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.

97 **METHODS**

98 **Reagents**

99 Anti-phospho-S6 (S235/236, 1:500), anti-S6 (1:1000), anti-phospho-4E-BP (T37/46,
100 1:500), anti-4E-BP (1:1000), anti-phospho-CaMKII (T286, 1:1000), anti-phospho-S6K
101 (T389, 1:500) and anti-S6K (1:500) antibodies were purchased from Cell Signaling
102 Technology (Danvers, MA). Anti-CaMKII (1:500) antibodies were purchased from Santa
103 Cruz Biotechnology (Santa Cruz, CA). Anti-Actin (1:10,000) antibodies, phosphatase
104 Inhibitor Cocktails 2 and 3, Dimethyl sulfoxide (DMSO), D-sucrose and NMDA were from
105 Sigma Aldrich (St. Louis, MO). Nitrocellulose membranes and anti-NeuN antibodies were
106 purchased from EMD Millipore (Billerica, MA, USA). Enhanced Chemiluminescence
107 (ECL) was purchased from GE Healthcare (Pittsburg, PA, USA). Donkey anti-rabbit
108 horseradish peroxidase (HRP), and donkey anti-mouse horseradish peroxidase (HRP) were
109 purchased from Jackson ImmunoResearch (West Grove, PA). EDTA-free complete mini
110 Protease Inhibitor Cocktails were purchased from Roche (Indianapolis, IN). NuPAGE Bis-
111 Tris precast gels and Phosphate buffered saline (PBS) were purchased from Life
112 Technologies (Grand Island, NY). Pierce Bicinchoninic Acid (BCA) protein assay kit was
113 obtained from Thermo Scientific (Rockford, IL), and ProSignal Blotting Film were
114 purchased from Genesee Scientific (El Cajon, CA). Ethyl alcohol (190 proof) was
115 purchased from VWR (Radnor, PA). Rapamycin was purchased from LC laboratories
116 (Woburn, MA). Ro25-6981 was purchased from Tocris Bioscience (Bristol, UK).

117

118 **Subjects**

119 Male Long Evans rats (Harlan, Indianapolis, IN) were one (*ex vivo* experiment) or two
120 months (*in vivo* experiments) old at their arrival. Animals were single-housed in a

121 temperature- and humidity-controlled colony room (22±2 °C, relative humidity: 50–60%)
122 under a normal 12h light/dark cycle (lights on at 7:00AM) with food and water available
123 ad libitum. Rats were given a week of habituation to the housing conditions before the
124 beginning of the experiments. All animal procedures were approved by the University of
125 California San Francisco Institutional Animal Care and Use Committee (IACUC) and were
126 conducted in agreement with the Association for Assessment and Accreditation of
127 Laboratory Animal Care (AAALAC).
128
129 **Preparation of solutions**
130 Alcohol was diluted to 20% (v/v) and sucrose to 1-8% (w/v) in tap water. Rapamycin
131 (50ng/μl) was dissolved in PBS. Ro25-6981 (10μg/μl) and NMDA (25μM) were dissolved
132 in 0.1% DMSO in PBS.
133
134 **Intermittent access to 20% alcohol using two bottle choice and drug infusion**
135 Rats underwent intermittent access to 20% alcohol in a 2-bottle choice (IA-20%2BC)
136 paradigm for 7 weeks as described in (36). Specifically, rats were given 24 hours of
137 concurrent access to one bottle of 20% alcohol (v/v) in tap water and one bottle of water.
138 Control rats had access to water only. Drinking sessions started at 12:00pm on Monday,
139 Wednesday and Friday, with 24- or 48 hours (weekend) of alcohol-deprivation periods in
140 which rats consumed only water. The placement (left or right) of the water or alcohol
141 solution was alternated between each session to control for side preference. Water and
142 alcohol bottles were weighed at the beginning and at the end of each alcohol drinking
143 session. Rats were weighed once a week. Rats drinking more than 3.5 g/kg/24h on the last
144 week of IA-20%2BC were selected for the study, approximately 70-80% of the cohort.

145

146 **Stereotaxic Surgery**

147 Following stable baseline of alcohol drinking, rats underwent stereotaxic surgery.
148 Specifically, rats were continuously anesthetized using isoflurane (Baxter) and bilateral
149 guide cannulae (Plastic One) were implanted in the ventrolateral OFC (anterioposterior
150 +3.5mm, mediolateral ±2mm and dorsoventral +3.9mm; all coordinates are from bregma),
151 secured with screws (Plastic One) and dental cement (Ortho-Jet, Lang Dental).

152

153 **Alcohol seeking and drug infusion**

154 First, rats underwent IA-20%2BC paradigm for 7 weeks, as described above. Rats drinking
155 more than 3.5 g/kg/24h on the last week of IA-20%2BC were selected for alcohol self-
156 administration training in an operant self-administration (OSA) paradigm (37) with
157 modification adapted from (21, 38, 39). Training was conducted in operant chambers
158 (Med-Associates; Georgia, VT) using a fixed ratio 3 (FR3) (i.e. three active lever presses
159 result in the delivery of one alcohol reward) during daily 30 minutes-session (Mon-Fri) as
160 previously described (37). An inactive lever was also extended during the operant sessions
161 but had no programmed consequence. Following stable baseline of alcohol responding, rats
162 underwent stereotaxic surgery for cannula placement in the OFC, as described above. After
163 a one-week recovery period, operant self-administration procedure was resumed and
164 handling for drug microinfusions began. Rats received infusion of either vehicle or
165 rapamycin (50ng/µl/side) 3 hours or R025-6981 (10µg/µl/side) 15 minutes before a single
166 30 minutes-extinction session. Drugs were injected with bilateral infusion needles (Plastics
167 One) that projected 0.5 mm past the end of the guide cannula, at a rate of 0.5µl/minute.

168 Drug testing was performed using a "within-subject" design in which rats received both
169 treatments in counterbalanced order.
170 Upon establishing a baseline of IA-20%2BC, a separate cohort of animals received an
171 infusion of R025-6981 (10 μg/μl/side) or vehicle 15 minutes prior to the end of the last
172 withdrawal session. The OFC was harvested at the end of the 24-hrs withdrawal session
173 and were processed for western blot analysis.
174
175 **Habitual and goal-directed alcohol seeking and drug infusion**
176 After 7 weeks of IA-20%2BC, high-drinking rats (alcohol intake >3.5 g/kg/24h) were
177 trained to self-administer 20% alcohol in an operant self-administration (OSA) paradigm
178 (37) with modification adapted from (21, 38, 39). Self-administration training was initiated
179 under FR1 for 3 consecutive overnight sessions. Afterwards, the sessions lasted for an hour.
180 Only one lever was available during the operant sessions. Rats were pseudo-randomly
181 assigned to 2 groups and were subjected to a random interval (RI), or a random ratio (RR)
182 schedule of reinforcement which biases responding towards habitual or goal directed
183 actions, respectively (16). Only rats that self-administered alcohol more than 0.3g/kg/hour
184 were included in the study.
185 RI training: During RI training, each reward was delivered following one lever press
186 occurring after variable reward delivery time intervals (16). Three sessions were under RI-
187 5 (i.e. inter-reward time was 5 seconds on average, ranging from 1 to 10 seconds), followed
188 by three RI-15 sessions (i.e. inter-reward time was 15 seconds on average ranging from 10
189 to 30 seconds). Reinforcement was then shifted to RI-30 (i.e. inter-reward time was 30
190 seconds on average ranging from 10 to 50s) for at least 20 sessions (**Timeline, Fig. 2a**).

191 RR training: During RR training, reward was delivered following a variable number of
192 lever responses (16). Six sessions under RR-2 (1 reward delivery following on average 2
193 lever presses with number of presses ranging from 1 to 3) followed by RR-3 (1 reward
194 delivery following on average 3 lever presses with number of presses ranging from 1 to 5)
195 for at least 20 sessions (**Timeline, Fig. 2a**).
196 Following stable RI-30 or RR-3 responding, rats underwent alcohol devaluation testing as
197 described in (38) with modifications. Rats were given home-cage 2BC access to 1%
198 sucrose on non-devalued (ND) days or 20% alcohol on devalued (D) days for 30 minutes
199 (**Fig. 2b**). On each day, immediately after home-cage pre-feeding, rats underwent a 10-
200 minutes extinction test during which unrewarded lever presses were recorded. A "within-
201 subject" design was used in which ND or D sessions were in a counterbalanced order, with
202 2 standard retraining sessions between the tests. Rats that did not acquire the behavior and
203 were pressing less than 5 lever presses per session on ND day were excluded from analysis.
204 To minimize the effects of individual variance in total lever pressing we normalized ND
205 and D lever presses (15, 16, 21). Normalized lever pressing was calculated as ND or D
206 lever presses/total number of lever presses, respectively. Normalizing devaluation lever
207 presses produces a distribution of data, where 50% is equivalent to equal lever pressing
208 between ND and D (16). Devaluation index was calculated as the ratio of lever presses on
209 ND day–lever presses on D day/total number of lever presses. At the end of the training
210 period and upon acquiring the behavior (RR, goal directed behavior and RI, habitual
211 behavior), rats underwent stereotaxic surgery to bilaterally implant a guide cannula in the
212 ventrolateral OFC, as described above. One week later, RI-30 or RR-3 training was
213 resumed for 2 weeks after which rats received microinjections of either vehicle or

214 rapamycin (50ng/µl/side) 3 hours or R025-6981 (5µg/µl/side) 15 minutes prior an alcohol
215 devaluation testing.
216
217 **Habitual sucrose seeking**
218 RI30 training was similar to the procedure described for alcohol except that 8% sucrose
219 was initially used and progressively reduced to 1% across training sessions. Only ten RI30
220 sessions (2 weeks) were conducted prior to the initial outcome devaluation test. Rats were
221 then implanted with a guide cannula in the OFC as described above. After one-week
222 recovery period, sucrose self-administration was resumed for two weeks, after which rats
223 received microinjections of vehicle or drug in a counter-balanced manner, prior to the
224 devaluation test. Sensitivity to changes in sucrose value was tested using a sucrose
225 devaluation procedure which was similar to alcohol devaluation except that rats had 2BC
226 access to 20% alcohol on ND day and 1% sucrose on D day (**Timeline, Fig. 3a-b**).
227
228 **Histology**
229 For verification of cannula placement, brains were post-fixed in 4% PFA for one week,
230 then rapidly frozen and sectioned into 50µm coronal slices using a Leica CM3050 cryostat
231 (Leica Biosystems). Slices were stained using cresyl violet and examined for cannula
232 placement using a bright-field microscope. All rats had correct guide cannula placement
233 and were included in the study.
234
235 ***Ex vivo* NMDA treatment**
236 Naïve rats were euthanized by isoflurane and decapitated. Brains were removed and
237 sectioned into 300 μm-thick slices at 4° C in a solution of aCSF containing (in mM) 200

238 sucrose, 1.9 KCl, 1.4 NaH_2PO^4, 0.5 $CaCl_2$, 6 $MgCl_2$, 10 glucose, 25 $NaHCO_3$, 4 ascorbic
239 acid; 310-330 mOsm. Immediately after sectioning, OFC-containing slices were recovered
240 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
241 glucose, 0.4 ascorbic acid) heated at 32° C for 30 minutes and then moved to an aCSF
242 solution containing 1 μM TTX at room temperature for 30 minutes (40). The OFC was
243 dissected and incubated for 30 minutes in aCSF containing 1 μM TTX at room temperature.
244 OFC sections were then treated for 3 minutes with vehicle (0.1% DMSO) or NMDA
245 (25μM) in TTX-containing aCSF , and western blot analysis was conducted as described
246 below.
247
248 **Western blot analysis**
249 Tissue were homogenized in ice-cold radio immunoprecipitation assay (RIPA) buffer (in
250 mM: 50 Tris-Cl, 5 EDTA, 120 NaCl, 1% NP-40, 0.1% deoxycholate, 0.5% SDS, protease
251 and phosphatase inhibitors), using a sonic dismembrator. Protein content was determined
252 using BCA kit. Tissue homogenates (30 μg per sample) were resolved on NuPAGE 10%
253 Bis-Tris gels at 100 V for 2 hours and transferred onto nitrocellulose membranes at 30V
254 for 2 hours. Blots were blocked with 5% milk-phosphate-buffered saline with 0.1% tween-
255 20 at room temperature and then probed with primary antibodies overnight at 4°C.
256 Membranes were washed and probed with HRP-conjugated secondary antibodies for 2
257 hours at room temperature, and bands were visualized using ECL. Band intensities were
258 quantified by ImageJ (National Institutes of Health, MD, USA).
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262 **Data analysis**

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

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287 **RESULTS**

288 **mTORC1 in the OFC contributes to alcohol seeking**

289 We first examined whether activation of mTORC1 in the OFC contributes to the
290 maintenance of heavy alcohol use. Rats that underwent 7 weeks of IA-20%2BC received
291 an intra-OFC bilateral infusion of vehicle or the mTORC1 inhibitor, rapamycin (50ng/μl),
292 3 hours prior to a drinking session (8) (**Fig. 1 - Supplement 1**). As shown in **Fig. 1 -**
293 **Supplement 2,** intra-OFC administration of rapamycin did not impact voluntary intake of
294 alcohol. Next, rats that underwent IA-20%2BC for 7 weeks were trained to self-administer
295 20% alcohol in an operant self-administration (OSA) paradigm (42). Upon establishing a
296 baseline of alcohol lever presses, rats received a bilateral infusion of vehicle or rapamycin
297 (50ng/μl) into the OFC 3 hours prior to an OSA session, and the number of lever presses
298 and alcohol consumption were measured (8). As shown in **Fig. 1a-c**, inhibition of
299 mTORC1 in the OFC failed to alter the number of lever presses (**Fig.1a-b**), and alcohol
300 consumed (**Fig. 1c**). Inactive lever pressing (**Fig. 1 - Supplement 3a**). The latency to the
301 first or last lever press were also unaltered (**Fig. 1 - Supplement 3b-c**). Next, we
302 determined whether mTORC1 plays a role in alcohol seeking by testing whether rapamycin
303 alters lever presses during an extinction session. Intra-OFC administration of rapamycin
304 suppressed both the number (**Fig. 1d-e**), and the rate (**Fig. 1f**) of rats' responding on the
305 lever that was previously associated with alcohol. The reduction in alcohol seeking was
306 not due to alterations in locomotion, as inter-response intervals were similar in vehicle and
307 rapamycin-treated rats (**Fig. 1 - Supplement 4**). Together, these data suggest that mTORC1
308 in the OFC does not contribute to alcohol drinking *per se,* but does promote alcohol
309 seeking.

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311 **mTORC1 in the OFC participates in habitual responding for alcohol**

312 Reward seeking can be driven by goal-directed or habitual compulsive behaviors
313 (43-45). We next examined whether mTORC1 in the OFC plays a role in goal-directed or
314 habitual alcohol seeking using a satiety outcome devaluation procedure. Rats that first
315 underwent 7 weeks of IA-20%2BC were then trained to lever press for 20% alcohol using
316 either an random ratio (RR) or random interval (RI) schedule of reinforcement, which
317 biases lever responding toward goal-directed (RR) or habitual (RI) actions (16, 21, 38)
318 (**Timeline. Fig. 2a**). RR and RI trained rats showed a similar number of presses throughout
319 the training (**Fig. 2 - Supplement 1a**), however alcohol consumption was slightly higher
320 during RI training (**Fig. 2 - Supplement 1b**), presumably due to fewer lever presses being
321 required to obtain the same volume of alcohol, compared with RR training. Rats trained to
322 self-administer alcohol in a goal-directed manner are sensitive to outcome devaluation,
323 while habitually-trained rats are not, e.g. animals who habitually press a lever to obtain a
324 reward will continue to press that lever, even when the relative value of the reward is
325 reduced due to satiety (16, 21, 38, 46). Thus, we conducted a devaluation procedure to
326 differentiate between goal-directed and habitual responding, (**Fig. 2b**). On the devalued
327 (D) day, rats had 30 minutes of home-cage access to 20% alcohol, the reward previously
328 associated with lever responding, and on the non-devalued (ND) day, rats had 30 minutes
329 of home-cage access to 1% sucrose, a reward not previously associated with lever
330 responding (**Fig. 2b**). Rats then underwent a 10-minute extinction test, during which lever
331 presses were recorded (**Fig. 2b**). In order to minimize the effects of individual variability
332 in overall lever pressing, and to graphically represent the distribution of lever presses
333 between ND and D, we normalized lever-pressing data such that 50% indicates identical
334 lever pressing on both days as shown in (15, 16, 21). As shown in **Fig. 2c-d,** rats trained

335 on an RR schedule exhibit a decrease in responding following alcohol devaluation, while
336 RI-trained rats remained insensitive to devaluation. These data show that RI training can
337 produce habitual responding for alcohol in rats.

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

351 To determine whether mTORC1 in the OFC plays a role in habitual responding to
352 a natural reward, animals were trained to self-administer sucrose under an RI schedule of
353 reinforcement, and lever presses were measured in rats treated with rapamycin (50ng/μl)
354 or vehicle (**Fig. 3 - Supplement 1**) during an extinction session in animals that were first
355 offered sucrose (devalued) or alcohol (non-devalued) in their home cage **(Timeline, Fig.**
356 **3a-b)**. Rapamycin administration did not alter home cage sucrose intake prior to the
357 extinction session (**Fig. 3 - Supplement 2**) As shown in **Fig. 3c-d**, habitual responding for
358 sucrose was similar in the vehicle- and rapamycin-treated animals. Together, these data

359 suggest that mTORC1's contribution to habitual behavior is not generalized to all
360 rewarding substances.
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362 **Alcohol-dependent mTORC1 activation in the OFC requires GluN2B**
363 Next, we set out to elucidate the mechanism by which long-term heavy alcohol use
364 activates mTORC1 in the OFC. Glutamatergic inputs from cortical and subcortical
365 structures project to the OFC (47, 48). Extracellular glutamate content is elevated in
366 cortical regions of humans and rats during alcohol withdrawal (49). N-methyl D-Aspartate
367 receptors (NMDAR) are targets of alcohol (50). For example, GluN2B-containing
368 NMDARs are activated by alcohol in the hippocampus and dorsal striatum (50).
369 Stimulation of GluN2B activates the guanine nucleotide releasing factor 1 (GRF1), the
370 activator of H-Ras (51) (**Figure 4 – Supplement 1**). Since H-Ras is upstream of mTORC1
371 (52) (**Figure 4 – Supplement 1**), we hypothesized that alcohol-dependent stimulation of
372 GluN2B activates mTORC1 in the OFC. We first tested whether NMDAR stimulation
373 activates mTORC1. To do so, OFC slices were briefly treated with NMDA (25μM), and
374 the activation of CaMKII and mTORC1 were tested by measuring the phosphorylation of
375 mTORC1 downstream targets, S6K and S6 (**Figure 4 – Supplement 1**). We found that *ex*
376 *vivo* treatment of the OFC with NMDA led to increased phosphorylation, and thus
377 activation, of CaMKII, a marker of NMDAR activation (**Fig. 4a-b**), as well as of mTORC1
378 (**Fig. 4a-b**). These data suggest that NMDAR stimulation activates mTORC1 in the OFC.
379 We previously showed that mTORC1 is activated in the OFC in response to a binge
380 drinking session which persists for at least 24 hours into alcohol abstinence (1). Thus, we
381 next tested whether GluN2B is required for alcohol-dependent mTORC1 activation. To do
382 so, rats that underwent 7-weeks of IA-20%2BC paradigm or that drank water only for the

383 same duration of time, received a bilateral intra-OFC infusion of the selective GluN2B
384 antagonist Ro-25-6981 (53) (10μg/ul) or vehicle, 15 minutes prior to the end of the last
385 alcohol drinking session, and mTORC1 activation was examined at the end of the last 24
386 hour water only session by measuring the phosphorylation of mTORC1 downstream
387 targets, 4-EBP and S6 (**Fig. 4 - Supplement 1, Timeleine Figure 4 - Supplement 2**). In
388 vehicle-treated rats, alcohol produced a robust activation of CaMKII (**Fig. 4c,f**) and
389 mTORC1, as measured by S6 (**Fig. 4d,g**), and 4-EBP phosphorylation (**Fig. 4e,h**). In
390 contrast, R025-6981-treated rats exhibited a marked reduction in both CaMKII and
391 mTORC1 activation during alcohol withdrawal **Fig. 4c-h**. In contrast, GluN2B protein
392 levels in the OFC were unchanged by alcohol (**Fig. 4i-j).** Together our data suggest that
393 GluN2B mediates mTORC1 activation following alcohol consumption.
394

395 **GluN2B in the OFC is required for alcohol seeking and habit**

396 We reasoned that, if GluN2B is upstream of mTORC1, then GluN2B should also
397 participate in habitual alcohol seeking. First, we examined whether inhibition of GluN2B
398 alters alcohol seeking during extinction. We found that intra-OFC administration of Ro25-
399 6981 (10μg/μl) 15 minutes prior to an extinction session (**Fig. 5 - Supplement 1**)
400 suppressed alcohol seeking, as indicated by a reduction in cumulative (**Fig. 5a**), active lever
401 presses (**Fig. 5b**), as well as the rate of lever pressing (**Fig. 5c**). The reduction of alcohol
402 seeking by Ro25-6981 was not due to alteration of locomotion as inter-response intervals
403 were similar in vehicle-treated and Ro25-6981-treated animals (**Fig. 5 - Supplement 2**).

404 Finally, we tested whether GluN2B in the OFC contributes to habitual alcohol
405 seeking. The timeline of the experiment is depicted in **Fig. 5d**. Intra-OFC infusion of R025-
406 6981 (5μg/μl) did not impact home cage alcohol consumption in a 2BC test during

407 devalued days (**Fig. 5 - Supplement 3**). In contrast, administration of Ro25-6981 (5μg/μl)
408 into the OFC reverted habitual responding for alcohol to goal directed responding (**Fig. 5e-**
409 **f**). Together, these data indicate that the recruitment of GluN2B signaling by alcohol is
410 required for alcohol seeking and habit.
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431 **DISCUSSION**

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

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

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

455 study is focused on habitual responding specifically for alcohol. It would be of interest to
456 determine the normal role of mTORC1 in the OFC, and whether mTORC1 suppresses other
457 decision-making tasks.

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

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

474 Interestingly, mTORC1 inhibition in the OFC does not alter habitual responding
475 for sucrose, indicating that mTORC1 plays a specific role in the formation of habits in
476 response to alcohol exposure and not natural rewards. The difference between the
477 consequences of mTORC1 inhibition on habitual alcohol vs. sucrose seeking is striking,

478 but it is important to note that, unlike alcohol, voluntary sucrose intake does not activate
479 mTORC1 in the OFC (1).

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

487 We previously reported that repeated cycles of binge alcohol drinking and
488 withdrawal activates mTORC1 in the OFC (1), and we demonstrate herein that mTORC1
489 activation in the OFC depends, at least in part, on GluN2B suggesting that GluN2B is
490 activated in response to alcohol use. The NMDAR is a target of alcohol (50), and alcohol
491 enhances GluN2B phosphorylation in brain regions such as the cerebellum, the DMS and
492 the hippocampus (50). GluN2B phosphorylation enhances the activity of the channel (69).
493 Thus, it is plausible that alcohol-dependent GluN2B phosphorylation in the OFC enhances
494 GluN2B function thereby enabling mTORC1 activation. Alternatively, the hyperactivity
495 of GluN2B could be a compensatory homeostatic plasticity resulting from the inhibition of
496 NMDAR by alcohol (50). In contrast to our findings that alcohol does not alter the
497 expression of GluN2B in the OFC, Nimitvilai and colleagues showed that chronic exposure
498 to alcohol vapor decreases in the expression of GluN2B (28). One important difference
499 between our study and Nimitvilai et al. as well as additional reports on the interaction
500 between the OFC and alcohol discussed herein, relates to the use of contingent vs. non-
501 contingent alcohol administration. Specifically, prior to the OSA, rats were subjected to 7

502 weeks of IA-20%2BC. The IA-20%2BC paradigm models "problem drinkers" e.g. human
503 subjects that suffer from AUD phenotypes such as binge drinking, compulsive drinking
504 and craving (64). In our case, rats achieve blood alcohol concentrations (BAC) of 80 mg/dl
505 (65) a BAC equivalent to binge drinking humans (66). In contrast, the majority of other
506 reports described herein involved passive exposure of alcohol vapor, which achieves BAC
507 of 150-250 mg/dl, and leads to physical dependence on alcohol (67). Humans who are
508 dependent on alcohol show profoundly degraded white matter and reduced neuronal
509 density in the OFC (31, 32), possibly an indication of alcohol-induced damage to this
510 structure. Thus, it is likely that cycles of binge drinking do not damage the OFC which may
511 be the case in animal models of alcohol dependence. As only a small percentage of alcohol
512 users exhibit physical dependence (68), we believe that our findings have important
513 implications for the understanding of the mechanisms underlying problem drinking and
514 AUD.

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

523 We show here that GluN2B activation in the OFC is sufficient to influence the
524 formation and/or maintenance of alcohol seeking and habit. In line with the possibility that
525 GluN2B activation contributes to the development of habit, DePoy et al. showed that

526 habitual behavior, developed upon subchronic cocaine treatment during adolescence (71),
527 is inhibited by the treatment of animals with the GluN2B inhibitor ifenprodil (71). DePoy
528 et al. further showed that cocaine exposure during adolescence produces structural changes
529 in the OFC which were attenuated by ifenprodil (71). While our data suggest that GluN2B
530 in the OFC participates in behavioral inflexibility, Brigman et al. reported that GluN2B in
531 the OFC plays a role in choice learning (72), It is plausible that alcohol-dependent
532 neuroadaptations tilt the function of GluN2B from choice learning, e.g. a goal directed
533 behavior, to habitual responding.

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

548

549

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792 **FIGURE LEGENDS**

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

794 **(a-c) Intra-OFC infusion of rapamycin does not alter self-administration of alcohol**.
795 Rats underwent 7 weeks of IA-20%2BC, and were then trained to self-administer 20%
796 alcohol using a FR3 schedule. Vehicle (white) or rapamycin (50ng/µl, red) was infused
797 bilaterally in the OFC 3 hours before a 30 min self-administration session. **(a)** RM ANOVA
798 of cumulative lever presses did not identify a significant main treatment effect ($F_{1,12}$=0.26,
799 p>0.05). Two-tailed paired t-test revealed that the number of active lever presses **(b)**
800 (t_6=1.40, p>0.05), and amount of alcohol consumed **(c)** (t_6=1.51, p>0.05) did not differ
801 between treatment groups. **(d-f) Intra-OFC infusion of rapamycin inhibits lever presses**
802 **during extinction.** Vehicle (white) or rapamycin (50ng/µl, red) was infused in the OFC 3
803 hours before a 30 min extinction session, and responses on the previously active lever were
804 recorded. (**d**). RM ANOVA revealed a significant main treatment effect ($F_{1,16}$=4.40,
805 p=0.05) of cumulative lever presses. Two-tailed paired t-test revealed that the number (per
806 5 min) (**e**) t_8=3.31, p<0.05, and rate of lever presses (**f**) (t_8=3.30, p<0.05) were reduced in
807 the rapamycin-treated animals. Data are presented as individual values and mean±SEM.
808 *p<0.05. **(a-c)** n=7, **(d-e)** n=9.

809

810 **Figure 1 – Supplement 1**

811 **Schematic drawing of cannulae placement.** Schematic illustration of coronal sections of
812 the rat brain showing approximate bilateral placements of cannulae in the OFC according
813 to Paxinos and Watson 2007.

814

815

816 **Figure 1 – Supplement 2**

817 **Inhibition of mTORC1 in the OFC does not alter alcohol consumption in a 2-bottle**
818 **choice paradigm.** Rats with a history of IA-20%2BC were infused with vehicle (white) or
819 rapamycin (50ng/µl, red) into the OFC 3 hours before the beginning of the final 2BC
820 session, and alcohol consumption was recorded at the 30 minutes and 24-hour time points.
821 Significance between treatment groups was determined using RM ANOVA. No
822 differences were observed in alcohol consumption at the 30 minutes (t_{11}=1.25, p>0.05), or
823 at the 24-hour time point (t_{11}=0.87, p>0.05). n=12.

824

825 **Figure 1 – Supplement 3**

826 **Inhibition of mTORC1 in the OFC does not impact inactive lever press or the latency**
827 **to the first and last active lever press.** Rats were trained to self-administer 20% alcohol
828 using an FR3 schedule. Vehicle (white) or rapamycin (50ng/µl, red) was infused into the
829 OFC 3 hours before an operant self-administration session and active and inactive lever
830 presses were recorded. Significance between treatment groups was determined using two-
831 tailed paired t-tests. **(a)** number of inactive lever presses (t_8=0.37, p>0.05), latency to first
832 lever press **(b)** (t_8=0.27, p>0.05), and latency to the last active lever press **(c)** (t_8=0.89,
833 p>0.05) did not differ between groups. n=9.

834

835 **Figure 1 – Supplement 4**

836 **Intra-OFC infusion of rapamycin does not alter locomotion**. Rats were trained to self-
837 administer 20% alcohol using a FR3 schedule. Vehicle (white) or rapamycin (50ng/µl, red)
838 was infused into the OFC 3 hours before a 30 minutes extinction session, and inter-response
839 intervals (IRIs) were measured. Relative number of IRIs expressed as % of total IRIs with

840 all intervals equal or smaller of 20 seconds presented in 1 second bins. Interval greater than
841 20 seconds are also shown. Two-way ANOVA of IRIs across the session did not identify
842 a significant main treatment effect ($F_{1,12}$=0.1412, p=0.7137). n=9.
843
844 **Figure 2. Inhibition of mTORC1 in the OFC attenuates habitual responding for**
845 **alcohol**
846 **(a) Timeline of experiment.** Rats underwent 7 weeks of IA20%-2BC. Rats were the
847 trained to operant self-administer 20% alcohol during 3 overnight FR1 sessions and were
848 pseudo-randomly assigned to 2 groups. One group was subjected to a progressive RI
849 reinforcement schedule, while the other was assigned a RR schedule. Following a stable
850 RI-30 or RR-3 responding, rats underwent alcohol devaluation testing depicted in **b**. Upon
851 establishing the behavior, rats underwent stereotaxic surgery to bilaterally implant a guide
852 cannula. One week later, RI-30 or RR-3 training was resumed for 2 weeks, after which rats
853 received microinjections of vehicle or drug in a counter-balanced manner, prior to the
854 devaluation test. **(b) Alcohol devaluation test.** On test day, rats were pre-fed with 20%
855 alcohol for 30 minutes in their home cage during devalued day (D) or with 1% sucrose in
856 the non-devalued day (ND), and normalized lever pressing (percent of total presses on D
857 or ND, see methods) was used to evaluate goal-directed or habitual responding during a
858 10-minuted extinction session. **(c-d) RR trained animals are goal directed whereas RI**
859 **trained animals show habitual behavior.** RR trained rats (green) press less during
860 devalued day as compared to RI trained animals (red). Two-way ANOVA with Sidak post
861 hoc analysis indicates a significant decrease in normalized lever pressing on D vs ND in
862 RR-trained rats (p=0.0018), but not in RI-trained rats (p=0.8014) (Main effect of
863 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). **(e-f) Intra-OFC infusion of rapamycin does not alter goal-directed alcohol seeking.** Rats that were trained using an RR 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 (**b**), 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 (**b**), 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. **(c-d)** n=8-10, **(e-f)** n=8-11, **(g-h)** n=8-9.

888 **Figure 2 – Supplement 1**

889 **Number of lever presses and alcohol consumed in RI and RR-trained animals.** Rats
890 were trained to respond for alcohol in an operant self-administration paradigm under RI
891 (red) or RR, (green) schedule of reinforcement. Significance between treatment groups was
892 determined using RM ANOVA. **(a)** Training groups did not differ in the number of lever
893 presses during training ($F_{1,20}$=2.01, p>0.05), **(b)** RI-trained rats exhibited a small but
894 significant increase of alcohol intake over the course of training ($F_{1,20}$=6.65, p<0.05).
895 *p<0.05. RR n=12. RI n=10.

896

897 **Figure 2 – Supplement 2**

898 **Schematic drawing of cannulae placement.** Schematic illustration of coronal sections of
899 the rat brain showing approximate bilateral placements of cannulae in the OFC according
900 to Paxinos and Watson 2007.

901

902 **Figure 2 – Supplement 3**

903 **Inhibition of mTORC1 in the OFC does not alter voluntary alcohol intake prior to**
904 **devaluation.** Rats were trained to respond for alcohol in an operant self-administration
905 paradigm under a random interval (RI, red) or random ration (RR, green) schedule of
906 reinforcement. Vehicle (white) or rapamycin (50ng/µl, red) was infused into the OFC 3
907 hours prior to home cage access to alcohol, and alcohol intake was recorded at the end of
908 the 30 minutes session. Significance between treatment groups was determined using RM
909 ANOVA. The amount of alcohol consumed did not differ by treatment ($F_{1,30}$=0.21, p>0.05)
910 or training history ($F_{1,30}$=1.48, p>0.05). *p<0.05. RR n=12. RI n=10.

911

912 **Figure 3. Habitual sucrose responding is not affected by mTORC1 inhibition in the**
913 **OFC**

914 **(a) Timeline of experiment.** Rats underwent 7 weeks of IA20%-2BC, and were then
915 trained to operant self-administer 8% sucrose. Sucrose concentration was progressively
916 reduced to 1% during 3 overnight FR1 sessions. Rats were then subjected to an RI
917 reinforcement schedule. Following a stable RI-30 responding, rats underwent alcohol
918 devaluation testing depicted in **b**. Upon establishing the behavior, rats underwent
919 stereotaxic surgery to bilaterally implant a guide cannula. One week later, RI-30 training
920 was resumed for 2 weeks, after which rats received microinjections of vehicle or rapamycin
921 (50ng/μl) in a counter-balanced manner, prior to the devaluation test. **(b) Sucrose**
922 **devaluation test.** Habitual behavior was probed in an identical way to the alcohol-trained
923 cohorts with the exception that sucrose pre-feeding was considered the devaluing context
924 and alcohol was the non-devalued substance. **(c)** Two-way RM ANOVA failed to show a
925 significant effect of repeated testing (Main effect of devaluation: $F(1,12)=0.006464$,
926 $p>0.9999$; treatment X devaluation: $F(1,12)=0.7257$, $p=0.4110$) on lever presses between
927 devalued and non-devalued days when habitually responding animals were pretreated with
928 either vehicle or rapamycin. n=7 **(d)** Devaluation index. Two-tailed paired t-test detected
929 no significant difference between the treatment groups ($t=0.7024$, $p=0.5087$). n=7.

930

931 **Figure 3 - Supplement 1**

932 Schematic drawing of cannulae placement. Schematic illustration of coronal sections of
933 the rat brain showing approximate bilateral placements of cannulae in the OFC according
934 to Paxinos and Watson 2007.

935

936 **Figure 3 - Supplement 2**

937 Inhibition of mTORC1 in the OFC does not alter voluntary sucrose intake prior to
938 devaluation. Rats were trained to respond for sucrose in an operant self-administration
939 paradigm under RI schedule of reinforcement. Vehicle (white) or rapamycin (50ng/µl, red)
940 was infused into the OFC 3 hours prior to home cage access to sucrose, and sucrose intake
941 was recorded at the end of the 30 minutes session. Significance between treatment groups
942 was determined using RM ANOVA. The amount of sucrose consumed did not differ by
943 treatment ($F_{1,30}$=0.06, p>0.05). n=8.

944

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

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

960 was dissected 24 hours later. Water only consuming rats were used as controls. (**c**-**e**)
961 Representative images of CaMKII (**c**), S6 (**d**) and 4E-BP (**e**) phosphorylation in water vs.
962 alcohol-exposed animals that were pre-treated with vehicle or Ro25-6981. (**f**-**h**) ImageJ
963 was used for optical density quantification. Data are expressed as the average ratio +/-
964 S.E.M of phospho-CaMKII to CaMKII (**f**), phospho-S6 to S6 (**g**), and phospho-4E-BP to
965 4E-BP (**h**), and are expressed as percentage of water + vehicle group. Two-way RM
966 ANOVA showed a significant effect of alcohol on pCaMKII ($F_{1,20}$=24.66, p<0.0001), pS6
967 ($F_{1,20}$=21.92, p=0.0001), and p4E-BP ($F_{1,20}$=48.23, p<0.0001). In addition, two-way RM
968 ANOVAs indicated a main effect of drug treatment for pCaMKII ($F_{1,20}$=48.51, p<0.0001),
969 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
970 interaction for pCaMKII ($F_{1,20}$=18.57, p<0.01), pS6 ($F_{1,20}$=7.559, p<0.05), and p4E-BP
971 ($F_{1,20}$=13.22, p<0.01). Tukey's multiple comparison post hoc analysis revealed that alcohol
972 increased pCaMKII (p<0.001), pS6 (p<0.001), and p4E-BP (p<0.001) in vehicle-treated
973 animals and that pretreatment with R025-6981 reduced the phosphorylation of pCaMKII
974 (p<0.0001), S6 (p<0.05), and 4E-BP (p<0.001). **(i-j) Chronic alcohol intake does not alter**
975 **the level of GluN2B in the OFC.** At the conclusion of the 7 weeks of IA-20%2BC, the OFC
976 was collected from rats at the end of the last 24 hours water session. GluN2B levels were
977 determined by western blot analysis, and actin was used as a loading control. **(i)**
978 Representative blots of GluN2B protein levels in the OFC of alcohol consuming vs. water
979 only consuming rats. **(j)** ImageJ was used for optical density quantification. Data are
980 expressed as the average ratio +/-S.E.M of GluN2B levels, expressed as percentage of
981 water. Significance was determined using two-tailed unpaired t-test. Alcohol had no effect
982 on GluN2B levels (t_{10}=0.85, p>0.05). *p<0.05, ***p<0.001. (**a-b**) n = 7 per group, (**c-j**) n
983 = 6 per group.

984 **Figure 4 - Supplement 1**

985 **GluN2B-dependent activation of mTORC1 signaling**

986 Model depicts the signaling cascade associated with Figure 4. GluN2B activation results
987 in calcium release which in turn activates CaMKII and GRF1. CamKII activation is
988 measured by phosphorylation (P) was used as a positive control. Calium binding to GRF1
989 activates H-Ras which activates AKT that in turn activates mTORC1. mTORC1 activation
990 is measured by the phosphorylation of 4E-BP, S6K and S6. Red arrows depicts the specific
991 proteins whose phosphorylation levels were tested.

992

993 **Figure 4 - Supplement 2**

994 **Time line of experiments depicted in Figure 4c-h**

995 Rats underwent 7-weeks of IA-20%2BC paradigm or drank water only for the same
996 duration of time. Ro-25-6981 (53) (10μg/ul) or vehicle was infused into the OFC infusion
997 15 minutes before the end of the last 24 hours alcohol drinking session, and mTORC1
998 activation was measured 24 hours later.

999

1000 **Figure 5. GluN2B in the OFC promotes alcohol seeking and habitual alcohol**
1001 **responding**

1002 (**a-c**) **Intra-OFC administration of Ro25-6981 decreases alcohol seeking.** Rats
1003 underwent 7 weeks of IA-20%2BC, and were then trained to self-administer 20% alcohol.
1004 Vehicle (white) or Ro25-6981 (Ro25, 10μg/μl, blue) was infused bilaterally in the OFC 15
1005 minutes prior to a 30 minutes extinction session. **(a)** Cumulative lever presses. Two-way
1006 RM ANOVA revealed a significant main treatment effect ($F_{1,12}$=10.89, $p<0.05$). Two-tailed
1007 paired t-test revealed that the number **(b)** and rate (per 5 minutes) **(c)** of lever press were

1008 significantly different in the vs. vs. Ro25-6981 groups ng (both t_6=2.62, p<0.05). **(d)**
1009 **Timeline of experiment.** Rats underwent 7 weeks of IA20%-2BC, and were then trained
1010 to operant self-administer 20% alcohol using a progressive RI reinforcement schedule.
1011 Following a stable RI-30 lever presses, rats underwent stereotaxic surgery to bilaterally
1012 implant guide cannulae in the OFC. One week later, RI-30 training was resumed for 2
1013 weeks, after which rats received microinjections of vehicle (white) or Ro25-6981 (5 μg/μl,
1014 blue) in a counter-balanced manner, 15 minutes prior to a 30 minutes home cage 20%2BC
1015 alcohol drinking session, and the number of lever presses were measured during extinction.
1016 **(e-f) Intra-OFC administration of Ro25-6981 attenuates habitual alcohol seeking**. (**e**)
1017 Two way RM ANOVA showed a main effect of devaluation ($F_{1,33}$=13.72, p<0.001) as well
1018 as a treatment x devaluation interaction ($F_{1,33}$=6.31, p<0.05). Sidak's multiple comparison
1019 test detected a significant difference for Ro25-6981 (p<0.001) on D compared to ND days.
1020 **(f)** Devaluation index. Two-tailed paired t-test indicated a significant difference (t_{15}=3.72,
1021 p<0.01). Individual results and mean±SEM are shown, (**a-c**) n=7, (**e-f**) n=12. *p<0.05, **
1022 p<0.01.
1023
1024 **Figure 5 - Supplement 1**
1025 **Schematic drawing of cannulae placement.** Schematic illustration of coronal sections of
1026 the rat brain showing approximate bilateral placements of cannulae in the OFC according
1027 to Paxinos and Watson 2007.
1028
1029 **Figure 5 - Supplement 2**
1030 **Inhibition of GluN2B in the OFC does not alter locomotion.** Vehicle (white) or Ro25-
1031 6981 (10μg/μl, blue) was infused in the OFC 15 minutes before a single 30 minutes

1032 extinction session and inter-response intervals (IRIs) were measured. Mean +/- SEM of the
1033 relative number of IRIs expressed as % of total IRIs with all intervals equal or smaller of
1034 20 seconds presented in 1 second bins. Intervals greater than 20 seconds are also shown.
1035 Two way RM ANOVA of IRIs across the session did not identify a significant main
1036 treatment effect ($F_{1,12}$=0.81, p=0.3858). n=7.
1037
1038 **Figure 5 - Supplement 3**
1039 **Inhibition of GluN2B in the OFC does not alter voluntary alcohol intake prior to**
1040 **devaluation.** Rats were trained to respond for alcohol in an operant self-administration
1041 paradigm under a random interval (RI) schedule of reinforcement. Vehicle (white) or
1042 Ro25-6981 (5μg/μl, blue) was infused into the OFC 15 minutes prior to home cage access
1043 to alcohol, and alcohol intake was recorded at the end of the 30 minutes session.
1044 Significance between treatment groups was determined using RM ANOVA. The amount
1045 of alcohol consumed did not differ by treatment (t_5=2.80, p>0.05). n=12.
1046

Figure 1

Inhibition of mTORC1 in the OFC reduces alcohol seeking

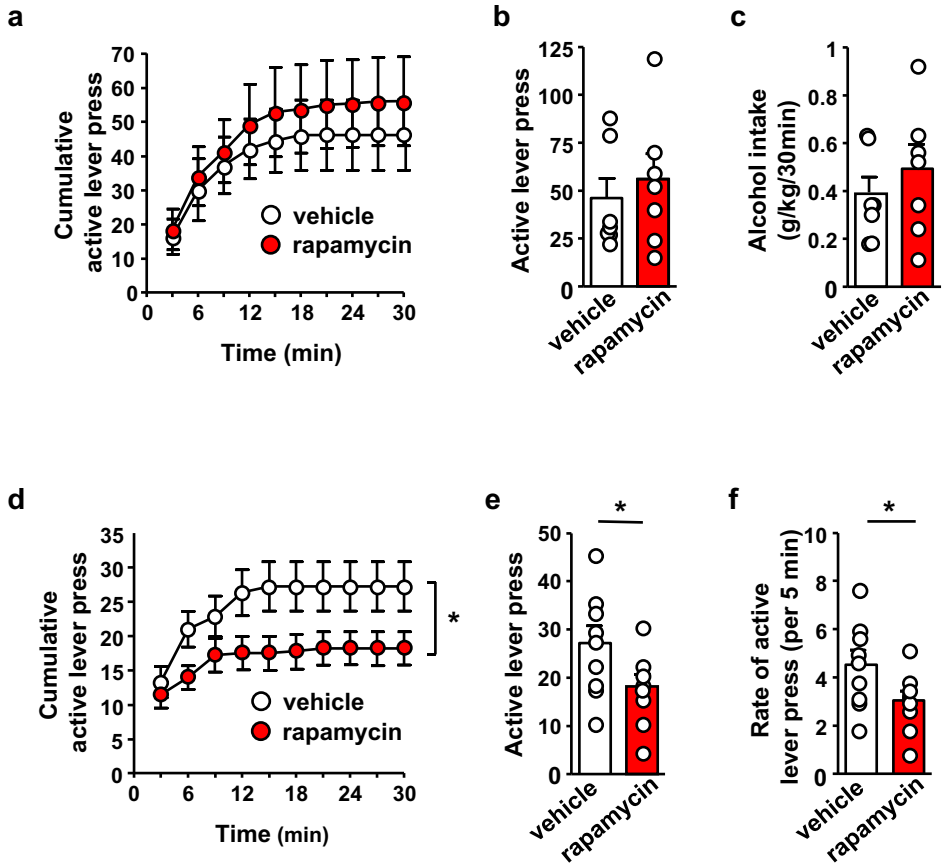


Figure 1 – Supplement 1

Schematic drawing of cannulae placement

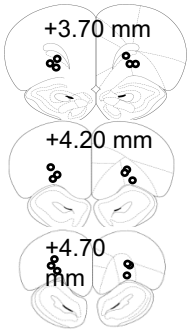


Figure 1 – Supplement 2

Inhibition of mTORC1 in the OFC does not affect alcohol consumption in a 2-bottle choice paradigm

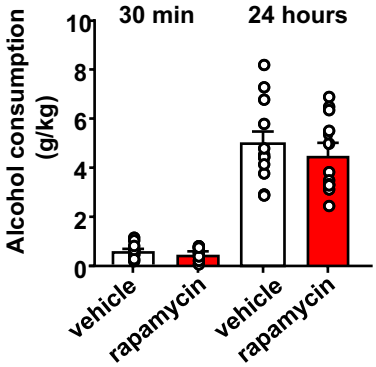


Figure 1 – Supplement 3

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

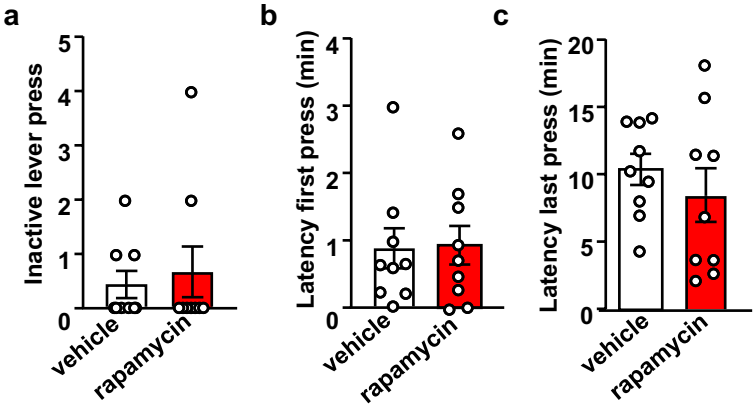


Figure 1 – Supplement 4

Inhibition of mTORC1 in the OFC does not alter locomotion

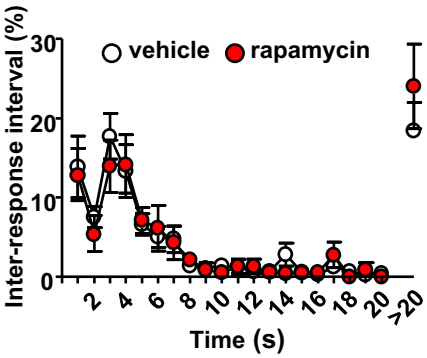


Figure 2

Inhibition of mTORC1 in the OFC attenuates habitual responding for alcohol

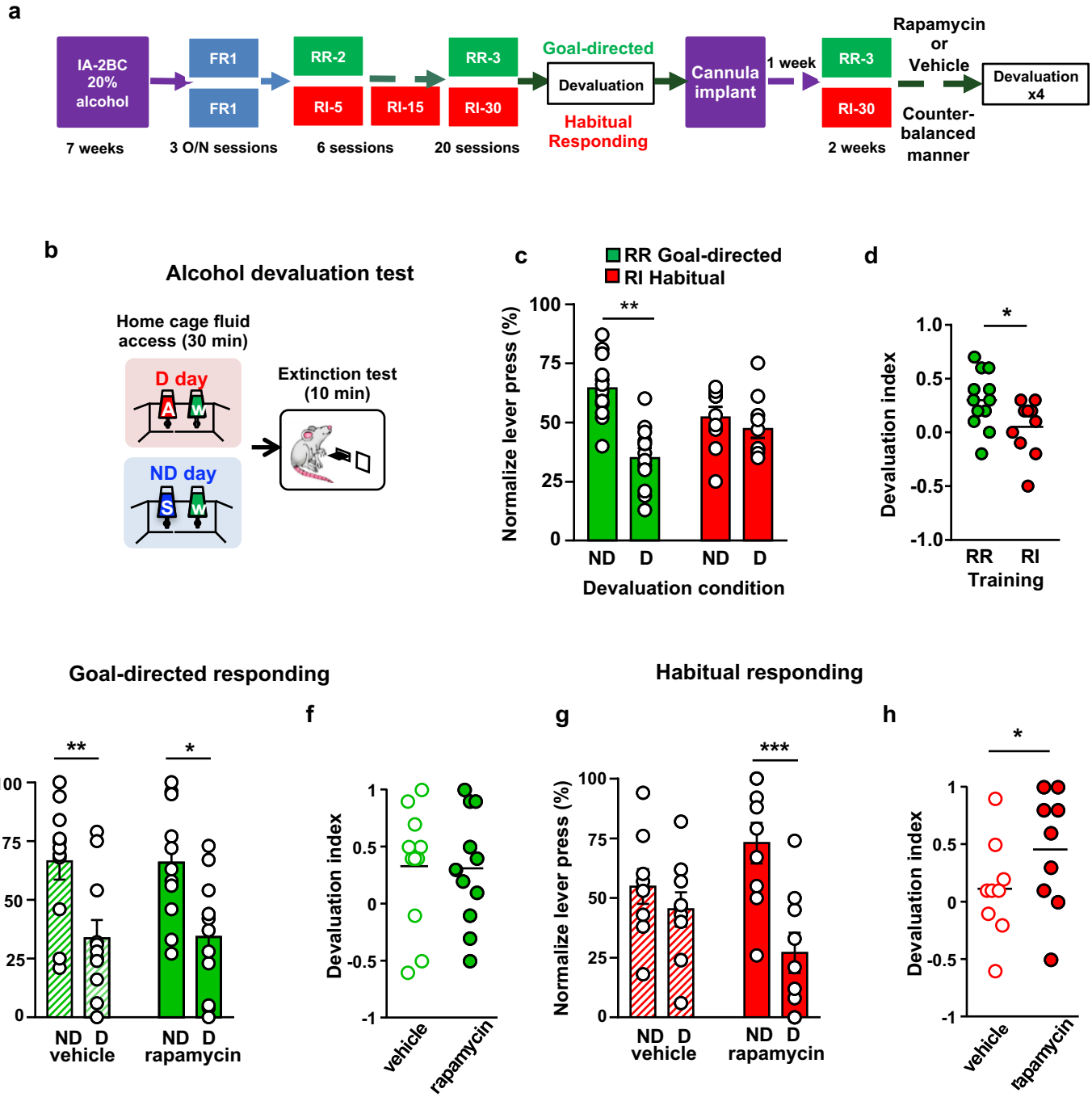


Figure 2 – Supplement 1

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

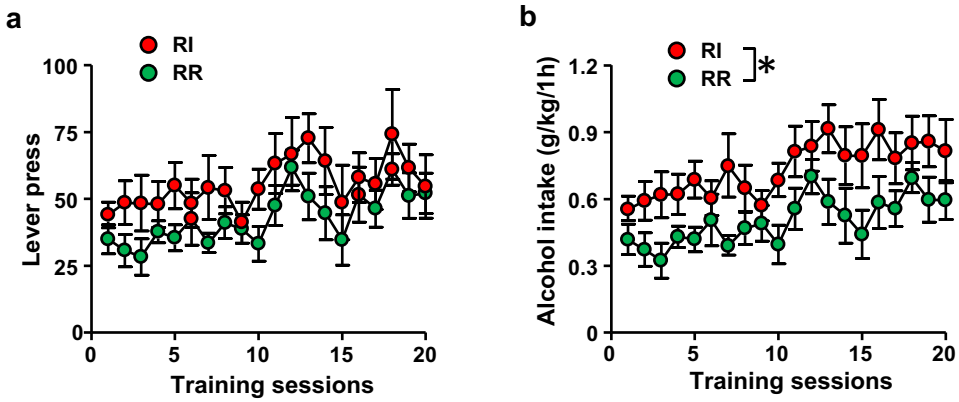


Figure 2 – Supplement 2

Schematic drawing of cannulae placement

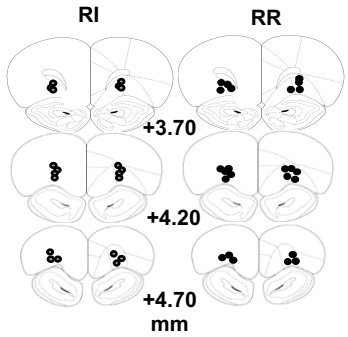


Figure 2 – Supplement 3

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

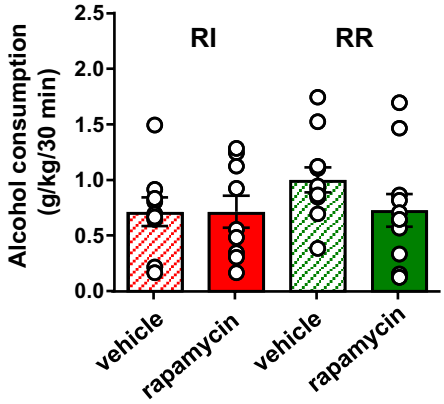


Figure 3

Habitual sucrose responding is not affected by mTORC1-OFC inhibition

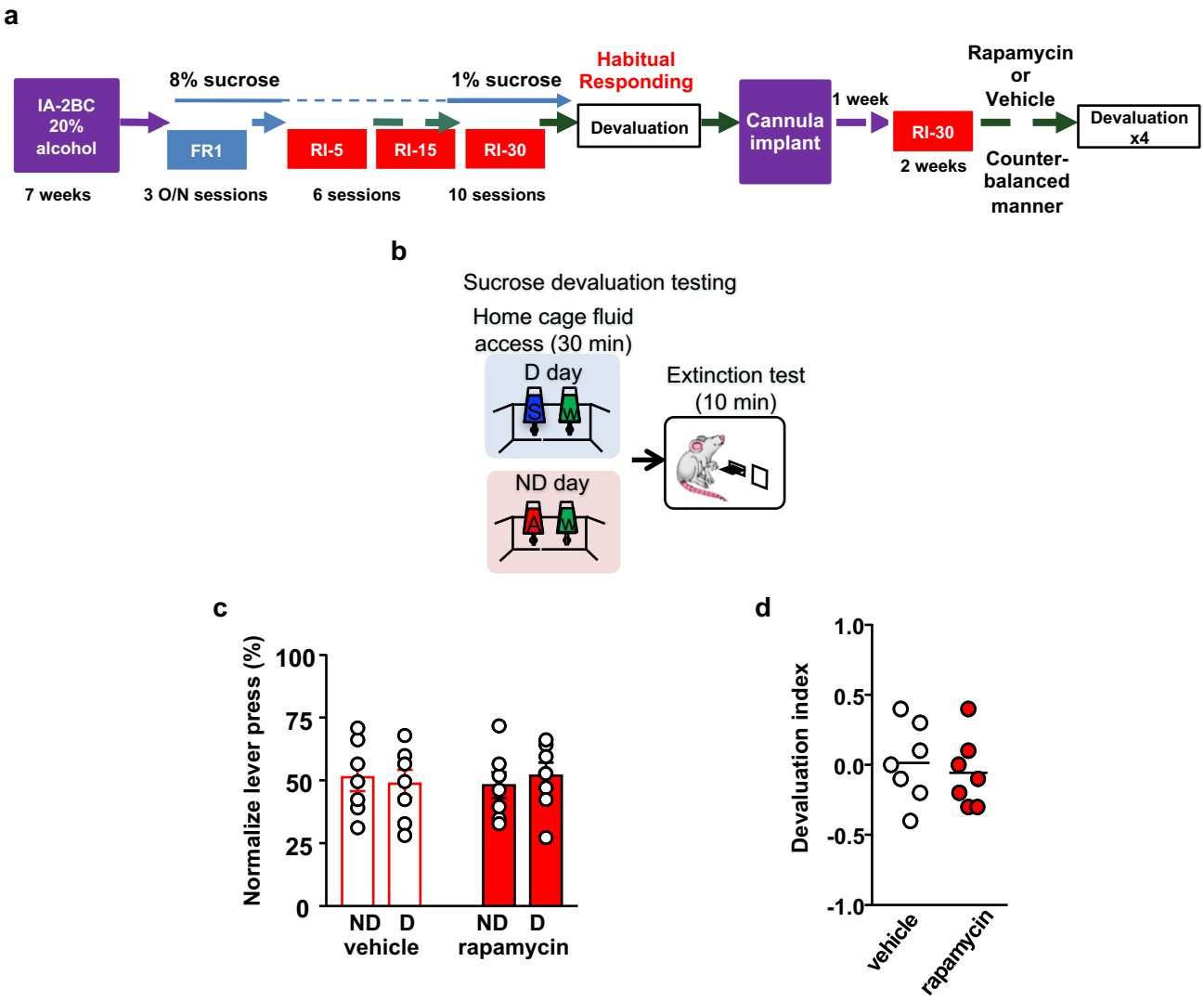


Figure 3 – Supplement 1

Schematic drawing of cannulae placement

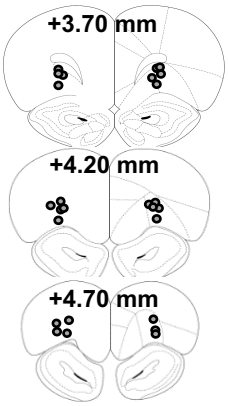


Figure 3 – Supplement 2

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

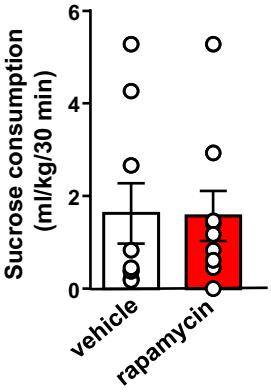


Figure 4

GluN2B is required for mTORC1 activation by alcohol

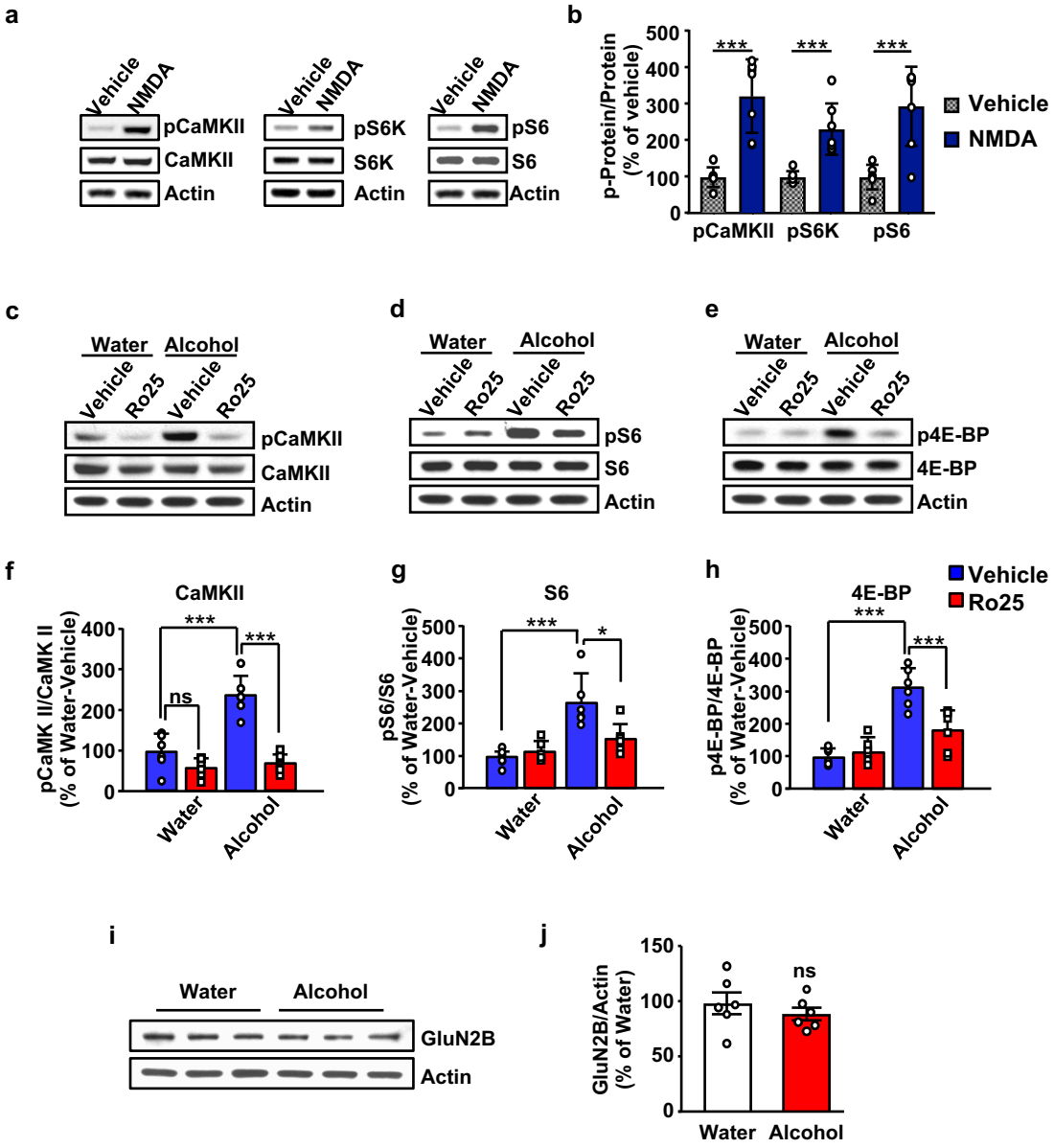


Figure 4 – Supplement 1

GluN2B-dependent activation of mTORC1 signaling

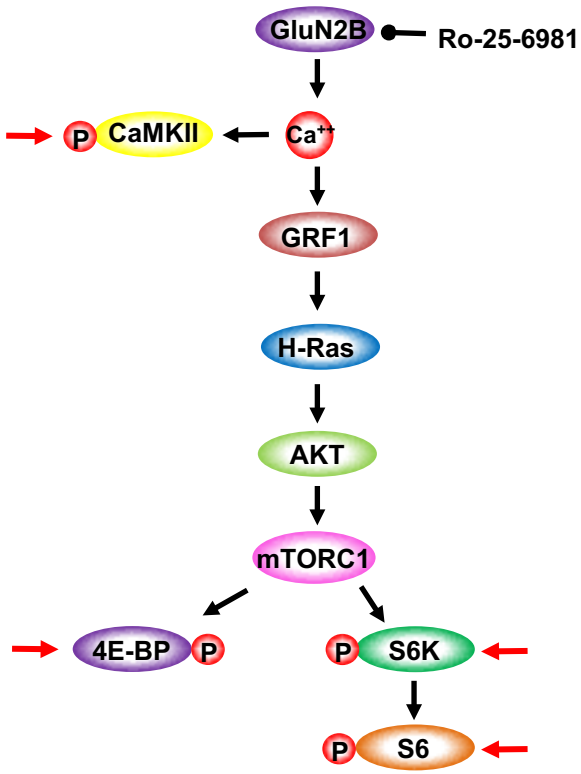


Figure 4 – Supplement 2

Timeline of experiments depicted in Figure 4c-h

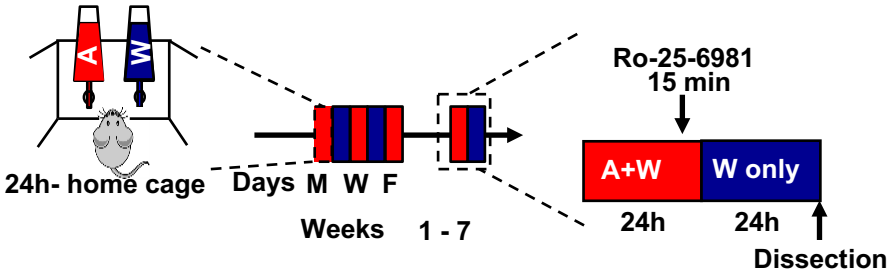


Figure 5

GluN2B in the OFC promotes alcohol seeking and habitual alcohol responding

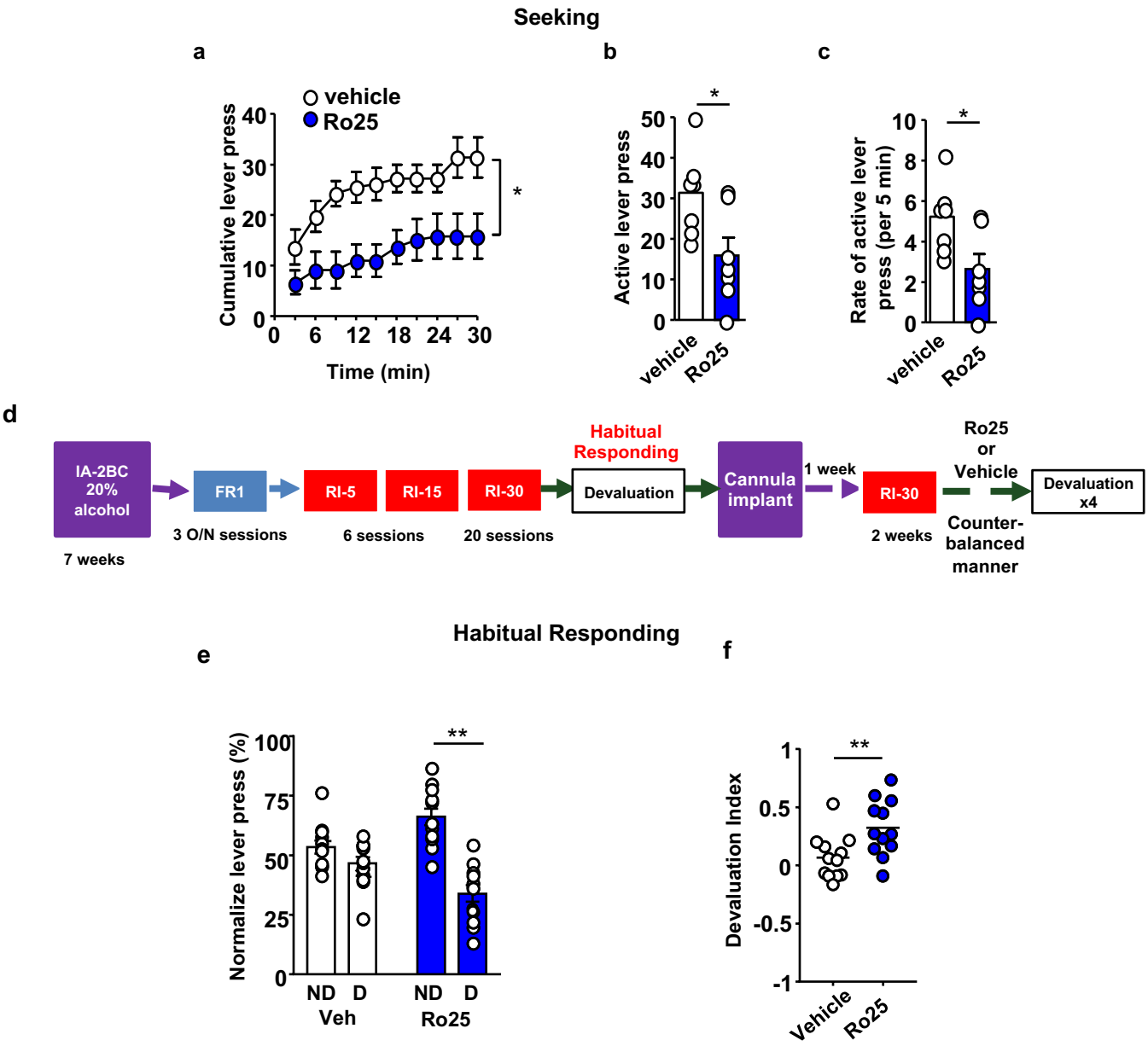


Figure 5 – Supplement 1

Schematic drawing of cannulae placement

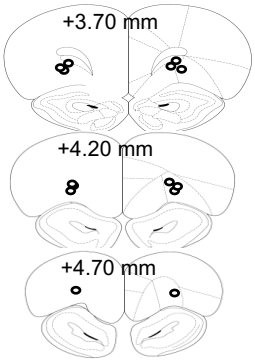


Figure 5 – Supplement 2

Inhibition of GluN2B in the OFC does not alter locomotion

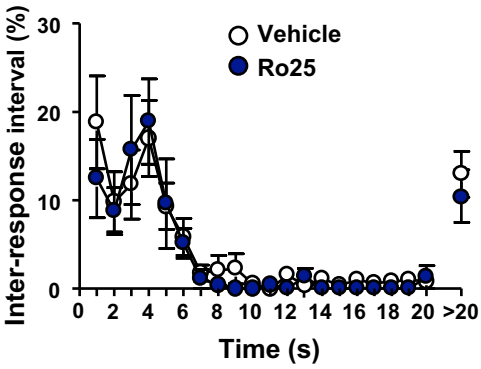
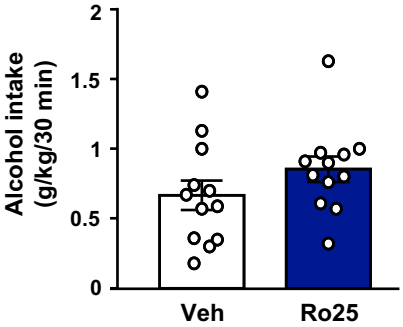


Figure 5 – Supplement 3

Inhibition of GluN2B in the OFC does not alter voluntary alcohol consumption prior to devaluation