

1 **Chronic alcohol drinking persistently suppresses thalamostriatal excitation of**
2 **cholinergic neurons to impair cognitive flexibility**

3 **Running Title:** Alcohol reduces striatal cholinergic modulation and cognitive flexibility

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27 **Abstract**

28 Exposure to addictive substances impairs flexible decision-making. Cognitive
29 flexibility is mediated by striatal cholinergic interneurons (CINs). However, how chronic
30 alcohol drinking alters cognitive flexibility through CINs remains unclear. Here, we report
31 that chronic alcohol consumption and withdrawal impaired reversal of instrumental
32 learning. Chronic alcohol consumption and withdrawal also caused a long-lasting (21 d)
33 reduction of excitatory thalamic inputs onto CINs and reduced pause response of CINs in
34 the dorsomedial striatum (DMS). CINs are known to inhibit glutamatergic transmission in
35 dopamine D1 receptor-expressing medium spiny neurons (D1-MSNs) but facilitate this
36 transmission in D2-MSNs, which may contribute to flexible behavior. We discovered that
37 chronic alcohol drinking impaired CIN-mediated inhibition in D1-MSNs and facilitation in
38 D2-MSNs. Importantly, *in vivo* optogenetic induction of long-term potentiation of
39 thalamostriatal transmission in DMS CINs rescued alcohol-induced reversal learning
40 deficits. These results demonstrate that chronic alcohol drinking reduces thalamic
41 excitation of DMS CINs, compromising their regulation of glutamatergic transmission in
42 MSNs, which may contribute to alcohol-induced impairment of cognitive flexibility. These
43 findings provide a neural mechanism underlying inflexible drinking in alcohol use disorder.

44 **Introduction**

45 Alcohol use disorder is a chronic brain disorder characterized by an inability to stop
46 drinking despite the resultant adverse consequences (1, 2). This inability is associated
47 with impaired flexibility in decision-making, which contributes to compulsive alcohol use
48 (1-5). Increasing evidence suggests that the dorsomedial striatum (DMS) is involved in
49 cognitive flexibility (6-11). Understanding whether and how chronic alcohol consumption
50 affects striatum-mediated cognitive flexibility will provide therapeutic strategies to treat
51 alcohol addiction.

52 In the DMS, cholinergic interneurons (CINs) are the major source of acetylcholine
53 and contribute to cognitive flexibility in response to salient stimuli (12-14). CINs play an
54 essential role in modulating striatal circuit activity, thereby regulating output from the
55 striatum (15-17). The medium spiny neurons (MSNs), which express either dopamine D1
56 receptors (D1R) or D2Rs, are the principal striatal projection neurons. D1-MSNs and D2-
57 MSNs play different roles in motor control and goal-directed behavior (18-24).
58 Accumulating evidence demonstrates that the characteristic burst-pause firing of CINs
59 regulates MSN activity; this firing pattern is triggered by excitatory inputs from the
60 thalamus, which is a critical modulator of striatal activity (14, 17, 25). MSN regulation by
61 CINs is mediated by the actions of acetylcholine on pre- and postsynaptic muscarinic
62 receptors. For example, burst-associated transient acetylcholine release produces a
63 muscarinic M2/M4 receptor-mediated reduction in glutamate release at corticostriatal
64 terminals on both D1- and D2-MSNs (17, 26). The more prolonged effects of acetylcholine
65 on postsynaptic excitability during the “pause window” are mediated by the preferential
66 activation of muscarinic M1 receptors on D2-MSNs, but not D1-MSNs. These studies

67 demonstrated that CIN burst-pause firing following thalamic activation is crucial for the
68 functional modulation of striatal MSNs. Since striatal D1- and D2-MSNs respectively give
69 rise to the direct (“Go”) and indirect (“No-Go”) pathway, CINs stand to allow cognitive
70 flexibility by modulating “Go” and “No-Go” actions (17, 25). Several studies have
71 demonstrated that alcohol preferentially increases glutamatergic transmission in D1-
72 MSNs, but not in D2-MSNs, an effect that potentiates the “Go” pathway (23, 24, 27-29).
73 However, it remains unclear how alcohol affects CIN-mediated modulation of D1- and D2-
74 MSNs.

75 In the present study, we demonstrated that chronic alcohol intake and withdrawal
76 impaired cognitive flexibility in reversing action-outcome contingency. We found that
77 chronic alcohol intake reduced thalamic inputs to CINs. In the meantime, chronic alcohol
78 consumption led to reduced pause responses of CINs along with increased spontaneous
79 firing activities. Moreover, chronic alcohol intake impaired both CIN-mediated inhibition of
80 glutamatergic transmission in D1-MSNs and CIN-mediated short-term facilitation of
81 glutamatergic transmission in D2-MSNs. These results indicate that alcohol consumption
82 is associated with distinctive CIN-mediated changes in different MSN circuits, providing a
83 potential neural mechanism driving the inflexible drinking underlying alcohol use disorder.

84

85 **Results**

86 **Chronic alcohol consumption and withdrawal impair reversal of operant learning** 87 **in rats**

88 Thalamic inputs to DMS CINs have been implicated in the reversal of instrumental
89 learning (9, 10, 30). We thus examined whether chronic alcohol intake and withdrawal
90 affected the acquisition and reversal of action-outcome contingencies. Rats that had been
91 exposed to water (controls) or 20% alcohol using an intermittent-access 2-bottle choice
92 drinking procedure (24, 31-34) for 8 weeks were trained to learn two action-outcome
93 contingencies involving food pellets or sucrose solution (9, 35) (Fig. 1A). The water and
94 alcohol groups both acquired action-outcome contingencies during the increased-effort
95 training schedule (Fig. 1B). The total number of lever presses was slightly lower in the
96 alcohol group than in the water group, but this difference was not statistically significant
97 (Fig. 1B; $F_{(1, 22)} = 3.55$, $p = 0.07$). Cumulative lever presses during the last session of the
98 initial learning period did not differ between the two groups (Fig. 1C; $F_{(1, 22)} = 0.13$, $p >$
99 0.05).

100 After the initial acquisition of this task, we investigated the sensitivity to outcome
101 devaluation. To achieve this goal, animals were fed with either food pellets or sucrose
102 solution before receiving extinction training, where lever presses were monitored. We
103 found that both alcohol-drinking and water control rats significantly decreased their
104 presses on the outcome-satiated (devalued) lever (Fig. 1D; $t_{(12)} = 2.20$, $p < 0.05$ for water
105 group; $t_{(10)} = 3.71$, $p < 0.01$ for alcohol group). Analysis of the devaluation index (the
106 difference between the proportion of non-devalued and devalued lever presses) did not

107 identify any statistically significant difference between the degree of goal-directed versus
108 habitual behavior in the alcohol-drinking and water control rats (Fig. 1E; $t_{(22)} = -1.02$, $p >$
109 0.05). These results indicated that alcohol-drinking and water control rats showed similar
110 levels of goal-directed behavior.

111 Next, we examined the flexibility of the rats' responses to a change in the action-
112 outcome contingency. We reversed the relationship between action and outcome so that
113 pressing the lever previously used to access sucrose solution now led to the delivery of
114 food pellets and vice versa (Fig. 1F). Following this contingency reversal, the total lever
115 presses were significantly lower in the alcohol group than in the control group (Fig. 1G;
116 $F_{(1,22)} = 6.28$, $p < 0.05$). Cumulative lever presses were also lower in the alcohol-drinking
117 rats than in water controls during the last session of reversal training (Fig. 1H; $F_{(1,20)} =$
118 4.68 , $p < 0.05$). These results indicated that chronic alcohol intake and withdrawal (at
119 least 10 d) impaired reversal learning in this task.

120 Lastly, our analysis of the relative contributions of goal-directed versus habitual
121 behavior following contingency reversal showed that the alcohol group pressed
122 indiscriminately on devalued and non-devalued levers, whereas the water control rats still
123 favored the non-devalued lever (Fig. 1I; $t_{(12)} = 2.87$, $p < 0.05$ for water group; $t_{(10)} = 0.18$,
124 $p > 0.05$ for alcohol group). The devaluation index was, therefore, significantly lower in
125 alcohol-drinking rats, as compared to their water controls (Fig. 1J; $t_{(22)} = 3.14$, $p < 0.01$).
126 We also compared the difference between the first and second devaluation indices in the
127 two study groups; the alcohol group showed a significantly larger decrease than did the
128 water group (Supplementary Fig. 1; $t_{(22)} = 2.88$, $p < 0.01$). These results indicated that the
129 water controls maintained a goal-directed strategy in response to the new action-outcome

130 association. However, alcohol-drinking rats failed to do so and instead used a strategy
131 more consistent with habitual behavior, suggesting that chronic alcohol intake and
132 withdrawal impaired cognitive flexibility in response to changes in action-outcome
133 associations in rats.

134

135 **Chronic alcohol consumption reduces glutamatergic thalamostriatal inputs onto**
136 **DMS CINs**

137 The striatum receives major glutamatergic inputs from both the cortex and
138 thalamus. Reduced flexibility in reversal learning is known to be associated with
139 thalamostriatal transmission in DMS CINs (9, 17, 36). We next investigated whether
140 alcohol consumption altered thalamic inputs to DMS CINs. To selectively induce
141 thalamostriatal transmission, we expressed channelrhodopsin 2 (ChR2) in thalamic
142 inputs (Fig. 2A) by crossing transgenic mice expressing Cre recombinase under the
143 control of the vesicular glutamate transporter 2 (VGLUT2) promoter (VGLUT2-Cre mice)
144 with transgenic mice with Cre-dependent ChR2-eYFP expression (Ai32 mice) (37). This
145 cross produced VGLUT2-Cre;Ai32 mice. Previous studies in VGLUT2-Cre mice reported
146 that VGLUT2-expressing inputs to the striatum mainly arose from the thalamus (38, 39).

147 CINs are easily distinguished from other striatal cell types because they have a
148 large diameter and unique electrophysiological characteristics (40, 41). We thus
149 distinguished CINs from MSNs by their larger size, spontaneous firing (Supplementary
150 Fig. 2A), higher resting membrane potential, characteristic voltage sag in response to
151 hyperpolarizing current injection, and greater excitability in response to depolarizing

152 current injection (Supplementary Fig. 2B, resting membrane potentials: $t_{(10)} = 4.75$, $p <$
153 0.001). Interestingly, repetitive light-mediated stimulation of thalamic inputs in VGlut2-
154 Cre;Ai32 mouse slices evoked distinct patterns of excitatory postsynaptic potentials
155 (EPSPs) in CINs and MSNs. We found that the second EPSP was larger than the first
156 EPSP in CINs, while MSNs showed the opposite pattern (Supplementary Fig. 2C; $t_{(10)} =$
157 6.87, $p < 0.001$). We used a combination of these approaches to identify CINs when these
158 neurons did not express fluorescent proteins.

159 We then explored how chronic alcohol intake influenced thalamostriatal
160 glutamatergic transmission onto DMS CINs. VGlut2-Cre;Ai32 mice were trained to
161 consume 20% alcohol for 8 weeks using the intermittent-access 2-bottle choice drinking
162 procedure (23, 34). Twenty-four hours after the last alcohol exposure, we prepared striatal
163 slices and measured optically-evoked excitatory postsynaptic currents (oEPSCs) in CINs.
164 We found that the oEPSC amplitude was significantly lower in CINs from the alcohol
165 group than those from the water control group (Fig. 2B; $F_{(1, 22)} = 5.39$, $p < 0.05$). This
166 result suggests that chronic alcohol intake reduced thalamostriatal inputs onto DMS CINs.
167 To further investigate the mechanism underlying this reduction, we measured the paired-
168 pulse ratio (PPR) of oEPSCs that were activated by two stimuli, delivered 100 ms apart.
169 This analysis found no difference between the alcohol group and the water group (Fig.
170 2C; $t_{(19)} = 0.72$, $p > 0.05$). These results suggested that the reduced thalamostriatal
171 transmission to CINs in mice with chronic alcohol exposure was unlikely to be caused by
172 a reduced probability of presynaptic glutamate release.

173 To further confirm the alcohol-associated suppression of thalamostriatal
174 transmission, we infused an adeno-associated virus (AAV)-Chrimson-tdTomato (tdT) into

175 a thalamic nucleus that is known to project to DMS CINs. Previous studies identified
176 dense inputs to the striatum from multiple thalamic nuclei, including the parafascicular
177 nucleus (PfN) (42, 43). To investigate this, we infused rabies helper viruses into the DMS
178 of ChAT-Cre mice, waited three weeks, and then infused rabies-GFP at the same location
179 3 weeks later (Fig. 2D). Two Cre-dependent (Flex) AAV serotype 8 vectors were
180 employed as helper viruses; one expressed rabies glycoprotein (RG) (AAV8-DIO-RG),
181 and the other expressed an avian membrane EnvA receptor protein (TVA) and mCherry
182 (AAV8-DIO-TVA-mCherry). This approach produced extensive GFP expression in the
183 PfN (Fig. 2E), indicating dense innervation of DMS CINs by thalamic PfN neurons. Next,
184 we infused AAV-Chrimson-tdT into the PfN of ChAT-eGFP mice and detected the tdT
185 fluorescent signal in the striatum (Fig. 2F). Animals were trained to consume alcohol as
186 described above. Twenty-four hours after the last alcohol exposure, striatal slices were
187 prepared to measure optically evoked excitatory postsynaptic currents (oEPSCs) in CINs.
188 Similar changes in the oEPSCs were observed (Supplementary Fig. 3C; $F_{(1, 22)} = 4.74$, p
189 < 0.05) as in Figure 2B. We did not observe significant changes in PPR measurements
190 (Supplementary Fig. 3D; $t_{(39)} = -1.44$, $p > 0.05$). Because we had previously observed
191 behavioral deficits weeks after stopping alcohol consumption (Fig. 1), we also measured
192 oEPSCs 21 d after the last alcohol exposure. Similar results were observed at this time-
193 point (oEPSCs: Fig. 2H; $F_{(1, 27)} = 10.91$, $p < 0.01$. PPR: Fig. 2I; $t_{(53)} = -0.72$, $p > 0.05$).

194 Taken together, these data suggest that chronic alcohol consumption causes a
195 long-lasting decrease in thalamostriatal inputs onto DMS CINs.

196

197 **Chronic alcohol consumption significantly increases the spontaneous firing of**
198 **DMS CINs and shortens their pause responses**

199 Having shown that chronic alcohol intake reduced thalamic inputs onto DMS CINs,
200 we asked whether alcohol also altered the spontaneous spiking of these tonically active
201 neurons. We trained ChAT-eGFP mice to consume alcohol for 8 weeks using the
202 intermittent-access 2-bottle choice drinking procedure. CINs were identified by their green
203 fluorescence (Fig. 3A), and spontaneous firing of DMS CINs was measured using cell-
204 attached recording, 24 h and 21 d after the last alcohol exposure. We found that chronic
205 alcohol consumption decreased the inter-spike interval (Fig. 3C) and significantly
206 increased the firing frequency over time (Fig. 3D; $F_{(2, 112)} = 5.69$, $p < 0.01$). In contrast,
207 our measurement of intrinsic excitability using whole-cell recording did not find any
208 difference in the evoked firing of DMS CINs from the water and alcohol groups (Fig. 3E;
209 $F_{(1, 27)} = 0.93$, $p > 0.05$). These results suggested that chronic alcohol consumption
210 increased the spontaneous activity of DMS CINs.

211 CINs exhibit characteristic burst-pause firing, which is important for regulating
212 MSN activity. Next, we investigated the effects of chronic alcohol intake on the burst-
213 pause firing of CINs. To induce burst-pause response of CINs, we expressed ChR2 in
214 CINs by crossing transgenic mice expressing Cre recombinase under the control of
215 choline acetyltransferase (ChAT) promoter (ChAT-Cre mice) with transgenic mice with
216 Cre-dependent ChR2-eYFP expression (Ai32 mice)(37). ChAT-Cre;Ai32 mice were
217 trained to consume 20% alcohol for 8 weeks using the intermittent-access 2-bottle choice
218 drinking procedure. Twenty-four hours after the last alcohol exposure, we prepared
219 striatal slices and measured optically evoked burst-pause responses of DMS CINs. We

220 found that the pause duration was significantly shorter in CINs from the alcohol group
221 than those from the water control group using cell-attached recording (Fig. 4 A, B; $t_{(40)} =$
222 2.32, $p < 0.05$). We also observed similar results with whole-cell recording (Fig. 4 C, D;
223 $t_{(31)} = 2.06$, $p < 0.05$).

224

225 **Chronic alcohol consumption impairs CIN-induced suppression of NMDAR-** 226 **mediated glutamatergic transmission in DMS D1-MSNs**

227 CINs regulate flexible behaviors by modulating MSN activity. After characterizing
228 the effects of chronic alcohol consumption on DMS CIN activity, we investigated how
229 alcohol intake might affect CIN-mediated modulation of MSNs, leading to changes in
230 striatal output. In striatal circuits, muscarine is known to modulate NMDA receptor
231 (NMDAR)-mediated synaptic responses in D1-MSNs by acting on muscarinic M4
232 receptors (26). We, therefore, examined whether endogenous acetylcholine release
233 induced by optogenetic excitation of CINs altered NMDAR-EPSCs in DMS D1-MSNs. To
234 achieve this, we generated triple transgenic ChAT-Cre;Ai32;D1-tdT mice, in which CINs
235 expressed ChR2-eYFP and D1-MSNs contained tdT (Fig. 5A). Stimulating electrodes
236 were placed within the striatum to elicit glutamatergic transmission, and we patched the
237 (red) D1-MSNs and excited CINs by delivering blue light through the objective lens (Fig.
238 5B). After NMDAR-mediated EPSCs were recorded for 5 min (baseline), blue light (2 ms,
239 10 pulses at 15 Hz) was delivered 1 sec prior to each electrical stimulation, and EPSCs
240 were continuously monitored for 10 min (Fig. 5C). We found that optogenetic excitation
241 of CINs significantly reduced the NMDAR-EPSC amplitude in D1-MSNs (Fig. 5D; $t_{(6)} =$
242 6.13, $p < 0.001$). We further confirmed that this effect was mediated by muscarinic M4

243 receptors, as subsequent application of an antagonist of this receptor, PD 102807 (1 μ M)
244 (44), completely abolished the CIN-mediated suppression of NMDAR-EPSCs (Fig. 5D; $t_{(6)}$
245 = -4.64, $p < 0.01$). We found that chronic alcohol consumption completely abolished this
246 CIN excitation-induced suppression of NMDAR-EPSCs in D1-MSNs (Fig. 5E; $t_{(6)}$ = -0.68,
247 $p > 0.05$). Taken together, these data indicated that excitation of DMS CINs activated
248 muscarinic M4 receptors to suppress NMDAR-EPSCs in DMS D1-MSNs and that chronic
249 alcohol consumption attenuated this suppression.

250

251 **Chronic alcohol consumption compromises CIN-mediated short-term facilitation of** 252 **glutamatergic transmission in DMS D2-MSNs**

253 Having found that chronic alcohol consumption impaired CIN-mediated regulation
254 of glutamatergic transmission in D1-MSNs, we next examined whether it altered CIN-
255 mediated regulation of glutamatergic transmission in another major MSN type, the D2-
256 MSN. We employed ChAT-Cre;Ai32;D1-tdT mice, in which putative D2-MSNs were
257 identified as non-fluorescent (Fig. 6A). Thalamic stimulation of cholinergic activity has
258 been shown to cause short-term facilitation of AMPAR-EPSPs in D2-MSNs (17). We thus
259 recorded electrically-evoked AMPAR-EPSPs in D2-MSNs using the current-clamp
260 recording. Five EPSPs were measured before and 1 sec after light-mediated stimulation
261 of CINs in mice that had been exposed to alcohol or water only. Compared to amplitudes
262 recorded before light stimulation, we found that direct light stimulation (15 Hz, 10 pulses,
263 1 sec before electrical stimulation) of CINs caused short-term facilitation of EPSP
264 amplitudes in the water group (Fig. 6B; $F_{(1, 8)} = 5.66$, $p < 0.05$), as expected. Interestingly,
265 there was also a main effect of pulse number ($F_{(4, 32)} = 3.89$, $p < 0.05$), in that later electrical

266 pulses generated higher relative EPSP amplitudes than earlier pulses (Fig. 6B; versus
267 pulse 1: $q = 4.53$, $p < 0.05$ (pulse 2); $q = 6.67$, $p < 0.001$ (pulse 4); $q = 7.41$, $p < 0.001$
268 (pulse 5)). In contrast, light stimulation of CINs failed to potentiate the EPSP amplitudes
269 in the alcohol group (Fig. 6C; $F_{(1, 11)} = 0.91$, $p > 0.05$). These results demonstrated that
270 chronic alcohol consumption compromised CIN-mediated short-term facilitation of
271 AMPAR-mediated transmission in DMS D2-MSNs.

272

273 **The alcohol-induced impairment of reversal learning is rescued by in vivo** 274 **optogenetic induction of long-term potentiation of PfN-to-CIN transmission**

275 The above evidence points to the key roles of DMS CINs in mediating the
276 detrimental effect of chronic alcohol intake on cognitive flexibility. Lastly, we aimed to
277 alleviate this detrimental effect by manipulating the PfN→CIN connectivity. It has been
278 shown that a global enhancement of the neuronal activity of CINs through
279 pharmacogenetics failed to rescue the impairment of reversal learning in aged mice (10),
280 indicating the need for a more targeted modulation of CINs by thalamostriatal processes.
281 Therefore, we infused AAV-Chronos-GFP into the PfN and AAV-FLEX-Chrimson-tdT into
282 the DMS for selective manipulation of PfN→CIN synapses. Optical fibers were implanted
283 into DMS (Fig. 7A). After recovery from surgery, rats were trained using the schedule
284 described in Figure 1. Once the rats acquired the initial A-O contingencies (Fig. 7C, D),
285 they were divided into two groups: Alcohol-Opto group received time-locked light
286 stimulation (Fig. 7B) during the reversal learning; Alcohol-Sham group underwent the
287 same procedure as Alcohol-Opto. group except the light lasers were not turned on. Both
288 groups showed similar acquisition of initial A-O contingencies and initial devaluations

289 (Supplementary Fig. 5). During reversal training, we delivered optogenetic high-frequency
290 stimulation (oHFS) of PfN inputs and optogenetic depolarization (oPSD) of DMS CINs, a
291 dual-channel optogenetic protocol that we recently developed to induce long-term
292 potentiation (LTP) in vivo (24). We found that there was no significant difference in terms
293 of lever presses between the two groups (Fig. 7E; $F_{(1,16)} = 0.002$, $p > 0.05$). However, our
294 analysis of the relative contributions of goal-directed versus habitual behavior following
295 contingency reversal showed that the sham group pressed more devalued levers,
296 indicating habitual behavior carrying over from initial learning; whereas the light
297 stimulation group still favored the non-devalued lever, indicating new goal-directed
298 behavior (Fig. 7F; $t_{(8)} = -1.52$, $p > 0.05$ for the sham group; $t_{(9)} = 1.91$, $p < 0.05$ for light
299 stimulation group). The devaluation index was therefore significantly higher in light-
300 stimulated rats, as compared to their sham controls (Fig. 7G; $t_{(17)} = -2.23$, $p < 0.05$). These
301 results indicated that the alcohol-induced impairment of cognitive flexibility was restored
302 by selectively potentiating thalamic inputs onto DMS CINs.

303

304

305 Discussion

306 In this study, we demonstrated that chronic alcohol exposure and withdrawal
307 reduced goal-directed cognitive flexibility and caused a long-lasting suppression of
308 thalamostriatal inputs onto DMS CINs and a shortened pause response along with the
309 increased spontaneous firing of these neurons. Furthermore, chronic alcohol
310 consumption and withdrawal impaired CIN-mediated downregulation of glutamatergic

311 transmission in D1-MSNs, as well as CIN-mediated short-term upregulation of
312 glutamatergic transmission in D2-MSNs. Our data suggest that chronic alcohol
313 consumption compromises the thalamostriatal regulation of glutamatergic transmission in
314 MSNs via CINs (Fig. 8), providing insight into how chronic alcohol consumption changes
315 from casual, flexible drinking to compulsive intake.

316 In individuals with alcohol use disorder, a progressive loss of cognitive flexibility
317 eventually results in compulsive alcohol-drinking behavior. There is increasing evidence
318 that the dorsal striatum is a key hub in the regulation of cognitive flexibility (6, 7). We
319 found that chronic alcohol consumption impaired the reversal of action-outcome
320 contingency, indicating behavioral inflexibility. It is highly likely that this behavioral change
321 is due to effects on the dorsal striatum (45, 46). Within this brain region, CINs play a
322 critical role in regulating reversal learning (9, 10), which is essential in the reversal phase
323 but not in the initial memory acquisition (9)—a fact that highlights the importance of CINs
324 activity for new state formation, or the revision of behavior to accommodate a new
325 situation (9, 47). Given that the present study found that chronic alcohol consumption and
326 withdrawal affected glutamatergic transmission from the thalamus to striatal CINs, it is
327 highly possible that this disruption induces a deficit in goal-directed action selection (9).
328 This prediction was supported by our devaluation results, which indicated that chronic
329 alcohol intake and withdrawal impaired devaluation during contingency reversal but did
330 not impair contingency acquisition. Taken together, these findings suggest that chronic
331 alcohol intake functionally impaired the flexibility of goal-directed behavior mediated by
332 striatal circuits.

333 Having observed these behavioral effects of alcohol, we investigated whether the
334 thalamic to CINs circuits were affected. Most previous *in vitro* and *in vivo* studies have
335 demonstrated that thalamic stimulation produces burst-pause activity in CINs, which
336 modulate D1-and D2-MSNs (17, 25). Thalamic glutamatergic transmission to CINs is,
337 therefore a key component of this circuit(17). By selectively activating thalamic inputs to
338 the DMS, the present study demonstrated that thalamic input modulated CIN activity and
339 thus controlled the striatal MSN network. Furthermore, we found that chronic alcohol
340 consumption decreased thalamic glutamatergic transmission to CINs. This result is
341 consistent with the acute alcohol impairing the ability of thalamostriatal inputs to modulate
342 a subsequent corticostriatal glutamatergic response in MSNs ⁽³⁾. Our study of paired-
343 pulse ratios found that the probability of glutamate release did not decrease, indicating
344 that this effect was not mediated by a decrease in thalamic activity (Fig. 2). A previous
345 acute alcohol exposure study reported a decrease in evoked GABAergic MSN responses,
346 with no change in the paired-pulse ratio (3). The mechanisms underlying this reduced
347 thalamic input to DMS CINs need further investigation.

348 Interestingly, the present study employed a chronic alcohol consumption
349 procedure, we found an increase in the spontaneous firing of CINs, in contrast to the
350 inhibiting effect of acute alcohol on CINs firing (3). It is not uncommon that chronic and
351 acute drugs have opposite effects due to the adaptation response of the organism. For
352 example, acute morphine administration increased the spontaneous firing of dopamine
353 neurons in the ventral tegmental area (VTA) (48, 49), while chronic morphine
354 administration and withdrawal greatly reduced the spontaneous activity of VTA dopamine
355 neurons (50, 51). We also found that acute alcohol suppressed the NMDA activity while

356 chronic alcohol consumption enhanced NMDA function (52). As for the function of CINs
357 it may not directly correlate with the baseline activity, in other words, increased baseline
358 firing does not mean enhanced function of CINs. It has been shown the aged mice have
359 increased CINs spontaneous firing and exhibit impairments in reversal learning of action-
360 outcome contingency (10). Pharmacogenetic direct stimulation of CINs in the DMS did
361 not alleviate the impairment of reversal learning in aged mice (10). It seems the extent
362 that CINs can be modulated rather than their baseline firing plays a more important role
363 in their function. With increased baseline firing, CINs could be less prone to be modulated,
364 such as the shortened pause response observed in our study (Fig. 4). By selectively
365 strengthening the thalamic inputs to DMS CINs, we were able to rescue the impairment
366 of reversal learning in chronic alcohol consumption rats (Fig. 7).

367 CINs are a major source of acetylcholine within the striatum, and their dense
368 terminals primarily synapse with MSNs. We generated triple transgenic mice in order to
369 induce selective optogenetic excitation of CINs and to allow fluorescent identification of
370 D1-MSNs. We found that direct optogenetic excitation of CINs elicited a stimulation-
371 evoked firing response followed by a pause (Fig. 4). The burst-pause firing of CINs is
372 intricately linked with dopamine activity in the striatum (53). Indeed, the pause duration
373 was reduced by blocking dopamine D2Rs (Supplementary Fig. 4). This result is consistent
374 with the finding that the thalamically-evoked pause is dependent upon dopamine release
375 and DR activation(17). Therefore, direct optogenetic stimulation of CINs might exert a
376 complex and powerful influence on specific types of striatal outputs. Cholinergic
377 muscarinic M4 receptors, functionally coupled with the NMDAR, are only expressed in
378 D1-MSNs(54). In addition, acetylcholine produced a prolonged enhancement of

379 postsynaptic responsiveness in D2-MSNs. Our findings showed that the burst stimulation
380 of CINs at 15 Hz, which is close to the burst firing frequency observed under physiological
381 conditions (17), suppressed NMDAR-mediated glutamatergic inputs onto D1-MSNs and
382 facilitated AMPAR-mediated glutamatergic transmission in D2-MSNs. The depression of
383 D1-MSNs and facilitation of D2-MSNs by our direct optical activation of CINs was
384 consistent with previous studies that employed electrical stimulation of the thalamus (17).
385 The integrated effect on DMS MSNs, namely a decrease in the D1-direct pathway output
386 and an increase in the D2-indirect pathway output, is to activate the striatopallidal network
387 to suppress action (“No-Go”). Our results showed that chronic alcohol intake disrupted
388 CIN-mediated depression of D1-MSNs and facilitation of D2-MSNs. A previous study also
389 found that glutamatergic transmission increased in D1-MSNs after alcohol consumption
390 (23). The effect of this disruption, which increases the relative activity of D1-MSNs and
391 reduces that of D2-MSNs, is to reduce action suppression and make a “Go” outcome
392 more likely. The discovery of these mechanisms provides a deep understanding of how
393 alcohol consumption impacts thalamostriatal-CIN-MSN connectivity and thus promotes
394 behavioral inflexibility.

395 In summary, DMS CINs modulate striatal circuits via burst-pause firing, which is
396 triggered by inputs from the thalamus. Alcohol consumption disrupts this modulation by
397 reducing thalamic excitation of CINs, and increasing spontaneous CIN activity. Our
398 research demonstrated that alcohol attenuated the CIN-mediated inhibition of
399 glutamatergic transmission in D1-MSNs and the CIN-mediated short-term facilitation of
400 glutamatergic transmission in D2-MSNs. These effects have the potential to impair

401 cognitive flexibility. Our findings provide an evidence base for the development of new
402 therapeutic strategies to enhance cognitive flexibility in alcohol use disorder.

403

404 **Methods**

405 **Animals.** ChAT-eGFP, VGluT2-Cre, ChAT-Cre, and D1-tdT mice were purchased from
406 the Jackson Laboratory. All mice were backcrossed onto a C57BL/6 background.
407 VGluT2-Cre or ChAT-Cre mice were crossed with Ai32 to generate VGluT2-Cre;Ai32 or
408 ChAT-Cre;Ai32 lines. VGluT2-Cre (or ChAT-Cre) and ChAT-eGFP (or D1-tdT) mice were
409 crossed with Ai32 to generate VGluT2-Cre;Ai32;ChAT-eGFP or ChAT-Cre;Ai32;D1-tdT
410 triple transgenic mice. Both male and female mice were used for electrophysiology
411 studies. Male Long-Evans rats (3 months old) purchased from Harlan Laboratories were
412 used for behavioral testing. Long Evans-Tg(ChAT-Cre) rats were purchased from Rat
413 Resource & Research Center. Animals were housed individually at 23°C under a 12-h
414 light:dark cycle, with lights on at 7:00 A.M. Food and water were provided *ad libitum*. All
415 animal care and experimental procedures were approved by the Institutional Animal Care
416 and Use Committee and were conducted in accordance with the National Research
417 Council *Guide for the Care and Use of Laboratory Animals*.

418

419 **Reagents.** PD 102807, dihydro- β -erythroidine hydrobromide, and DNQX (6,7-
420 dinitroquinoxaline-2,3-dione) were purchased from Tocris. LY367385, mecamylamine
421 hydrochloride, methyllycaconitine citrate, sulpiride, scopolamine, picrotoxin, and others
422 were obtained from Sigma.

423

424 **Behavioral Procedures**

425 **Intermittent-access to 20% alcohol 2-bottle choice drinking procedure.** This
426 procedure was conducted as described previously (23, 24, 31-34, 55). Briefly, animals
427 were given concurrent access to one bottle of alcohol (20%, in water) and one bottle of
428 water for 24-h periods, which were separated by 24- or 48-h periods of alcohol
429 deprivation. Alcohol intake (g/kg/day) was calculated by determining the weight of 20%
430 alcohol solution consumed and multiplying this by 0.2. Water control animals only have
431 access to water.

432 **Magazine training.** This procedure was adapted from Bradfield et al. (35). After 5 days
433 of food restriction, rats were trained for magazine entries for 20 min on two consecutive
434 days. During these training sessions, a reinforcer (either a food pellet or 0.1 mL sucrose
435 solution) was delivered along with illumination of the magazine light for 1 sec with a
436 random interval between each reinforcer (on average 60 sec). The house light was
437 illuminated throughout the session, and no levers were available during magazine
438 training. An equal number of rats received either 20 food pellets or 20 sucrose deliveries
439 during the first training session and were then switched to receive the other reward in the
440 second training session.

441 **Acquisition of initial contingencies.** Following magazine training, rats were trained to
442 access different reinforcers via lever pressing over the next 10 days. Each session
443 consisted of 4 blocks (2 blocks per lever), separated by a 2.5-min timeout during which
444 no levers were available, and all lights were extinguished. Only one lever was available

445 during each block (pseudorandom presentation), which lasted for 10 min or until 10
446 reinforcers had been earned. For half of the animals in each group, the left lever was
447 associated with food pellet delivery and the right lever with sucrose solution delivery. The
448 remaining animals were trained using the opposite pairs of action-outcome contingencies.
449 Lever training started with a fixed ratio 1 (FR1) schedule in which every lever press
450 resulted in the delivery of a reinforcer. After 2 days of FR1 training, the training schedule
451 was elevated to a random ratio 5 (RR5) schedule for the next 3 days, during which a
452 reinforcer was delivered after an average of 5 lever presses. An RR10 training schedule
453 was then employed for 3 days, followed by an RR20 schedule for the final 2 days.

454 **Devaluation test.** After the final RR20 training, devaluation testing was performed for 2
455 days. On both days, rats were habituated in a dark, quiet room (different from the operant
456 room) for 30 min, then were given *ad libitum* access to either the food pellets (25 g placed
457 in a bowl) or the sucrose solution (100 mL in a drinking bottle) in a devaluation cage for
458 1 h. The devaluation cage was similar to their home cage but did not contain bedding.
459 The rats were then placed in the operant chamber for a 10-min extinction choice test.
460 Both levers were extended during this test, but no outcomes were delivered in response
461 to any lever press. On the second devaluation day, the rats were pre-fed, as described,
462 with the other reward before repeating the same extinction test. Lever presses (LP) were
463 recorded, and those on the lever that the rat had learned to associate with the non-
464 devalued reward were termed LP_{valued} , while those on the lever associated with the
465 devalued reward were termed LP_{devalued} . The devaluation index $[(LP_{\text{valued}} -$
466 $LP_{\text{devalued}})/(LP_{\text{valued}} + LP_{\text{devalued}})]$ was then used to determine the extent of goal-directed
467 versus habitual behavior.

468 **Contingency reversal and devaluation testing.** After the devaluation test, rats were
469 retrained on their current action-outcome contingencies for 1 day. The contingencies were
470 then reversed so that the lever that previously delivered food now delivered sucrose, and
471 the rats were trained using the RR20 schedule. All other procedures were unchanged.
472 The contingency reversal training lasted for 4 days. The rats then underwent devaluation
473 testing again using the procedure described above.

474

475 **Electrophysiology**

476 Slice electrophysiology was performed as previously described (24, 55). Animals were
477 sacrificed 24 h after their last alcohol (or control water) consumption, and 250- μ m coronal
478 sections containing the striatum were prepared in an ice-cold cutting solution containing
479 (in mM): 40 NaCl, 148.5 sucrose, 4 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂,
480 10 glucose, 1 sodium ascorbate, 3 sodium pyruvate, and 3 myoinositol, saturated with
481 95% O₂ and 5% CO₂. Slices were then incubated in a 1:1 mixture of cutting solution and
482 external solution at 32°C for 45 min. The external solution contained the following (in mM):
483 125 NaCl, 4.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 15 sucrose, and
484 15 glucose, saturated with 95% O₂ and 5% CO₂. Slices were then maintained in external
485 solution at room temperature until use.

486 Slices were perfused with the external solution at a flow rate of 3-4 mL/min at 32°C.
487 The CINs and MSNs in the DMS were identified either by differential interference contrast
488 or by fluorescence. Whole-cell patch-clamp and cell-attached recordings were made
489 using a MultiClamp 700B amplifier controlled by pClamp 10.4 software (Molecular

490 Devices). For cell-attached and whole-cell current-clamp recordings, we used a K⁺-based
491 intracellular solution containing (in mM): 123 potassium gluconate, 10 HEPES, 0.2 EGTA,
492 8 NaCl, 2 MgATP, 0.3 NaGTP (pH 7.3), with an osmolarity of 270–280 mOsm. For whole-
493 cell voltage-clamp recordings, we used a Cs-based solution, containing (in mM): 119
494 CsMeSO₄, 8 TEA.Cl, 15 HEPES, 0.6 EGTA, 0.3 Na₃GTP, 4 MgATP, 5 QX-314.Cl, 7
495 phosphocreatine. The pH was adjusted to 7.3 with CsOH.

496 For measurement of spontaneous CIN firing, cell-attached recordings were
497 conducted in the voltage-clamp mode. In whole-cell current-clamp recordings, evoked
498 action potentials were elicited by 500-ms stepped current injections at 30-pA increments
499 from -120 pA to +120 pA. Optogenetically-evoked CIN firing was induced by light
500 stimulation (473 nm, 2 ms, 15 Hz, 10 pulses) through the objective lens. Bipolar
501 stimulating electrodes were positioned 100-150 μm away from the recording electrode
502 that was used to record glutamatergic transmission in MSNs. To measure NMDAR-
503 EPSCs, the neurons were recorded in the presence of DNQX and with magnesium-free
504 external solution. All of the measurements were conducted in the presence of the GABA_A
505 receptor antagonist, picrotoxin (100 μM). The experiments in Figure 5 were conducted in
506 the presence of the mGluR1/5 antagonists, LY367385 (10 μM).

507

508 **Stereotaxic surgery and Histology.**

509 The rabies helper viruses (AAV8-DIO-RG and AAV8-DIO-TVA-mCherry), AAV-
510 Chrimson-tdT, AAV-FLEX-Chrimson-tdT, and AAV-Chronos-GFP were purchased from
511 the University of North Carolina Vector Core. The pseudotyped rabies viruses, EnvA-

512 SADΔG-mCherry and EnvA-SADΔG-GFP (2.04×10^8 TU/mL), were obtained from the
513 Salk Institute Vector Core.

514 Stereotaxic viral infusions were performed as described previously (23, 24, 28, 56).
515 Briefly, mice were anesthetized using isoflurane and mounted in a rodent stereotaxic
516 frame (Kopf). The skin was opened to uncover the skull and expose Bregma and Lambda,
517 and the location of the desired injection site. A three-axis micromanipulator was used to
518 measure the spatial coordinates for Bregma and Lambda. Small drill holes were made in
519 the skull at the appropriate coordinates, according to the Paxinos atlas (57). Two
520 microinjectors were loaded with 0.5 μ L of a 1:1 mixture of AAV8-DIO-RG and AAV8-DIO-
521 TVA-mCherry, and then lowered into the pDMS (AP: 0.0 mm, ML: \pm 1.87 mm, DV: -2.90
522 mm). This helper virus mixture was infused into the brain at a rate of 0.1 μ L/min. To avoid
523 backflow of the virus, microinjectors were left in place for 10 min after the infusion was
524 complete. Following their removal, the skin was sutured and the mice were allowed to
525 recover for 3 weeks prior to the infusion of pseudotyped rabies virus (EnvA-SADΔG-
526 mCherry or EnvA-SADΔG-eGFP). The rabies virus was injected at the same site and
527 using the same injection volume as the initial helper virus injection. To prevent coincident
528 rabies infection along the injection tract, the rabies virus was infused into adapted
529 coordinates (AP, 0.0 mm; ML, \pm 2.42 mm; DV, -2.94 mm) at an angle of 10 degrees (58)
530 to the previous injection. The modified coordinates were calculated by measuring from
531 the midline and parallel to the dorsal-ventral axis. The coordinates for mice AAV-
532 Chrimson-tdT (0.5 μ L) PfN injection were AP, -2.2 mm; ML, \pm 0.7 mm; and DV, -3.5 mm.
533 ChAT-Cre rats, DMS (AAV-FLEX-Chrimson-tdT): AP, 0.0 mm; ML, \pm 2.8 mm; and DV, -
534 4.85 mm; PfN (AAV-Chronos-GFP): AP, -4.2 mm; ML, \pm 1.25 mm; and DV, -6.2 mm. For

535 rats, 1 μ l to 1.2 μ l of the virus was infused in each hemisphere. After virus injections,
536 bilateral optical fiber implants (300- μ m core fiber secured to a 1.25-cm ceramic ferrule
537 with 5 mm of fiber extending past the end of the ferrule) were lowered into the DMS right
538 on the top of virus injection sites. Coordinates: AP, 0.0 mm; ML, \pm 2.8 mm; and DV, -4.8
539 mm. Implants were secured on the skull using metal screws and dental cement (Henry
540 Schein) and covered with denture acrylic (Lang Dental). The incision was closed around
541 the head cap and the skin vet-bonded to the head cap. Rats were monitored for 1 week
542 or until they resumed normal activity.

543

544 The histology procedure was performed as described previously (24, 56). Briefly,
545 mice were anesthetized and perfused intracardially with 4% paraformaldehyde in
546 phosphate-buffered saline (PBS). Whole brains were taken out and placed into 4%
547 paraformaldehyde in PBS for post-fixation overnight (4°C), then moved to 30% sucrose
548 in PBS (4°C) and allowed to sink to the bottom of the container before preparing for
549 sectioning. Frozen brains were cut into 50- μ m coronal sections on a cryostat. A confocal
550 laser-scanning microscope (Fluorview-1200, Olympus) was used to image these sections
551 with a 470-nm laser (to excite eYFP and GFP) and a 593-nm laser (to excite tdT). All
552 images were processed using Imaris 8.3.1 (Bitplane, Zurich, Switzerland).

553

554 **Statistical analysis.** All data are expressed as the mean \pm SEM. Statistical significance
555 was assessed using the unpaired or paired *t* test or two-way RM ANOVA followed by the
556 *Tukey* test for *post hoc* comparisons. Statistical significance was set at $p < 0.05$.

557

558 **Author contributions**

559 J.W. conceived, designed, and supervised all the experiments in the study. T.M. and Z.H.
560 contribute equally to this research. The order of co-first author is determined by who
561 completed the first draft of the manuscript. T.M. wrote the first draft of the manuscript and
562 J.W., T.M., Z.H., Y.C., L.S., R.S., and Y.Z revised the manuscript. Z.H., T.M., and X.Z.
563 designed and performed electrophysiology experiments and analyzed the data. Z.H.,
564 X.X., and M.C. designed and performed the behavior experiments and analyzed the data.
565 H.G. and X.W. conducted histology experiments.

566

567

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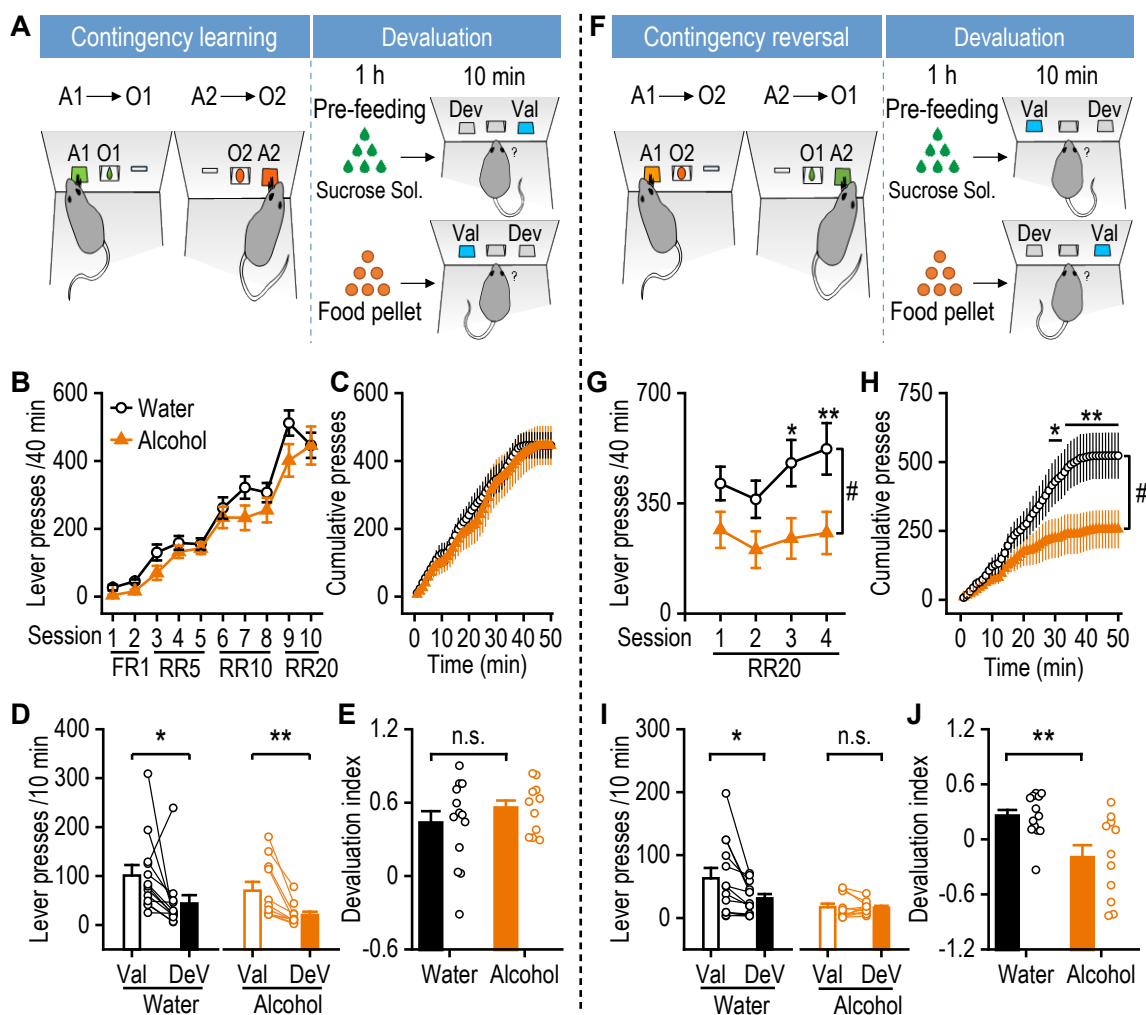
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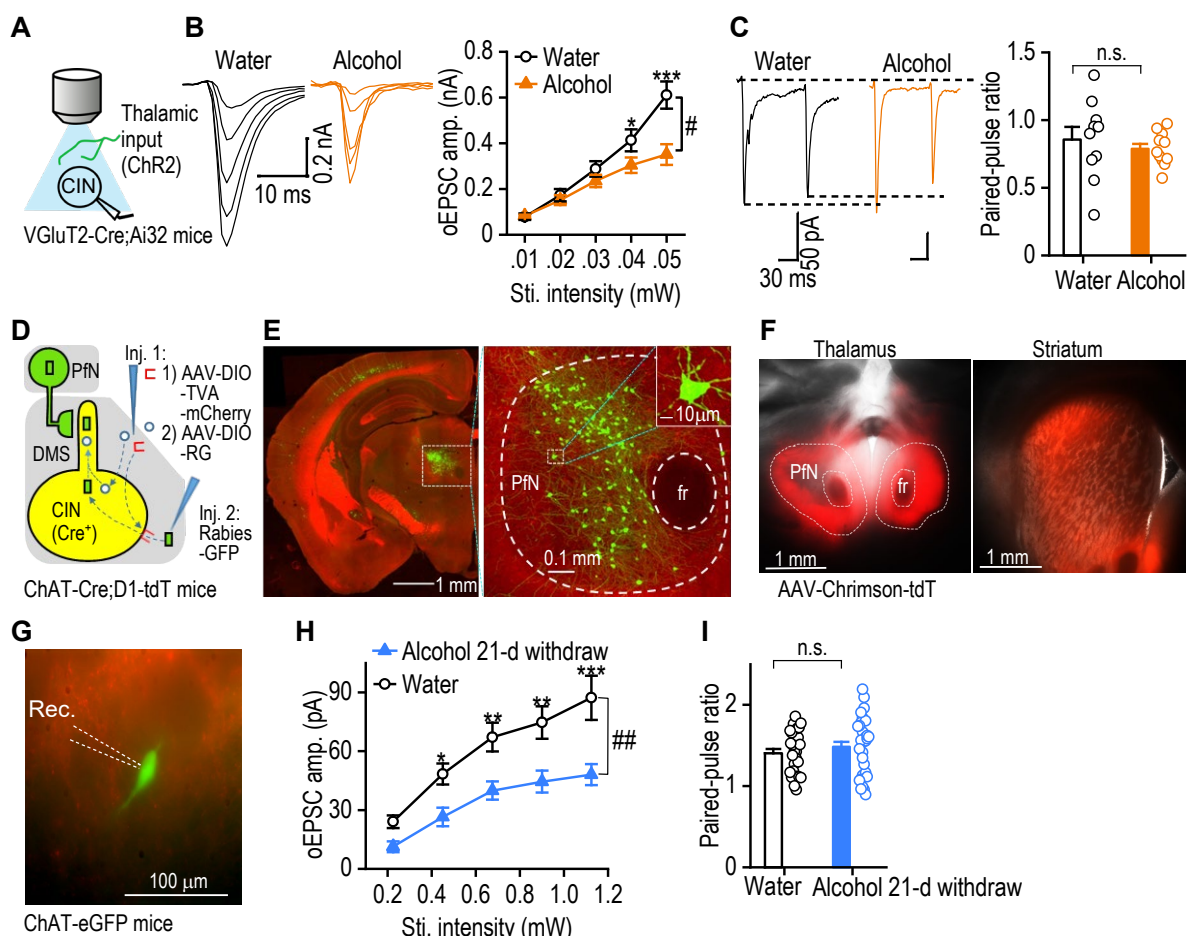
743 **Figures**



744

745 **Figure 1** Chronic alcohol intake impairs reversal of instrumental learning. **(A)** Schematic
 746 diagram depicting the instrumental learning procedure and subsequent devaluation
 747 testing. Long-Evans rats were trained to consumed 20% alcohol using the intermittent-
 748 access 2-bottle choice drinking procedure or received water for 8 weeks prior to operant
 749 training. Left, alcohol-exposed rats and water controls were trained to acquire the first set
 750 of action-outcome (A-O) contingencies, where pressing one of two available levers (A1
 751 or A2) provided a reward of either a food pellet (O1) or sucrose solution (O2). Right,
 752 outcome-specific devaluation tests involved pre-feeding the rats with O1 or O2 for 1 h
 753 prior to extinction choice testing (A1 versus A2). The lever associated with the pre-fed
 754 reward was defined as devalued (DeV), and the other lever was defined as valued (Val).
 755 **(B)** The alcohol and water groups showed no significant difference in total lever presses
 756 during the acquisition of the initial contingencies, moving from a fixed ratio 1 (FR1)
 757 schedule to random ratio 5 (RR5), RR10, and RR20 schedules as indicated; two-way RM
 758 ANOVA, n = 13 rats (Water) and 11 rats (Alcohol). **(C)** The alcohol and water groups

759 showed no difference in cumulative lever presses during the last initial training session
760 (session 10); two-way RM ANOVA, $n = 13$ rats (Water) and 11 rats (Alcohol). **(D)**
761 Outcome-specific devaluation testing showed that both water and alcohol groups pressed
762 the DeV lever significantly fewer times than the Val lever; $*p < 0.05$ (Water) and $**p <$
763 0.01 (Alcohol) by paired t test, $n = 13$ rats (Water) and 11 rats (Alcohol). **(E)** The
764 devaluation index, defined as $(Val - DeV)/(Val + DeV)$, did not differ significantly between
765 the two groups; n.s., not significant by unpaired t test, $n = 13$ rats (Water) and 11 rats
766 (Alcohol). **(F)** Schematic diagram showing the next round of instrumental learning, with
767 reversed contingencies and subsequent devaluation testing. Left, alcohol rats and water
768 controls were trained to acquire the reversed set of A-O contingencies for 4 days using
769 the RR20 schedule. Right outcome-specific devaluation testing was performed as
770 described above. **(G)** The alcohol group showed significantly reduced lever pressing
771 during the reversed contingency training sessions, as compared to the water group; $\#p <$
772 0.05 by two-way RM ANOVA; $*p < 0.05$, $**p < 0.01$ versus the same session in the alcohol
773 group by *Tukey post-hoc* test; $n = 13$ rats (Water) and 11 rats (Alcohol). **(H)** The alcohol
774 group showed significantly fewer cumulative lever presses in the last reversal learning
775 session (session 4), as compared to the water group; $\#p < 0.05$ by two-way RM ANOVA;
776 $*p < 0.05$, $**p < 0.01$ for group comparisons at the indicated time points by *Tukey post-*
777 *hoc* test; $n = 12$ rats (Water) and 10 rats (Alcohol). **(I)** Outcome-specific devaluation after
778 the reversed A-O contingency learning showed that the water group interacted less with
779 the DeV lever, but this devaluation was not observed in the alcohol group; $*p < 0.05$
780 (Water) and n.s., not significant, $p > 0.05$ (Alcohol) by paired t test, $n = 13$ rats (Water)
781 and 11 rats (Alcohol). **(J)** The devaluation index was significantly lower in the alcohol
782 group than in the water group; $**p < 0.01$ by unpaired t test; $n = 13$ rats (Water) and 11
783 rats (Alcohol).

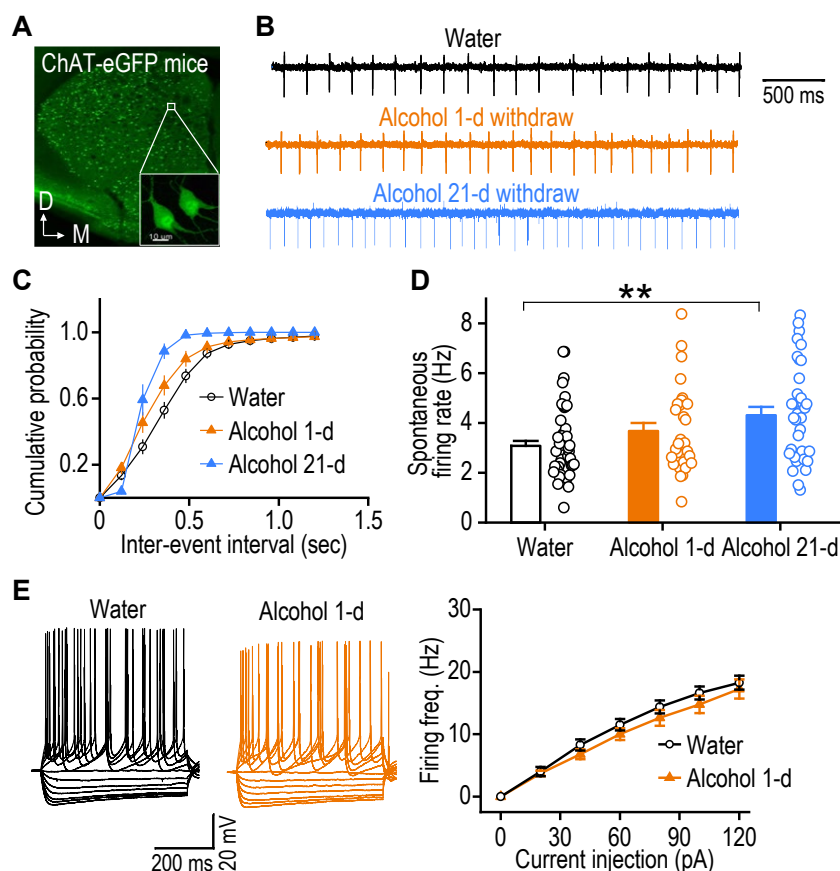


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785 **Figure 2** Chronic alcohol consumption reduces thalamostriatal glutamatergic inputs onto
 786 DMS CINs. **(A)** Schematic diagram showing light stimulation of ChR2-expressing
 787 thalamic inputs and whole-cell recording of CINs. **(B)** Chronic alcohol consumption
 788 suppressed thalamostriatal transmission onto CINs in DMS slices prepared 24 h after the
 789 last alcohol exposure. Left and middle, sample traces of EPSCs evoked by the indicated
 790 optical stimulation (oEPSCs). Right, input-output curves of oEPSC amplitudes in CINs
 791 from mice exposed to alcohol or water; # $p < 0.05$ by two-way RM ANOVA; * $p < 0.05$, *** p
 792 < 0.001 versus the same stimulation intensity in the alcohol group by *Tukey post-hoc* test,
 793 $n = 11, 4$ (Water) and $13, 3$ (Alcohol). **(C)** Chronic alcohol consumption did not alter the
 794 glutamate release probability at thalamostriatal synapses. Left and middle, representative
 795 traces of oEPSCs induced by paired-pulse optical stimulations in the alcohol and water
 796 groups. Right, the paired-pulse ratios in the indicated groups; $p > 0.05$ by unpaired *t* test,
 797 $n = 10, 3$ (Water) and $11, 3$ (Alcohol). **(D)** Schematic showing viral infusions. We injected
 798 rabies helper viruses (AAV-DIO-TVA-mCherry and AAV-DIO-RG) into the DMS of ChAT-
 799 Cre;D1-tdT mice, leading to selective expression of TVA and RG in CINs (Inj. 1). D1-tdT
 800 was used to show the background. After 2 weeks, we injected rabies-GFP into the same
 801 DMS site (Inj. 2). This approach caused selective TVA-mediated infection of CINs with
 802 rabies-mCherry, followed by RG-mediated retrograde transsynaptic infection of
 803 presynaptic neurons, including those in the thalamic parafascicular nucleus (Pfn). **(E)**

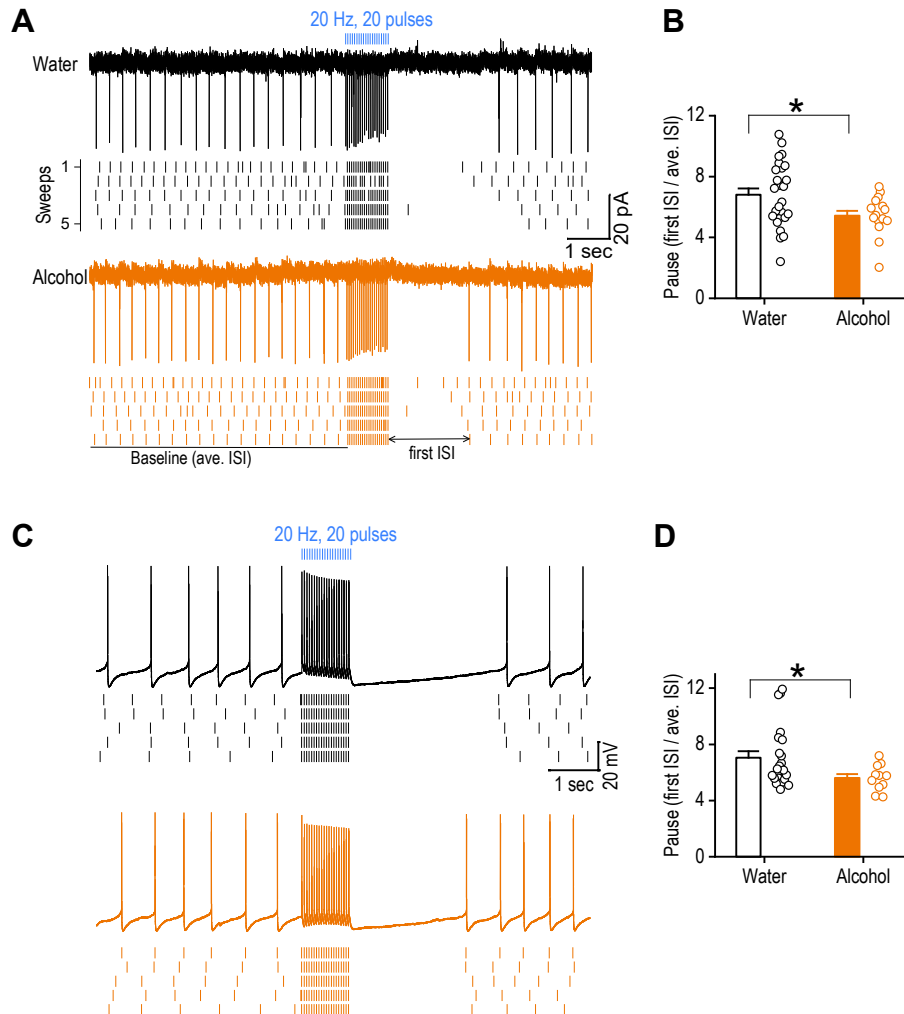
804 Sample coronal images showing that many rabies-GFP-labeled PfN neurons projected to
805 DMS CINs. Similar results were observed in 4 mice; fr, fasciculus retroflexus. **(F)** Optical
806 images of tdT fluorescence in striatal slices from ChAT-eGFP mice that were infused with
807 AAV-Chrimson-tdT in the PfN of the thalamus. **(G)** High magnification optical image
808 showing an GFP expressing CIN and its surround tdT fluorescence. **(H)** Input-output
809 curves of oEPSC amplitudes in CINs from mice injected with AAV-Chrimson-tdT and
810 exposed to alcohol or water; $^{##}p < 0.01$ by two-way RM ANOVA; $^*p < 0.05$, $^{**}p < 0.01$,
811 $^{***}p < 0.001$ versus the same stimulation intensity in the alcohol group by *Tukey post-hoc*
812 test, $n = 15, 4$ (Water) and $14, 4$ (Alcohol). **(I)** Paired-pulse ratios in mice injected with
813 AAV-Chrimson-tdT and exposed to alcohol or water; $p > 0.05$ by unpaired *t* test, $n = 27,$
814 5 (Water) and $32, 5$ (Alcohol).

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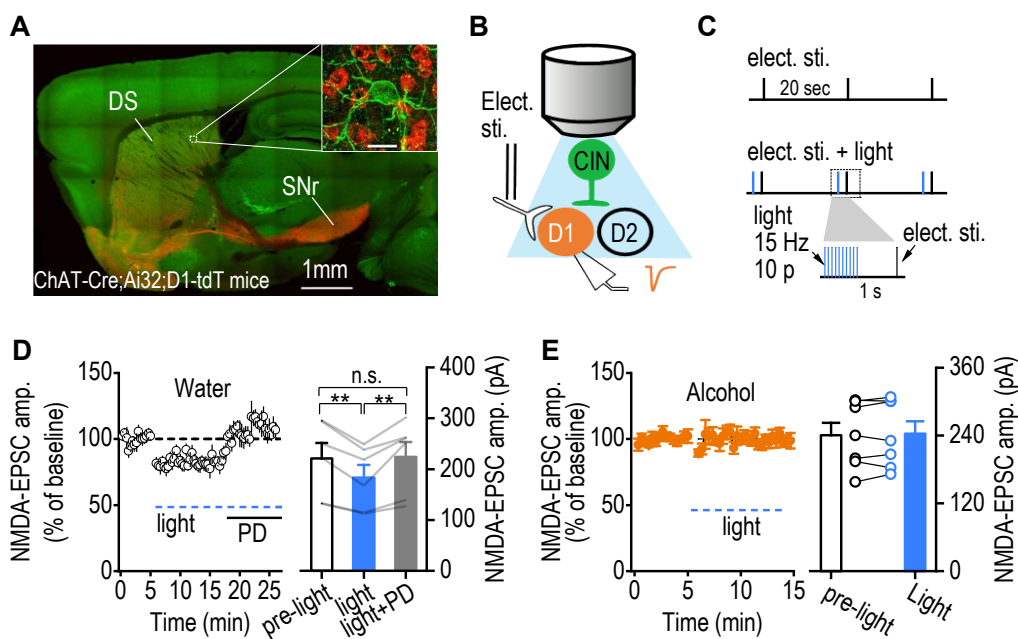
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817 **Figure 3** Chronic alcohol consumption increases spontaneous, but not evoked, firing of
 818 DMS CINs. ChAT-eGFP mice were trained to consume 20% alcohol for 8 weeks and
 819 DMS slices were prepared 24 h and 21-d after the last alcohol exposure. **(A)** Sample
 820 image showing green CINs in the striatum. D, dorsal; M, medial. **(B)** Sample traces of
 821 spontaneous CIN firing in the water and alcohol groups using the cell-attached recording.
 822 **(C)** Cumulative plots of the inter-event intervals and **(D)** the spontaneous firing rates of
 823 CINs in the indicated groups; $###p < 0.01$ by one-way ANOVA, $**p < 0.01$ versus water
 824 group by *Tukey post-hoc* test; $n = 49, 7$ (Water), $31, 6$ (Alcohol 1-d), and $36, 4$ (Alcohol
 825 21-d). **(E)** Chronic alcohol did not change evoked CIN firing. Left and middle, sample
 826 traces of membrane potentials generated in the indicated groups in response to a series
 827 of 500-ms current injections. Right, the input-output relationship between the injected
 828 current magnitude and the CIN firing frequency in water and alcohol groups; $p > 0.05$ by
 829 two-way RM ANOVA, $n = 16, 4$ (Water) and $13, 3$ (Alcohol).



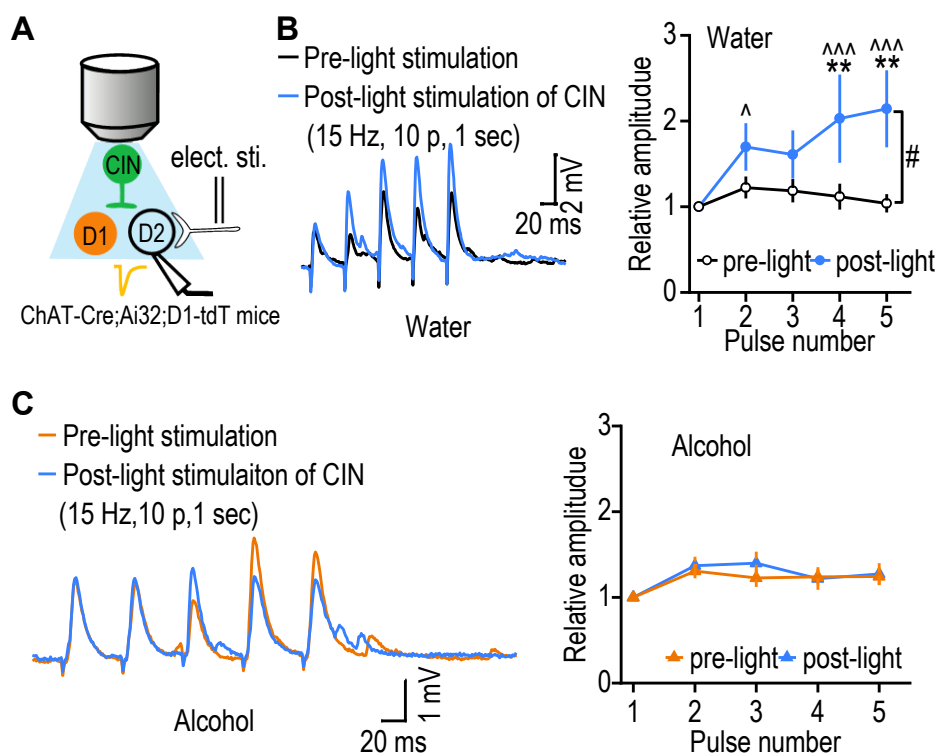
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831 **Figure 4** Chronic alcohol consumption shortens pause response of DMS CINs. ChAT-
832 Cre;Ai32 mice were trained to consume 20% alcohol for at least 8 weeks. Then DMS
833 slices were prepared 24 h after last alcohol exposure and optically-evoked burst-pause
834 responses of CINs were measured. **(A)** Sample traces of burst-pause responses of a CIN
835 from the water (top) and alcohol (bottom) groups using the cell-attached recording. ISI:
836 inter-spike interval. **(B)** The pause durations in the indicated groups; $p < 0.05$ by unpaired
837 t test, $n = 26, 5$ (Water) and $16, 4$ (Alcohol). The pause duration is defined by the first ISI
838 right after optical stimulation divided by baseline average ISI before the optical stimulation.
839 **(C)** Sample traces of burst-pause responses of a CIN from the water (top) and alcohol
840 (bottom) groups using whole-cell recording. **(D)** The pause durations in the indicated
841 groups; $p < 0.05$ by unpaired t test, $n = 22, 5$ (Water) and $11, 3$ (Alcohol).



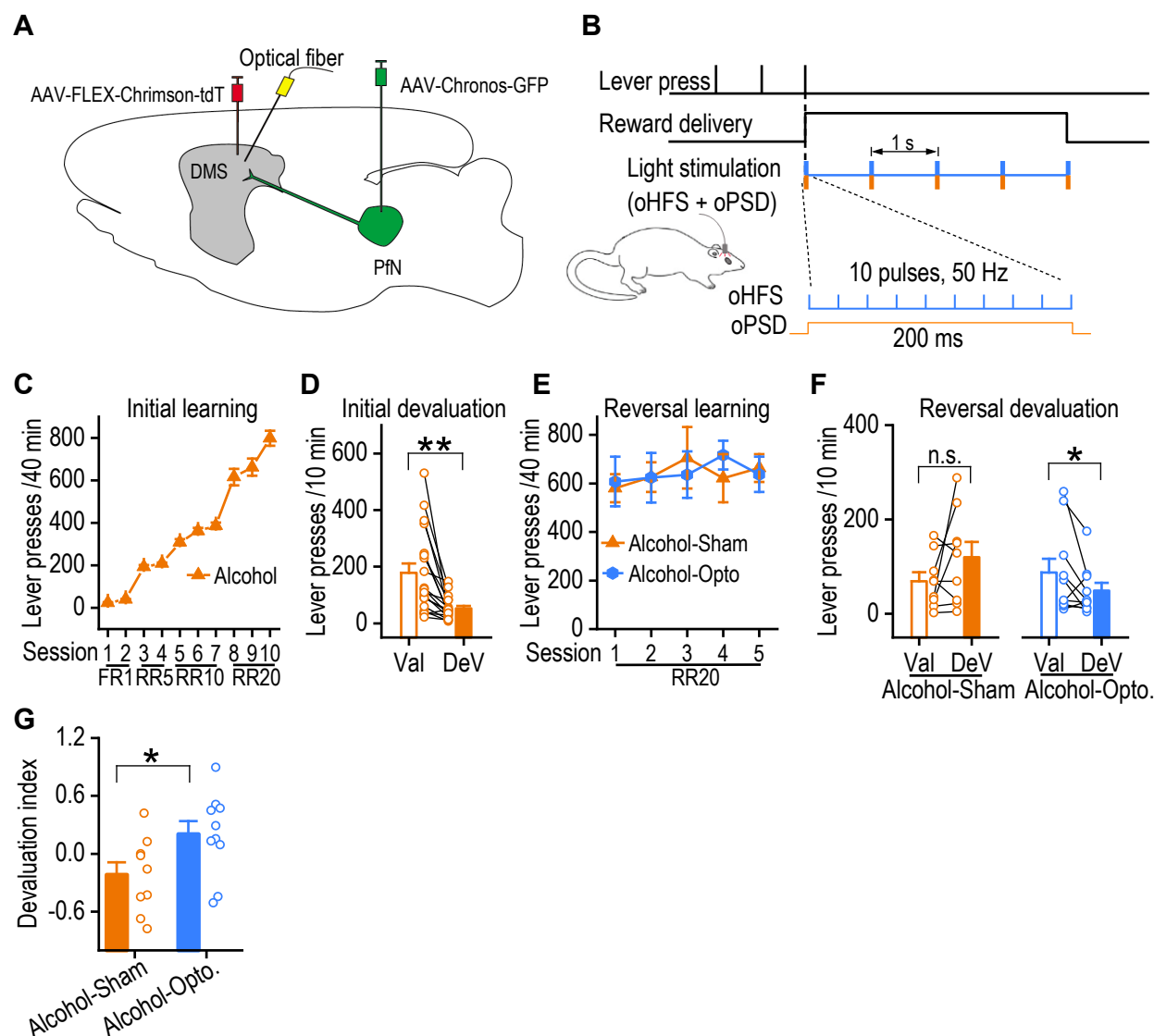
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843 **Figure 5** Chronic alcohol consumption impairs CIN-mediated suppression of
 844 glutamatergic transmission in DMS D1-MSNs. **(A)** Sample image of a sagittal section from
 845 a ChAT-Cre;Ai32;D1-tdT mouse. Inset shows a green CIN with several red D1-MSNs
 846 (scale bar: 20 μ m). **(B)** Schematic illustration of the electrical and optical stimulation and
 847 selective recording of D1-MSNs. The stimulating electrodes were placed in the DMS close
 848 to the recording pipette. **(C)** Schematic of the electrical and light stimulation protocols.
 849 Electrical stimulation (top) was delivered every 20 sec, 1 sec after the delivery of a burst
 850 of 473-nm light (2 ms of 10 pulses at 15 Hz) (middle and bottom). **(D)** The amplitude of
 851 NMDAR-mediated EPSCs before light stimulation, during light stimulation, and during
 852 light stimulation in the presence of the muscarinic M4 antagonist, PD 102807 (PD, 1 μ M),
 853 showed that optogenetic excitation of DMS CINs caused an M4 receptor-dependent
 854 suppression of NMDAR activity in D1-MSNs; $**p < 0.01$, unpaired t test, $n = 7$, 5 per group.
 855 **(E)** Chronic alcohol consumption abolished CIN-induced suppression of NMDAR-EPSCs;
 856 $p > 0.05$ by paired t test, $n = 7$, 4 per group.



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858 **Figure 6** Chronic alcohol intake compromises CIN-mediated short-term facilitation of
 859 glutamatergic transmission in DMS D2-MSNs. **(A)** Schematic illustration of the electrical
 860 and light stimulation and whole-cell recording of D2-MSNs in ChAT-Cre;Ai32;D1-tdT mice.
 861 Putative D2-MSNs were identified by their absence of fluorescence. **(B)** Left,
 862 sample traces showing that electrical stimulation led to five EPSPs in D2-MSNs before and after
 863 light-mediated excitation of CINs. Electrical stimulation was delivered every 20 sec, 1 sec
 864 after the delivery of a burst of 473-nm light (2 ms of 10 pulses at 15 Hz). Right, calculation
 865 of the relative amplitudes of five EPSPs detected short-term facilitation in water control
 866 mice after light-mediated excitation of CINs. EPSPs were normalized to the first one; # p
 867 < 0.05 by two-way RM ANOVA; ** $p < 0.01$ versus the same pulse number in the pre-light
 868 group by *Tukey post-hoc* test; ^ $p < 0.05$, ^^ $p < 0.001$ versus pulse number 1 within the
 869 post-light group by *Tukey post-hoc* test; $n = 9$, 6 per group. **(C)** Left, sample traces
 870 showing the EPSPs before and after light stimulation of CINs in the alcohol group. Right,
 871 calculation of the relative amplitudes of EPSPs in the alcohol group did not identify any
 872 change after light stimulation of CINs; $p > 0.05$; two-way RM ANOVA, $n = 12$, 4 per group.



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 874 **Figure 7** Optogenetic stimulation PfN-to-CIN pathway in the DMS rescues the
 875 impairment of reversal learning. **(A)** Schematic diagram of viruses injection and optical
 876 fiber implantation. ChAT-Cre rats were bilaterally injected with AAV-FLEX-Chrimson-tdT
 877 and AAV-Chronos-GFP into DMS and PfN, respectively. Optical fibers were bilaterally
 878 implanted into DMS. After recovery from surgery rats were trained by the schedule as
 879 Figure 1. **(B)** Optical light stimulation protocol used during the reversal learning. Briefly,
 880 rats press the lever to get the reward. Light stimulation is time-locked to reward delivery.
 881 Light stimulation contains five repeats of dual light stimulus within 5 s reward delivery
 882 period. Each repeat consists of blue light high frequency stimulation (10 pulses, 50 Hz)
 883 and yellow light (continuous, 200 ms) delivering at the same time. oHFS: optical high-
 884 frequency stimulation; oPSD: optical postsynaptic depolarization. **(C)** The initial
 885 acquisition learning curve. $n = 19$. **(D)** Outcome-specific devaluation testing showed that
 886 rats pressed the DeV lever significantly fewer times than the Val lever; $**p < 0.01$ by
 887 paired t test, $n = 19$. **(E)** There was no significantly difference in terms of lever pressing

888 between two groups during the reversed contingency training sessions; # $p > 0.05$ by
889 two-way RM ANOVA; $n = 9$ rats (Alcohol-Sham) and 10 rats (Alcohol-Opto). **(F)**
890 Outcome-specific devaluation after reversed A-O contingency learning showed that the
891 sham group still interacted more with the DeV lever (which is Val lever during initial
892 learning), while the group received light stimulation showed successful devaluation after
893 the reversed A-O contingency; n.s., not significant, $p > 0.05$ and * $p < 0.05$ by paired t
894 test, $n = 9$ rats (Alcohol-Sham) and 10 rats (Alcohol-Opto). **(G)** The devaluation index
895 was significantly higher in the opto group than in the sham group; * $p < 0.05$ by unpaired
896 t test; $n = 9$ rats (Alcohol-Sham) and 10 rats (Alcohol-Opto).

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906 **Figure 8** Schematic diagram showing the effects of chronic alcohol intake on the thalamic
907 inputs to CINs and their modulation of glutamatergic transmission to D1-MSN and D2-
908 MSN in the striatum. Chronic alcohol consumption reduces thalamic excitatory inputs to
909 DMS CINs and increases their spontaneous firing, which makes them less prone to be
910 modulated by external signals. In the meantime, the CIN-mediated inhibition of
911 glutamatergic transmission in D1-MSNs and the CIN-mediated short-term facilitation of
912 glutamatergic transmission in D2-MSNs are comprised after chronic alcohol intake, which
913 could change striatal outputs and lead to behavioral inflexibility.

