 attenuates habitual alcohol seeking**. Rats that were trained to lever press for alcohol using an RI schedule of reinforcement, received an intra-OFC infusion of vehicle (hatched blue) or (Ro25, 5μg/μl, blue) 15 minutes before the alcohol devaluation procedure as described in **Figure 2a-c**, and the number of lever presses were measured during extinction. (**d**) Two way RM ANOVA showed a main effect of devaluation ($F_{1,33}=13.72$, $p<0.001$) as well as a treatment x devaluation interaction ($F_{1,33}=6.31$, $p<0.05$). Sidak's multiple comparison test detected a significant difference for Ro25-6981 ($p<0.001$) on D compared to ND days. **(e)** Devaluation index. Two-tailed paired t-test indicated a significant difference ($t_{15}=3.72$, $p<0.01$). Individual results and mean±SEM are shown, (**a-c**) n=7, (**d-e**) n=12. *$p<0.05$, ** $p<0.01$.

Figure 1

Inhibition of mTORC1 in the OFC reduces alcohol seeking

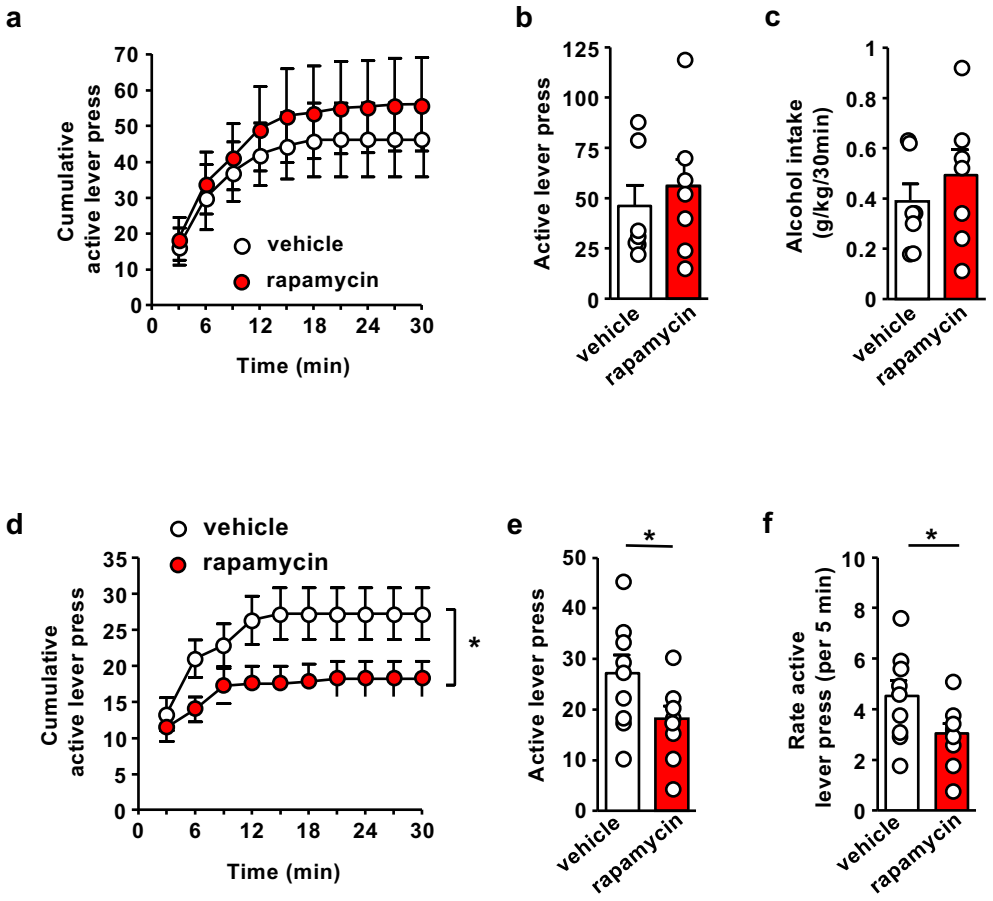


Figure 2

Inhibition of mTORC1 in the OFC attenuates habitual responding for alcohol

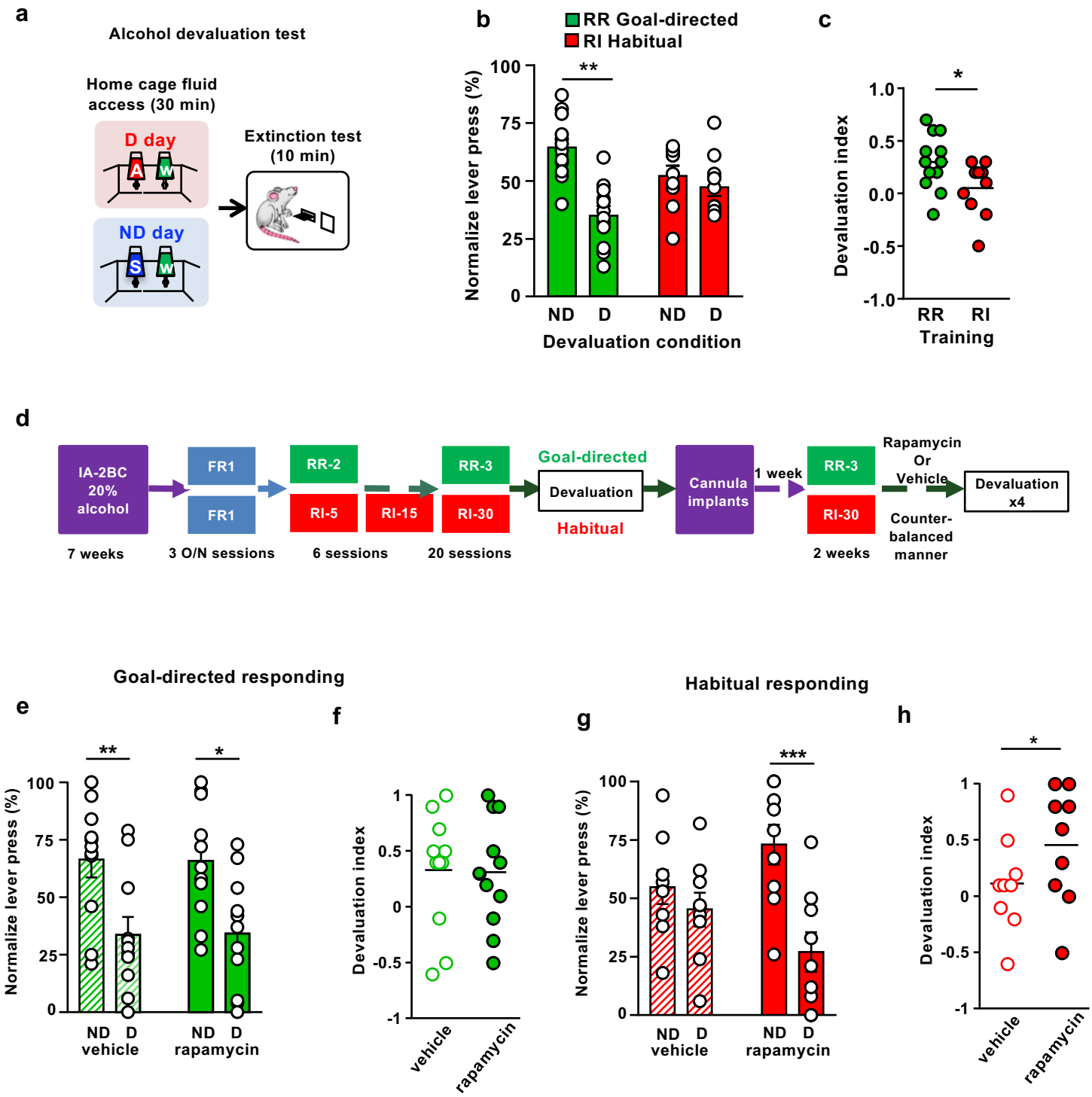
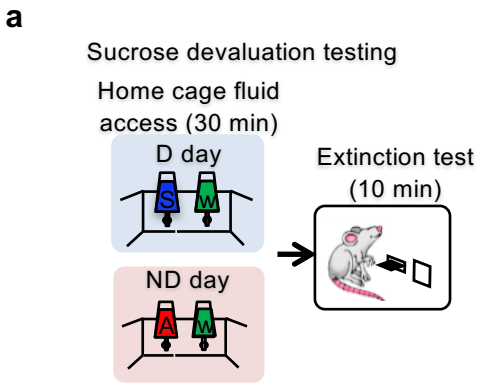
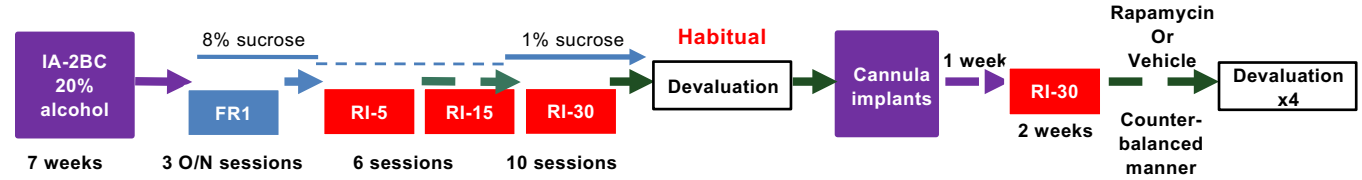
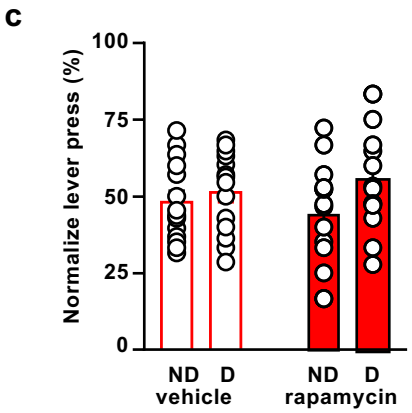


Figure 3

Habitual sucrose responding in rats with a history of excessive alcohol drinking is not affected by mTORC1-OFC inhibition







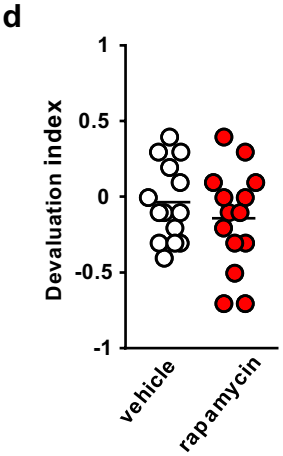


Figure 4

GluN2B is required for mTORC1 activation by alcohol

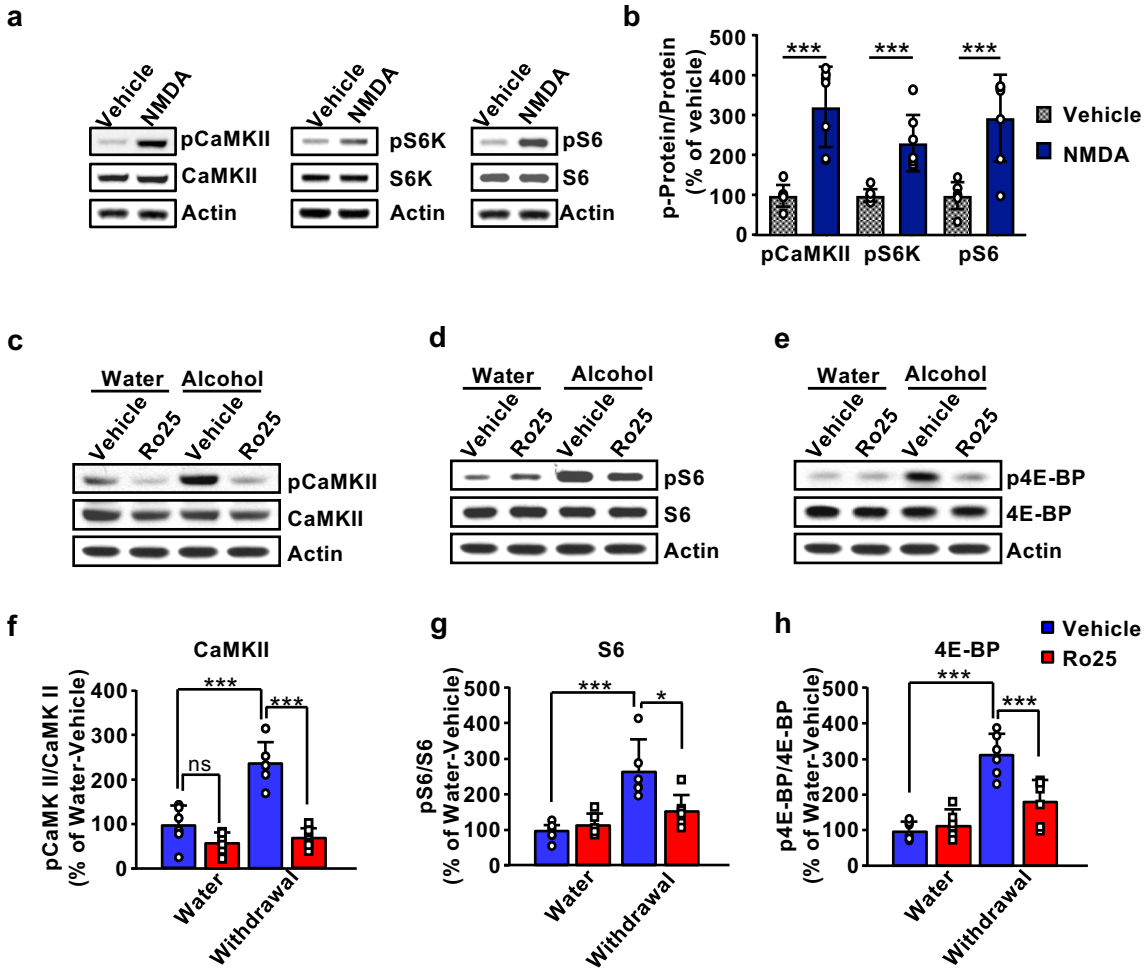
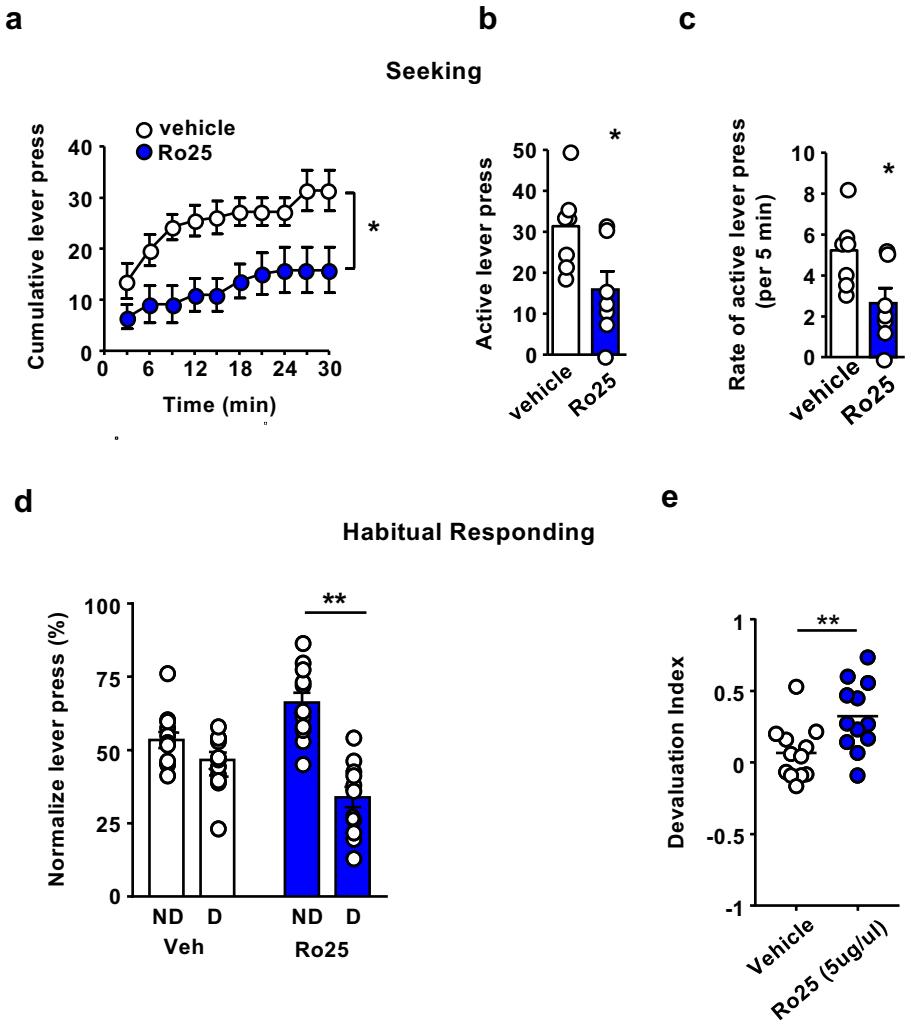


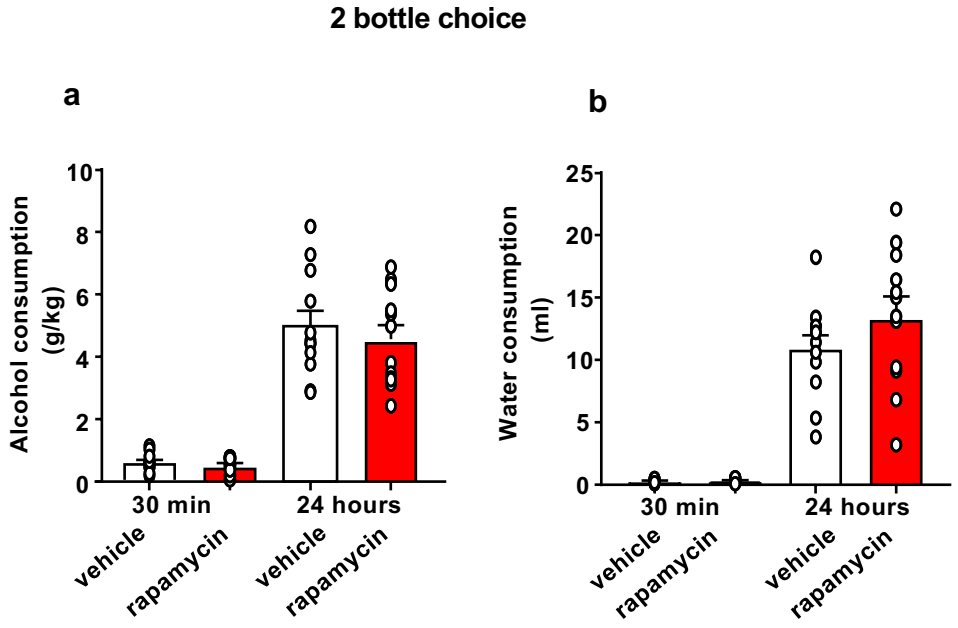
Figure 5

GluN2B in the OFC promotes alcohol seeking and habitual alcohol responding



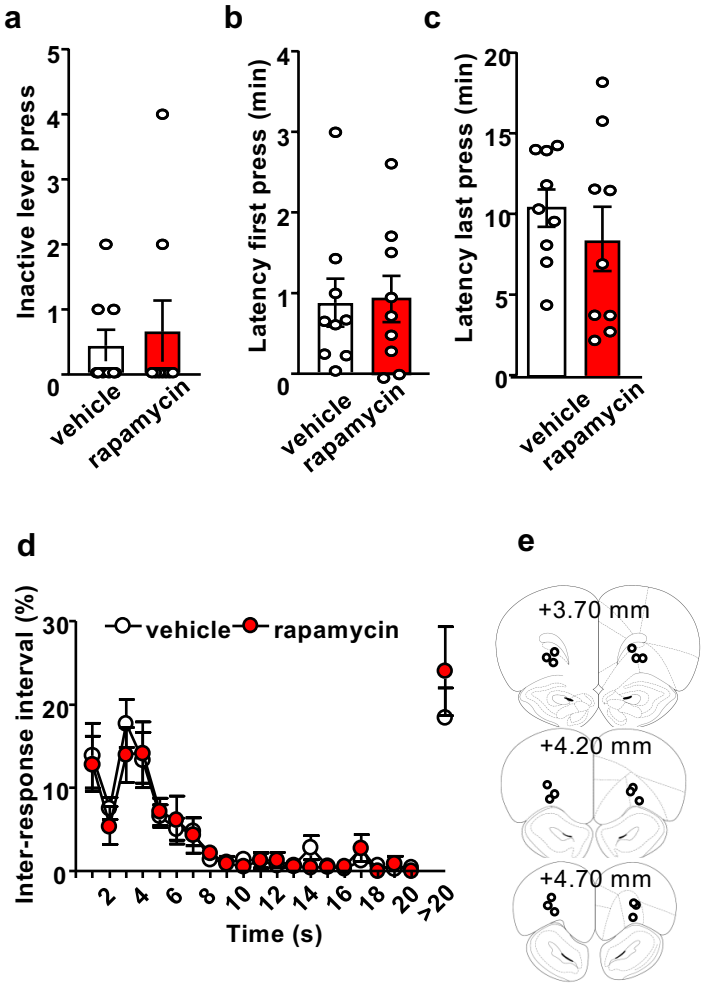
Supplemental Figure 1

Inhibition of mTORC1 in the OFC does not impact alcohol consumption



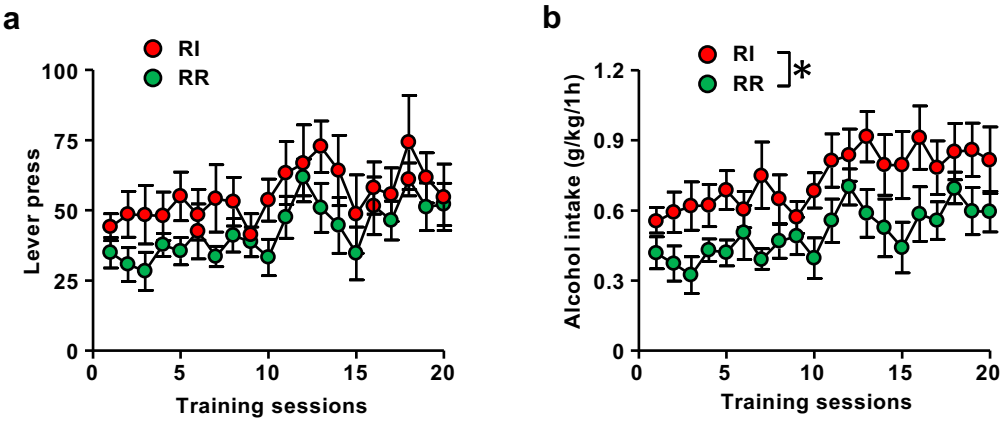
Supplemental Figure 2

Inhibition of mTORC1 in the OFC does not impact inactive lever presses, latency to the first and last lever press and locomotion



Supplemental Figure 3

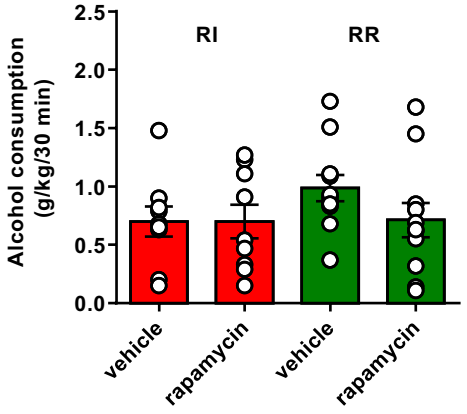
Number of lever presses and alcohol consumed in RI and RR-trained animals



Supplemental Figure 4

Inhibition of mTORC1 in the OFC does not alter voluntary intake of alcohol prior to devaluation in RR and RI-trained animals

a



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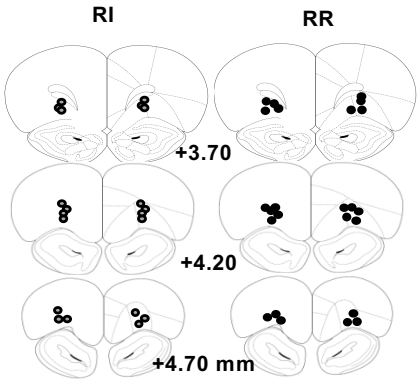
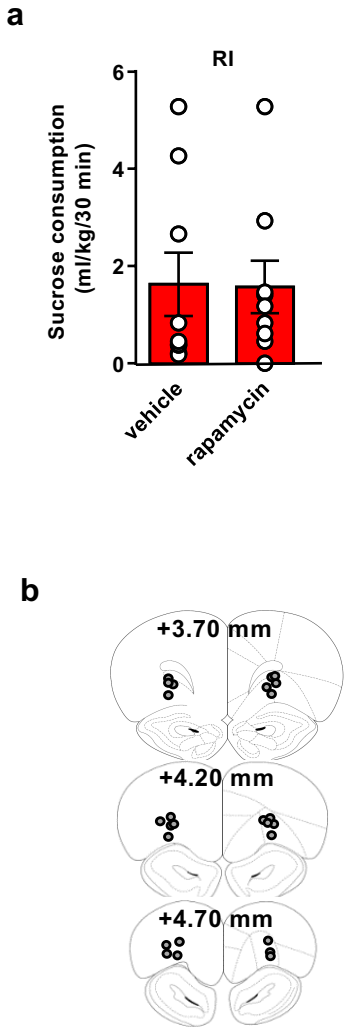


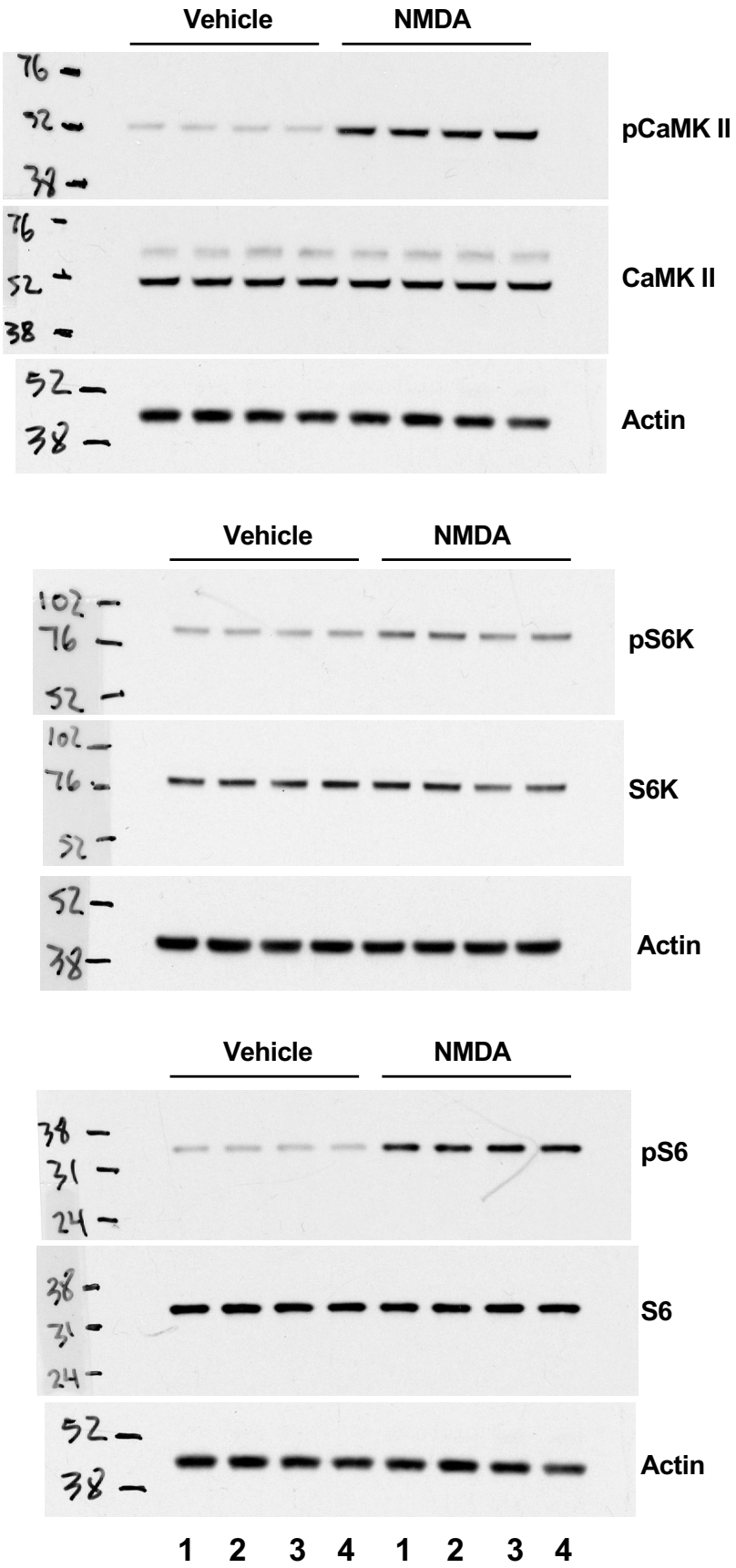
Supplemental Figure 5

Inhibition of mTORC1 in the OFC does not alter voluntary intake of sucrose prior to devaluation in RI-trained animals



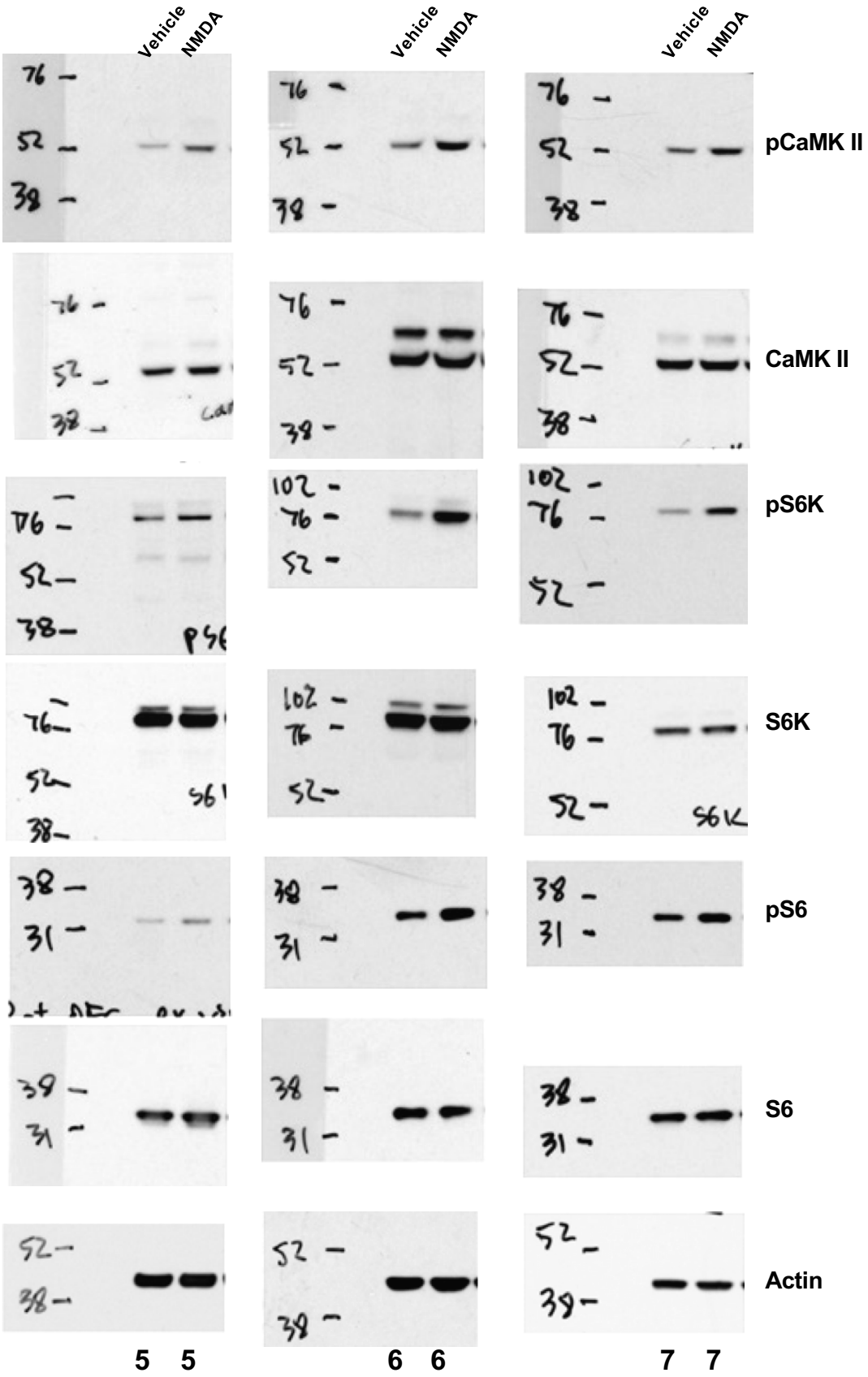
Supplemental Figure 6

Stimulation of the NMDA receptors activates mTORC1 in the OFC



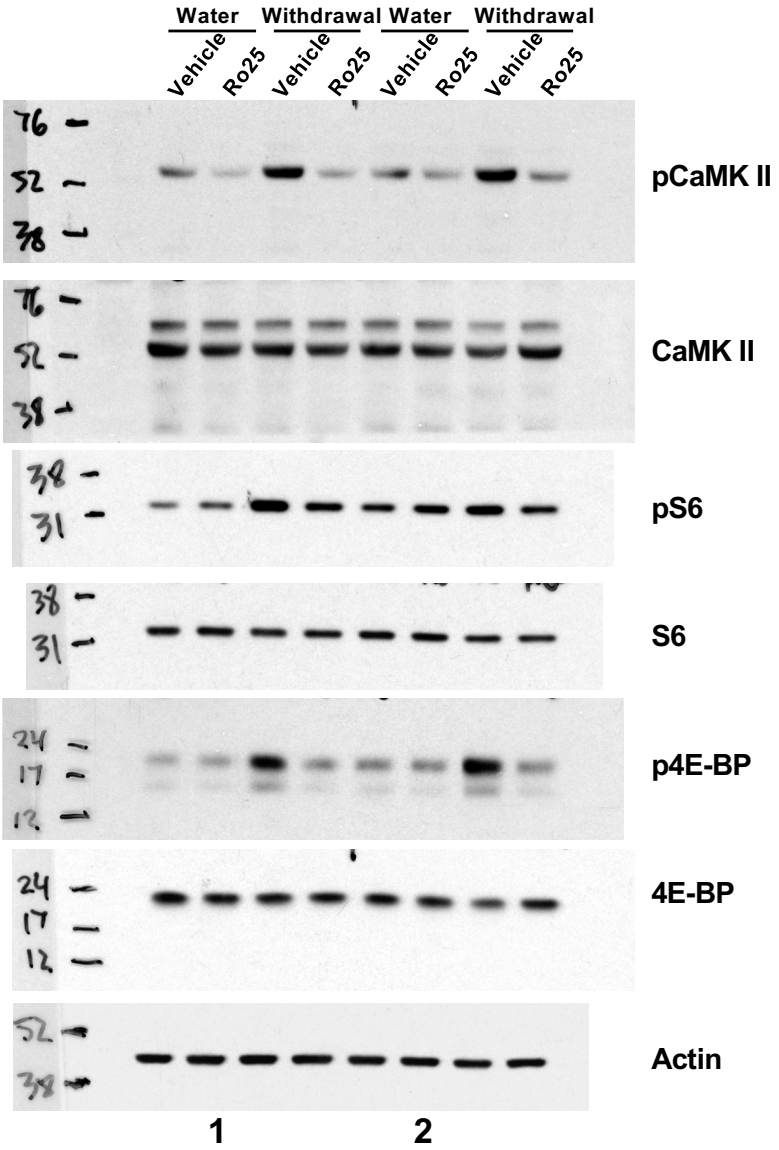
Supplemental Figure 6

Stimulation of the NMDA receptors activates mTORC1 in the OFC



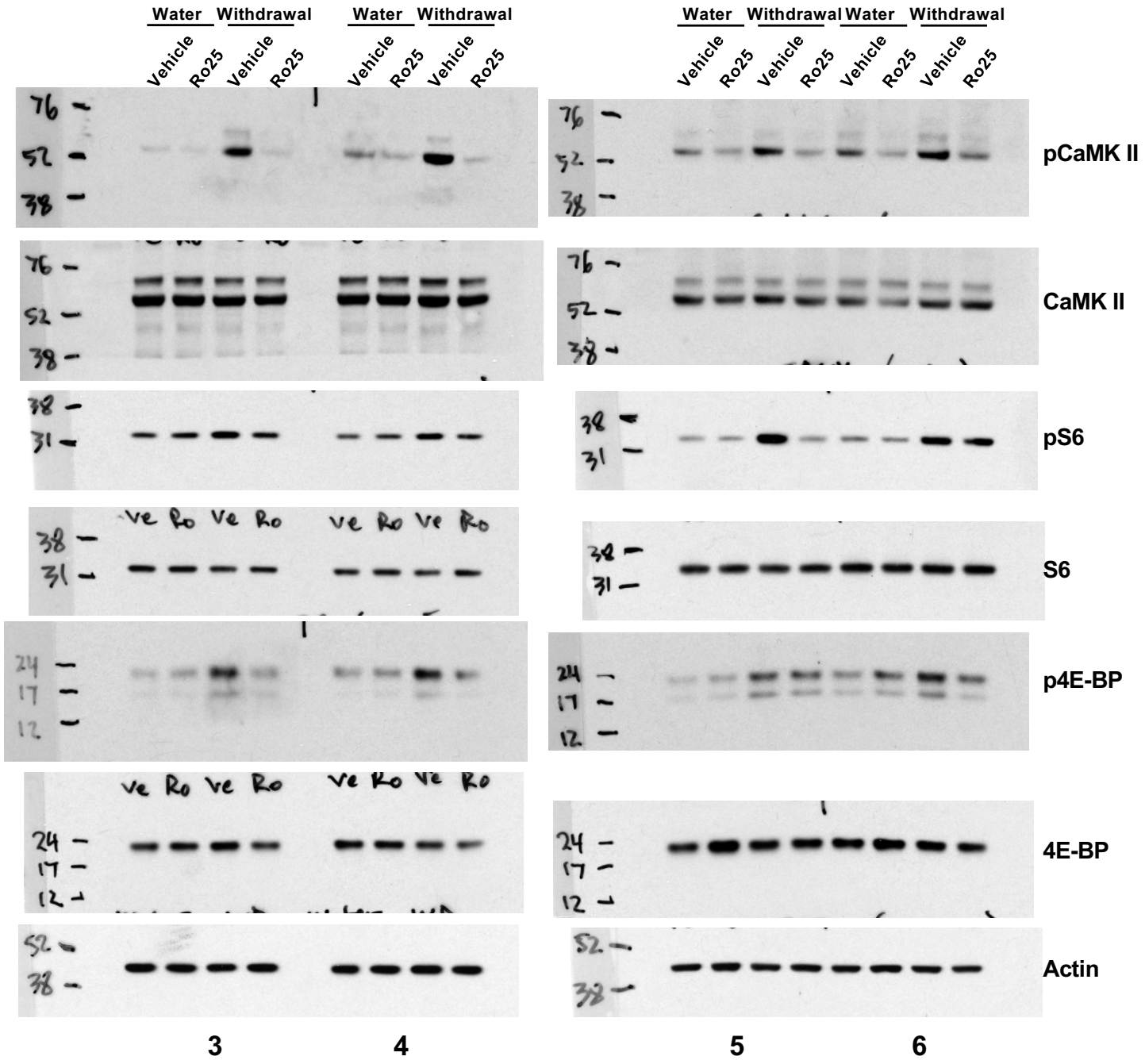
Supplemental Figure 7

GluN2B is required for mTORC1 activation by alcohol



Supplemental Figure 7

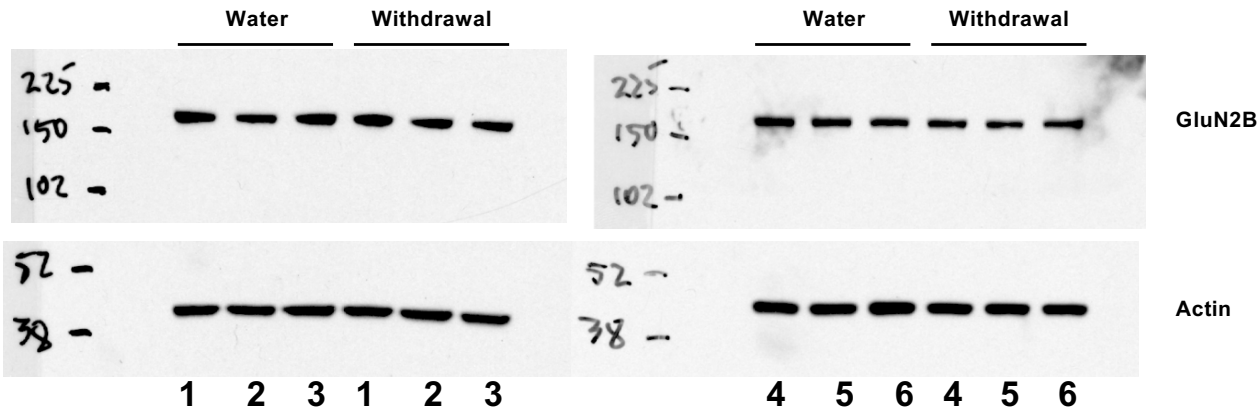
GluN2B is required for mTORC1 activation by alcohol



Supplemental Figure 8

GluN2B protein levels are not altered by alcohol in the OFC

a



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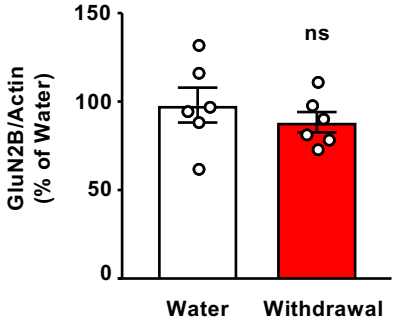
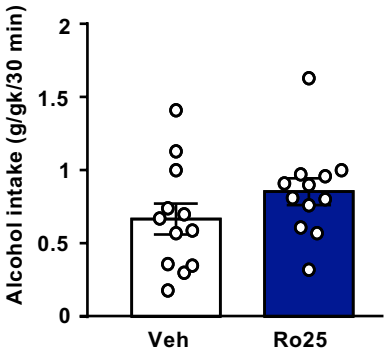


Supplemental Figure 9

Inhibition of GluN2B in the OFC does not alter voluntary alcohol or sucrose intake prior to devaluation



Supplemental Figure 10

Inhibition of GluN2B in the OFC does not alter alcohol locomotion

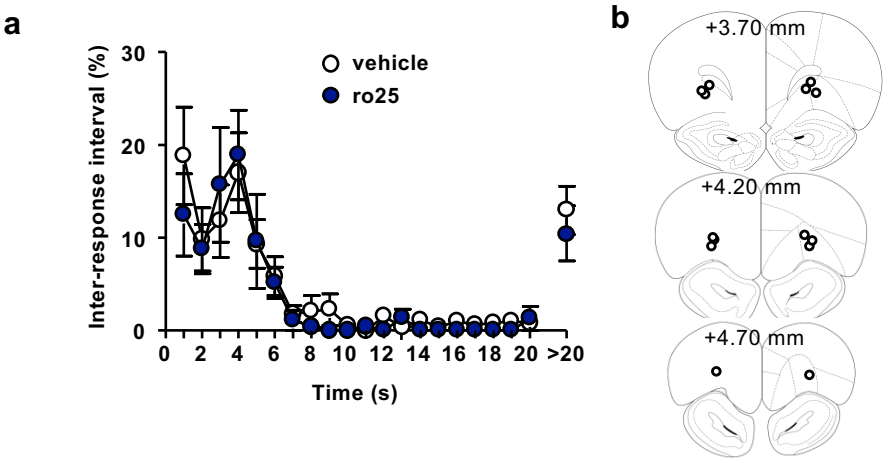


Table 1 Mean consumption of sucrose, alcohol and water during outcome devaluation

Alcohol Devaluation							
	Training	RI		RR		RI	
	Group	Vehicle	rapamycin	Vehicle	rapamycin	Vehicle	Ro25
Non-devalued day	1% Sucrose (ml/kg)	1.4	2.0	1.2	1.9	2.7	4.5
		±	±	±	±	±	±
		0.5	0.8	0.4	0.6	1.5	0.9
	Water (ml/kg)	0.7	0.6	1.2	0.8	1.0	2.5
		±	±	±	±	±	±
		0.3	0.1	0.7	0.1	0.2	1.2
Devalued day	20% Alcohol (g/kg)	0.7	0.7	1.0	0.7	0.9	1.0
		±	±	±	±	±	±
		0.1	0.1	0.1	0.1	0.1	0.2
	Water (ml/kg)	0.4	0.6	0.7	0.6	1.0	0.9
		±	±	±	±	±	±
		0.1	0.1	0.1	0.1	0.4	0.2

Sucrose Devaluation			
	Training	RI+RR	
	Group	Vehicle	rapamycin
Non-devalued day	20% Alcohol (g/kg)	0.9	0.8
		±	±
		0.1	0.1
	Water (ml/kg)	0.9	1.1
		±	±
		0.1	0.1
Devalued day	1% Sucrose (ml/kg)	4.5	5.1
		±	±
		0.7	1.4
	Water (ml/kg)	1.2	0.9
		±	±
		0.2	0.1

Table 2

Individual alcohol-drinking data in rodent used for biochemical experiments			
Animals drinking data			
Figure 4c-h	*Drinking paradigm IA20%-2BC 24h-withdrawal*	*Rat number*	*Last 4 drinking session (g kg-1 24h-1)*
	n=12	1	5.35
		2	5.52
		3	5.54
		4	4.84
		5	6.41
		6	3.16
		7	4.37
		8	3.45
		9	3.48
		10	6.24
		11	2.71
		12	5.72
		Mean ± S.E.M	4.73 ± 0.37

SUPPLEMENTARY FIGURES LEGENDS

Supplemental Figure 1. Inhibition of mTORC1 in the OFC does not alter alcohol consumption

(a-b) Rats with a history of IA-20%2BC were infused with vehicle (white) or rapamycin (50ng/µl, red) into the OFC 3 hours before the beginning of the final 2BC session. Alcohol and water consumption were recorded at the 30 minutes and 24-hour time points. No differences were observed in alcohol consumption at the 30 minutes (t_{11}=1.25, p>0.05), or at the 24-hour time point (t_{11}=0.87, p>0.05). There was no difference in water intake between groups at the 30 minutes (t_{11}=0.95, p>0.05), or at the 24-hour time point (t_{11}=1.73, p>0.05). n=12.

Supplemental Figure 2. Inhibition of mTORC1 in the OFC does not impact inactive lever presses, latency to the first and last lever press or locomotion

(**a-c**) **Intra-OFC infusion does not alter inactive lever presses or latency to the first and last active lever press.** Rats were trained to self-administer 20% alcohol using a FR3 schedule. Vehicle (white) or rapamycin (50ng/µl, red) was infused into the OFC 3 hours before a single 30-minute extinction session. **(a)** number of inactive lever presses (t_8=0.37, p>0.05), **(b)** latency to first lever press (t_8=0.27, p>0.05), and **(c)** latency to last lever press (t_8=0.89, p>0.05) did not differ between groups. **(d) Intra-OFC infusion of rapamycin does not alter inter-response interval**. Relative number of inter-response intervals (IRIs) expressed as % of total IRIs with all intervals equal or smaller of 20 seconds presented in 1 second bins. Interval greater than 20 seconds are also shown. Two-way ANOVA of IRIs across the session did not identify a significant main treatment effect ($F_{1,12}$=0.1412, p=0.7137). **(e)** Schematic drawing of coronal sections of the rat brain showing

approximate bilateral placements of cannulae in the OFC according to Paxinos and Watson 2007. n=9.

Supplemental Figure 3. Number of lever presses and alcohol consumed in RI and RR-trained animals

Rats with a history of IA-20%2BC were trained to respond for alcohol in an operant self-administration paradigm under a random interval (RI) or random ration (RR) schedule of reinforcement. **(a)** Training groups did not differ in the number of lever presses during training ($F_{1,20}$=2.01, p>0.05), **(b)** RI-trained rats exhibited a small but significant increase of alcohol intake over the course of training ($F_{1,20}$=6.65, p<0.05). Significance between treatment groups was determined using RM ANOVA and two-tailed paired t-tests *p<0.05. RR n=12. RI n=10.

Supplemental Figure 4. Inhibition of mTORC1 in the OFC does not alter voluntary alcohol intake prior to devaluation

Rats with a history of IA-20%2BC were trained to respond for alcohol in an operant self-administration paradigm under a random interval (RI) or random ration (RR) schedule of reinforcement. Prior to a 10-minute devaluation probe session, animals were allowed access to alcohol in their home case. **(a)** The amount of alcohol consumed did not differ by treatment ($F_{1,30}$=0.21, p>0.05) or training history ($F_{1,30}$=1.48, p>0.05). **(b)** Schematic drawing of coronal sections of the rat brain showing approximate bilateral placements of the cannulae in the OFC according to Paxinos and Watson 2007. (**a**) RR n=12. RI n=10.

Supplementary Figure 5. Inhibition of mTORC1 in the OFC does not alter voluntary sucrose intake prior to devaluation

Rats with a history of IA-20%2BC were trained to respond for sucrose in an operant self-administration paradigm under a random interval schedule of reinforcement. Prior to a 10-minutes devaluation probe session, animals were allowed access to sucrose in their home cage. **(a)** The amount of sucrose consumed did not differ by treatment ($F_{1,30}$=0.06, p>0.05) or training history ($F_{1,30}$=0.55, p>0.05). n=8. **(b)** Schematic drawing of coronal sections of the rat brain showing the approximate bilateral placements of cannulae in the OFC according to Paxinos and Watson 2007.

Supplemental Figure 6. Stimulation of NMDARs in the OFC activates CaMKII and mTORC1 signaling. Raw data of results depicted in **Figure 4a-**b. Vehicle (0.1% DMSO) or NMDA (25μM) was applied to OFC slices of naïve rats for 3 minutes. Phosphorylation of CaMKII, S6K and S6 were determined by western blot analysis. Individual replicates are numbered at the bottom of the blots. n = 7.

Supplemental Figure 7. Alcohol-dependent mTORC1 activation is attenuated by the GluN2B inhibitor, Ro25-6981. Raw data of results depicted in **Figure 4c-h**. Rats underwent 7 weeks of IA-20%2BC resulting in an average of alcohol intake of 4.37 g/kg/24 hours (**Table 2**). Rats received bilateral intra-OFC infusions of vehicle (saline) or Ro25-6981 (Ro25, 10μg/μl) 30 minutes prior to the end of the 24-hours alcohol deprivation session. Brains were harvested 24-hours after the last alcohol drinking session, and phosphorylation of CaMKII, S6 and 4E-BP were measured. Individual animals are numbered at the bottom of the blots. n = 6.

Supplemental Figure 8. GluN2B protein levels are not altered by alcohol in the OFC

At the conclusion of IA-20%2BC, the OFC was collected 24-hour after the last IA-20%2BC, and GluN2B levels were determined by western blot analysis, with actin used as a loading control. **(a)** Raw data depicting GluN2B protein levels in the OFC of alcohol exposure rats compared with rats given only water. Individual animals are numbered at the bottom of the blots. **(b)** ImageJ was used for optical density quantification. Data are expressed as the average ratio +/- S.E.M of the GluN2B/Actin ratio, expressed as percentage of water. Alcohol had no effect on GluN2B levels OFC (t_{10}=0.85, p>0.05) Significance was determined using two-tailed unpaired t-test. n=6.

Supplemental Figure 9. Inhibition of GluN2B in the OFC does not alter voluntary alcohol intake prior to devaluation

Rats with a history of IA-20%2BC were trained to respond for alcohol in an operant self-administration paradigm under a random interval (RI) schedule of reinforcement. Prior to the devaluation test, rats had free access alcohol. Vehicle or Ro25-6981 (10µg/µl) was infused in the OFC 30 minutes before a 10-minutes extinction session. Intra-OFC pretreatment with Ro25-6981 did not impact alcohol (t_5=2.80, p>0.05). n=12.

Supplemental Figure 10. Inhibition of GluN2B in the OFC does not alter locomotion

Vehicle or Ro25-6981 (10µg/µl) was infused in the OFC 30 minutes before a single 30-minutes extinction session. **(a)** Mean +/- SEM of the relative number of IRIs expressed as % of total IRIs with all intervals equal or smaller of 20 seconds presented in 1 second bins. Intervals greater than 20 seconds are also shown. RM ANOVA of IRIs across the session did not identify a significant main treatment effect ($F_{1,12}$=0.81, p>0.05). **(b)** Schematic drawing of coronal sections of the rat

brain showing the bilateral placements of the injections in the OFC (Paxinos and Watson 2007). (**a**) n=7.

Table 1 Mean consumption of sucrose, alcohol and water during outcome devaluation

Rats undergoing alcohol seeking probe trials were pre-fed with sucrose (non-devalued test) or alcohol (devalued test), and water 30 minutes prior to a 10 minutes extinction session. The mean amount consumed is reported, split between treatment groups and labeled according to training history and experimental manipulation.

Table 2 Individual alcohol-drinking data in rodents used for biochemical experiments

Individual alcohol drinking data of the final 4 sessions of IA-2BC20%. Alcohol intake is expressed as mean +/- S.E.M.