mTORC1 IN THE ORBITOFRONTAL CORTEX PROMOTES HABITUAL ALCOHOL SEEKING

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Abstract

The mammalian target of rapamycin complex 1 (mTORC1) plays an important role in dendritic translation, synaptic plasticity, and learning and memory. We previously showed that heavy alcohol use activates mTORC1 in the orbitofrontal cortex (OFC) of rodents. Here, we set out to determine the consequences of alcohol-dependent mTORC1 activation in the OFC. We found that although inhibition of mTORC1 in the OFC does not alter rat alcohol intake *per se*, it attenuates alcohol seeking. We then tested whether mTORC1 in the OFC is required for goal-directed or habitual alcohol seeking. To do so, rats were trained self-administer alcohol under a random ratio (RR) or a random interval (RI) schedule of reinforcement, which biases toward goal-directed or habitual responding, respectively, and tested whether mTORC1 inhibition alters lever presses following alcohol devaluation. We found that pharmacological inhibition of mTORC1 or knockdown of the adaptor protein, Raptor, did not affect goal-directed alcohol responding but restored sensitivity to devaluation in RI-trained rats. In contrast, habitual responding for sucrose was unaltered by mTORC1 inhibition. These data suggest that mTORC1 in the OFC drives alcohol habit. We then elucidate the mechanism by which mTORC1 is activated by alcohol, and found that the recruitment of GluN2B during alcohol withdrawal stimulates mTORC1 in OFC cFos-positive neurons. Finally, we show that inhibition of GluN2B in the OFC attenuates both alcohol seeking and habitual responding for alcohol. Together, our data suggest that alcohol withdrawal promotes an NMDAR-dependent activation of mTORC1 which in turn drives habitual alcohol seeking.

Introduction

The mechanistic target of rapamycin complex 1 (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[1]. Upon activation, mTORC1 phosphorylates eIF4E-binding protein (4E-BP), and the ribosomal protein S6 kinase (S6K) which in turn phosphorylates its substrate, S6[2]. These phosphorylation events lead to the translation of a subset of mRNAs to proteins[1, 2]. In the CNS, mTORC1 is responsible for the local dendritic translation of synaptic proteins[3, 4]. As such, mTORC1 plays an important role in synaptic plasticity, and learning and memory[5].

Increasing lines of evidence in rodents suggest that mTORC1 is a key player in mechanisms underlying alcohol use disorder (AUD)[6]. For instance, excessive alcohol intake, and reinstatement of alcohol place preference activate mTORC1 in the nucleus accumbens (NAc) of rodents [7-10] resulting in the translation of synaptic proteins that in turn produce synaptic and structural adaptations that drive and maintain heavy alcohol use and relapse[7-11]. Chronic alcohol intake also produces a robust and sustained activation of mTORC1 in the OFC[9], however the behavioral consequences of this activation are unknown.

The OFC integrates sensory and reward information [12] and is an essential player in decision making[12-14]. The OFC plays a role in stimulus-outcome association[15-17], in updating the value of predicted outcomes[13, 18], in goal-directed behaviors[16, 19, 20], and compulsive behavior[21].

Exposure to drugs of abuse impairs performance of OFC-dependent behavioral tasks[22], and aberrant neuroadaptations induced by drugs of abuse in the OFC contribute to drug-seeking and compulsive drug taking[22-24]. Furthermore, withdrawal from alcohol vapor exposure produces morphological alterations[25], and neuronal hyper-excitability in the OFC[26]. Inactivation of the OFC

enhances alcohol consumption in dependent mice[27]. Chronic alcohol use also alters the protein landscape in the OFC of primates[28]. In humans, degraded OFC white matter and reduced neuronal density are associated with alcohol-dependent patients[29, 30], and presentation of alcohol-associated cues to alcohol-dependent subjects elicits OFC activation[31-33].

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

Methods

Details regarding reagents, subjects, molecular and behavioral procedures can be found in the Supplementary Information section.

Habitual and goal-directed alcohol seeking

After 7 weeks of intermittent access to 20% alcohol using a 2 bottle choice paradigm (IA20%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[34] with modification adapted from[20, 35, 36]. Only one lever was available during the operant sessions. Self-administration training was initiated under FR1 for 3 consecutive overnight sessions. Afterwards, the sessions lasted for an hour. 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].

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.

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 2 lever presses with number of presses ranging from 1 to 5) for at least 20 sessions.

Only rats that self-administered alcohol more than 0.3g/kg/hour were included in the study. Training data are shown in **Supplemental Fig. 3**. Following stable RI-30 or RR-3 responding, rats underwent alcohol devaluation testing as described in[35] with small 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 were excluded from analysis. 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, 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. RI- and RR-trained rats also received bilateral intra-OFC infusions of Ltv-SCR or Ltv-shRaptor (1×10^8 pg/ml). Following recovery, RI-30 or RR-3 training was resumed for four weeks, and alcohol devaluation was conducted.

Data analysis

Data were analyzed using two-tailed paired *t*-test, one- or two-ways analysis of variance (ANOVA) with repeated measures. Significant main effects and interactions of the ANOVAs were further investigated with the Student–Newman–Keuls *post hoc* test or method of contrast analysis. Statistical significance was set at $p<0.05$ unless indicated otherwise. 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[7, 37].

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 IA20%2BC received bilateral intra-OFC infusion of vehicle or the mTORC1 inhibitor, rapamycin (50ng/μl) 3 hours prior to a drinking session[7]. As shown in **Supplementary Fig. 1,** intra-OFC administration of rapamycin did not impact voluntary intake of alcohol. Next, rats that underwent IA20%2BC for 7 weeks were trained to self-administer a 20% alcohol in an OSA paradigm[38]. 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 intake were measured[7]. 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 also were 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 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[39-41]. We next examined weather mTORC1 in the OFC plays a role in goal-directed or habitual alcohol seeking by using a satiety outcome devaluation procedure (**Fig. 2a**). Rats that first underwent 7 weeks of IA20%2BC were then trained to lever press for 20% alcohol using RR or RI schedule of reinforcement which biases lever responding toward goal-directed (RR) or habitual (RI) actions[16, 20, 35] (**Supplementary Fig. 3a-b**). Goal-directed responding is sensitive whereas habitual responding is insensitive outcome to devaluation[16, 20, 35]. Thus, a devaluation procedure was conducted to differentiate between goal-directed vs. 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-minutes extinction test during which lever presses were recorded (**Fig. 2a**). As shown in **Fig.2b-c**, RR-trained rats showed a decrease in responding after alcohol devaluation, whereas RI-trained rats were insensitive to devaluation, confirming that RR training produced goal directed behavior, whereas RI training produced habitual responding.

Next, to test whether mTORC1 in the OFC participates in goal-directed alcohol or habitual alcohol seeking, rapamycin (50ng/μl) or vehicle was infused bilaterally 3 hours prior to a devaluation session, and lever presses were examined in the RR- and RI-trained groups. Rapamycin administration did not alter home cage alcohol or sucrose intake prior to the extinction session in either groups (**Supplementary Fig. 4**). Intra-OFC administration of rapamycin did not impact responding in RR (goal-directed)-trained cohorts, as lever presses were reduced in rats that were pre-fed with alcohol and treated with either vehicle or rapamycin (**Fig. 2d-e**). In contrast,

inhibition of mTORC1 in the OFC reduced responding in the RI (habitual)-trained rats in the devalued condition (**Fig.2f-g**). Critically, the reduction in responding in the rapamycin-treated RI-trained rats was parallel to the responding of RR-trained animals (**Fig.2f-g**). These data suggest that mTORC1 inhibition in the OFC restores goal-directed alcohol seeking in animals trained to habitually self-administer alcohol (**Fig.2f-g**).

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 during an extinction session in animals that were first offered sucrose (devalued) or alcohol (none-devalued) in their home cage **(Supplementary Fig. 5a)**. As shown in **Supplementary Fig. 5b-c** habitual responding for sucrose was similar in the vehicle and rapamycin-treated animals. Together, these data suggest that the contribution of mTORC1 in the OFC to habitual behaviors is specific for alcohol.

Next, to solidify the conclusion that mTORC1 in the OFC contributes to habitual responding for alcohol, rats were trained to self-administer alcohol using the RR or RI schedule of reinforcement. Afterwards, OFC neurons were infected bilaterally with a lentivirus expressing a short hairpin sequence targeting Raptor (shRaptor, 1×10^8 pg/ml), an essential protein in the mTORC1 complex[1], or a lentivirus expressing a scrambled sequence (SCR, 1×10^8 pg/ml). Intra-OFC administration of lenti-shRaptor resulted in a high level of infected OFC neurons (**Supplementary Fig. 6a**), and a significant knockdown of the protein (**Supplementary Fig. 6b**). Knockdown of raptor did not impact alcohol self-administration (**Supplementary Fig. 6c-f**), nor did it alter goal-directed alcohol responding (**Fig. 2h-i**). Although the SCR infected rats exhibited a small but significant reduction in habitual lever pressing (which is likely due to two outliers) (**Fig. 2j**), RI-trained animals infected with shRaptor exhibited a robust and highly significant

decrease in alcohol seeking following devaluation in RI trained animals (**Fig. 2j-k**). Together our data suggest that mTORC1 in the OFC is a molecular transducer alcohol habit.

mTORC1 activation in the OFC during alcohol withdrawal depends on GluN2B

Next, we set out to elucidate the mechanism by which long-term heavy alcohol use activates mTORC1 in the OFC. Nimitvilai et al. reported that OFC neurons are hyperexcitable during withdrawal from chronic intermittent alcohol vapor exposure in mice[26]. We therefore first examined whether OFC neurons are activated during withdrawal from IA20%2BC, and if so, we aimed to determine whether mTORC1 activation is localized to the same neurons. We used cFos as a marker for neuronal activation, and as shown in **Fig.3A-B**, cFos immunoreactivity was significantly increased in the ventral (vOFC) and lateral OFC (lOFC) 24 hours after the last IA20%2BC session suggesting that OFC neurons are activated during withdrawal from excessive alcohol use. To determine whether mTORC1 is activated in cFos positive neurons, we co-stained slices with anti-phosphoS6 antibody a marker of mTORC1 activation. We found that S6 phosphorylation was elevated in cFos positive neurons (**Fig.3A-B),** suggesting that mTORC1 is stimulated in OFC neurons that are activated by alcohol during withdrawal.

Glutamatergic inputs from cortical and subcortical structures project to the OFC[42, 43]. Extracellular glutamate content is elevated in cortical regions of alcohol-dependent patients as well as in rats during early withdrawal[44]. In addition, NMDA receptors (NMDAR), and specifically GluN2B containing NMDARs, are activated in the striatum during alcohol withdrawal in rodents[45]. Stimulation of GluN2B activates the guanine nucleotide releasing factor 1 (GRF1), the activator of H-Ras[46]. As H-Ras is upstream of mTORC1[47], we hypothesized that GluN2B stimulation activates mTORC1 in the OFC during alcohol withdrawal.

We first tested whether NMDAR stimulation activates mTORC1. OFC slices were briefly treated with NMDA (25μM), and the activation of CaMKII and mTORC1 were measured. 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. 3C-D**), as well as of mTORC1, as measured by S6K, and S6 phosphorylation (**Fig. 3C-D**). These data suggest that NMDAR stimulation activates mTORC1 in the OFC.

Next we tested whether GluN2B is required for alcohol-withdrawal-dependent mTORC1 activation. To do so, rats that underwent a 7-weeks period of IA20%2BC or water only received bilateral intra-OFC infusion of the GluN2B antagonist R025-6981 (10μg/ul) or vehicle 30 minutes prior to the end of the last withdrawal session, and mTORC1 activation was examined. In vehicle-treated rats, alcohol withdrawal produced a robust activation of CamKI (**Fig. 3C, right panel**), and mTORC1 as measured by S6 and 4-EBP phosphorylation (**Fig. 3C, middle and left panels**). In contrast, as shown in **Fig. 3C**, R025-6981-treated rats exhibited a marked reduction of both CaMKII and mTORC1 activation during alcohol withdrawal. Together our data suggest that withdrawal from alcohol use, recruits glutamatergic signaling in OFC neurons which activates the GluN2B/mTORC1 axis.

GluN2B in the OFC is required to 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 R025-6981 (10μg/ul) 30 minutes prior to an extinction session suppressed alcohol seeking behavior, as indicated by a reduction in cumulative (**Fig. 4a**), active (**Fig. 4b**), and the rate (**Fig. 4c**) of lever presses.

Importantly, intra-OFC administration of Ro25-6981 (10μg/μl) reverted habitual responding for alcohol to goal directed responding in rats trained under the RI (habitual) schedule (**Fig.4d-e**). In contrast, intra-OFC infusion of R025-6981, did not impact alcohol or sucrose consumption in a 2BC test during the devalued or non-devalued days (**Supplementary Fig. 7**). Finally, as shown in **Supplementary Fig. 8a,** inhibition of GluN2B in the OFC did not change locomotion. Together, these data indicate that the recruitment of GluN2B signaling during alcohol withdrawal is required for alcohol seeking and habit.

Discussion

In this study we present data implicating mTORC1 in the OFC in mechanisms underlying habitual alcohol seeking. This conclusion stems from the findings that inhibition of mTORC1 activity or disruption of the mTORC1 complex in the OFC attenuates alcohol seeking and habit. 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 drives alcohol seeking and habit.

mTORC1 in the OFC and alcohol seeking

We found that inhibition of mTORC1 in the OFC attenuates presses during extinction suggesting that mTORC1 is responsible for alcohol seeking. The OFC has been implicated in updating reward anticipation based on the value of an expected outcome[15, 48], and O'Doherty et al. showed that the lOFC is activated during anticipation of reward in humans[49]. We show that mTORC1 is activated in cFos positive OFC neurons during withdrawal when alcohol is not on board. 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.

mTORC1 in the OFC and habit

We found that blockade of mTORC1 activity converts habitual alcohol responding to 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[35, 50, 51]. The OFC sends projections to the dorsal striatum (DS), including the DLS[20]. In addition, Renteria and colleagues showed that alcohol vapor exposure generated habitual

behavior via the disruption of OFC to DMS circuit [36]. Thus, it is plausible that mTORC1 activation is strengthening OFC projections to the DLS and or through weakening of OFC to DMS projection. Although a caveat in comparing our study to Retina et al. is the fact that we measured habitual vs. goal directed responding for alcohol, whereas Retina et al. measured responding for food. It is plausible that Retina et al. tested whether alcohol alters decision making in general whereas we focused on habitual responding for alcohol *per se*. It would be of interest to determine the normal role of mTORC1 in the OFC and whether mTORC1 suppresses other decision-making tasks.

Interestingly, Pascoli et al. recently reported that OFC neurons projecting to the dorsal striatum (DS) are associated with compulsive lever pressing in response to optogenetic activation of dopaminergic ventral tegmental area (VTA) neurons[21]. Compulsive drug use is associated with maladaptive habitual responding[51, 52]. Thus, it is plausible that mTORC1 signaling is driving both habitual and compulsive alcohol seeking.

Alcohol-dependent mTORC1 activation and glutamatergic signaling

We show that mTORC1 activation following 24 hours of withdrawal from heavy alcohol use is localized to cFos positive, and thus activated, OFC neurons. We further demonstrated that mTORC1 activation in the OFC depends on glutamatergic signaling, and specifically on the activity of GluN2B. Finally, we reported that GluN2B activation in the OFC is sufficient to influence the formation and/or maintenance of alcohol seeking and habit. Brigman et al. reported that GluN2B in the OFC plays a role in choice learning[53], whereas we report herein the GluN2B in this brain region participates in an inflexible behavior. It is plausible that alcohol-dependent neuroadaptations tilt the function of GluN2B from choice learning, e.g. a goal directed behavior to habitual responding.

Activation of mTORC1 triggers the translation of a subset of mRNA to proteins in the cell body and in dendrites[2]. Activation of NMDARs in mouse hippocampal dendritic spines promotes mRNA translation[54], raising the possibility that the consequences of GluN2B-dependent mTORC1 activation is the translation of synaptic proteins. Furthermore, mTORC1 plays an important role in synaptic plasticity[4, 5, 55, 56]. Thus, a remaining question is whether, and if so, what kind of mTORC1-dependent synaptic alterations initiate and/or maintain alcohol habit formation.

Gremel et al. showed that the endocannabinoid/CB1 receptor system in in OFC to DS circuit promotes habit formation by weakening OFC to DS circuit required for goal directed behavior[20]. Cannabinoid signaling was shown to activate mTORC1 in hippocampal neurons[57]. Furthermore, THC-dependent impairment of novel object recognition was reversed by rapamycin[57]. Thus, it is possible that endocannabinoids and mTORC1 are part of the same signaling cascade that gates goal-directed behaviors thereby promoting habit.

Finally, Gourely and colleagues reported that the brain-derived neurotrophic factor (BDNF) in the OFC participates in goal-directed behaviors[17, 58, 59]. BDNF and mTORC1 play opposing roles in AUD, i.e. BDNF keeps alcohol intake in moderation, and mTORC1 is associated with alcohol abuse[60]. As breakdown of the BDNF signaling cascade results in compulsive alcohol use[61], it is tempting to speculate that the balance between BDNF and mTORC1 signaling in the OFC determines whether alcohol use becomes habitual.

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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 IA20%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 (t_8=3.31, p<0.05 and t_8=3.30, p<0.05, respectively). Data is presented as individual data points 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, 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 goal-directed or habitual responding were examined during a 10-minuted extinction session. **(b) 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). One-way ANOVA with Bonferroni post hoc analysis indicates a significant decrease in lever pressing on D vs ND in RR-trained rats ($p<0.05$), but not in RI-trained rats ($p>0.999$). (**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 t-test shows a significant difference in devaluation index ($t_{16}=3.266$, $p=0.0049$). **(d-e) 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. (**d**). Two-tailed paired t-tests indicate significant decreases in lever pressing between ND and D days in both vehicle- and rapamycin-treated, RR-trained rats ($t_{13}=4.254$, $p=0.0009$ and $t_{14}=3.266$, $p=0.0056$, respectively). **(e)** Devaluation index. No significant difference was detected (two-tailed paired t-test, $t_{13}=1.135$, $p=0.2768$)**. (f-g) 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) e OFC 3 hours before the alcohol devaluation procedure as in (**a-c**), and the number of lever presses were measured during extinction. **(f)** Two-tailed paired t-tests indicate a significant difference for rapamycin- ($t_{14}=2.941$, $p=0.0107$), but not vehicle-treated ($t_{13}=1.953$, $p=0.0727$), RI trained rats on D compared to ND days. **(g)** Devaluation index. No significant difference between rapamycin and vehicle-treated rats was determined using two-tailed paired t-test ($t_{12}=2.068$, $p=0.0609$). **(h-i) Knockdown of Raptor in the OFC does not alter goal-directed alcohol seeking.** Rats that were trained to lever press for alcohol using an RR (green) schedule of reinforcement, received an intra-OFC infusion of a lentivirus expressing an shRNA sequence

targeting Raptor (Raptor, 1×10^8 pg/ml, green) or a scrambled sequence (SCR, 1×10^8 pg/ml, hatched green). Rats underwent alcohol devaluation testing as in (**a**-**c**), and the number of lever presses were measured during extinction. (**h**) Two-tailed paired t-tests detected a significant decrease in lever pressing in both shSCR (t_8=4.188, p=0.0030) and shRaptor-treated (t_7=3.332, p=0.0126). (**i**) No significant difference in devaluation index for RR-trained rats (t_{15}=0.8277, p=0.4208) **(j-k) Knockdown of Raptor in the OFC alters habitual alcohol seeking.** OFC neurons of rats that were trained using an RI (red) schedule of reinforcement were infected with shRaptor (1×10^8 pg/ml, green) or SCR (1×10^8 pg/ml, hatched green). Rats underwent alcohol devaluation testing as in (**a**-**c**), and the number of lever presses were measured during extinction. (**j**) Two-tailed paired t-tests detected a significant decrease in lever pressing in both shSCR (t_6=2.74, p=0.0338) and shRaptor-treated (t6=7.298, p=0.0003). (**k**) No significant difference in devaluation index for RI-trained rats (t_{12}=1.830, p=0.0922). Individual results and mean±SEM are shown. **(b-c)** n=8-10, **(d-g)** n=11-15, **(h-i)** n=8-9, and **(j-k)** n=7-8. *p<0.05, **p<.01 ***p<0.001, ****p<0.0001.

Figure 3. Alcohol withdrawal-dependent GluN2B stimulation activates mTORC1

(a-b) Alcohol withdrawal increased the number of c-Fos positive neurons and phosphoS6 positive neurons. Rats underwent 7 weeks of IA20%2BC reaching alcohol intake of 3.75 g/kg/24 hours. Control rats consumed water only. Brains were harvested at the end of the last 24-hours withdrawal session and immunoreactivity of cFos and phosphoS6 (pS6) were examined. (**a**) Representative images of OFC labeled with cFos (red, left) and pS6 (green, middle) of rats consuming only water or at the end of the last 24 hours of withdrawal. Colocalization of cFos and pS6 immunoreactivity is depicted in yellow (right). Scale bar is 1mm. LO: lateral OFC, VO:

ventral OFC, MO: medial OFC. (**b**) Quantification was conducted using the cell counter plugin in ImageJ software (NIH). Labeled cells are expressed as percentage of water controls. Significance was determined using two-tailed unpaired *t*-test. cFos: t_{11}=3.88, p<0.01, phospho-S6: t_{11}=4.91, p<0.001, merge: t_{11}=4.51, p<0.001. (**c-d**) **Stimulation of NMDARs in the OFC activates CaMKII and mTORC1 signaling.** 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. (**c**) Representative blots of S6 (left), S6K (middle) and CaMKII (right) phosphorylation in OFC slices treated with vehicle or NMDA. (**d**) ImageJ was used for optical density quantification. Data are expressed as the average ratio +/-S.E.M of phospho-S6 to S6, phospho-S6K to S6K and phospho-CaMKII to CaMKII, 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). **(e-j) Alcohol withdrawal-dependent stimulation of GluN2B in the OFC activates CaMKII and mTORC1 signaling.** Rats underwent 7 weeks of IA-20%2BC resulting in alcohol intake of 4.37 g/kg/24 hours. Rats received bilateral intra-OFC infusions of vehicle (saline) or Ro25-6981 (Ro25, 10μg/μl) 30 minutes prior to the end of the withdrawal session. Brains were harvested at the end of the 24-hours withdrawal session. Water only consuming rats were used as controls. (**e**-**g**) Representative images of S6 (**e**), 4E-BP (**f**) and CaMKII (**g**) phosphorylation in water vs. alcohol-withdrawal animals that were pre-treated with vehicle or Ro25-6981. (**h**-**j**) ImageJ was used for optical density quantification. Data are expressed as the average ratio +/- S.E.M of phospho-S6 to S6 (**h**), phospho-4E-BP to 4E-BP (**i**), and phospho-CaMKII to CaMKII (**j**) and are expressed as percentage of water + vehicle group. Two-way RM ANOVA showed a significant effect of alcohol withdrawal

on pS6 ($F_{1,20}$=21.92, p=0.0001), p4E-BP ($F_{1,20}$=48.23, p<0.0001), and pCaMKII ($F_{1,20}$=24.66, p<0.0001). In addition, two-way RM ANOVAs indicated a main effect of drug treatment on pS6 ($F_{1,20}$=4.58, p<0.05), p4E-BP ($F_{1,20}$=8.49, p<0.01), and pCaMKII ($F_{1,20}$=48.51, p<0.0001). Tukey's multiple comparison post hoc analysis revealed that alcohol withdrawal increased pS6 (p<0.001), p4E-BP (p<0.001), and pCaMKII (p<0.001) in vehicle-treated animals and that pretreatment with R025-6981 reduced the phosphorylation of S6 (p<0.05), 4E-BP (p<0.001), and pCaMKII (p<0.0001). (**a-b**) n = 6-7 per group, (**c-d**) n = 7 per group, (e-j) n = 6 per group. *p<0.05, **p<0.01, ***p<0.001.

Figure 4. 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 IA20%2BC, and were then trained to self-administer 20% e. Vehicle (white) or Ro25-6981 (Ro25, 10μg/μl, blue) was infused bilaterally in the OFC 30 min prior to a 30 min self-administration session. **(a)** Cumulative lever press. RM ANOVA revealed a significant main treatment effect ($F_{1,12}$=10.89, p<0.05). **(b)** Number and **(c)** rate (per 5 min) 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, 10μg/μl, blue) 3 hours before the alcohol devaluation procedure as described in **Figure 2a-c**, and the number of lever presses were measured during extinction. (**d**) 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=16. *$p<0.05$, ** $p<0.011$.

Figure 1

Inhibition of mTORC1 in the OFC reduces alcohol seeking

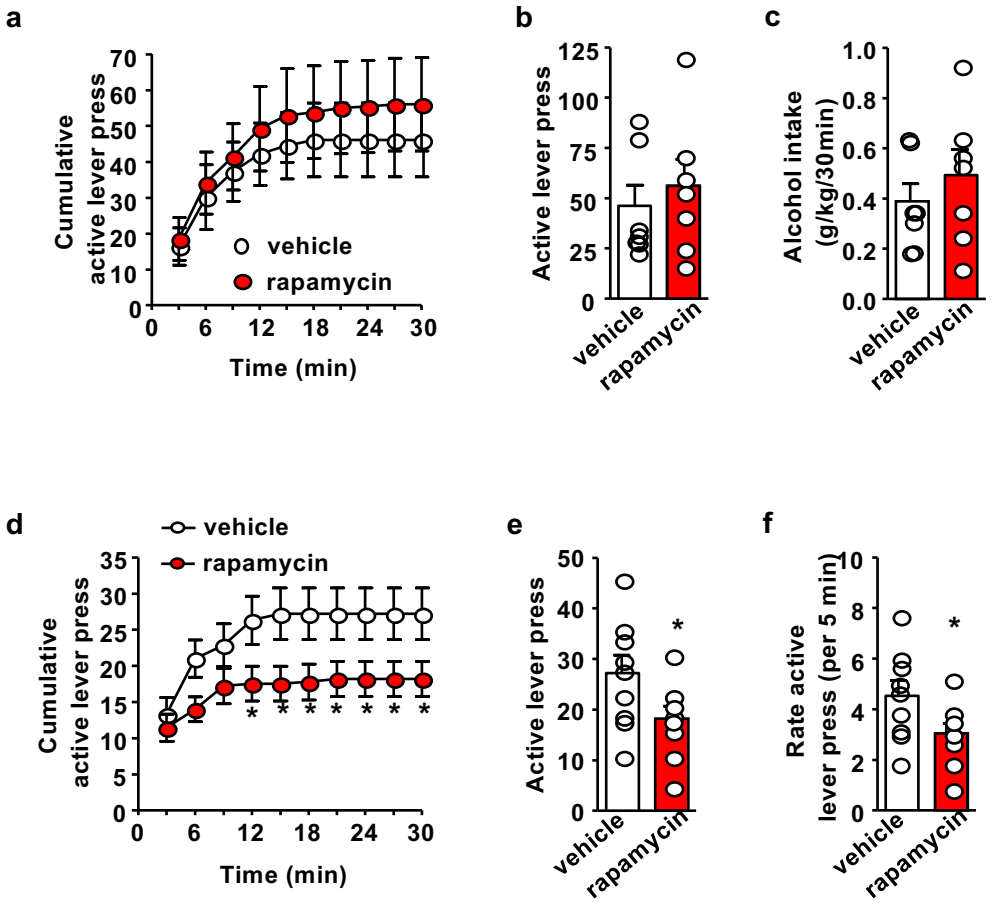


Figure 2

Inhibition of mTORC1 in the OFC attenuates habitual responding for alcohol

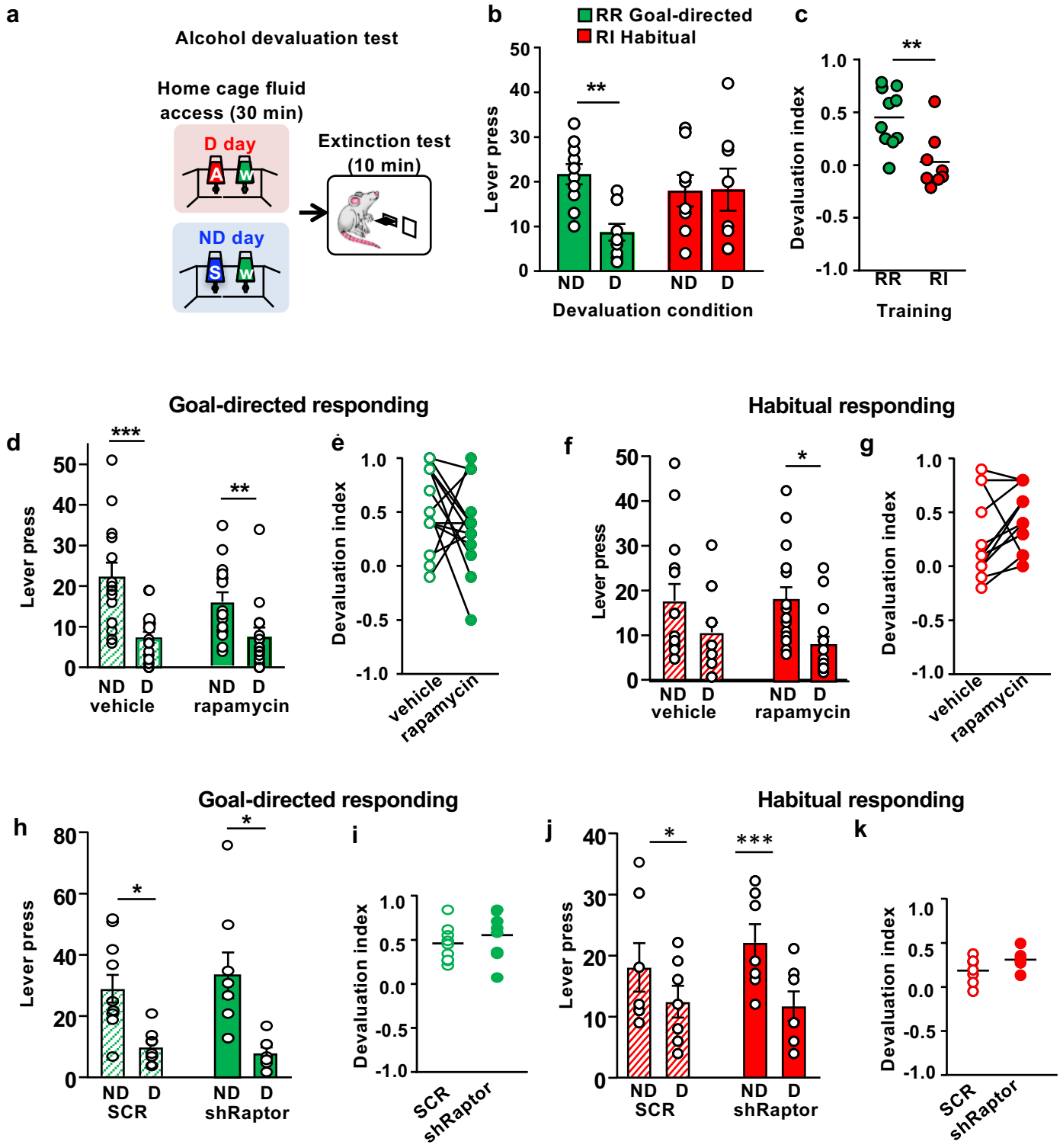


Figure 3

Alcohol-withdrawal-dependent GluN2B stimulation in the OFC activates mTORC1

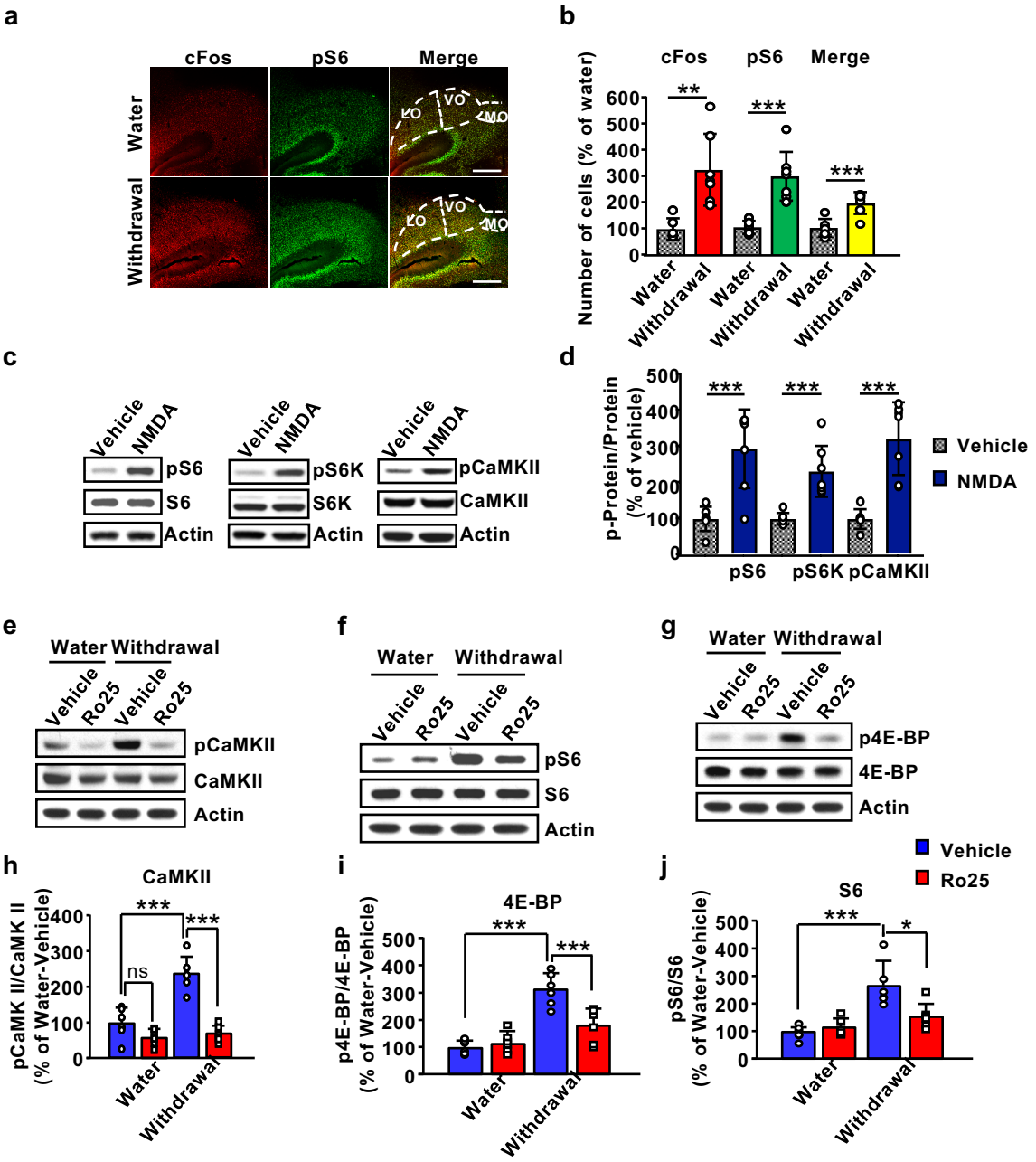
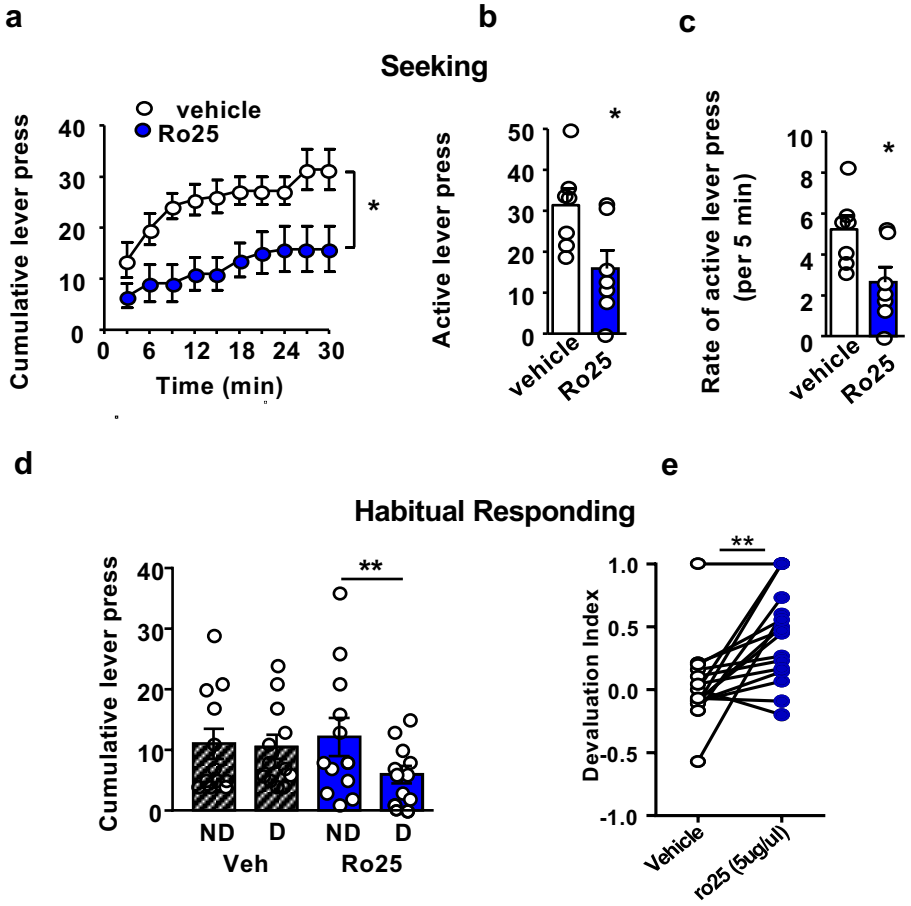


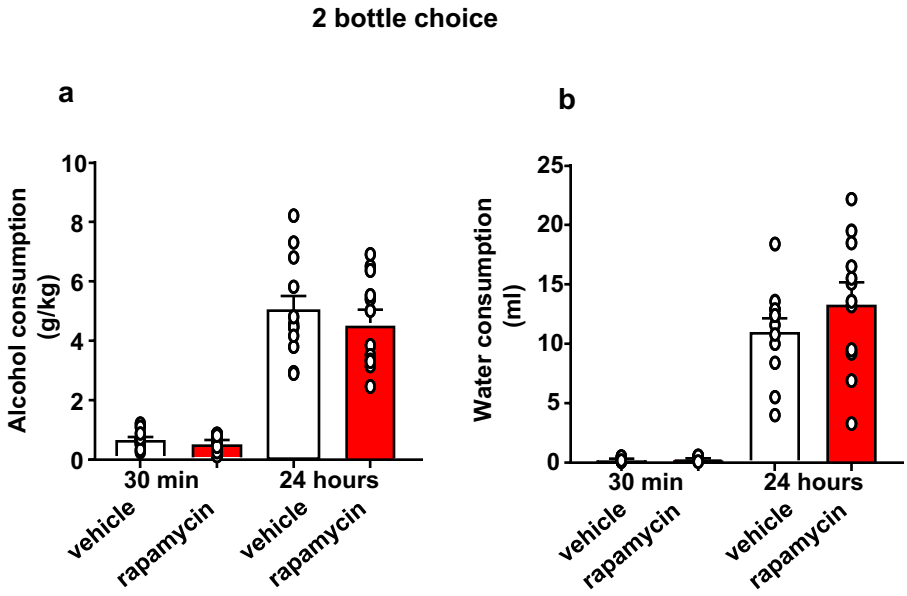
Figure 4

GluN2B in the OFC promotes alcohol seeking and habitual alcohol responding



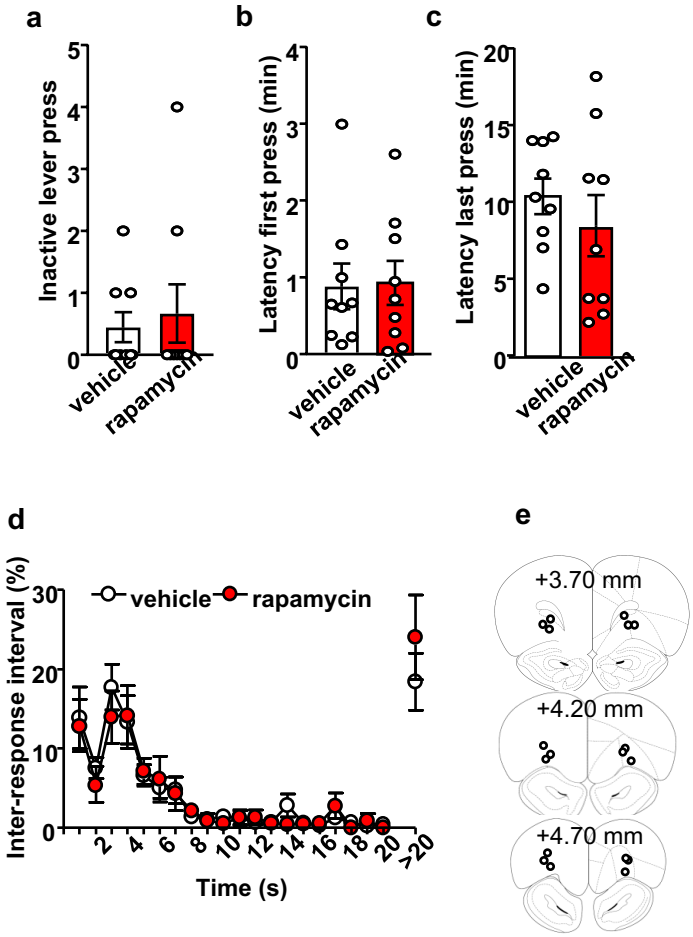
Supplemental Figure 1

Inhibition of mTORC1 in the OFC does not impact alcohol consumption



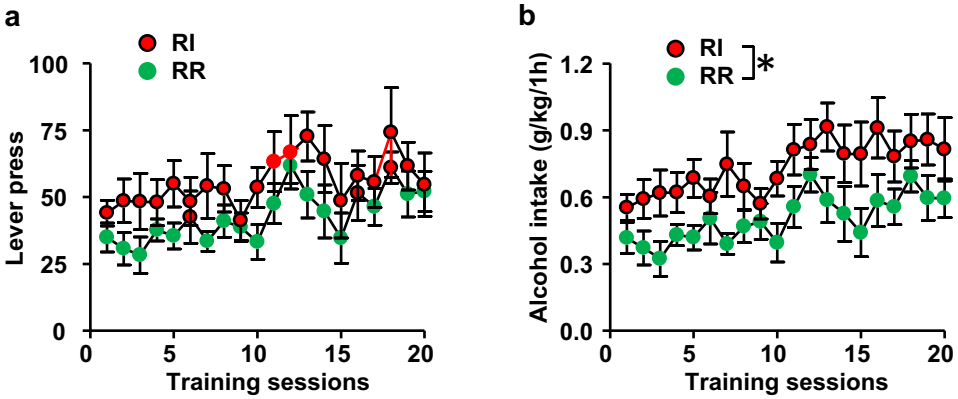
Supplemental Figure 2

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



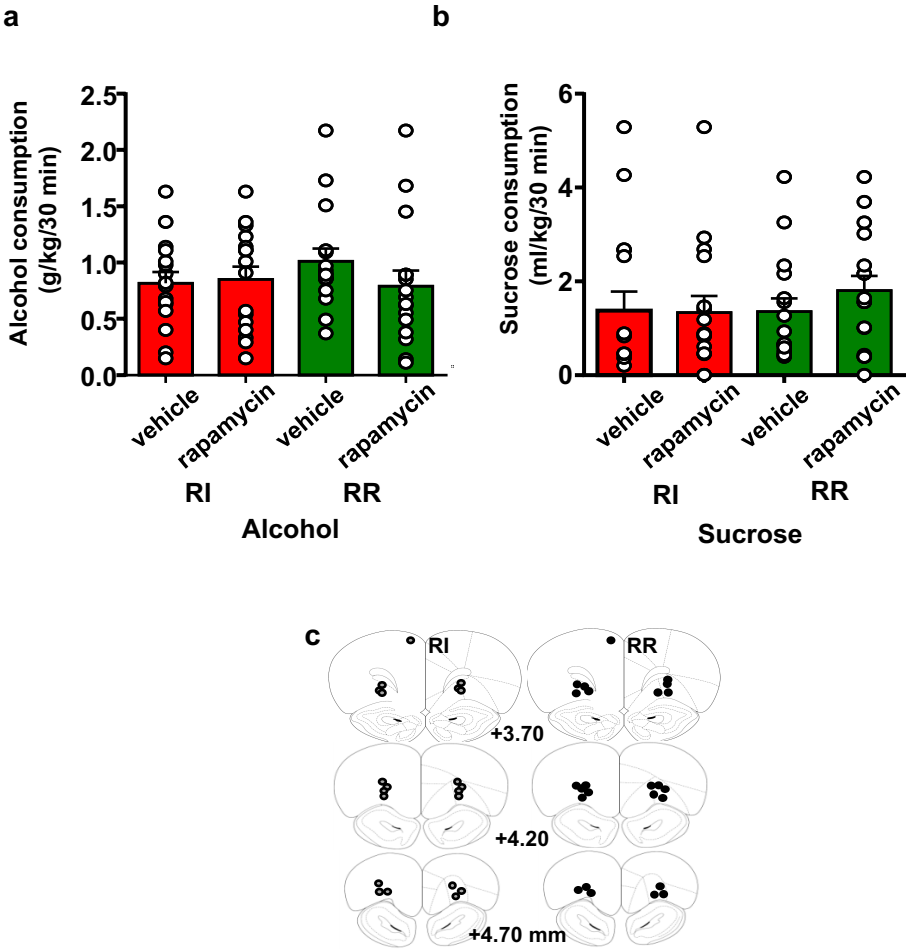
Supplemental Figure 3

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



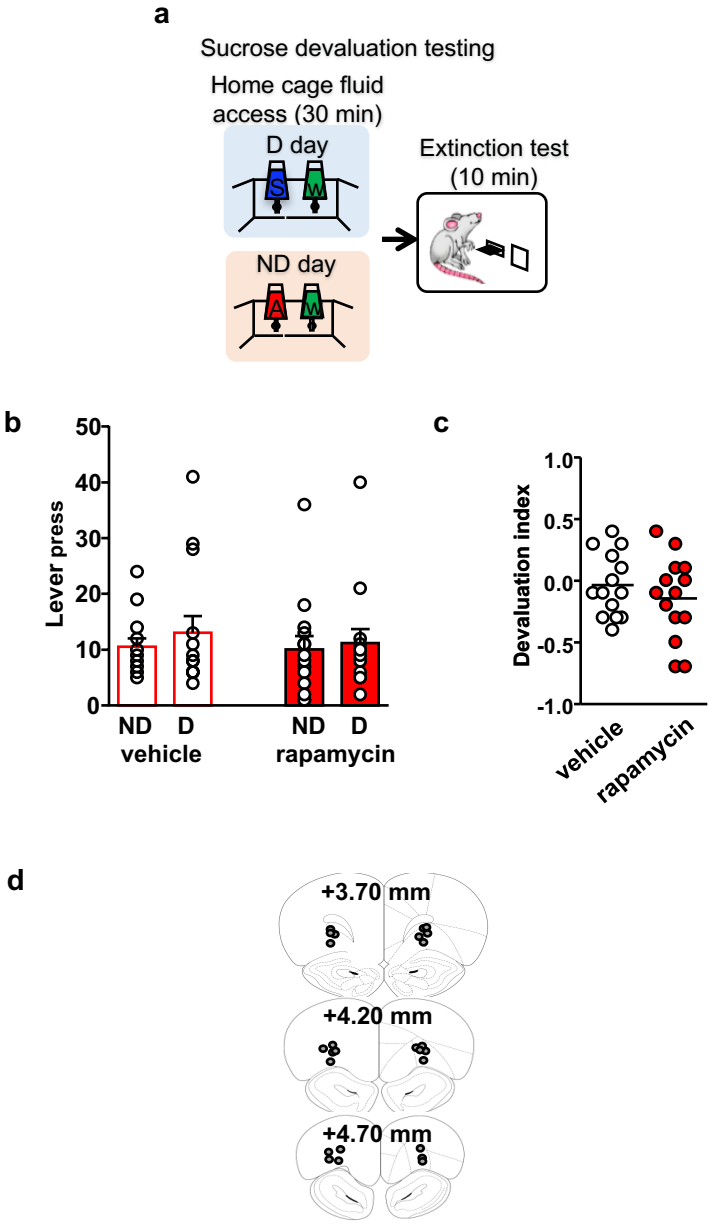
Supplemental Figure 4

Inhibition of mTORC1 in the OFC does not alter voluntary alcohol or sucrose intake prior to devaluation



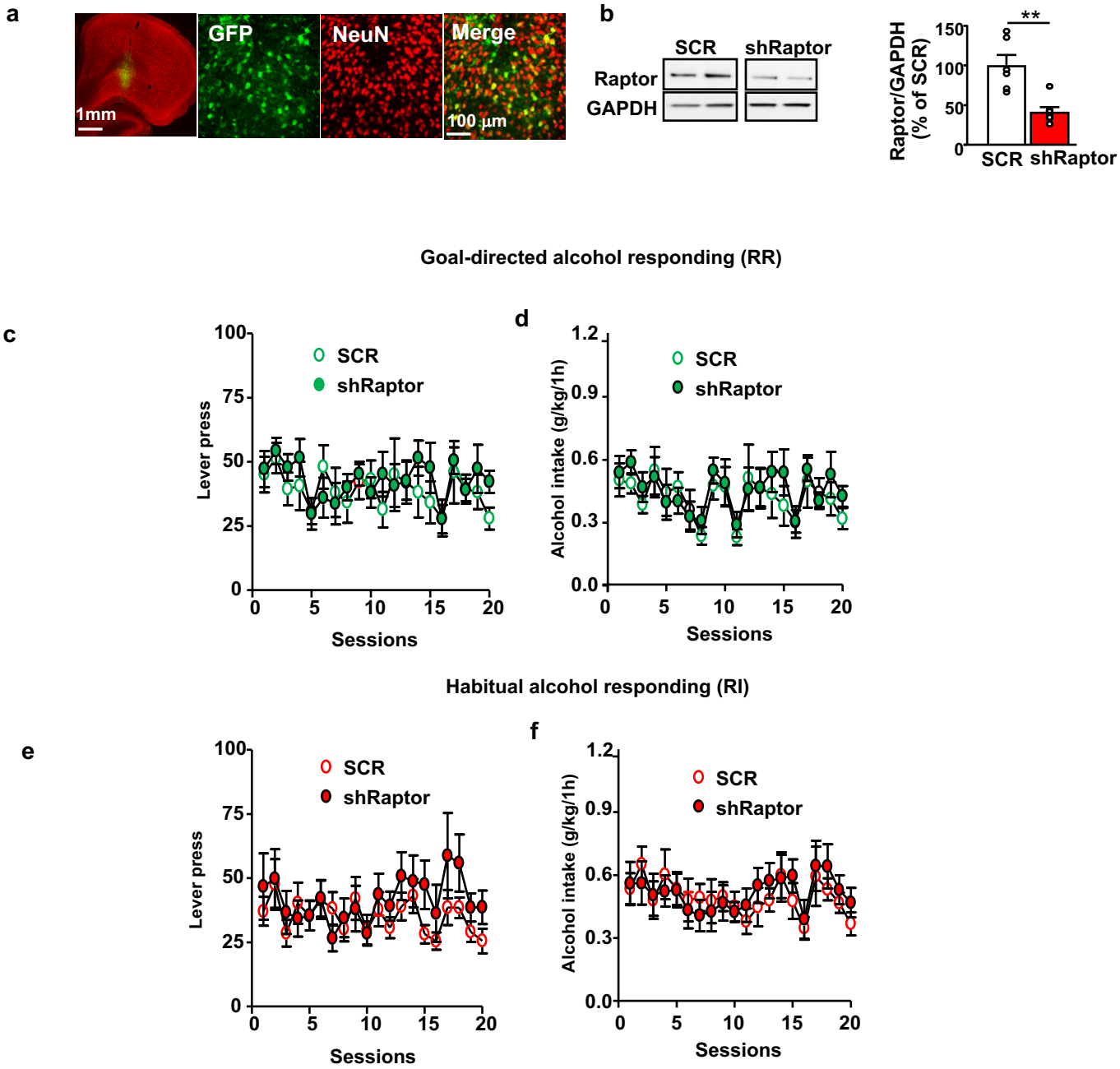
Supplemental Figure 5

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



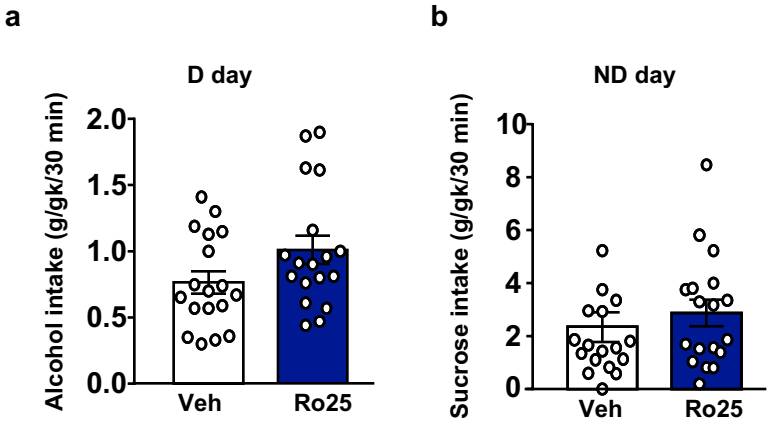
Supplemental Figure 6

shRNA-mediated knockdown of Raptor in the OFC does not affect alcohol self-administration in RI- and RR-trained rats or drinking during outcome devaluation



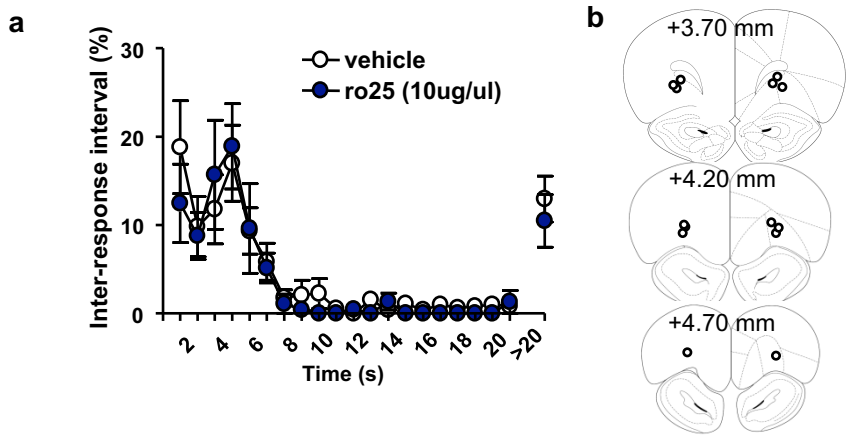
Supplemental Figure 7

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



Supplemental Figure 8

Inhibition of GluN2B in the OFC does not alter alcohol locomotion



Supplementary Information

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 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 and AP-5 were purchased from Tocris Bioscience (Bristol, UK).

Subjects Male Long Evans rats (Harlan, Indianapolis, IN) were one (*ex vivo* experiment) or two months (behavioral 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.

Construction, preparation and infusion of lentivirus expressing shRNA targeting Raptor

The strategy for generating shRaptor or a SCR control was as described in{Laguesse, 2017 #855}. Specifically, To target Raptor by shRNA, the 19 nucleotides short hairpin RNA (shRNA) sequence 5'- GCG AGC TGA TGT AGA ATT A -3' was selected using siRNA Wizard v3.1 (InvivoGen, San Diego, CA). The scrambled (SCR) 19 nucleotides sequence 5'-GCG CUU AGC UGU AGG AUU C-3' was used as a control. Synthesized DNA oligos containing the above sequences were

annealed and inserted into pLL3.7 vector (Addgene) at HpaI and XhoI sites. Plasmids DNA were prepared using a Plasmid Maxi Kit (Qiagen). All constructs were verified by sequencing. Production of lentivirus was conducted as described in{Laguesse, 2017 #855}. Specifically, HEK lentiX cells (Clontech) were transfected with the lentiviral packaging vectors psPAX2 and pMD2.G, together with the pLL3.7 shRaptor or pLL3.7 SCR using lipofectamine 2000 (Invitrogen) in OPTIMEM medium (Sigma-Aldrich). Six hours after transfection, medium was changed to DMEM-FBS 10%. Sixty hours after transfection, supernatant containing the viral particles was collected, filtered into 0.22μm filters and purified by ultracentrifugation at 26,000g for 90 minutes at 4ºC. Virus pellet 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. Intra-OFC infusion of lentivirus was conducted as described in{Ben Hamida, 2012 #47}. Briefly, rats were continuously anesthetized using isoflurane (Baxter) and bilateral microinfusions were made using stainless steel injectors (33 gauges, Hamilton) into the ventrolateral OFC (anterioposterior +3.5mm, mediolateral ±2mm and dorsoventral +4.4mm; all coordinates are from bregma). Ltv-SCR or Ltv-shRaptor (1×10^8 pg/ml, 1μl/side) were infused at an injection rate of 0.2μl/minutes. After each infusion, the injectors were left in place for an additional 10 minutes to allow diffusion of the virus. A cohort of naïve animals was used verification of virus expression by immunohistochemistry (**Supplemental Fig. 6a**). Efficacy of shRNA-mediated knockdown of Raptor was measured by western blot analysis (**Supplemental Fig. 6b-c**).

Ex vivo NMDA treatment

Naïve rats were euthanized by isoflurane and decapitated. Brains were removed and sectioned into 300 μm-thick slices at 4ºC in a solution of aCSF containing (in mM) 200 sucrose, 1.9 KCl, 1.4

NaH_2PO_4, 0.5 $CaCl_2$, 6 $MgCl_2$, 10 glucose, 25 $NaHCO_3$, 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 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgCl_2$, 25 glucose, 0.4 ascorbic acid) heated at 32°C for 30 minutes and then moved to an aCSF solution containing 1μM TTX at room temperature for 30 minutes. The OFC was dissected and incubated for 30 minutes in aCSF containing 1μM TTX at room temperature. OFC sections were then treated for 3 minutes with vehicle (0.1% DMSO) or NMDA (25μM) in TTX-containing aCSF{Sutton, 2002 #3224}, 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 (30μg 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{Ben Hamida, 2012 #47}. 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).

Intermittent access to 20% alcohol using two bottle choice

Rats underwent intermittent access to 20% alcohol in a 2-bottle choice (IA-20%2BC) paradigm for 7 weeks as described in{Neasta, 2011 #3046}. 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 12:00 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 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-2BC were selected for the study, approximately 70-80% of the cohort. Following stable baseline of alcohol drinking, rats underwent stereotaxic surgery as described previously{Jeanblanc, 2009 #3045}. Briefly, 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). After a one-week recovery period, IA20%2BC 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/lμ/side) 30 minutes prior to an alcohol drinking 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/minutes. Drug testing was performed using a "within-subject" design in which rats received both treatments in counterbalanced order, with at least one week of IA20%2BC between tests. Upon establishing a baseline of IA20%2BC, a separate cohort of animals received an infusion of R025-6981 (10μg/lμ/side) or vehicle 30 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 westernblot analysis.

Alcohol self-administration

First, rats underwent intermittent access to 20% alcohol in a 2-bottle choice (IA-2BC) paradigm for 7 weeks. Rats drinking more than 3.5 g/kg/24h on the last week of IA-2BC were selected for alcohol self-administration training. 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{Jeanblanc, 2009 #3045}. 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, and were treated with rapamycin as described above. An independent cohort of rats was used to test the effect of intra-OFC infusion of Ro25-6981 on alcohol seeking. To do so, rats received microinjections of either vehicle or Ro25-6981 (10μg/μl/side) in the OFC, in a counter-balanced manner, 30 minutes before a single 30 minutes-extinction session.

Habitual sucrose seeking

RI30/RR3 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/RR3 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 (**Supplemental Fig. 5a**). 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 (**Supplementary Figure 5b, vehicle**), revealing a rapid shift toward habitual sucrose responding as previously described {Corbit, 2012 #146}.

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.

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Supplementary Figures

Supplemental Figure 1 Inhibition of mTORC1 in the OFC does not impact 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 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 and 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

the bilateral placements of the injections in the OFC according to Paxinos and Watson 2007. Data are presented as mean±SEM. n=7.

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 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 n=10-12. *p<0.05.

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

Rats with a history of IA-20%2BC were trained to respond for alcohol or sucrose in an operant self-administration paradigm under a random interval (RI) or random ration (RR) schedule of reinforcement Prior to a 10 min devaluation probe session, animals were allowed access to alcohol or sucrose 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)** 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=16. **(e)** Schematic drawing of coronal sections of the rat brain showing the bilateral placements of the injections in the OFC according to Paxinos and Watson 2007.

Supplemental Figure 5 Habitual sucrose responding in rats with a history of excessive alcohol drinking is not affected by mTORC1 inhibition in the OFC

A cohort of rats were trained to respond for sucrose in an operant self-administration paradigm under a random interval (RI). **(a)** Habitual behavior was probed in an identical way to previous alcohol-trained cohorts with the exception that sucrose pre-feeding was considered the devaluing context and alcohol was the non-devalued substance. **(b)** RM ANOVA failed to show a significant effect of repeated testing ($F_{3,39}$=1.32, p=0.28) on lever presses between devalued and non-devalued days when habitually responding animals were pretreated with either vehicle or rapamycin. **(c)** Devaluation index. No significant difference was detected (t_{14}=0.96, p=0.36). n=14. **(d)** Indicates histological confirmation of injections into the OFC during pharmacological manipulations. Significance between treatment groups was determined using ANOVAs and two-tailed paired t-tests

Supplemental Figure 6 shRNA-mediated knockdown of Raptor in the OFC does not affect alcohol self-administration in RI- and RR-trained rats or alcohol drinking during outcome devaluation

(a-b) The OFC of rats was infected with a lenti-virus expressing shRaptor (1×10^8 pg/ml) or SCR (1×10^8 pg/ml), and viral infection (**a**) and protein knockdown (**b**) were evaluated 4 weeks later. (**a**) **shRaptor is expressed in OFC neurons**. Immunohistochemistry was used to confirm viral infection of shRaptor. GFP was used as a reporter tag. Slices from naïve rats were stained with anti-GFP antibodies and NeuN. Left panel depicts the specificity of the site of infection. Scale bar, 1mm, middle and right panels depicts shRaptor infection in neurons. Scale bar. 100μm. (**b**) **shRaptor reduces Raptor protein levels**. The OFC was dissected at the end of the behavioral experiment and raptor levels were detected using westernblot analysis. The Membranes were stained with anti-Raptor antibodies and GAPDH was used as a loading control. t_{11}=4.28, p<0.01.

(**c**). **Knockdown of raptor does not alter alcohol self- administration in RR-trained rats**. The OFC of was infected with shRaptor or SCR. Rats were then trained to self-administer alcohol using RR schedule of reinforcement and alcohol lever presses were monitored. Alcohol self-administration was similar in shRaptor vs. SCR infected animals (RM ANOVA $F_{1,18}$=0.63, p>0.05). **(d) Knockdown of raptor does not alter alcohol home cage intake prior to devaluation in RR-trained rats**. The OFC was infected with shRaptor or SCR. Rats were then trained to self-administer alcohol using RR schedule of reinforcement and alcohol intake prior to devaluation was measured. Knockdown of Raptor does not alter alcohol intake in RR-trained rats (RM ANOVA $F_{1,18}$=0.05, p>0.05). (**e-f**) **Knockdown of Raptor does not alter alcohol self-administration and home cage alcohol intake in rats trained under an RI schedule of reinforcement.** Alcohol intake was measured as above in RI-trained rats. There was no difference in **(e)** lever pressing (RM ANOVA $F_{1,15}$=0.30, p>0.05) or **(f)** alcohol intake (RM ANOVA $F_{1,15}$=0.21, p>0.05) in RI-trained animals infected with shRaptor or SCR. Significance was determined using RM ANOVA and two-tailed paired t-tests **(a-b)** n=6-7, **(c-f)** n=8-9. **p<0.001.

Supplemental Figure 7 Inhibition of GluN2B in the OFC does not alter voluntary alcohol or sucrose 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 to sucrose or alcohol. Vehicle or Ro25-6981 (10μg/μl) was infused in the OFC 30 min before a 10 minutes extinction session. Intra-OFC pretreatment with Ro25-6981 did not impact alcohol (t_5=2.80, p>0.05) or sucrose intake (t_5=0.20, p>0.05) on devalued (D) or non-devalued (ND) days, respectively. n=18.

Supplemental Figure 8 Inhibition of GluN2B in the OFC does not alter locomotion

Vehicle or Ro25-6981 (10μg/μl) was infused in the OFC 30 min before a single 30 min 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 Pre-exposure consumption on non-devaluation and devaluation trials of sucrose, alcohol, and water

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.