

1 **Title: Dopaminergic learning and arousal circuits mediate opposing effects on**
2 **alcohol consumption in *Drosophila*.**

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28

29 **ABSTRACT**

30

31 The response to drugs of abuse is a combination of aversive and reinforcing reactions.

32 While much is known about the role of dopamine in mammalian drug reinforcement, we

33 know little about the brain circuits mediating drug aversion. Here we show that two

34 distinct dopaminergic circuits mediate reinforcing and acute aversive responses to

35 alcohol consumption in *Drosophila*. Protocerebral anterior medial dopamine neurons

36 projecting to the mushroom bodies are required for flies to acquire alcohol preference.

37 Conversely, a bilateral pair of dopamine neurons projecting to the dorsal fan-shaped

38 body (dFSB) mediates acute alcohol avoidance. Alcohol consumption can be reduced

39 by decreasing the activity of the appetitive reinforcement-circuit to the mushroom bodies,

40 or by increasing activity in the dopamine neurons projecting to the dFSB. Thus, distinct

41 dopaminergic pathways can be targeted to reduce the intake of harmful drugs.

42

43 INTRODUCTION

44 Alcohol exposure causes both pleasurable and negative responses in humans.
45 People displaying increased sensitivity to alcohol's rewarding effects or resistance to
46 the acute aversive responses are at increased risk for alcohol use disorder (AUD)¹. The
47 development of AUD involves circuits mediating the reinforcing effects of alcohol,
48 including dopaminergic projections from the ventral tegmental area to the nucleus
49 accumbens². However, much less is known about the neurons mediating the acute
50 aversive responses to this drug.

51 Similar to mammals, *Drosophila* show naïve aversion to alcohol when given a
52 choice between liquid food with or without alcohol³. This initial alcohol aversion
53 transforms into experience-dependent preference after an alcohol pre-exposure³.
54 Dopaminergic neurons are involved in the recall of ethanol-conditioned odor preference⁴.
55 However, it is not known how dopamine is involved in voluntary ethanol consumption in
56 *Drosophila*—including naïve aversion and the transformation from aversion to
57 experience-dependent alcohol preference. Here we show that distinct dopamine
58 circuits mediate the acute aversive and reinforcing effects upon ethanol exposure. We
59 also show that normal ethanol responses require the *Drosophila* dopamine D1R1
60 receptor in the respective target neurons of these two dopamine circuits, and that
61 manipulations of either circuit can lead to reduction in voluntary alcohol consumption.

62

63

64 RESULTS

65 **Distinct dopamine neurons mediate opposing consummatory reactions to alcohol**

66 To determine the involvement of dopamine in alcohol-consumption behavior, we
67 fed flies the dopamine precursor L-DOPA (L-3,4-dihydroxyphenylalanine), or 3-IY (3-
68 iodo-tyrosine), an inhibitor of the rate-limiting dopamine synthesis enzyme tyrosine
69 hydroxylase (TH, Fig. 1a). Flies with increased dopamine levels (Supplementary Figure
70 1a) showed enhanced naïve aversion, while dopamine depletion (Supplementary Figure
71 1a) led to naïve preference in an abbreviated 16-hour “2-bottle choice” CAFÉ assay (for
72 capillary feeder, Fig. 1B⁵). As expected (see Fig. 1a), feeding of the TH product L-
73 DOPA was able to restore the naïve alcohol preference caused by TH inhibition back to
74 aversion (Supplementary Figure 1b). After a 20-min alcohol pre-exposure a day prior to
75 testing, control flies developed experience-dependent preference for alcohol, whereas
76 flies with increased dopamine levels did not (Fig. 1b). These results suggested that
77 dopamine is crucial for alcohol aversion rather than acquired preference in flies. To
78 confirm that levels of dopamine signaling correlated with alcohol aversion, we
79 genetically altered DAN activity using a *TH-Gal4* driver, which is expressed in most
80 DAN (Fig. 1c,⁶⁻⁹). Enhancing *TH-Gal4* DAN activity (using the heat-activated TrpA cation
81 channel) during pre-exposure and during the CAFÉ assay preference test (Fig. 1d)
82 caused alcohol aversion regardless of pre-exposure (Fig. 1e). Conversely, using a
83 heat-activated dominant negative dynamin (*shi^{ts}*) to silence these *TH-Gal4* DAN led to
84 alcohol preference, regardless of pre-exposure (Fig. 1e). Therefore, changing the
85 activity of the majority of DAN using *TH-Gal4* had the same effect as pharmacologically
86 altering dopamine levels (Fig. 1b).

87 The PAM cluster (protocerebral anterior medial) of DAN expresses TH, but lacks
88 *TH-Gal4* expression^{8,9}. These PAM-DAN are required for appetitive olfactory

89 conditioning. We therefore wanted to know whether these PAM-DAN contributed to
90 alcohol aversion, as indicated by our above pharmacological results, or whether they
91 were involved in alcohol preference as suggested by their requirement for appetitive
92 olfactory learning. When we activated the PAM-DAN subpopulation, using the heat-
93 sensitive cation channel TrpA, flies showed enhanced experience-dependent alcohol-
94 preference (Fig. 1f). Conversely, silencing these PAM-DAN using a dominant-negative
95 dunamin, *sh¹^{ts}*, abolished alcohol preference (Fig. 1f). These data suggested that *TH-*
96 *Gal4* DAN mediate alcohol aversion, while PAM dopaminergic activity is required for
97 experience-dependent alcohol preference. We confirmed the requirement for
98 dopaminergic activity by knocking down the TH enzyme specifically in PAM-DAN (using
99 *TH-RNAi*), which also abolished alcohol preference upon pre-exposure (Fig. 2b). This
100 result also suggested that it is dopamine in these PAM neurons that is involved in
101 experience-dependent alcohol preference rather than other putative co-transmitters.

102

103 **PAM dopamine neurons are required for the acquisition of experience-dependent** 104 **alcohol preference**

105 Because our behavioral paradigm consists of an alcohol pre-exposure followed
106 by a test of flies' consumption preference a day later, we were able to ask whether
107 PAM-DAN are required during the acquisition or the expression of alcohol preference.
108 Limiting the heat-induced activation and silencing of the PAM-DAN to the alcohol pre-
109 exposure or to the CAFÉ test revealed that these neurons had no effect during the
110 testing (Fig. 2e). However, PAM-DAN activity was required during pre-exposure to
111 acquire alcohol preference (Fig. 2d), and activating those neurons showed a trend

112 towards facilitated acquisition of preference (Fig. 2e, $P = 0.056$). PAM-DAN project to
113 the mushroom bodies (MB) a known center for associative learning in flies (Fig. 2a^{8,9}).
114 We therefore asked whether MB neurons are involved in experience-dependent alcohol
115 preference, and, if so, when? Inhibiting activity of the α/β and γ lobes of the MB during
116 the pre-exposure abolished preference acquisition (Fig. 2f), while we found no effect of
117 the MB α/β and γ lobe neurons during the CAFÉ preference test (Fig. 2g). MB-
118 projecting PAM-DAN activity is therefore required for the acquisition of alcohol
119 preference, similar to the nucleus accumbens-projecting mammalian ventral tegmental
120 area DAN².

121

122 **A PPL1-to-fan-shaped body circuit mediates naïve alcohol aversion**

123 We next investigated the role of the *TH-Gal4* DAN population (Fig. 3a) in naïve
124 alcohol aversion. Knocking down *TH* expression in these neurons resulted in naïve
125 alcohol preference (Fig. 3b), suggesting that dopamine is the relevant neurotransmitter
126 mediating naïve alcohol aversion. Activation or silencing of the *TH-Gal4* DAN during
127 ethanol pre-exposure had no effect on alcohol preference (Fig. 3c), but activating them
128 during the CAFÉ preference test enhanced naïve aversion and abolished experience-
129 dependent preference (Fig. 3d). Together with the data from Fig. 1b, e, these results
130 suggested that alcohol acutely induces aversion via *TH-Gal4* DAN during the CAFÉ
131 preference choice. This could be mediated by alcohol odor and/or taste sensation, but
132 in addition, *TH-Gal4* DAN might also be directly activated by alcohol's acute direct effect
133 on these DAN.

134 We tested this latter hypothesis by performing *ex vivo* dopamine voltammetry
135 experiments, while using the red light-activated CsChrimson cation channel¹⁰. Light-
136 induced activation of *TH-Gal4* DAN caused robust release of dopamine from larval brain
137 explants (Fig. 3e). Exposure to increasing amounts of alcohol revealed that even a low
138 alcohol concentration of 5 mM potentiated the release of dopamine (Fig. 3e,f). This level
139 is less than one third of the legal blood alcohol limit for driving. Acute direct exposure of
140 *TH-Gal4* DAN to alcohol may therefore contribute to their activation, and underlie or
141 potentiate acute sensory-induced aversion to alcohol at the behavioral level. Similarly,
142 PAM-DAN might also be activated at these doses and thereby cause an appetitive
143 reinforcing signal to the MB, ‘teaching the flies’ to like alcohol and override its aversive
144 effects. This model would also explain how a sub-threshold ethanol pre-exposure could
145 induce preference when paired with TrpA-mediated activation of PAM-DAN (Fig.2d,
146 30/120 EtOH/air flow; below threshold to turn aversion into preference in control flies).

147 One of the *TH-Gal4* DAN clusters activated by aversive stimuli is PPL1
148 (protocerebral posterior lateral 1)⁶, and these DAN might therefore also be involved in
149 alcohol aversion. We thus followed up on other Gal4 drivers expressing in PPL1 to ask
150 which of the *TH-Gal4* DAN are important in naïve, acute alcohol aversion. Using a
151 number of Gal4 lines expressed in smaller subsets of *TH-Gal4* and PPL1 DAN
152 (Supplementary Fig. 2)^{11,12}, we found that silencing of both *TH-D'-Gal4* and *439B-Gal4*
153 DAN caused naïve alcohol preference (Fig. 3g). *439B-Gal4* is expressed in 3 DAN per
154 hemisphere (from the PPL1 DAN cluster, Fig. 3h), two projecting to the MB (Fig. 3i, red
155 example), and one projecting to the dorsal fan-shaped body (dFSB; Fig. 3i, green). The
156 two MB-projecting *439B-DAN* are also expressed in line *504B-Gal4*¹², which had no

157 effect on acute alcohol aversion (Fig. 3g). This suggests that the dFSB-projecting 439B-
158 DAN is involved in acute alcohol aversion. We further tested the involvement of dFSB in
159 alcohol aversion by activating the PPL1-dFSB target neurons¹³ and found that this also
160 caused naïve alcohol preference (Fig. 3g). Interestingly, this PPL1-dFSB circuit is
161 involved in arousal from sleep^{11,13}. Note that flies with silenced PPL1-DAN, or activated
162 dFSB neurons consumed the same total amount of food as did controls (Supplementary
163 Fig. 3), arguing that these manipulations did not cause pervasive ‘sleepiness’ or
164 inactivity (Supplementary Fig. 4).

165

166 **A Dopamine D1-like receptor is required in dopamine target neurons for alcohol** 167 **aversion and preference**

168 Together, the above data suggested that the PPL1-dFSB circuit mediates acute
169 alcohol aversion, while the PAM-MB circuit participates in the acquisition of experience-
170 dependent alcohol preference. To further strengthen our evidence for a critical role of
171 dopamine in both these circuits, we investigated the involvement of two major dopamine
172 receptors, D1 and D2. Pan-neuronal knock down (Fig. 4a) of the D1-like Dop1R1 (*D1R*)
173 receptor led to naïve alcohol preference (Fig. 4d), while knock down of the D2-like
174 receptor (*D2R*) did not affect naïve aversion or experience-dependent preference (Fig.
175 4e). Furthermore, knock down of *D1R* in the dFSB was sufficient to cause naïve alcohol
176 preference (Fig. 4g). MB-specific knock down revealed that *D1R* is required in MBs for
177 experience-dependent preference (Fig. 4f).

178 To ascertain that our results were not caused by RNAi off-target effects, we also
179 tested the *D1R*^{UAS} mutation. These flies lack functional *D1R* expression, but the

180 presence of a Gal4-binding UAS site in the *D1R* gene allows expression to be restored
181 by a Gal4 driver¹⁴. As with pan-neuronal RNAi knock down of *D1R* (Fig. 4d), *D1R*^{UAS}
182 flies showed naïve alcohol preference (Fig. 4h). When we conditionally restored adult
183 *D1R* expression in neurons using an inducible pan-neuronal Gal4-driver (*elavGS+*), flies
184 showed normal naïve alcohol aversion, which turned into experience-dependent
185 preference upon pre-exposure (Fig. 4h). These data provide evidence for an acute
186 requirement of dopamine signaling during adult behavior (as suggested by Figs. 2, 3)
187 and argue against developmental defects causing the behavioral deficits¹⁵. When we
188 restored MB-specific *D1R* expression in *D1R*^{UAS} flies, we observed no change from the
189 *D1R*^{UAS} mutants, i.e., naïve preference with intact experience-dependent preference
190 (Fig. 4i). Conversely, dFSB-specific *D1R* expression rescued *D1R*^{UAS} naïve preference
191 back to normal naïve avoidance, but these dFSB-rescued *D1R*^{UAS} mutants did not
192 acquire experience-dependent preference (Fig. 4i), presumably due to the lack of D1R
193 in the MB. Indeed, when we restored D1R expression in both the MB and dFSB,
194 *D1R*^{UAS} flies displayed normal naïve alcohol aversion, followed by experience
195 dependent preference upon alcohol pre-exposure (Fig. 4i). These data confirm that
196 dopaminergic signaling from the PAM to the MB is required for acquired alcohol
197 preference, and PPL1 dopaminergic signaling to the dFSB is required for naïve alcohol
198 avoidance (Fig. 4j).

199

200 **DISCUSSION**

201 Here we demonstrate the multifaceted involvement of dopamine in voluntary
202 alcohol consumption. We show that PAM dopamine neurons are required for the

203 acquisition of experience-dependent alcohol preference. Consummatory preference
204 also requires the dopamine D1R1 receptor in the *Drosophila* MB. This PAM to MB
205 circuit has previously been found to be important in the acquisition of appetitive sucrose
206 learning^{8,9}, while D1R1 is well known to be involved in *Drosophila* learning and
207 memory¹⁶. Furthermore, the MB are involved in ethanol-reinforced odor preference⁴, as
208 well as preferential alcohol consumption¹⁷. Our findings that acute alcohol exposure
209 potentiates dopaminergic release suggest a mechanism of how an acute vapor
210 exposure of ethanol might cause reinforcement: as alcohol rises in the brain,
211 dopaminergic neurons, including reinforcing PAM neurons, get activated and impart an
212 association of cues with behavioral reinforcement. These cues might involve the smell
213 of alcohol itself, but this remains to be determined. Still, our data suggest that the
214 *Drosophila* PAM neurons act analogously to the ‘classical’ mammalian dopamine
215 neurons projecting from the ventral tegmental area to the nucleus accumbens, thought
216 to be important in mediating the reinforcing actions of drug of abuse.

217 Our data also suggests that a single bilateral pair of PPL1 dopamine neurons
218 mediates acute aversive effects of ethanol, and silencing these neurons causes
219 abolished alcohol aversion. Dopaminergic neurons in the PPL1 cluster are acutely
220 activated by a number of aversive stimuli⁶, consistent with their involvement in alcohol
221 aversion. Thus in our acute choice paradigm, the smell¹⁸, or taste^{19,20} of ethanol might
222 activate these neurons and contribute to acute avoidance of alcohol consumption. In
223 addition, acute drinking and alcohol’s pharmacodynamic potentiation of these neurons
224 might also contribute to acute avoidance. Interestingly, the PPL1-dFSB circuit is

225 involved in arousal (from sleep)^{11,13}, and our findings suggest that this circuit might be
226 more broadly involved in the processing and salience of aversive cues.

227 Taken together, our data show that *Drosophila* dopamine neurons are required
228 for both reinforcing and acute aversive reactions to alcohol. This is similar to recent
229 findings in the mammalian brain, where dopaminergic neurons are involved in opposing
230 reactions to drugs of abuse^{21,22}. We show that alterations in either of these dopamine
231 circuits can lead to a reduction in ethanol intake, emphasizing that acute sensitivity to
232 the aversive aspects of a drug are protective against the development of addiction. This
233 is in line with human genetic findings, where the strongest genetic factors that protect
234 people from developing alcohol abuse disorders are alcohol dehydrogenase variants,
235 which are involved in triggering acutely unpleasant reactions²³. Furthermore, people
236 perceiving alcohol as acutely more bitter tasting, are also less likely to develop
237 alcoholism^{24,25}. *Drosophila* thus show complex reactions to ethanol that are similar to
238 humans. In addition, distinct *Drosophila* dopaminergic circuits mediate these diverse
239 reactions. Thus, while the structural architecture of the fly brain is clearly different from
240 that of mammals, key sub-circuits are conserved in their logic and in their multifaceted
241 use of the same neurotransmitter—dopamine—which can mediate opposing behavioral
242 outcomes, depending on the specific dopaminergic circuit engaged.

243

244

245

246 **MATERIALS AND METHODS**

247 **Fly husbandry and Genetics**

248 *Drosophila melanogaster* were raised in a 12:12 hr. Light/Dark cycle on a
249 standard cornmeal/molasses diet at 25°C with 70% humidity, except for temperature
250 sensitive experiments, which used 30°C during the experiments. The genetic
251 background for all experiments used was *white** *Berlin* (unless explicitly stated).
252 Transgenic Gal4 driver lines containing different regions of the TH genomic locus (*TH-*
253 *C¹*, *D¹*, *D4*, *F2*, and *C1-Gal4*) were obtained from Dr. Mark Wu (John's Hopkins).
254 PPL1 specific-Gal4 lines were obtained from Dr. Karla Kaun (Brown University). Other
255 transgenic lines were obtained from the Bloomington stock center.

256

257 **Drug feeding protocols**

258 Pharmacological treatment with 3-iodo tyrosine (3IY, Sigma) and L-DOPA
259 (Sigma) were carried out as previously described²⁶. 3IY (10mg/ml) or L-Dopa (1mg/ml)
260 were dissolved in solutions containing 250 mM sucrose. Flies were pre-fed in a modified
261 CAFÉ assay in rectangular 4-well plates (128 x 85.5 mm, Thermo Scientific; Fig. 5).
262 Food was provided in 0.2 ml tubes with a 27 G needle hole at the bottom for drinking
263 access, a 27 G hole atop for pressure equilibration and a 25 G hole atop for filling with
264 solution. Flies were fed 3IY for a period of 48-hours and L-DOPA for 24-hours in the
265 modified CAFÉ apparatus. For the *elav-GeneSwitch* Gal4 experiments, food-deprived
266 flies were fed with 0.5 mM mifepristone (RU486) for 3 hours prior to pretreatment to
267 ethanol.

268

269 **Booz-o-mat exposure**

270 Exposure paradigms used are as previously described³. The day before ethanol
271 vapor exposure, male flies were collected in groups of 30 and put on un-yeasted food.
272 The following day, flies were transferred into the Booz-o-mat apparatus for a 20- minute
273 exposure at desired ethanol to air ratio (E/A) as described²⁷. For temperature-sensitive
274 experiments, *UAS-shi^{ts}*, *UAS-TrpA^{ts}*, and control flies were allowed to acclimate at 30°C
275 for 20 min in the Booze-o-mat before starting the 20-min exposure at 30°C. Flies were
276 then transferred to vials and placed into a 25°C/70% humidity incubator for a 24-hour
277 recovery period.

278

279 **Capillary Feeder (Café) assay**

280 24 hr after ethanol pre-exposure, 15 flies were placed into each well of the Café
281 assay apparatus as described²⁸. Preference testing was carried out for 16-hours.

282

283 **Immunohistochemistry**

284 Immunohistochemistry was performed as previously described²⁹. To visualize
285 *439B-PPL1-GAL4* expression in the brain, a *pJFRC225-5xUAS-IVS-myr::smGFP-FLAG*
286 (*smGFP-FLAG*) reporter probe³⁰ was utilized. The smGFP-FLAG transgene was
287 visualized with an anti-FLAG antibody. The presynaptic marker mouse anti-nc82
288 antibody was used to label general neuropil/brain structure. The multicolor Flip-out
289 approach³¹ was used for stochastic labeling of 439B-PPL1 neurons.

290

291 **Light-induced stimulation of DA neurons and fast-scan voltammetry**

292 All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and all

293 solutions were made with Milli-Q water (Millipore, Billerica, MA). Dissections, recordings,
294 and calibrations were performed in a simple buffer solution (131.3 mM NaCl, 3.0 mM
295 KCl, 10 mM NaH₂PO₄ monohydrate, 1.2 mM MgCl₂ hexahydrate, 2.0 mM Na₂SO₄
296 anhydrous, 1.2 CaCl₂ dihydrate, 11.1 mM glucose, 5.3 mM trehalose, pH = 7.4). Carbon
297 fiber microelectrodes were fabricated from T-650 carbon fibers (a gift of Cytec
298 Engineering Materials, West Patterson, NJ) and were used for fast-scan cyclic
299 voltammetry as previously described³². Virgin females with *UAS-CsChrimson* (a
300 chimera of CsChR and Chrimson) inserted in *attp18*³³ (a gift of Vivek Jayaraman) were
301 crossed with *TH-GAL4* (a gift of Jay Hirsh). Resulting heterozygous larvae were
302 shielded from light and raised on standard cornmeal food mixed 250:1 with 100 mM all-
303 trans-retinal. A small amount of moistened Red Star yeast (Red Star, Milwaukee, WI)
304 was placed on top of the food to promote egg laying.

305 For the protocerebrum recordings, brains were isolated into dissection buffer
306 from larvae using forceps under a dissection microscope, and the electrode was
307 implanted from the lateral edge of the tissue into the dorsal medial protocerebrum. The
308 electrode equilibrated in the tissue for 10 minutes prior to data collection and a baseline
309 recording was taken for 10 seconds prior to stimulation. Red light estimated at 0.75 mW
310 from a 617 nm fiber-coupled high-power LED with a 200 μm core optical cable
311 (ThorLabs, Newton, NJ) was used to stimulate the CsChrimson ion channel. The
312 TarHeel CV software (a gift of Mark Wightman) was used to control the light stimulation
313 and to record the current from the applied voltage. After taking a baseline 2 second
314 stimulation, 5 mM ethanol (10% in buffer) was added to the solution of fly buffer and
315 then another stimulation was recorded after 5 minutes. The concentration of ethanol

316 was increased to 15 mM and then to 45 mM. Stimulations were performed at each
317 concentration five minutes after the ethanol was added to allow for equilibration. Adding
318 increasing amounts of dissection buffer instead of ethanol was performed as a vehicle
319 control. Data are presented at mean +/- standard error of the mean (SEM) and graph
320 error bars are SEM.

321

322 **HPLC measurement of brain dopamine levels**

323 Flies were immobilized on ice (5/sample). Brains were dissected and
324 homogenized in ice-cold 0.02 M HClO₄ and 0.00025 M ascorbate solution using a
325 Kontes micro tissue grinder. Homogenate was centrifuged at 13,000 rpm for 5 min at
326 4°C. The supernatant was collected and centrifuged at 13,000 rpm for 30 sec at 4°C
327 using a 0.22 µm Ultrafree PVDF filter (MilliporeSigma, Billerica, MA). Samples (approx.
328 25 µL final volume) were stored at -80°C until analysis. The HPLC-ECD system
329 consisted of a Luna 3 µm C18 (2) 100 Å, 50 x 1 mm, LC column (Phenomenex,
330 Torrance, CA) and SenCell2 electrochemical cell at +450 mV (Antec Leyden,
331 Netherlands) with Ag/AgCl reference electrode. Aqueous mobile phase with ion-pairing
332 agents (0.50 g OSA, 0.05 g DSA, 0.13 g EDTA, 11.08 g NaH₂PO₄, 100-150 mL MeOH
333 in 1L H₂O; pH adjusted to 5.6) was delivered to the column using a LC110S piston
334 pump (Antec Leyden, Netherlands). Column and electrochemical cell were kept at 35°C
335 inside a controller (Intro model, GBC Separations, Hubbardson, MA) with a Rheodyne
336 8125 manual injector. Analog responses from the electrochemical detector were
337 digitized using an analog-to-digital converter (SS240x model, Scientific Software,
338 Pleasanton, CA). EZ Chrome Elite software was used to collect chromatograms

339 (Scientific Software, Pleasanton, CA). Sample dopamine content was estimated via the
340 calibration curve method using external dopamine standards prepared in 0.1 M H₃PO₄.
341 Reported estimates are derived from chromatograms with signal-to-noise ratio ≥ 3 that
342 met in-house quality criteria.

343

344 **Statistics**

345 Statistical significance of results in this manuscript was established using
346 analyses of variance (ANOVAs) tests with GraphPad Prism software for Mac. For the
347 post-hoc analyses, Dunnett's test was applied to control for the multiple comparison
348 when several groups were compared to the same control. Error bars in all experiments
349 represent SEM. Significance was only attributed to experimental lines that were
350 statistically different from their respective controls. Significance in all graphs shown are
351 defined as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

352

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366

367 **AUTHOR CONTRIBUTIONS**

368 S.A.O. and A.R. conceived the study. S.A.O., E.P.C., Y.N., D.W., R.A.G
369 performed experiments and analyzed the data with the other authors. R.A.G., G.M.R.,
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372

373 **COMPETING INTERESTS**

374 The authors declare no competing financial interests.

375

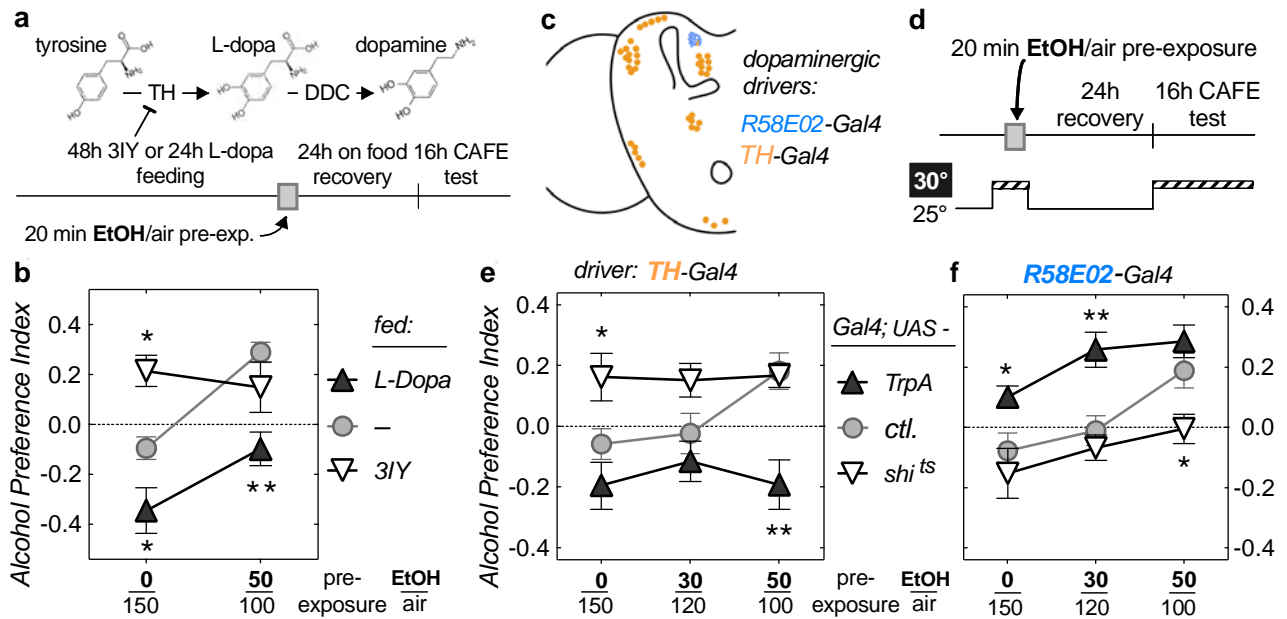
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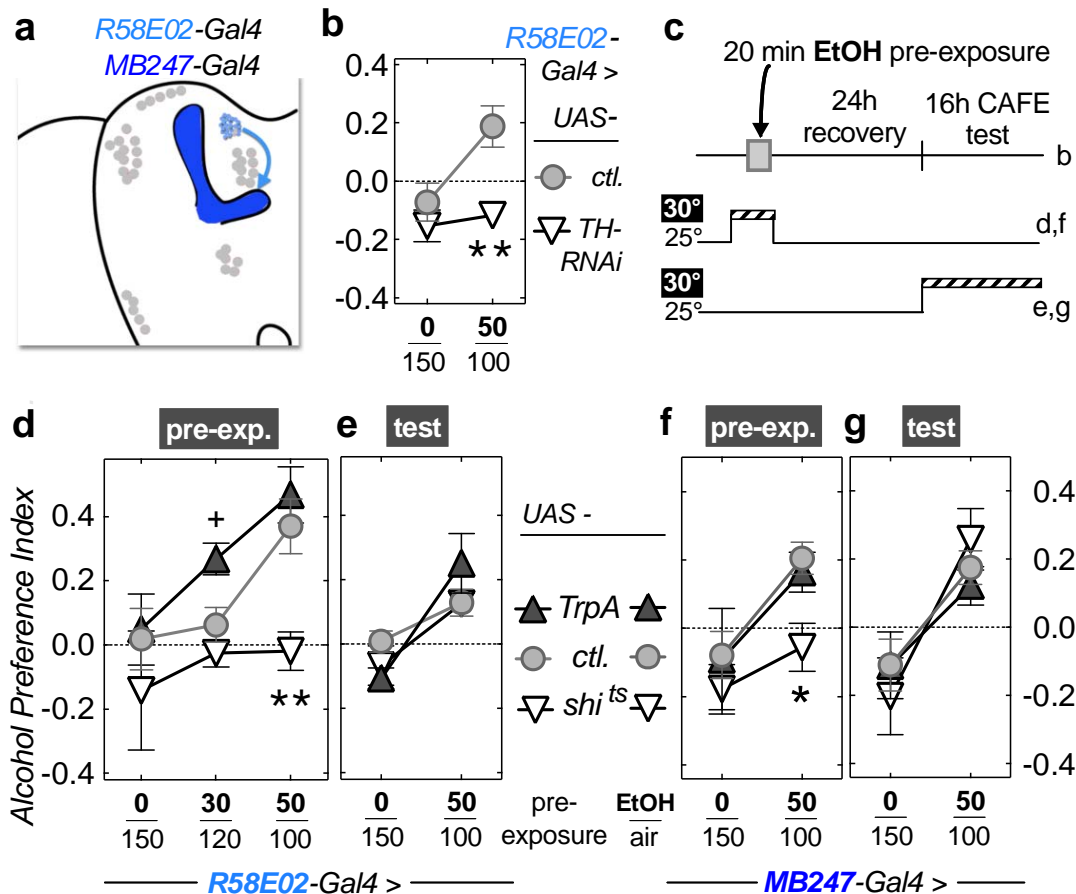
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452 **FIGURES**

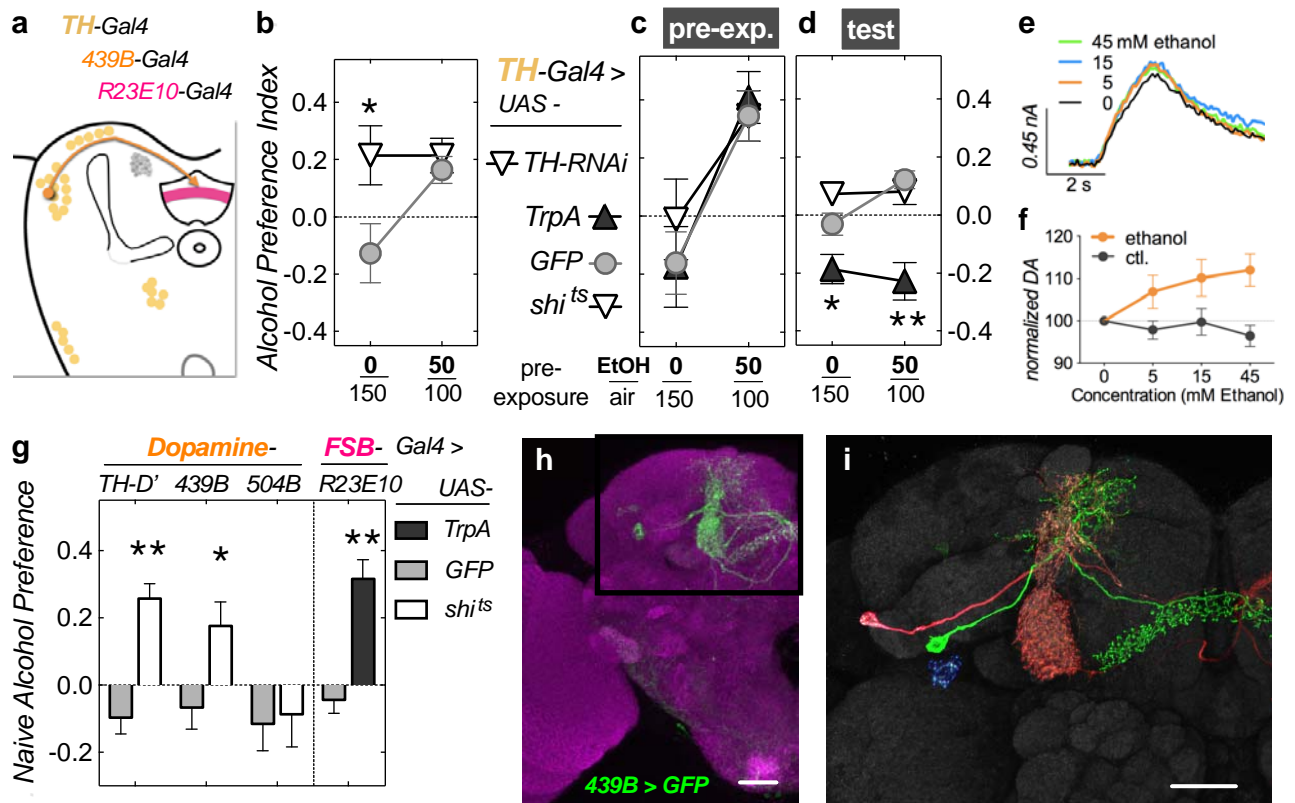


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454
455 **Fig. 1. Distinct dopamine neurons are required for naïve avoidance and experience-**
456 **dependent alcohol preference.** (a) Experimental paradigm describing drug feeding followed by
457 alcohol-induced consumption preference assay (CAFÉ stands for capillary feeder). (b) Naïve
458 control flies (0/150 alcohol/air flow mock pre-exposure, fed plain food: – grey) show slight aversion
459 to alcohol (Preference Index < 0); naïve flies with increased dopamine levels (fed L-DOPA: black)
460 show enhanced aversion; flies with reduced dopamine levels (fed 3IY: white) show naïve
461 preference. Alcohol pre-exposure (to 50/100 alcohol/air flow for 20 min the day before) induces
462 preference in control flies, but not in L-DOPA-fed flies (** $P < 0.01$, * $P < 0.05$, two-way ANOVA with
463 Dunnett's post-hoc comparisons vs. – control). (c) Schematic indicating cell bodies of dopamine
464 neurons in the fly brain and the two dopaminergic drivers used in e,f. (d) Experimental paradigm,
465 where 30° C is the effective temperature causing silencing (*shi^{ts}*), or activation (*TrpA*) of neurons.
466 (e) Silencing *TH-Gal4* dopamine neurons (white) causes naïve preference, and activating them
467 (black) suppresses experience-dependent preference, similar to the pharmacological intervention in
468 **b** (** $P < 0.01$, * $P < 0.05$; here, and in following Figures *ctl.* are *UAS-GFP* flies). (f) Conversely,
469 activating PAM dopamine neurons (*R58E02-Gal4* driver, black) facilitates experience-dependent
470 preference, while silencing these neurons (white) prevents preference acquisition (** $P < 0.01$, * $P <$
471 0.05).

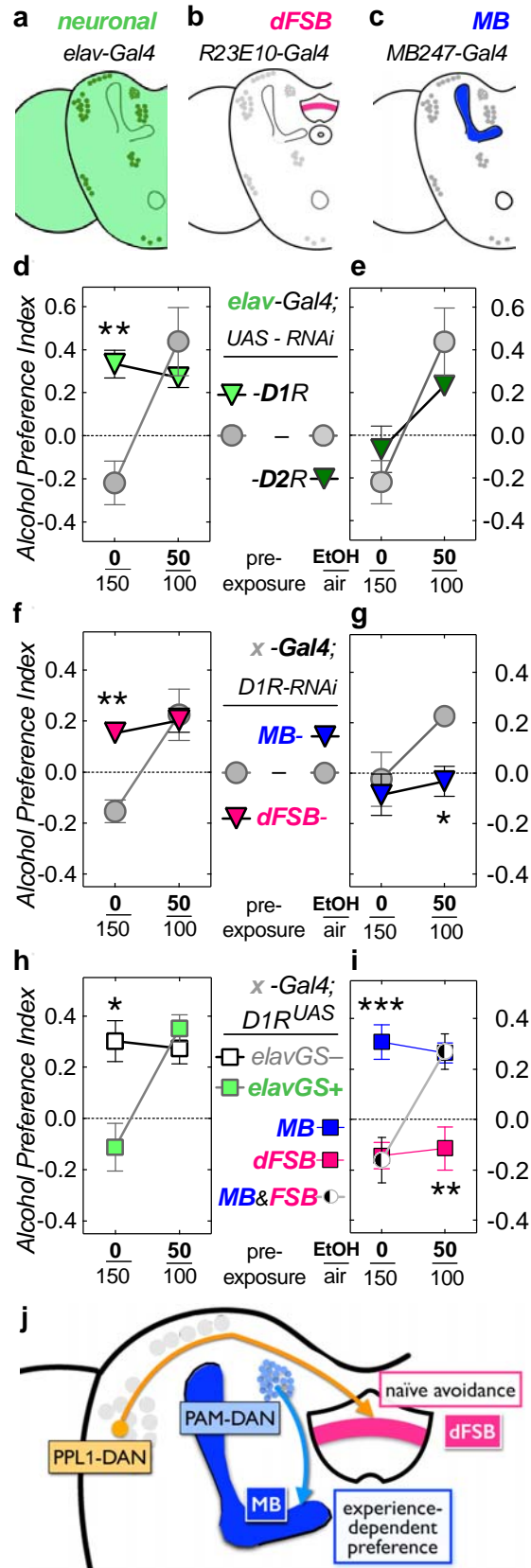


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473 **Figure 2: PAM dopamine neurons innervating the mushroom bodies are required for the**
 474 **acquisition of experience-dependent alcohol preference.** (a) Fly brain schematic indicating the
 475 two drivers used. The mushroom body is indicated by dark blue. (b) RNAi-mediated knockdown of
 476 TH (*UAS-TH-RNAi*) in *PAM-Gal4* dopamine neurons (white) prevents experience-dependent
 477 alcohol preference (** $P < 0.01$). (c) Experimental paradigm used in d-g. (d,e) Silencing *PAM-Gal4*
 478 dopamine neurons with *UAS-shi^{ts}* during pre-exposure (d, white), but not during testing (e) leads to
 479 loss of experience-dependent ethanol preference (** $P < 0.01$). Activating PAM neurons during pre-
 480 exposure with *UAS-TrpA* (d, black) also shows a trend for facilitation of preference acquisition ($^+P =$
 481 0.056). (f,g) Similar to PAM neurons, silencing the target mushroom body neurons (*MB247-Gal4*
 482 driver) with *UAS-shi^{ts}* during pre-exposure (f, white), but not during testing (g) also prevented
 483 formation of experience-dependent alcohol preference ($^*P < 0.05$).



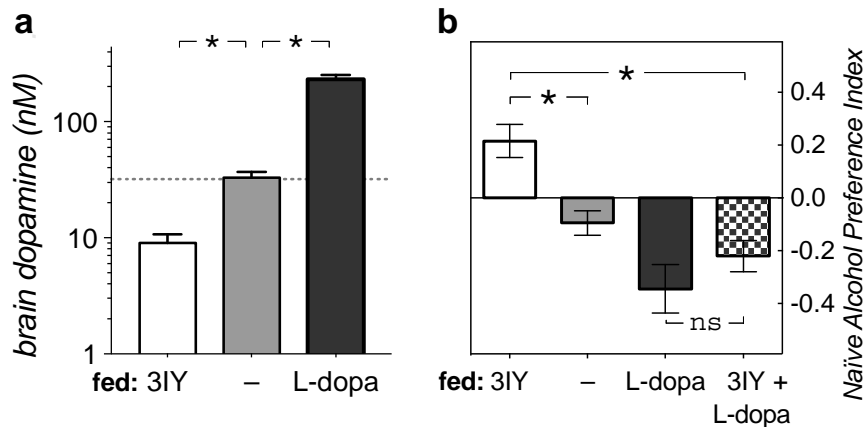
484
 485 **Figure 3: PPL1 dopamine neurons projecting to the fan-shaped body mediate acute naïve**
 486 **alcohol avoidance.** (a) Schematic highlighting dopamine cells (*TH*-, *439B-Gal4*) and their target,
 487 the fan-shaped body (*R23E10-Gal4*). (b) Knockdown of TH (*UAS-TH-RNAi*, white) in *TH-Gal4*
 488 neurons leads to naïve preference ($*P < 0.05$). (c) Activating or silencing *TH-Gal4* dopamine
 489 neurons during ethanol pre-exposure does not affect preference, while activating them during the
 490 test (d) increases alcohol avoidance, regardless of pre-exposure. (e) Representative voltammetry
 491 traces from a larval brain and (f) total dopamine released upon stimulation of *TH-Gal4* neurons
 492 (with red light, via *UAS-CsChrimson*) shows that even low levels (5 mM) of acute ethanol potentiate
 493 dopamine release. Dopamine was measured from the median protocerebrum, close to the fan-
 494 shaped body. (g) A subset of *TH-Gal4* dopamine neurons affects naïve alcohol avoidance when
 495 silenced during naïve preference testing ($**P < 0.01$, $*P < 0.05$), including *TH-D'-Gal4*, which
 496 expressed in the PPL1 cluster, and *439B-Gal4*, which expresses in three PPL1 dopamine neurons
 497 (h). (i) Higher magnification picture of two (pseudocolored) *439B-Gal4* neurons. Two dopamine
 498 neurons PPL1- $\gamma 2\alpha'1$ (red) and PPL1- $\alpha'2\alpha 2$ (cell body not visible in this picture) project to the
 499 mushroom bodies, and are also contained in the *504B-Gal4* driver, which does not affect naïve
 500 alcohol avoidance (see g). The green dopamine neuron (PPL1-dFSB) projects to the dorsal FSB,
 501 but not the MB. Activating layer 6 dFSB neurons (*R23E10-Gal4* driver) leads to naïve alcohol
 502 preference (see g).



504 **Figure 4. The dopamine D1R1 receptor is required in the mushroom bodies for experience-**
505 **dependent alcohol preference and in the fan-shaped body for naïve avoidance. (a-c)** Fly brain
506 schematics showing Gal-4 drivers used expressing in all neurons (a), dorsal fan-shaped body layer
507 6 (dFSB, b), and mushroom bodies (MB, c). (d) Pan-neuronal, RNAi-mediated knockdown of
508 dopamine D1R1 receptor (*D1R*), but not D2 receptor (*D2R*, e) abolishes naïve alcohol avoidance
509 (***P* < 0.01). (f,g) D1R knockdown in the dFSB also abolishes naïve alcohol avoidance (***P* < 0.01,
510 magenta in f), whereas, D1R knockdown in the MB abolishes experience-dependent preference
511 (***P* < 0.05, blue in g). (h,i) *D1R^{UAS}* flies are mutants that lack *D1R* expression, but this can be
512 restored by the introduction of a Gal4-driver . Together with an RU486-inducible pan-neuronal Gal4
513 driver (*elavGS*, for RU486-Gene-Switch), these *D1R^{UAS}* mutants show naïve alcohol preference
514 when Gal4 is not induced (*elavGS*–, white in h). This is rescued when adult flies are fed RU486
515 before the ethanol-pre-exposure and D1R expression is restored (*elavGS*+, green in h). When *D1R*
516 expression is restored in the MB, *D1R^{UAS}* flies still show naïve alcohol preference (*MB247-*
517 *Gal4>D1R^{UAS}*, blue in i). Conversely, restoring *D1R* expression in the FSB only rescues naïve
518 alcohol avoidance, but now reveals a loss of experience-dependent preference (*R23E10-*
519 *Gal4>D1R^{UAS}*, magenta in i), presumably due to lack of *D1R* in the MB. Indeed, when *D1R*
520 expression is restored in both the MB and the FSB, *D1R^{UAS}* mutants show normal naïve alcohol
521 avoidance succeeded by preference upon an alcohol pre-exposure (*MB247-Gal4;R23E10-*
522 *Gal4>D1R^{UAS}*, black and white in i). (j) Schematic highlighting the two dopamine circuits involved in
523 naïve alcohol avoidance (PPL1–dFSB) and the acquisition of experience-dependent alcohol
524 preference (PAM–MB).
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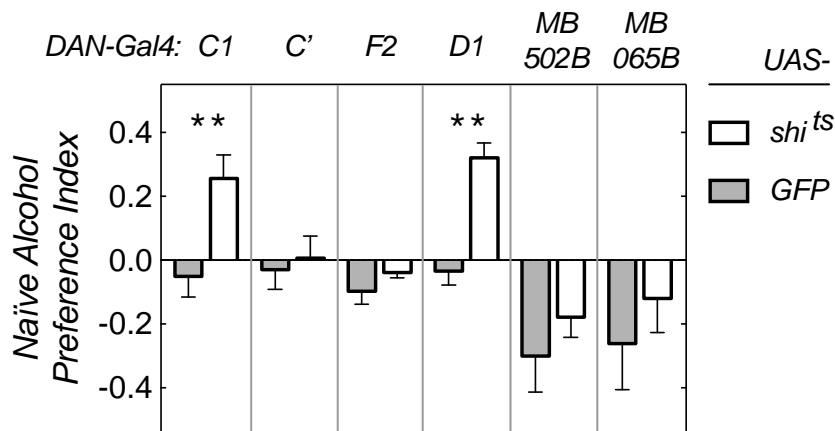
526 **Supplementary Materials:**

527 **Figures S1-4**



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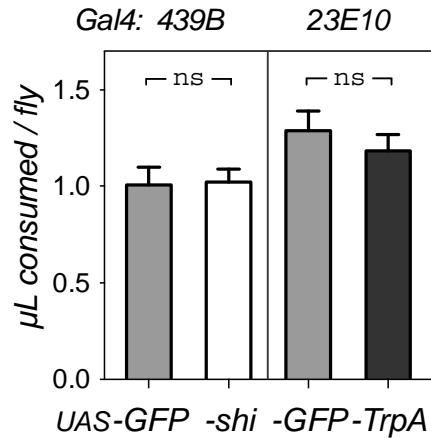
529 **Supplementary Figure 1. Dopamine pharmacology.** (a) Brain dopamine concentrations in
 530 pooled fly brain homogenates after drug feeding, as measured by HPLC-ECD ($*P < 0.05$, $n = 3$
 531 replicates of 5 brains homogenized). (b) Simultaneous feeding of 3IY and L-dopa converts 3IY-
 532 induced naïve preference back to naïve avoidance. (Data as in main Fig. 1B, with the addition of
 533 the co-feeding, checkered; $*P < 0.05$ one-way ANOVA with Dunnett's post-hoc comparison).



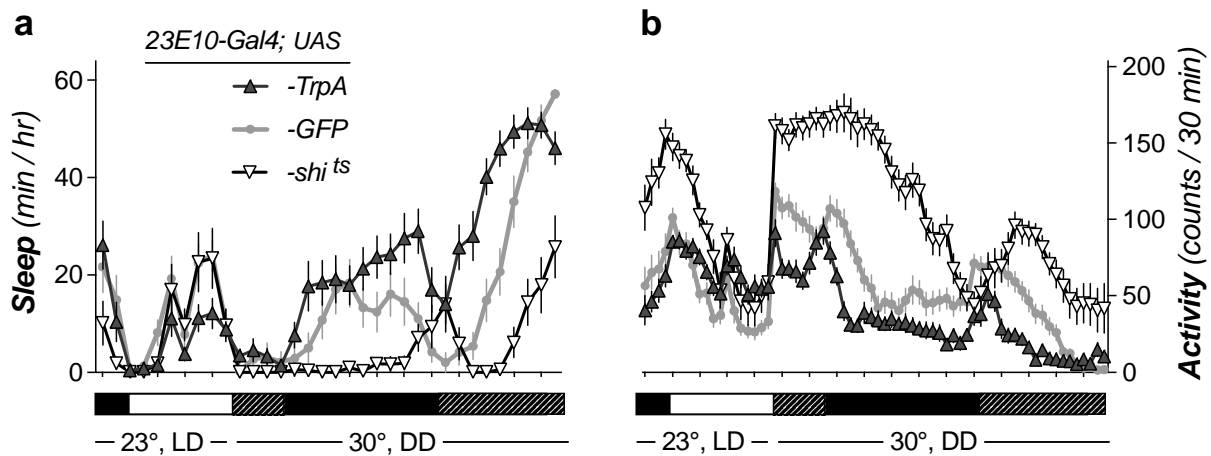
534

535 **Supplementary Figure 2. Silencing of different DAN causes naïve alcohol aversion.** 2 of 6
 536 transgenic *DAN-Gal4* driver lines (in addition to the ones shown in Fig. 3g) caused naïve
 537 preference to alcohol when silenced with *UAS-shi^{ts}* compared to their respective *UAS-GFP*
 538 controls ($*P < 0.05$, one-way ANOVA with Bonferroni correction, $n = 6-12$).

539



540
541 **Supplemental Figure 3. Naïve alcohol preference caused by manipulation of the PPL1-**
542 **dFSB circuit does not alter total food consumption.** Total food consumption (sucrose +
543 sucrose/ethanol mixture) per fly is indicated over the 16-hour CAFÉ test. No significant
544 differences were observed between experimentals and controls (grey). This was true for other
545 DAN-Gal4 lines affecting naïve alcohol aversion too (data not shown).
546



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549 **Supplemental Figure 4. Sleep induction by activation of dFSB.** (a) Shifting *23E10-Gal4;*
550 *UAS-TrpA* to 30°C (black) caused an increase in total sleep duration, but did not just put the
551 flies to sleep: considerable levels of activity remained (b). Note that this is the same
552 temperature and light regimen as used for the CAFÉ preference test.

553
554