Supplementary materials

Generation of silent synapses in dentate gyrus correlates with development of alcohol addiction

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22 **Materials and Methods**

23 **Animal model of alcohol addiction in the IntelliCages.**

24 *IntelliCages*. After 1 week of acclimatization, the mice were injected (s.c.) with unique 25 microtransponders (11.5 mm length, 2.2 mm diameter; Trovan, ID-100) under brief isoflurane 26 anesthesia. The mice were then allowed to recover for 3 days, and the animals with properly 27 located microtransponders were introduced to the IntelliCage system (NewBehavior AG, Zürich, 28 Switzerland) (http://www.newbehavior.com/), 15 animals per system. The IntelliCage consists of 29 a large standard rat cage (20.5 cm high, 40 cm x 58 cm at the top, 55 cm x 37.5 cm at the base). 30 In each corner, a triangular learning chamber is located with two bottles. To drink, only one 31 mouse can go inside a plastic ring (outer ring: 50 mm diameter; inner ring: 30 mm diameter; 20 32 mm depth into outer ring) that ends with two 13 mm holes (one on the left, one on the right) that 33 provide access to bottle nozzles. Each visit to the corner, nose-poke at the doors governing access 34 to the bottles, and licks were recorded by the system and ascribed to a particular animal.

35 Mice underwent either short training (to study mice behavior during withdrawal and cue 36 relapse) or full training (to identify addict and non-addict mice in five tests). The *short* training 37 was composed of the following tests: (1) adaptation to a new cage (days 1-4); (2) initiation of 38 alcohol drinking (days 5-17); (3) free alcohol access (days 18-90), (3) withdrawal (days 91-97); 39 (4) cue-induced-relapse (day 98); (5) alcohol relapse (day 99); (6) free alcohol consumption (days 40 100-115); (7) withdrawal (days 116-122); (8) cue-induced-relapse (day 122+90 min). Each test 41 was programmed and turned on and off automatically, without intrusion of an experimenter, at 42 the beginning of the dark phase.

43 The *full* training was composed of the following tests: (1) adaptation to a new cage (days 1-4); 44 (2) initiation of alcohol drinking (days 5-17); (3) free alcohol access (days 18-68); (4) motivation 45 to alcohol test (days 69-73); (5) free alcohol consumption (days 74-81); (6) persistence in alcohol

46 seeking test (days 82-84); (7) free alcohol consumption (days 85-91); (8) withdrawal (days 92-
47 98); (9) cue-induced-relapse (day 99); (10) alcohol relapse (day 100); (11) free alcohol
48 consumption (days 101-115); (12) withdrawal (days 115-122); (13) cue-induced-relapse (day
49 122).

50 *Adaptation phase.* All mice had free access to all bottles with water in both active corners. All
51 doors were open. After 24 hours, when all mice visited and licked from both corners, the doors
52 were closed. Under fixed ratio of reinforcement (FR 1), each nose-poke was rewarded by a 5
53 second access to the bottles with water.

54 *Initiation of alcohol consumption*. During the test, 2 corners were active, each with two bottles
55 available. In one corner, the animals had access to water ("water corner"), and in the other
56 ("reward corner") animals had access to ethanol solution at increasing concentrations (4, 8, and
57 12% ethanol changed every 3 days, prepared from 96% ethanol and tap water). When alcohol
58 was available, it was signaled by a green light turn on in the "reward corner" each time a mouse
59 entered the corner. All liquids were available under an FR1 schedule. The position of the "reward
60 corner" was altered every 3 days. During *free alcohol access phases* mice had unlimited access to
61 water in one corner and 4, 8 and12% ethanol in the "reward corner" (each concentration tested
62 for 3-5 days). Access to water and alcohol was under FR1. The position of the "reward corner"
63 was altered every 3-5 days to assure that mice followed alcohol in whichever corner it was
64 presented. 12% alcohol was chosen based on maximal alcohol consumption in g/kg/day during
65 initiation of alcohol consumption. Daily alcohol consumption (g/kg/day) was calculated with the
66 following formula: (number of licks of 12% alcohol per day x lick volume x 0.12 x 1 g/ml) /
67 animal weight. To calculate the average lick volume, water consumption (in µl) was measured for
68 3 consecutive days. The average volume of one lick was measured as total volume consumed /

69 number of licks. According to these calculations, an average lick volume was established as 1.94
70 ± 0.2 µl. In the control groups, 5% sucrose (in tap water) or tap water was presented in the reward
71 corner through the whole experiment.

72 *Motivation for alcohol tests*. During the test, two corners were active and available to animals.
73 The animals had to perform an increasing number of nosepokes (2, 4, 8, 12, 16, 20, 24, 28, 32,
74 and 36) spaced by less than 1 s during one visit to open the door and be allowed for a 5 s access
75 to the reward bottles. The number of required instrumental responses (nosepokes) increased when
76 an animal performed 10 sets of responses of a given ratio. The tests were terminated when 90%
77 of animals did not change FR level during the last 24 hours. The FR level reached during the test
78 was used as an index of motivation.

79 *Persistence in reward seeking tests.* The persistence in reward seeking was measured as a number
80 of reward nosepokes during signaled “no-reward” periods (nR). “Reward” periods (R) were
81 signaled by the green cue light in the reward corner. During the “reward” periods, nosepokes at
82 all doors opened the door for 5 s (FR 1). The “no-reward” periods were signaled by elimination
83 of the green cue light. During the “no-reward” periods, nose-pokes on the reward side were not
84 followed by any scheduled consequences. Each persistence test lasted 3 days, starting at the
85 beginning of the light phase and was composed of six, 6-hour long “reward periods” (R) altered
86 with 6-hour long “no-reward periods” (nR). Number of “reward” nose-pokes performed during
87 the test, as well as ratio of nose-pokes performed during no-reward to reward periods were used
88 as an index of persistence.

89 *Alcohol withdrawal followed by cue and alcohol relapse*. The withdrawal periods were signaled
90 as the “no-reward” periods and lasted 7 days. Door to reward was closed and nosepokes to the
91 reward corner were without scheduled consequences. Each withdrawal was followed by a 24-

92 hour cue-relapse. A green cue light (reward-associated cue) in the reward corner was presented
93 each time a mouse entered the reward corner. However, nose-pokes to the reward doors had no
94 scheduled consequences. Number of nosepokes performed in "reward corner" was used as an
95 index of cue-induced relapse. This test was followed by reward relapse when bottles with reward
96 (alcohol or sucrose) were added into the active "reward corner". During the test each nose-poke
97 into reward door opened the door for 5 s. Amount of reward drank during the first day of relapse
98 (number of licks) was used as an index of relapse.

99 For the electrophysiological analysis, all mice were trained according to the same protocol
100 till the last free access period. The length of the last alcohol consumption period was adjusted for
101 each mouse (from 15 to 30 days) so only one mouse per day finished the training and was used
102 for electrophysiological recordings. The length of the last free access period was balanced
103 between the experimental groups.

104 **Electrophysiology.**

105 Mice were anesthetized with isoflurane and decapitated. The brains were cut in half. One
106 hemisphere was used for electrophysiological recordings, another for DiI staining. For
107 electrophysiological recordings, acute, 250 μm-thick slices were prepared using Leica VT1000S
108 vibratome in ice-cold NMDG cutting solution (135 mM N-methyl-D-glucamine, 1 mM KCl, 1.2
109 mM KH_2PO_4, 1.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 mM choline bicarbonate, 10 mM D-glucose (all
110 chemicals purchased from Sigma)). Slices containing hippocampus were collected and
111 transferred to ACSF solution (119 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$,
112 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 10 mM D-glucose (all chemicals purchased from Sigma)) at
113 RT and incubated for 1h. All solutions were bobbled with carbogene (95% O2, 5% CO2). Patch-
114 clamp technique was used to analyze silent synapses as previously described (Stefaniuk *et al*,
115 2017). Slices were transferred to the recording chamber, perfused with ACSF solution heated up

116 to 31°C. Stimulating electrode (septum theta glass capillary with two chlorinated silver wires)
117 was placed in the perforant path. Granule cells of the upper blade of dorsal DG were identified
118 visually and patched with borosilicate glass capillaries (4-6 MΩ resistance) filled with internal
119 solution (130 mM Cs gluconate, 20 mM HEPES, 3 mM TEA-Cl, 0.4 mM EGTA, 4 mM Na_2ATP,
120 0.3 mM NaGTP, and 4 mM QX-314Cl, pH = 7.0-7.1, osmolarity: 290-295 mOsm). Series and
121 input resistances were monitored throughout the experiment. Electrical stimulation was elicited
122 by TTL pulse every 5 s. Recorded currents were filtered at 2 kHz (npi amplifiers) and digitized at
123 10 kHz (ITC-18 InstruTECH/HEKA). All recordings were performed in the presence of 50 µM
124 picrotoxin (Abcam) in ACSF, to pharmacologically block inhibitory neurotransmission and focus
125 on excitatory pathway specifically.
126 *Frequency of silent synapses:* for assessing the frequency of silent synapses on the neurons in
127 DG, the minimal stimulation protocol was used (Huang *et al*, 2009; Liao *et al*, 1995). The
128 strength of the electric pulse was decreased and adjusted to obtain both responses and failures of
129 AMPAR- and NMDAR-mediated EPSCs, recorded at -60 and +45 mV respectively. The noise
130 level of our recording setup allows to clearly distinguish responses as small as 5 pA above the
131 baseline. This is a crucial matter, as successes and failures are distinguished visually. During data
132 analysis, the experimenter was blinded to the experimental groups. The percentage of silent
133 synapses was calculated from the following equation:
134 % silent synapses $=1-\ln F_{-60mV}/\ln F_{+45mV}$,
135 where F_{-60mV} and F_{+45mV} are failure rates at -60 mV and +45 mV respectively.
136
137 **DREADD experiment.**

Mice were anaesthetized with isoflurane (5% for induction, 1.5-2.0% after) in the stereotaxic frame for the entire surgery and their body temperatures were maintained using a heating pad. We bilaterally injected into dorsal DG (stereotactic coordinates from bregma: ML, ±1.0 mm; AP, -2.0mm; DV, -2.0mm) adeno-associated viral vectors (AAV, serotype 1 and 2) coding inhibitory and activatory designer receptors exclusively activated by designer drugs (DREADD) (9) (pAAV-hSyn-HA-hM4D(Gi)-mCherry, Addgene Plasmid #50475; pAAV-hSyn-HA-hM3D(Gq)-mCherry, Addgene Plasmid #50474) (0.5 µl/ site, viral titer 1.30-1.9 x10^{9}/µl) or control mCherry (pAAV-CaMKIIa-mCherry (0.5 µl/ site, viral titer 1.35 x10^{9}/µl). The viruses were prepared by Animal Model Core Facility at Nencki Institute. The viruses were infused by a glass capillary (disposable BLAUBRAND micropipettes, intraMark) mounted to the custom-made pump attached to the Digital New Standard Stereotaxic system from Stoelting (51500D).

After surgery, animals were allowed to recover for 14 days before the training in the IntelliCages. After the initial period of free access to alcohol (day 1-30), the training was composed of the following phases: withdrawal 1 (day 31-37); cue relapse 1 (day 38); free access to alcohol (day 39-90); withdrawal 2 (day 91-97) and cue relapse 2 (day 98); free access to alcohol (day 99-105). Animals were injected with saline 20 minutes before the first cue relapse. Next, mice were injected with CNO (0.5 mg/kg in saline) or saline 20 minutes before second cue relapse. Mice were split into CNO and saline groups, based on their performance during the cue relapse 1 (alcohol seeking during the first cue relapse did not differ between the groups).

At the end of the behavioral training, mice were anesthetized and perfused (PBS/4%PFA). Next the brains were immersed in antifreeze solution (15% sucrose, 30% ethylene glycol in PBS with NaNO3) and cut on a cryostat (Leica CM1850) in 40-µm thick sections. Sections from dorsal hippocampus were mounted on slides and covered with DAPI Fluoromount-G

161 (Southernbiotech, 00-4959-52). Zeiss Spinning Disc confocal microscope (lens: 63x oil
162 immersion, pixel size: 132x132x260 nm) was used to photograph the dorsal hippocampus and
163 assess the extent of the expression of the viruses. Due to the lack of visible viral expression, one
164 animal was excluded from the behavioral analysis.

165

166 **Immunostaining**

167 Mice were anesthetized (i.p. sodium pentobarbital; 50 mg/kg) and perfused transcardially with
168 phosphate-buffered saline (PBS (Medicago), 3 min at RT), followed by 4 % paraformaldehyde
169 (PFA, Sigma-Aldrich), 6 min at RT. Brains were removed and placed in 4% PFA at 4° C for 24
170 hours and next in 30% sucrose at 4° C until sunk. Coronal sections (40 µm) were cut on cryostat
171 (Cryostats Leica CM1950) and stored at -20° C in PBSAF [PBS, 20% sucrose (Sigma–Aldrich),
172 15% ethylene glycol (Sigma–Aldrich), 0.05% NaN_3 (Sigma–Aldrich)]. The slices were incubated
173 overnight at 4°C with primary antibody directed against P-T286-CaMKII (R&D Systems,
174 PPS002, 1:500) and PSD95 (Millipore, MAB1598, 1:500) in 5% normal donkey serum in 0.3%
175 Triton in PBS. Next day slices were washed in 0.3% Triton X-100 in PBS and incubated with
176 secondary antibody bound with Alexa Fluor 555 (Invitrogen, A21447, 1:500), or Alexa Fluor 647
177 (Invitrogen, A31570. 1:500). Finally, the sections were washed in PBS and mounted on slides.

178

179 **Microscopy and image analysis**

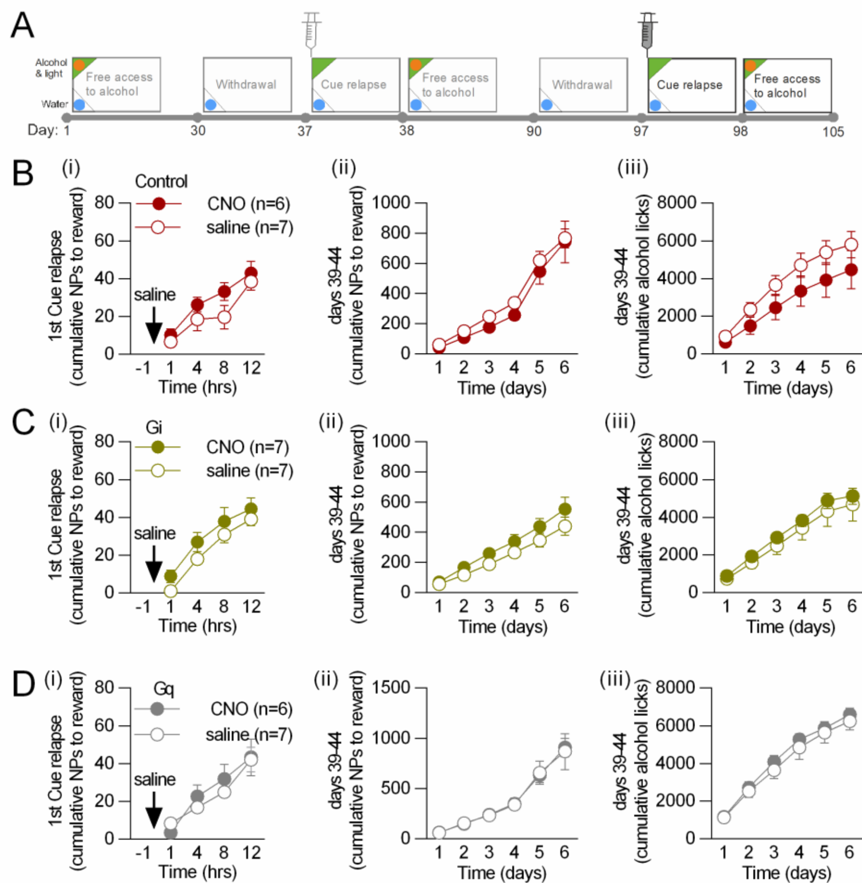
180 The immunostaining was photographed with Leica SP5 microscope (63x oil immersion objective
181 and 1.66x digital zoom), equipped with digital camera. Every sixth section through DG (approx..
182 6 sections per animal) were photographed bilaterally with the same microscope settings. Images
183 were analyzed using Fiji software. Intensity of the staining was measured using mean grey value

184 parameter (MGV) for 8-bit images ranging from 0 to 255. All microphotographs were analyzed
185 by a person blind to the experiment groups.
186

187 **Data analysis.**

188 The results are expressed as mean ± SEM. Differences between groups were considered
189 significant at $p < 0.05$. The statistical analysis of data was performed in GraphPad Prism. The
190 information on statistical tests is in figures legends.
191
192
193

194 **Results**



195

196 **Supplementary Figure 1. DREADD expression had no effect on mice behaviour during the**
197 **first cue relapse and the following period of free access to alcohol.**

198 **(A)** Experimental timeline. Before the first cue relapse all mice were injected with saline. “CNO”
199 groups were injected with CNO only before 2nd cue relapse (see Fig. 3).

200 **(B)** There was no difference between “CNO” and “saline” groups of mCherry-expressing mice in
201 alcohol seeking during the first cue relapse (i); and the following period of free access to alcohol
202 (ii-iii).

203 **(C)** There was no difference between “CNO” and “saline” groups of Gi-expressing mice in
204 alcohol seeking during the first cue relapse (i); and the following period of free access to alcohol
205 (ii-iii).

206 **(D)** There was no difference between “CNO” and “saline” groups of Gq-expressing mice in
207 alcohol seeking during the first cue relapse (i); and the following period of free access to alcohol
208 (ii-iii).

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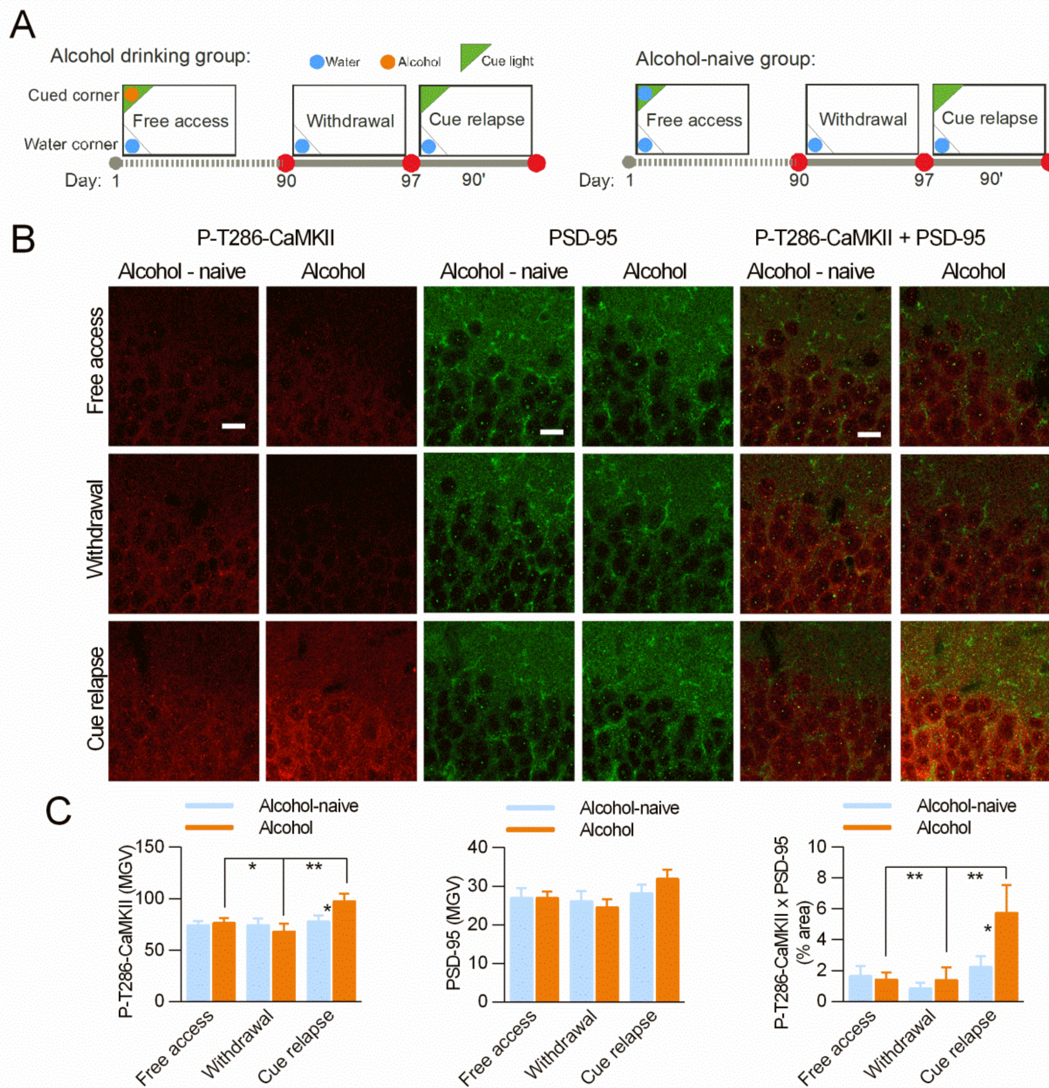

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211 **Suplementary Figure 2. Cue relapse incraeses the levels of P-T286-CaMKII in DG.**

212 (A) Experimental timeline and IntelliCage setups for alcohol drinking and alcohol-naïve mice.
213 Mice went through period of free access to alcohol (90 days), withdrawal (7 days) and cue
214 relapse (90 min). Two groups of alcohol drinking (n=7) and naïve mice (n=7) were sacrificed
215 after each stage of the training (black dots) (N=42). The brain section were prepared to perform
216 immunostaining.

217 (B) Representative microphotographs of the upper blade (granular and molecular layer) of dorsal
218 DG fluorescent immunostaining performed with specific antibodies recognizing
219 autophosphorylated calcium and calmodulin-dependent kinase (P-T286-CaMKII) (used as a
220 marker of ongoing synaptic plasticity (Giese et al. 1998; Shonesy et al. 2014; Coultrap et al.
221 2014) and synaptic marker, PSD95 (Broadhead et al. 2016). Scale bars, 100 μm.

222 (C) Summary of data showing that presentation of alcohol-, but not water-, associated cues
223 increases the levels of P-T286-CaMKII (2-way ANOVA, training: $F(2, 33)=3.748$, $P=0.034$,
224 alcohol: $F(1, 33)=1.008$, $P=0.322$), and colocalization of P-T286-CaMKII with PSD95 (training:
225 $F(2, 32)=5.802$, $P=0.007$, alcohol: $F(1, 32)=2.996$, $P=0.093$), but has no effect on PSD95
226 expression (training: $F(2, 33)=2.141$, $P=0.133$; alcohol: $F(1, 33)=0.1427$, $P=0.708$) .*$p<0.05$,
227 **$p<0.01$ by Tukey's multiple comparisons test

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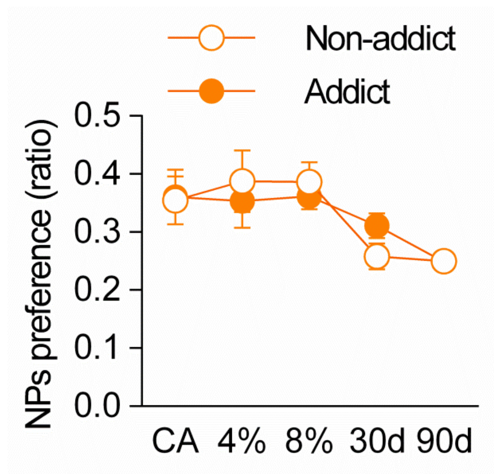
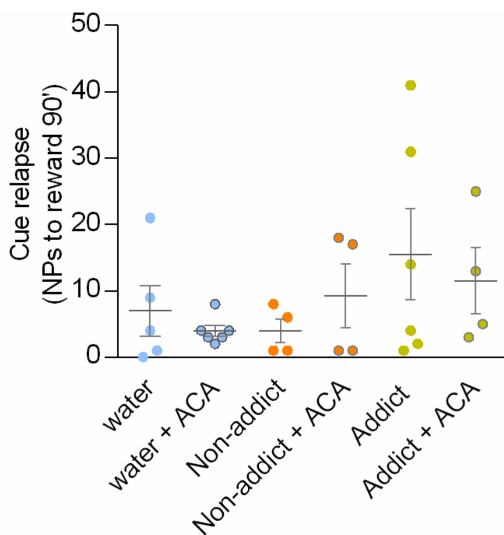
230 

231 **Supplementary Figure 3. Non-addict and addict mice do not differ in nose-poke preference**
232 **for the reward corner.** RM ANOVA, addiction index: F(1, 174)=0.0001, p=0.9900. CA, cage
233 adaptation; 4% & 8%, alcohol initiation; 30d, the first 30 days of free access to alcohol; 90d, free
234 access to alcohol, days 31-90.

235 

236 **Supplementary Figure 4. Acamprosate does not affect alcohol seeking during initial 90**
237 **minutes of cue relapse.** ACA, acamprosate. Kruskal-Wallis test, H=2.833, p=0.725.

238

239 **Literature**

240 Broadhead MJ, Horrocks MH, Zhu F, et al (2016) PSD95 nanoclusters are postsynaptic building
241 blocks in hippocampus circuits. Sci Rep 6:24626 . doi: 10.1038/srep24626

242 Coultrap SJ, Freund RK, O'Leary H, et al (2014) Autonomous CaMKII mediates both LTP and
243 LTD using a mechanism for differential substrate site selection. Cell Rep 6:431–7 . doi:
244 10.1016/j.celrep.2014.01.005

245 Giese KP, Fedorov NB, Filipkowski RK, Silva a J (1998) Autophosphorylation at Thr286 of the
246 alpha calcium-calmodulin kinase II in LTP and learning. Science 279:870–873 . doi:
247 10.1126/science.279.5352.870

248 Shonesy BC, Jalan-Sakrikar N, Cavener VS, Colbran RJ (2014) CaMKII: A molecular substrate
249 for synaptic plasticity and memory. Prog Mol Biol Transl Sci 122:61–87 . doi:
250 10.1016/B978-0-12-420170-5.00003-9

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