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1 **Genetics of Cocaine and Methamphetamine**

2 **Consumption and Preference in *Drosophila melanogaster***

3

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17 Running title: Genetics of Drosophila drug consumption

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19 Keywords: *Drosophila melanogaster* Genetic Reference Panel; genome-wide association

20 analysis; RNAi; genetic interaction network; mushroom bodies; dopamine

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21 **ABSTRACT**

22 Illicit use of psychostimulants, such as cocaine and methamphetamine, constitutes a significant
23 public health problem. Whereas neural mechanisms that mediate the effects of these drugs are
24 well-characterized, genetic factors that account for individual variation in susceptibility to
25 substance abuse and addiction remain largely unknown. *Drosophila melanogaster* can serve as
26 a translational model for studies on substance abuse, since flies have a dopamine transporter
27 that can bind cocaine and methamphetamine, and exposure to these compounds elicits effects
28 similar to those observed in people, suggesting conserved evolutionary mechanisms underlying
29 drug responses. Here, we used the *D. melanogaster* Genetic Reference Panel to investigate the
30 genetic basis for variation in psychostimulant drug consumption, to determine whether similar or
31 distinct genetic networks underlie variation in consumption of cocaine and methamphetamine,
32 and to assess the extent of sexual dimorphism and effect of genetic context on variation in
33 voluntary drug consumption. Quantification of natural genetic variation in voluntary
34 consumption, preference, and change in consumption and preference over time for cocaine and
35 methamphetamine uncovered significant genetic variation for all traits, including sex-, exposure-
36 and drug-specific genetic variation. Genome wide association analyses identified both shared
37 and drug-specific candidate genes, which could be integrated in genetic interaction networks.
38 We assessed the effects of ubiquitous RNA interference (RNAi) on consumption behaviors for
39 34 candidate genes: all affected at least one behavior. Finally, we utilized RNAi knockdown in
40 the nervous system to implicate dopaminergic neurons and the mushroom bodies as part of the
41 neural circuitry underlying experience-dependent development of drug preference.

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43 **AUTHOR SUMMARY**

44 Illicit use of cocaine and methamphetamine is a major public health problem. Whereas the
45 neurological effects of these drugs are well characterized, it remains challenging to determine
46 genetic risk factors for substance abuse in human populations. The fruit fly, *Drosophila*
47 *melanogaster*, presents an excellent model for identifying evolutionarily conserved genes that
48 affect drug consumption, since genetic background and exposure can be controlled precisely. We
49 took advantage of natural variation in a panel of inbred wild derived fly lines with complete genome
50 sequences to assess the extent of genetic variation among these lines for voluntary consumption
51 of cocaine and methamphetamine and to explore whether some genetic backgrounds might show
52 experience-dependent development of drug preference. The drug consumption traits were highly
53 variable among the lines with strong sex-, drug- and exposure time-specific components. We
54 identified candidate genes and gene networks associated with variation in consumption of cocaine
55 and methamphetamine and development of drug preference. Using tissue-specific suppression
56 of gene expression, we were able to functionally implicate candidate genes that affected at least
57 one consumption trait in at least one drug and sex. In humans, the mesolimbic dopaminergic
58 projection plays a role in drug addiction. We asked whether in Drosophila the mushroom bodies
59 could play an analogous role, as they are integrative brain centers associated with experience-
60 dependent learning. Indeed, our results suggest that variation in consumption and development
61 of preference for both cocaine and methamphetamine is mediated, at least in part, through a
62 neural network that comprises dopaminergic projections to the mushroom bodies.

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65 **INTRODUCTION**

66 Illicit use of cocaine and methamphetamine constitutes a significant public health problem that
67 incurs great socioeconomic costs in the United States and worldwide [1-3]. Cocaine and the
68 amphetamine class of drugs are potent central nervous system stimulants that act by raising
69 synaptic concentrations of biogenic amines. Cocaine inhibits neurotransmitter reuptake at
70 dopaminergic, serotonergic and noradrenergic synapses [4,5]. Amphetamine increases
71 neurotransmission by promoting the release of dopamine from presynaptic vesicles through its
72 actions on the vesicular monoamine transporter and subsequent reverse flux of dopamine via
73 the dopamine transporter and through the plasma membrane into the synaptic cleft [6,7].

74 Amphetamine, methamphetamine, and methylphenidate are used clinically to treat
75 attention deficit hyperactivity disorder and narcolepsy. Long term use of these compounds,
76 however, can lead to addiction, and ultimately death [8]. The addictive properties of these drugs
77 are mediated through the dopaminergic mesolimbic reward pathway, which projects from the
78 ventral tegmental area via the nucleus accumbens to prefrontal cortex [9]. Although most
79 studies on psychostimulants focus on addiction, addiction represents only one facet of the
80 diverse organismal effects that result from psychostimulant drug abuse. These drugs exert a
81 wide range of physiological and behavioral effects, including suppression of appetite, which can
82 result in malnutrition, and severe cardiovascular, respiratory and renal disorders. Use of cocaine
83 and amphetamine can also cause mental disorders, including paranoia, anxiety, and psychosis
84 [10,11].

85 Susceptibility to the effects of cocaine and methamphetamine is likely to vary among
86 individuals and be determined both by environmental and genetic factors. However, there is
87 limited information regarding the genetic basis of susceptibility to the effects of these drugs in
88 human populations [12]. Twin and adoption studies have focused primarily on alcohol abuse
89 and illicit drugs, such as cannabis, with heritability estimates ranging from ~30-70% [13,14].
90 Most studies on psychostimulant addiction to date have centered on candidate genes

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91 associated with neurotransmission in the mesolimbic projection [12], and many of these are
92 inconclusive or contradictory. For example, some studies reported that alleles of the dopamine
93 D2 receptor were associated with substance abuse [15-18], whereas others did not replicate
94 this finding [19-24]. Similar contradictory results have been obtained for association analyses
95 between polymorphisms in the dopamine transporter gene and cocaine-related phenotypes [24-
96 28]. These contradictory findings may be due in part to failure to account for multiple testing or
97 population structure [29]. However, genetic studies of substance abuse and addiction in human
98 populations are challenging due to diverse social conditions and physical environments,
99 confounding factors with comorbid conditions such as alcoholism or psychiatric disorders, and
100 difficulty to recruit large numbers of study subjects due to criminalization.
101 *Drosophila melanogaster* is an excellent model for identifying genes that affect drug
102 consumption behaviors since both the genetic background and environment, including exposure
103 to drugs, can be controlled precisely. These results have translational potential since 75% of
104 disease-causing genes in humans have a fly ortholog [30]. High resolution X-ray crystallography
105 has shown that the *D. melanogaster* dopamine transporter has a central conformationally pliable
106 binding site that can accommodate cocaine, methamphetamine and their closely related
107 analogues [31]. Similar to its effects in humans, methamphetamine suppresses sleep, causes
108 arousal and suppresses food intake in flies [32-34]. In addition, cocaine, amphetamine and
109 methylphenidate exert quantifiable locomotor effects in flies [35-41]. Thus, despite profound
110 differences between the neuroanatomical organization of the fly and vertebrate brains, it is likely
111 that behavioral and physiological effects of methamphetamine and cocaine are mediated, at
112 least in part, by evolutionarily analogous mechanisms.
113 Here, we used the inbred, sequenced lines of the *D. melanogaster* Genetic Reference
114 Panel (DGRP [42,43]) to investigate the genetic basis for variation in psychostimulant drug
115 consumption. We used a two-capillary Capillary Feeding (CAFE) assay [44-46] to quantify
116 voluntary consumption, preference and change of consumption and preference over time for

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117 cocaine and methamphetamine. Since cocaine and methamphetamine both target
118 dopaminergic synaptic transmission, but through different mechanisms, we asked to what extent
119 genetic networks that underlie variation in consumption of cocaine and methamphetamine
120 incorporate the same or different genes. We also sought to determine the extent of sexual
121 dimorphism for naïve and experience-dependent voluntary drug intake. In addition, we asked
122 how much variation in voluntary drug consumption exists among different DGRP lines and what
123 fraction of that variation is accounted for by genetic variation. We showed that there is naturally
124 occurring genetic variation for all drug consumption traits with strong sex-, drug- and exposure
125 time-specific components. We performed genome wide association (GWA) analyses to identify
126 candidate genes associated with the drug consumption behaviors that could be mapped to a
127 genetic interaction network. We tested the effects of RNAi mediated suppression of gene
128 expression [47] on all consumption behaviors for 34 candidate genes and found that all affected
129 at least one behavior in at least one drug and sex. Finally, we used RNAi to suppress gene
130 expression in neurons, glia, the mushroom bodies and dopaminergic neurons in a subset of
131 genes and showed that innate preference and the development of preference for
132 psychostimulant drugs involves dopaminergic neurons and the mushroom bodies, neural
133 elements associated with experience-dependent modulation of behavior.
134
135 **RESULTS**

136 **Quantitative genetic analysis of drug consumption behaviors in the DGRP**

137 We used a two-capillary CAFE assay [44-46] to enable flies to choose to consume either
138 sucrose or sucrose supplemented with 0.2 mg/ml cocaine (or 0.5 mg/ml methamphetamine),
139 analogous to the two-bottle choice assay used in rodent studies [48] (Fig.1). We quantified
140 consumption for three consecutive days for males and females from each of 46 DGRP lines that
141 were unrelated, free of chromosomal inversions, and free of infection with the endosymbiont
142 *Wolbachia pipientis* [43; Table S1]. These data enabled us to assess whether there is naturally

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143 occurring genetic variation in this population for naïve consumption of each solution and
144 preference, and change of consumption and preference upon repeated exposures (*i.e.*,
145 experience-dependent modification of behavior).

146 We performed four-way mixed model analyses of variance (ANOVA) to partition variation
147 in consumption between DGRP lines, males and females, drug *vs.* sucrose, and the three
148 exposures. All main effects were significant for both drugs (Table 1), indicating genetic variation
149 for consumption, difference between amount of sucrose and drug consumed, sexual
150 dimorphism, and experience-dependent modulation of behavior. We are most interested in the
151 two- and three-way interaction terms involving Line, as they indicate genetic variation in sexual
152 dimorphism ($L \times X$), change of consumption between exposures ($L \times E$), preference for sucrose or
153 drug solution ($L \times S$), and change of preference for sucrose or drug between exposures ($L \times E \times S$).
154 With the exception of $L \times S$, these interaction terms were significant for both the cocaine and
155 methamphetamine analyses (Table 1).

156 We next performed reduced ANOVA models to quantify broad sense heritabilities (H^2)
157 for consumption and change in consumption traits (Table S2). We found significant genetic
158 variation in consumption of both drugs and sucrose alone within each sex and exposure, with H^2
159 ranging between 0.20 and 0.38 for cocaine consumption and between 0.22 and 0.30 for
160 methamphetamine consumption (Fig. 2, Table S2). Further, there was significant genetic
161 variation for the change in consumption of sucrose alone or drug in both sexes between the
162 third and first exposures, with H^2 ranging between 0.14 and 0.18 for cocaine and between 0.17
163 and 0.22 for methamphetamine (Fig. 2, Table S2). Thus, there is genetic variation for both
164 consumption and experience-dependent consumption of both drugs and sucrose alone in the
165 DGRP.

166 Finally, we defined preference in two ways: as the difference between amount of drug
167 and sucrose alone consumed (Preference A), and as this difference scaled by the total amount
168 of both solutions consumed (Preference B). Preference values of 0 indicate equal consumption

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169 of sucrose alone and sucrose containing drug; values > 0 represent preference for the drug and
170 values < 0 indicate drug avoidance. Both preference metrics were significantly genetically
171 variable for each sex and exposure for cocaine, with H^2 ranging from 0.06-0.16; while for
172 methamphetamine, both preference metrics were significantly genetically variable in females for
173 all exposures (H^2 from 0.05-0.18) and for males in the second and third exposures (H^2 from
174 0.08-0.11) (Table S2). For cocaine, the difference in preference A between exposures 3 and 1
175 was significant only in females (H^2 = 0.11) while the difference in Preference B was significant
176 for females (H^2 = 0.13) and males (H^2 = 0.05). For methamphetamine, the difference in
177 Preference A was significant in males (H^2 = 0.04) and the difference in Preference B was
178 significant in females (H^2 = 0.04) (Table S2). Thus, there is genetic variation for both innate drug
179 preference and experience-dependent drug preference in the DGRP.

180 The heritabilities of consumption traits are low, as is typical for behavioral traits,
181 indicating that environmental factors, including previous experience, predominantly contribute to
182 the observed phenotypic variation. The advantage of performing multiple replicate
183 measurements of each DGRP line is that the broad sense heritabilities of line means (Table S3)
184 used in the GWA analyses (see below) are much greater than heritabilities based on individual
185 vial replicates (Table S2).

186 We computed the genetic and phenotypic correlations between males and females for
187 the consumption behaviors, between exposures for consumption and preference, and between
188 solutions (Table S4). Cross-sex genetic correlations for consumption tended to decrease with
189 the number of exposures for both cocaine and methamphetamine, suggesting that the
190 experience-dependent modification of consumption is sex-specific. Consumption of drugs and
191 sucrose is highly correlated across the three exposures (albeit significantly different from unity),
192 while the correlations of drug preference across exposures are low to moderate for both cocaine
193 and methamphetamine in both sexes. Although the consumption of drugs and sucrose for
194 cocaine and methamphetamine are genetically and phenotypically correlated in both sexes,

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195 preference for the two drugs is not significantly correlated. Finally, Preference A and Preference
196 B within each exposure are nearly perfectly correlated, as expected since the difference in
197 consumption is in both metrics.
198 In summary, we found that there is extensive genetic variation in consumption and
199 preference as well as change in consumption and preference with repeated exposures for both
200 cocaine and methamphetamine across different genetic backgrounds, and that genetic variation
201 for these traits has significant sex- and drug-specific components.
202
203 **Genome wide association analyses of drug consumption in the DGRP**
204 Our quantitative genetic analyses of consumption in the DGRP indicate that there is genetic
205 variation for all traits assessed, and that the traits have a complex correlation structure
206 indicating partially common and partially distinct genetic bases. Therefore, we performed single
207 variant GWA analyses for 12 traits (drug and sucrose consumption exposure 1, drug and
208 sucrose consumption exposure 3, change in drug and sucrose consumption, preference A
209 exposure 1, preference A exposure 3, preference B exposure 1, preference B exposure 3,
210 change in preference A, and change in preference B) for cocaine and methamphetamine,
211 separately for males and females. We performed association tests for 1,891,456 DNA sequence
212 variants present in the 46 DGRP lines with minor allele frequencies greater than 0.05 [43].
213 At a lenient significance threshold of $P < 5 \times 10^{-5}$, we identified 1,441 polymorphisms in
214 or near (within 1 kb of the start and end of the gene body) 725 genes for all consumption
215 behaviors related to cocaine, and 1,413 polymorphisms in or near 774 genes for
216 methamphetamine exposure (Table S5). The majority of these variants had sex-specific effects.
217 A total of 40 variants and 141 genes overlapped between cocaine and methamphetamine. The
218 variants in or near genes implicate candidate genes affecting consumption behaviors, while the
219 intergenic variants could potentially contain regulatory motifs for transcription factor-binding
220 sites or chromatin structure regulating these traits. Only two variants are formally significant

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221 following a Bonferroni correction for multiple tests ($P < 2.64 \times 10^{-8}$). *2L*_10179155_SNP is
222 located within an intronic region in *CG44153* and affects experience-dependent development of
223 methamphetamine preference in both sexes. Its human homolog *ADGRB3* encodes a G-protein
224 coupled receptor, which contributes to the formation and maintenance of excitatory synapses
225 [49] and has been implicated in GWA studies on human addiction [50]. *3R*_27215016_SNP is a
226 synonymous SNP in the coding sequence of *CG1607* and affects naïve consumption of
227 sucrose. *CG1607* encodes an amino acid transmembrane transporter. One of its human
228 orthologs, *SLC7A5*, is an amino acid transporter, mutations in which are associated with autism
229 spectrum disorder and defects in motor coordination [51].

230 While not formally significant, we identified genes previously associated with cocaine-
231 related behaviors (*Bx* [*Lmo*], *loco*, *Tao*) and ethanol-related behaviors (*Bx*, *DopR*, *Egfr*, *hppy*,
232 *Tao*, *Tbh*) [52] in *D. melanogaster*. In addition, the genes implicated by the GWA analyses are
233 enriched for multiple gene ontology (GO) categories and pathways [53,54] at a false discovery
234 rate < 0.05 (Table S5). GO terms involved in nervous system development and function were
235 among the most highly enriched, consistent with the known neurobiological mechanisms of
236 action of these drugs. Finally, we note that ~ 70% of the candidate genes from the GWA
237 analyses have human orthologs, and many of these genes have previously been associated
238 with cocaine or methamphetamine abuse in humans or with behaviors associated with intake
239 and response to various psychoactive substances (alcohol, cannabis, nicotine, opioids) in
240 humans as well as zebrafish, mouse and rat models (Table S6). This suggests that cocaine and
241 methamphetamine exert their effects in flies and humans through evolutionarily conserved
242 neural mechanisms.

243 These results suggest a highly polygenic architecture for variation in consumption and
244 drug preference, and that the genetic underpinnings for variation in consumption or preference
245 are both shared and distinct for cocaine and methamphetamine, consistent with the quantitative
246 genetic analyses.

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247

248 **A genetic interaction network for consumption behaviors**

249 We next asked whether the genes we identified in the GWA analyses belonged to a known
250 genetic interaction network. Since the consumption behaviors are highly inter-correlated, we
251 queried whether all 1,358 candidate genes from the GWA analyses for both cocaine and
252 methamphetamine combined could be clustered into significant sub-networks based on curated
253 genetic interactions in Drosophila. If we do not allow any missing genes, we find a significant (P
254 $= 9.99 \times 10^{-4}$) network of 81 candidate genes (Fig. 3, Table S7), most of which (88.9%) are
255 predicted to have human orthologs [55].

256 We performed enrichment analyses [53,54] to gain insight in the biological context for
257 genes in the network using a false discovery rate < 0.05. Surprisingly, many canonical signaling
258 pathways are highly enriched, including the Wingless (Wnt), Cadherin, Cholecystokinin
259 Receptor (CCKR), Transforming Growth factor beta (TGF), and Fibroblast Growth Factor (FGF)
260 signaling pathways. Concomitantly, we find high enrichment of molecular function GO terms
261 associated with regulation of transcription and DNA and protein binding, and biological function
262 GO terms associated with development (including the development of the nervous system;
263 Table S7). These results suggest that naturally occurring genetic variation in nervous system
264 development is associated with variation in propensity to consume psychostimulant drugs.
265 Furthermore, our results indicate that natural variants in key genes regulating all aspects of fly
266 development and function can be associated with variation in drug consumption behaviors.

267

268 **Functional evaluation of candidate genes**

269 We used RNA interference (RNAi) to functionally test whether reduced expression of candidate
270 genes implicated by the GWA analyses affect consumption phenotypes. We selected 34
271 candidate genes for RNAi mediated suppression of gene expression. A total of nine of the
272 candidate genes were in the network; the others were chosen based on gene expression in the

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273 nervous system and their known role in nervous system function, as well as belonging to
274 enriched pathways and gene ontology categories. We measured consumption of cocaine and
275 sucrose (Table S8) and methamphetamine and sucrose (Table S9) for three consecutive days,
276 separately for males and females, for each of the RNAi and control genotypes, exactly as
277 described for the DGRP lines.

278 We performed three-way fixed effect ANOVAs for each *UAS*-RNAi and control genotype,
279 separately for males and females (Tables S10, S11). The main effects in these models are
280 genotype (L, RNAi and control), solution (S, sucrose and drug) and exposure (E, first and third).
281 A significant L effect denotes a difference in overall consumption between the RNAi and control
282 genotypes; a significant S effect indicates a difference in preference between sucrose alone and
283 sucrose with drug; and a significant E effect indicates a difference in consumption between
284 exposures 1 and 3. Significant $L \times S$ and $L \times E$ interaction terms denote, respectively, a difference
285 in preference between the RNAi and control genotypes, and a difference in consumption
286 between exposures 1 and 3 between the two genotypes. A significant $L \times S \times E$ interaction
287 indicates a change in preference with repeated exposure between the RNAi and control
288 genotypes. We are most interested in the main effect of genotype and interactions with
289 genotype; *i.e.*, consumption, preference, change of consumption and change of preference.

290 First, we used a weak ubiquitous *GAL4* driver crossed to all 34 *UAS*-RNAi genotypes
291 and their respective controls. All candidate genes had a significant ($P < 0.05$) effect on at least
292 one of the consumption traits in at least one drug or sex combination. A total of 22 (25) genes
293 affected consumption of cocaine (methamphetamine), 21 (23) affected a change of
294 consumption with exposure to cocaine (methamphetamine), 16 (10) affected cocaine
295 (methamphetamine) preference, and 11 (11) affected a change in cocaine (methamphetamine)
296 preference with exposure in males and/or females (Tables S10, S11, Fig.s S1-S3). There were
297 pronounced sex- and drug-specific effects for all drug-related traits. The majority of RNAi
298 genotypes showed reduced consumption of cocaine and/or methamphetamine compared to

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299 their controls, dependent on exposure and sex. If consumption is positively associated with
300 gene expression, this suggests that the products of these genes contribute to drug
301 consumption. On the other hand, several RNAi constructs caused increased drug consumption,
302 suggesting that naturally occurring variants that decrease expression of these genes could
303 predispose to drug preference. Finally, several RNAi-targeted genes exhibit a relative increase
304 or decrease in drug consumption compared to the control at the third exposure, indicating
305 experience-dependent change in preference.

306 To extend and refine our RNAi analysis, we next selected 10 genes (*Dop1R1*, *Ect4*, *ed*,
307 *mld*, *msi*, *Oct-TyrR*, *olf413*, *Snoo*, *Vha100-1*, *wmd*) from among those that showed phenotypic
308 effects when targeted by RNAi under the ubiquitous driver and which have known effects on the
309 nervous system. We assessed functional effects of these genes on consumption traits when
310 their corresponding RNAi constructs were expressed under the control of the neuronal-specific
311 *elav* driver or glial-specific *repo* driver. All of these genes had a significant ($P < 0.05$) effect on at
312 least one of the consumption traits in at least one drug or sex combination under the *elav* driver,
313 and all but *Snoo* had significant effects on at least one of the consumption traits in at least one
314 drug or sex combination under the *repo* driver. With neuronal-specific suppression of gene
315 expression, 9 (10) genes affected consumption of cocaine (methamphetamine), 6 (7) affected a
316 change in consumption with exposure to cocaine (methamphetamine), 2 (7) affected cocaine
317 (methamphetamine) preference, and 3 (6) affected a change in cocaine (methamphetamine)
318 preference with exposure in males and/or females (Tables S10, S11, Fig.s 4, S4). With glia-
319 specific suppression of gene expression, 4 (7) genes affected consumption of cocaine
320 (methamphetamine), 7 (6) affected a change in consumption with exposure to cocaine
321 (methamphetamine), 3 (0) affected cocaine (methamphetamine) preference, and 2 (3) affected
322 a change in cocaine (methamphetamine) preference with exposure in males and/or females
323 (Tables S10, S11, Fig.s 4, 5, S4). These effects were largely sex-, drug- and driver-specific. We

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324 infer from these results that variation in gene expression in both neurons and glia contributes to
325 phenotypic variation in drug intake behaviors.
326 In humans, the mesolimbic dopaminergic projection plays a role in drug addiction. In
327 Drosophila, the mushroom bodies could play an analogous role, as they are integrative centers
328 in the fly brain associated with experience-dependent learning [56,57], dependent on
329 dopaminergic input. To test whether the mushroom bodies and dopaminergic projection neurons
330 could serve as neural substrates that contribute to variation in drug consumption or preference,
331 we focused on four genes (*Dop1R1*, *ed*, *msi*, *Snoo*,) that showed robust phenotypic effects
332 when targeted with a corresponding *elav*-driven RNAi. Knockdown of all four genes with a
333 mushroom body specific driver resulted in significant effects on consumption of cocaine and/or
334 methamphetamine for at least one drug and sex combination (Tables S10, S11, Fig.s 4, 5, S5).
335 Expression of RNAi in mushroom bodies affected change in consumption of cocaine and
336 methamphetamine for *Dop1R1*; cocaine preference and change of methamphetamine
337 preference for *ed*; change in consumption of cocaine for *msi*; and cocaine and
338 methamphetamine preference, cocaine preference, change of cocaine preference and change
339 of consumption of methamphetamine for *Snoo*. Expression of RNAi in dopaminergic neurons
340 affected change of consumption of cocaine and change in methamphetamine preference for
341 *Dop1R1*; consumption for cocaine and methamphetamine, change of consumption of
342 methamphetamine and cocaine preference for *ed*; consumption of cocaine and
343 methamphetamine, change of consumption of cocaine, and cocaine preference for *msi*; and all
344 four traits for *Snoo* (Tables S10, S11, Fig.s 4, 5, S5). These effects are largely sex-, drug- and
345 driver-specific.
346 These results suggest that, despite differences in the genetic underpinnings of
347 susceptibility to cocaine and methamphetamine, phenotypic manifestation of genetic variation in
348 consumption and development of preference for both drugs is channeled through a neural
349 network that comprises dopaminergic projections to the mushroom bodies.

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350

351 **DISCUSSION**

352 Although studies using mice [58,59], rats [60,61], primates [62] and humans [63] provide
353 important information about the cellular, developmental, physiological, and behavioral effects of
354 psychostimulants, these systems are less suited to dissecting the relationship between naturally
355 occurring genetic variation and phenotypic variation in individual susceptibility to drug
356 consumption and/or preference. Here, we show that *D. melanogaster* harbors substantial
357 naturally occurring variation for all consumption-related behaviors, including experience-
358 dependent change in consumption, innate drug preference and experience-dependent change
359 in preference, under conditions where we can obtain replicated measurements of consumption
360 for each genotype in a choice assay performed over three successive days under controlled
361 environmental conditions. We show that genetic variation for consumption and preference
362 metrics is both shared between males and females and the different exposures, but is also sex-,
363 exposure- and drug-specific. Sex differences in drug self-administration and addiction have also
364 been shown in humans and mammalian animal models [64-72].

365 The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) defines
366 11 criteria for substance use disorder in humans, all related to continuing to use of the
367 substance despite adverse social and physiological effects and the development of tolerance
368 with repeated exposure. The DSM-V also recognizes that there is individual variability of
369 unknown etiology for the propensity both to experiment with psychostimulants and to develop
370 symptoms of substance abuse following initial exposure. Previous studies of effects of cocaine
371 [35,37-39,73-76] and methamphetamine [77] in Drosophila examined mutations and
372 pharmacological interventions using locomotor-based assays, clearly demonstrating an adverse
373 effect of these substances. However, previous Drosophila studies have not assessed naturally
374 occurring variation in drug self-administration and change in this behavior on repeated

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375 exposure, which may better model the genetic basis of individual susceptibility – or resistance –
376 to substance abuse and the development of tolerance (increased drug preference over time).
377 To begin to understand the nature of the genetic basis for variation in drug consumption
378 and preference, we performed GWA analyses for all consumption traits, separately for cocaine
379 and methamphetamine, using 1,891,456 DNA sequence variants present in the 46 DGRP lines
380 with minor allele frequencies greater than 0.05 [43]. We identified 1,358 unique candidate genes
381 using a lenient significance threshold of 5×10^{-5}. We hypothesized that these candidate genes
382 would be enriched for true positive associations despite the low power of the GWA analyses
383 and that choosing genes for functional evaluation from this list would be more productive than
384 choosing genes at random. Observations supporting this hypothesis are that mutations in
385 several candidate genes have previously been shown to affect cocaine or ethanol-related
386 phenotypes in Drosophila [52], that the candidate genes are highly enriched for GO terms
387 involved in the development and function of the nervous system, and that 81 candidate genes
388 can be assembled into a known genetic interaction network (Fig. 3), which is highly unlikely (P =
389 9.9×10^{-3}) to occur by chance. The candidate genes in the significant genetic interaction
390 network are enriched for several canonical signaling pathways as well as all aspects of
391 development, including nervous system development. These observations suggest that subtle
392 genetic variation in nervous system development is associated with variation in propensity for
393 consumption of psychostimulant drugs. Nearly 90% of the genes in the network have human
394 orthologs and are candidates for future translational studies.
395 We selected nine candidate genes in the significant genetic network and 25 additional
396 candidate genes to assess whether RNAi reduction using a weak ubiquitous *GAL4* driver
397 affected consumption traits, using the same experimental design as for the DGRP lines. All of
398 these genes affected at least one consumption trait/sex/drug. However, there is considerable
399 variation in the effects of different drivers on consumption, preference and change in preference
400 for cocaine and methamphetamine, which likely reflects variation in the effects of RNA

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401 interference on different neural elements of a complex integrated neural circuitry. Indeed,
402 several candidate genes, functionally implicated by RNAi, are associated with neural
403 development and represent several early developmental signaling pathways. *Snoo* has been
404 identified as a negative regulator of the decapentaplegic signaling pathway [78,79] and has
405 been implicated in dendritic patterning [80]. Echinoid, the gene product of *ed*, is an
406 immunoglobulin domain containing membrane protein of adherens junctions that interacts with
407 multiple developmental signaling pathways, including Egfr, Notch and Hippo signaling [81-83].
408 Musashi, encoded by *msi*, is a neural RNA binding protein that interacts with Notch signaling to
409 determine cell fate [84]. RNAi targeting of expression of these genes under *MB-GAL4* or *TH-*
410 *GAL4* drivers show different effects on consumption, change in consumption, preference and
411 change in preference for the two drugs (Fig. S5).
412 Among the functionally validated candidate genes, *Oct-TyrR* and *Dop1R1* are of special
413 interest. *Oct-TyrR* encodes an octopamine-tyramine receptor expressed in mushroom bodies
414 [85], and *Dop1R1*, which encodes a dopamine receptor enriched in the mushroom bodies, has
415 previously been implicated in aversive and appetitive conditioning [86], innate courtship
416 behavior [87] and sleep-wake arousal [88]. Loss-of-function mutations of *Dop1R1* increase
417 sleep and these effects are reversed by administration of cocaine [88]. Octopamine and
418 tyramine act on astrocytes via the Oct-Tyr1 receptor and this activation of astrocytes can in turn
419 modulate dopaminergic neurons [89]. Thus, we can hypothesize that combinations of
420 octopaminergic and dopaminergic signaling in the mushroom bodies can modulate drug
421 consumption and/or experience-dependent changes in consumption or preference following
422 repeated exposure to cocaine or methamphetamine.
423 Finally, genes which were functionally validated with RNAi represent evolutionarily
424 conserved processes. Future studies can assess whether their human counterparts play a role
425 in variation in susceptibility to psychostimulant drug use in human populations.
426

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427 **MATERIALS AND METHODS**

428 **Drosophila stocks**

429 The DGRP, *UAS*-RNAi and *GAL4* driver lines used are listed in Table S12. The DGRP lines are
430 maintained in the Mackay laboratory. RNAi lines [47] were obtained from the Vienna Drosophila
431 Resource Center and the *GAL4* driver lines from the Bloomington, Indiana Drosophila stock
432 center. All lines were maintained on standard cornmeal/yeast/molasses medium at 25°C on a
433 12 hour light/dark cycle with constant humidity of 50%.

434

435 **Consumption assay**

436 We used a two-capillary Capillary Feeder (CAFE) assay [44-46] to measure drug consumption.
437 Briefly, five 3-5 day old flies per genotype/sex were anesthetized using CO_2 and placed on
438 cornmeal/yeast/molasses/agar medium one day prior to the assay. Flies were transferred
439 without anesthesia 45 minutes prior to the assay to vials containing 4-5ml of 1.5% agar (Sigma
440 Aldrich). Two capillaries (VWR International: 12.7 cm long, 5 µl total volume) containing 4%
441 sucrose (Sigma Aldrich) + 1% yeast (Fisher Scientific) or 4% sucrose + 1% yeast + drug, with a
442 mineral oil (Sigma Aldrich) overlay (to minimize evaporation), were inserted in the top of each
443 vial. Cocaine and methamphetamine were obtained from the National Institute on Drug Abuse
444 under Drug Enforcement Administration license RA0443159. Flies were allowed to feed for 16-
445 18 hours with the vials placed in an enclosed plastic chamber wrapped in a plastic bag under a
446 12 hour light/dark cycle with constant humidity of 50%. For each experiment, an identical set of
447 vials without flies was included in each chamber to determine evaporation loss. The capillaries
448 were then removed and the volume of food consumed (1 mm = 0.067 µl) in each calculated as
449 described previously [90]. The capillaries were replaced with a Drosophila activity monitor tube
450 (TriKinetics, Inc. Waltham, MA) containing standard cornmeal/yeast/molasses medium for a
451 recovery period of 4-6 hours. The assay was performed on three consecutive days for each vial
452 of flies. A total of 10 replicate vials were tested for each genotype and sex.

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453 We defined four behaviors: total amount of each solution consumed, drug preference,
454 and change in consumption and change of preference between exposures 3 and 1. Preference
455 was quantified in two ways: as the difference between the amount of drug and sucrose
456 consumed (Preference A), and as this difference scaled by the total amount consumed
457 (Preference B).
458

459 **Genetic variation in drug consumption behaviors in the DGRP**

460 We performed four-way factorial mixed model analyses of variance (ANOVA) to partition
461 variation in consumption in the DGRP: $Y = \mu + L + E + S + X + (L \times E) + (L \times S) + (L \times X) + (E \times$
462 $S) + (E \times X) + (S \times X) + (L \times E \times S) + (L \times E \times X) + (L \times S \times X) + (E \times S \times X) + (L \times E \times S \times X) +$
463 ε, where Y is consumption; μ is the overall mean; L is the random effect of line; E, S, and X are
464 the fixed effects of exposure (day 1-3), solution (drug, sucrose), and sex (males, females); and ε
465 is the residual variation between replicate vials. The main effect of L and all interaction terms
466 with L are genetic factors affecting drug consumption. We also ran the same ANOVA models to
467 compare the effects of cocaine and methamphetamine on consumption, separately for males
468 and females. The full model for variation in change in consumption over time is $Y = \mu + L + S +$
469 $X + (L \times S) + (L \times X) + (S \times X) + (L \times S \times X) + \varepsilon$. We assessed variation in the development of
470 preference using the model $Y = \mu + L + E + X + (L \times E) + (L \times X) + (E \times X) + (L \times E \times X) + \varepsilon$. We
471 also assessed whether there is natural variation in the change of preference over time using the
472 model $Y = \mu + L + X + (L \times X) + \varepsilon$. We also ran reduced models for each trait. All ANOVAs were
473 performed using the PROC GLM function in SAS. We used the R function pf to assign exact *P*-
474 values.
475

476 **Quantitative genetic analyses in the DGRP**

477 We used the SAS PROC MIXED function to estimate variance components for each of the
478 random effect terms in the full and reduced models. The R package lmer and lmerTest were

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479 utilized in combination with the pchisq function to assign *P*-values for the segregating genetic
480 variation for each trait. We computed broad sense heritabilities as the sum of all genetic
481 variance components divided by the total phenotypic variance for each model, and broad sense
482 heritabilities of line means as the sum of all genetic variance components divided by the sum of
483 all genetic variance components plus the environmental variance/10, where 10 is the number of
484 replicate vials per line, sex, exposure and treatment. We computed pairwise genetic correlations
485 as $r_G = \sigma_L^2/\sigma_{L1}\sigma_{L2}$, where σ_L^2 is the among line variance from the appropriate two-way factorial
486 ANOVA and σ_{L1} and σ_{L2} are the among line standard deviations from the one-way ANOVA for
487 each condition. We computed Pearson product-moment correlations of line means to estimate
488 phenotypic correlations between different traits.
489

490 **Genome wide association mapping in the DGRP**

491 We performed GWA analyses on line means for all consumption traits using the DGRP pipeline
492 (http://dgrp2.gnets.ncsu.edu/). This pipeline accounts for effects of Wolbachia infection status,
493 major polymorphic inversions and polygenic relatedness [43] and implements single-variant
494 tests of association for additive effects of variants with minor allele frequencies ≥ 0.05. We
495 tested effects of 1,891,456 DNA sequence variants on each trait.
496

497 **Network analysis**

498 We annotated candidate genes identified by the GWA analyses using Flybase release 5.57 [56]
499 and mapped gene-gene networks through the genetic interaction database downloaded from
500 Flybase. We then constructed a subnetwork using Cytoscape 3.5.1 where candidate genes
501 directly interact with each other. We evaluated the significance (α = 0.05) of the constructed
502 subnetwork by a randomization test [91-93].
503

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504

505 **Gene Ontology analysis**

506 We carried out gene ontology (GO) enrichment analysis with PANTHER 11.1
507 (http://pantherdb.org/) [53,54].

508

509 **RNAi knockdown of gene expression**

510 We used the binary *GAL4-UAS* system for RNAi-targeted knockdown of expression of
511 candidate genes associated with variation in consumption of cocaine or methamphetamine with
512 a weak ubiquitous driver (*Ubi156-GAL4*) and drivers specific for neurons (*elav-GAL4*), glia
513 (*repo-GAL4*), mushroom bodies (*201Y-GAL4*) and dopaminergic neurons (*TH-GAL4*). We
514 crossed 3 homozygous *GAL4* driver males to 5-7 homozygous females harboring a unique
515 *UAS-RNAi* transgene or the progenitor control to generate F1 *GAL4-UAS-RNAi* and *GAL4*
516 control progeny. We assessed the consumption traits exactly as described above for the DGRP
517 lines. Differences between RNAi lines and their corresponding control lines for consumption
518 were assessed with a fixed-effect ANOVA, separately for males and females. The full model
519 was: $Y = \mu + L + E + S + (L \times E) + (E \times S) + (L \times S) + (L \times E \times S) + \varepsilon$, where Y denotes the
520 mean consumption, E denotes the different exposures, L is the line (Control or RNAi), S
521 denotes the different solutions (sucrose or cocaine/methamphetamine), and ε the error
522 variance. Differences between RNAi lines and controls for change in consumption and
523 preference were also assessed with fixed-effect ANOVAs. The full model for change in
524 consumption was: $Y = \mu + L + S + (L \times S) + \varepsilon$, while the full model for preference was $Y = \mu + L$
525 $+ E + (L \times E) + \varepsilon$. All ANOVAs were run using R.

526

527 **ACKNOWLEDGMENTS**

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528 We thank Mathew J. Eddinger, Emily L. Davis, Kishan N. Patel, and Shaunaci Stevens for
529 technical assistance. This work was supported by grants U01 DA041613 and R01 AA016560
530 from the National Institutes of Health to TFCM and RRHA.

531

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800 **Table 1. Analyses of variance of consumption measured over three exposures.** Exposure,
801 Sex, Solution, and their interaction are fixed effects, the rest are random. E: Exposure; X: Sex;
802 S: Solution; L: DGRP Line; ε: residual; df: degrees of freedom; MS: Type III mean squares; F: F-
803 ratio test; P: P-value; σ2: variance component estimate; SE: standard error; H2: Broad sense
804 heritability. Significant P-values are highlighted in bold font.
805
806
807
808

Source	df	MS	F	*P*	σ^2 (SE)	H^2
A. Cocaine						
L	45	9822	4.55	**2.44E-08**	63.86 (17.55)	0.29
E	2	12579	14.85	**2.67E-06**	Fixed	
S	1	15088	16.21	**2.15E-04**	Fixed	
X	1	223732	169.93	**6.95E-17**	Fixed	
L×E	90	846.8	1.57	**3.09E-02**	7.71 (4.00)	
L×S	45	930.9	1.41	1.08E-01	4.52 (3.85)	
E×S	2	812.9	1.53	2.23E-01	Fixed	
L×X	45	1317	3.28	**4.00E-04**	15.24 (4.93)	
E×X	2	3530	12.83	**1.25E-05**	Fixed	
S×X	1	955.5	2.41	1.28E-01	Fixed	
L×E×S	90	533	1.98	**7.00E-04**	13.16 (4.45)	
L×E×X	90	275.1	1.02	4.63E-01	0.27 (2.87)	
L×S×X	45	396.7	1.47	6.13E-02	4.23 (3.09)	
E×S×X	2	917.1	3.4	**3.77E-02**	Fixed	
L×E×S×X	90	269.8	1.01	4.56E-01	0.25 (4.06)	
ε	4968	267.3			267.28 (5.36)	
B. Methamphetamine						
L	45	7426.77	3.27	**2.41E-06**	42.71 (13.40)	0.28
E	2	21687.00	17.33	**3.00E-07**	Fixed	
S	1	31338.00	37.3	**2.16E-07**	Fixed	
X	1	179509.00	162.17	**1.60E-16**	Fixed	
L×E	90	1251.40	2.32	**8.72E-05**	18.48 (5.08)	
L×S	45	840.12	1.34	1.37E-01	4.03 (3.43)	
E×S	2	208.98	0.47	6.29E-01	Fixed	
L×X	45	1106.92	2.32	**2.30E-03**	10.98 (4.18)	
E×X	2	3333.61	11.16	**4.68E-05**	Fixed	
S×X	1	806.85	2.1	1.54E-01	Fixed	
L×E×S	90	448.30	2.17	**2.00E-04**	10.69 (3.35)	
L×E×X	90	298.62	1.44	**4.18E-02**	3.20 (2.24)	
L×S×X	45	384.45	1.86	**6.50E-03**	5.00 (2.71)	
E×S×X	2	880.99	4.26	**1.71E-02**	Fixed	
L×E×S×X	90	206.94	0.88	7.82E-01	0 (0)	
ε	4968	234.92			243.42 (4.66)	

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809 **Figure Captions**

810

811 **Fig. 1. Consumption and preference assay.** (A) Cartoon illustrating the four capillary CAFÉ
812 assay. Each of the three exposures consists of an 18 hour feeding trial with sucrose or drug +
813 sucrose, followed by 6 hours recovery with standard culture medium. (B) Positions of capillaries
814 with the two solutions (indicated by red and yellow).

815

816 **Fig. 2. Variation in drug consumption among 46 DGRP lines.** (A) Initial exposure. Lines are
817 from lowest to highest consumption in females. (B) Third exposure. The line order is the same
818 as in (A). (C) Change in consumption between exposures 3 and 1. Positive values indicate
819 increased drug consumption in Exposure 3. The line order is the same as in (A). Pink denotes
820 females, blue indicates males, and purple is overlap of both sexes. Error bars are ± 1SD.

821

822 **Fig. 3. Significant genetic interaction network of genes identified in the GWA analyses for**
823 **all cocaine and methamphetamine related traits combined.** Borders indicate the strength of
824 the evidence for a human ortholog. Black: DIOPT score < 3; Blue: DIOPT score 3-6; Green:
825 DIOPT score 7-9; Orange: DIOPT score 10-12; Red: DIOPT score 13-15. Grey boxes have
826 effects on at least one drug-seeking behavior from RNAi knockdown of gene expression.

827

828 **Fig. 4. Differences in cocaine preference and change in cocaine preference between the**
829 **third and first exposures between RNAi and control genotypes.** (A) Female preference. (B)
830 Male preference. (C) Female change of preference. (D) Male change of preference. Red, black,
831 blue, and green bars denote *elav-GAL4*, *repo-GAL4*, *201Y-GAL4* and *TH-GAL4* drivers,
832 respectively. Asterisks represent significant $L\times S$ terms (A, B) or significant $L\times S\times E$ terms from
833 the full ANOVA models. Exact P-values are given in Table S11.

834

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835 **Fig. 5. Differences in methamphetamine preference and change in methamphetamine**
836 **preference between the third and first exposures between RNAi and control genotypes.**
837 (A) Female preference. (B Male preference. (C) Female change of preference. (D) Male change
838 of preference. Red, black, blue, and green bars denote *elav-GAL4*, *repo-GAL4*, *201Y-GAL4* and
839 *TH-GAL4* drivers, respectively. Asterisks represent significant $L \times S$ terms (A, B) or significant
840 $L \times S \times E$ terms from the full ANOVA models. Exact *P*-values are given in Table S12.
841
842 **Supplementary Table Captions**
843
844 **Table S1. DGRP raw consumption data.** (A) Cocaine experiment. (B) Methamphetamine
845 experiment. F: female; M: male.
846
847 **Table S2. Analyses of variance of consumption, change in consumption, preference and**
848 **change in preference of cocaine and methamphetamine.** Exposure, Sex, Solution, and their
849 interaction are fixed effects, the rest are random. Mixed model three-way factorial ANOVAs are
850 given for males and females, as well as reduced models by Exposure, Sex, and Solution. *E*:
851 Exposure; *X*: Sex; *S*: Solution; *L*: DGRP Line; df: degrees of freedom; MS: Type III mean
852 squares; F: F-ratio test; *P*: *P*-value; σ^2: variance component estimate; SE: standard error; H^2:
853 Broad sense heritability. Significant *P*-values are shown in red font. (A) Cocaine experiment. (B)
854 Methamphetamine experiment.
855
856 **Table S3. DGRP line means for all traits.** (A) Cocaine experiment. (B) Methamphetamine
857 experiment. Means are given in mm; 1 mm = 0.067 μl.
858
859 **Table S4. Genetic and phenotypic correlations between traits.** (A) Cross-sex, cross-
860 exposure and cross-solution genetic correlations. Significant *P*-values are indicated in red font.

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861 (B) Pair-wise phenotypic correlations. Entries in the cells are the correlation coefficients and the
862 cell color denotes the P-value. Red: $P < 0.0001$; orange: $P < 0.001$; yellow: $P < 0.01$; green: $P <$
863 0.05; white: $P > 0.05$.

864

865 **Table S5. Results of genome wide association (GWA) analyses for consumption**
866 **behaviors.** (A) Top variants ($P < 5 \times 10^{-5}$) and associated genes for each trait. (B) Variants and
867 genes for the cocaine traits, the methamphetamine traits, and variants and genes overlapping
868 between the two experiments. (C) Pathway and gene ontology enrichment analysis for the
869 cocaine GWA analyses. (D) Pathway and gene ontology enrichment analysis for the
870 methamphetamine GWA analyses.

871

872 **Table S6. DGRP candidate genes and human orthologs.** The references indicate which of
873 the human orthologs have been associated with addictive phenotypes.

874

875 **Table S7. A significant genetic interaction network with no missing genes.** (A) Genes in
876 network. (B) Pathway and gene ontology enrichment analysis.

877

878 **Table S8. Raw cocaine and sucrose consumption data for RNAi and control genotypes.**
879 (A) *Ubi156-GAL4*. (B) *elav-GAL4*. (C) *repo-GAL4*. (D) *201Y-GAL4*. (E) *TH-GAL4*. Data are
880 given in mm; 1 mm = 0.067 μl.

881

882 **Table S9. Raw methamphetamine and sucrose consumption data for RNAi and control**
883 **genotypes.** (A) *Ubi156-GAL4*. (B) *elav-GAL4*. (C) *repo-GAL4*. (D) *201Y-GAL4*. (E) *TH-GAL4*.
884 Data are given in mm; 1 mm = 0.067 μl.

885

886 **Table S10. Analyses of variance of consumption, change in consumption, preference and**

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887 **change in preference of cocaine and sucrose in RNAi lines and their controls.** Fixed effect
888 three-way factorial ANOVAs are given for males and females as well as reduced models by
889 Exposure and Solution. E: Exposure; S: Solution; L: RNAi or control genotype; df: degrees of
890 freedom; MS: Type III mean squares; F: F-ratio test; *P*: *P*-value. Significant *P*-values are shown
891 in red font. (A) *Ubi156-GAL4*. (B) *elav-GAL4*. (C) *repo-GAL4*. (D) *201Y-GAL4*. (E) *TH-GAL4*.
892
893 **Table S11. Analyses of variance of consumption, change in consumption, preference and**
894 **change in preference of methamphetamine and sucrose in RNAi lines and their controls.**
895 Fixed effect three-way factorial ANOVAs are given for males and females as well as reduced
896 models by Exposure and Solution. E: Exposure; S: Solution; L: RNAi or control genotype; df:
897 degrees of freedom; MS: Type III mean squares; F: F-ratio test; *P*: *P*-value. Significant *P*-values
898 are shown in red font. (A) *Ubi156-GAL4*. (B) *elav-GAL4*. (C) *repo-GAL4*. (D) *201Y-GAL4*. (E)
899 *TH-GAL4*.
900
901 **Table S12. Drosophila lines used in this study.** (A) DGRP lines. (B) RNAi lines and control
902 genotypes. (C) *GAL4* driver lines.
903
904 **Supplementary Fig. Captions**
905
906 **Fig. S1. *P*-value summary from three-way ANOVA models of consumption for *UAS*-RNAi**
907 **and control genotypes of candidate genes crossed to a weak ubiquitous *GAL4* driver**
908 **(*Ubi156-GAL4*).** Red: $P < 0.0001$; orange: $P < 0.001$; yellow: $P < 0.01$; green: $P < 0.05$; white: P
909 > 0.05.
910
911 **Fig. S2. Differences between *Ubi156-GAL4* RNAi and control genotypes for 34 candidate**
912 **genes.** (A) Cocaine preference, females. (B) Cocaine preference, males. (C) Change in cocaine

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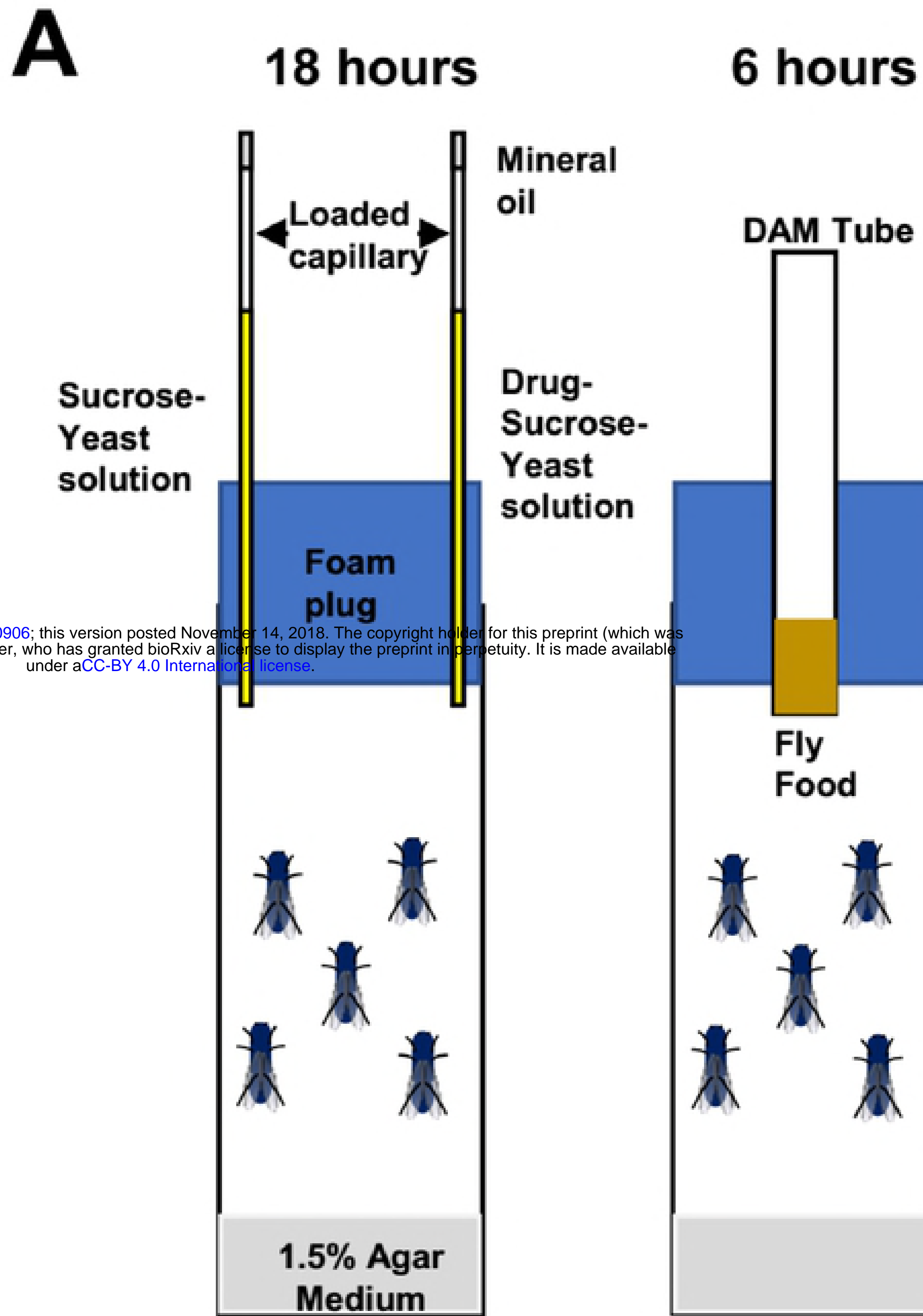
913 preference between third and first exposures, females. (D) Change in cocaine preference
914 between third and first exposures, males. Asterisks represent significant $L \times S$ terms (A, B) or
915 significant $L \times S \times E$ terms from the full ANOVA models. Exact *P*-values are given in Table S11.
916
917 **Fig. S3. Differences between *Ubi156-GAL4* RNAi and control genotypes for 34 candidate**
918 **genes.** (A) Methamphetamine preference, females. (B) Methamphetamine preference, males.
919 (C) Change in methamphetamine preference between third and first exposures, females. (D)
920 Change in methamphetamine preference between third and first exposures, males. Asterisks
921 represent significant $L \times S$ terms (A, B) or significant $L \times S \times E$ terms from the full ANOVA models.
922 Exact *P*-values are given in Table S12.
923
924 **Fig. S4. *P*-value summary from three-way ANOVA models of consumption for *UAS*-RNAi**
925 **and control genotypes of candidate genes crossed to neuronal (*elav-GAL4*) and glial**
926 **(*repo-GAL4*) *GAL4* drivers.** Red: $P < 0.0001$; orange: $P < 0.001$; yellow: $P < 0.01$; green: $P <$
927 0.05; white: $P > 0.05$.
928
929 **Fig. S5. *P*-value summary from three-way ANOVA models of consumption for *UAS*-RNAi**
930 **and control genotypes of candidate genes crossed to mushroom body (*201Y-GAL4*) and**
931 **dopaminergic (*TH-GAL4*) *GAL4* drivers.** Red: $P < 0.0001$; orange: $P < 0.001$; yellow: $P <$
932 0.01; green: $P < 0.05$; white: $P > 0.05$.



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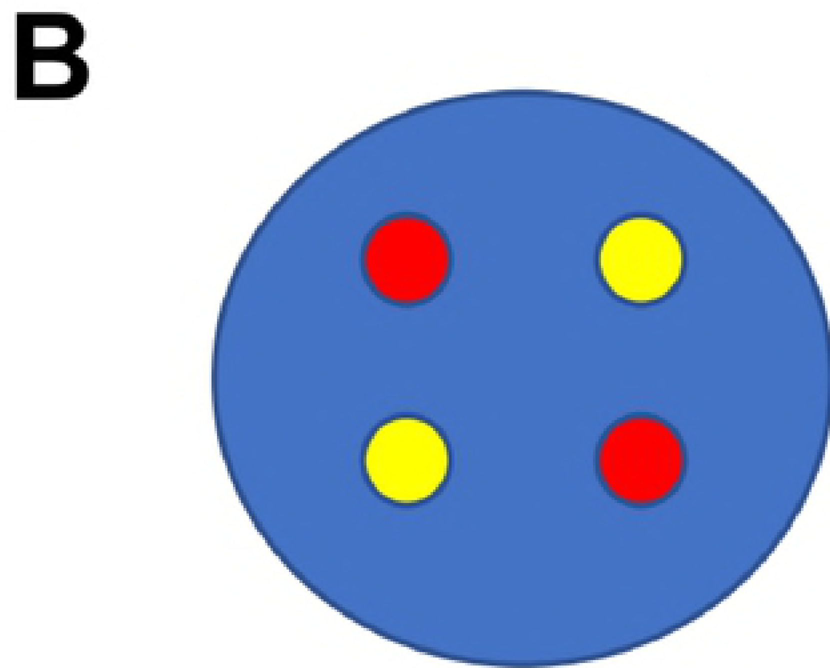

Figure 1

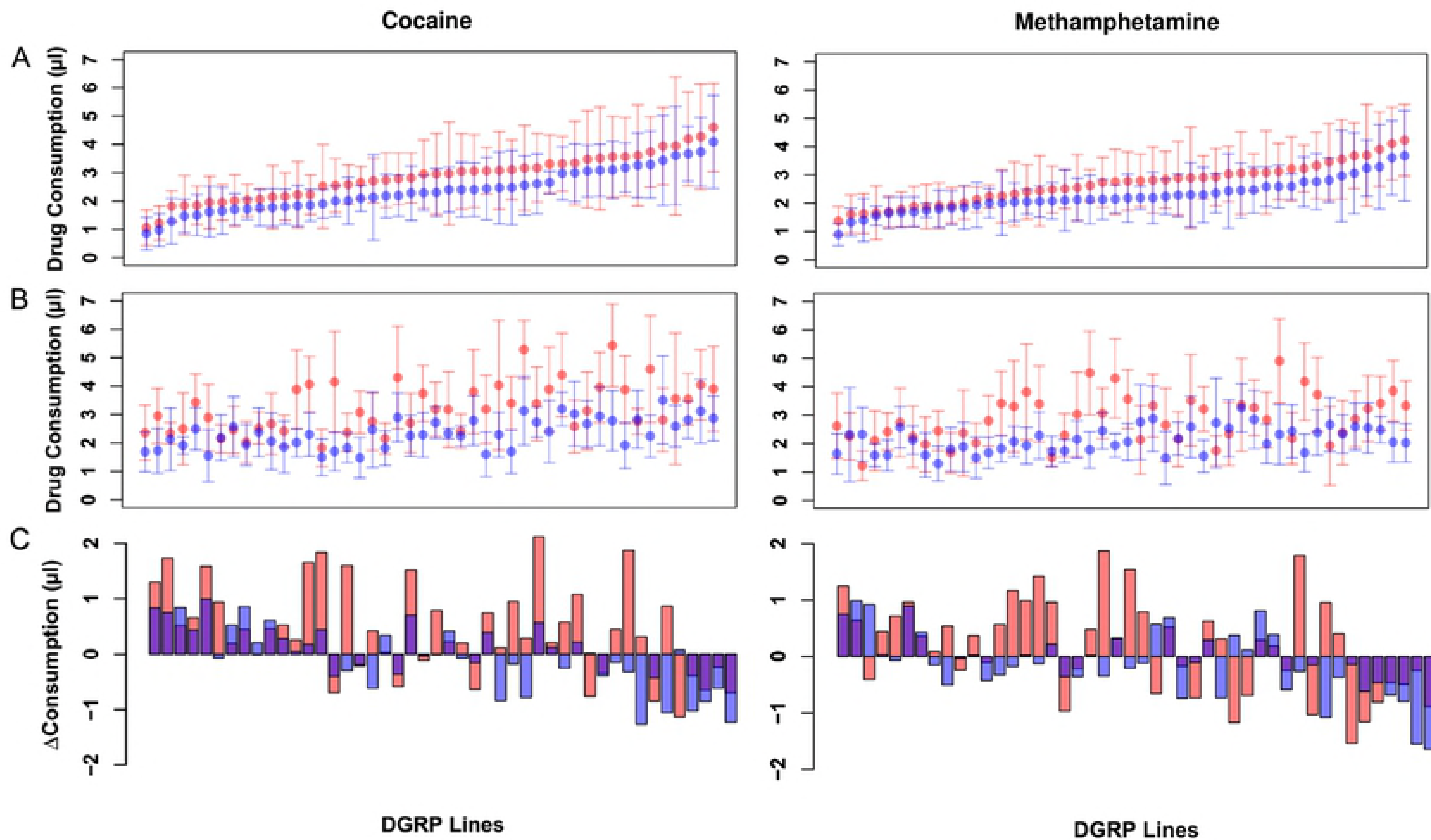

Figure 2

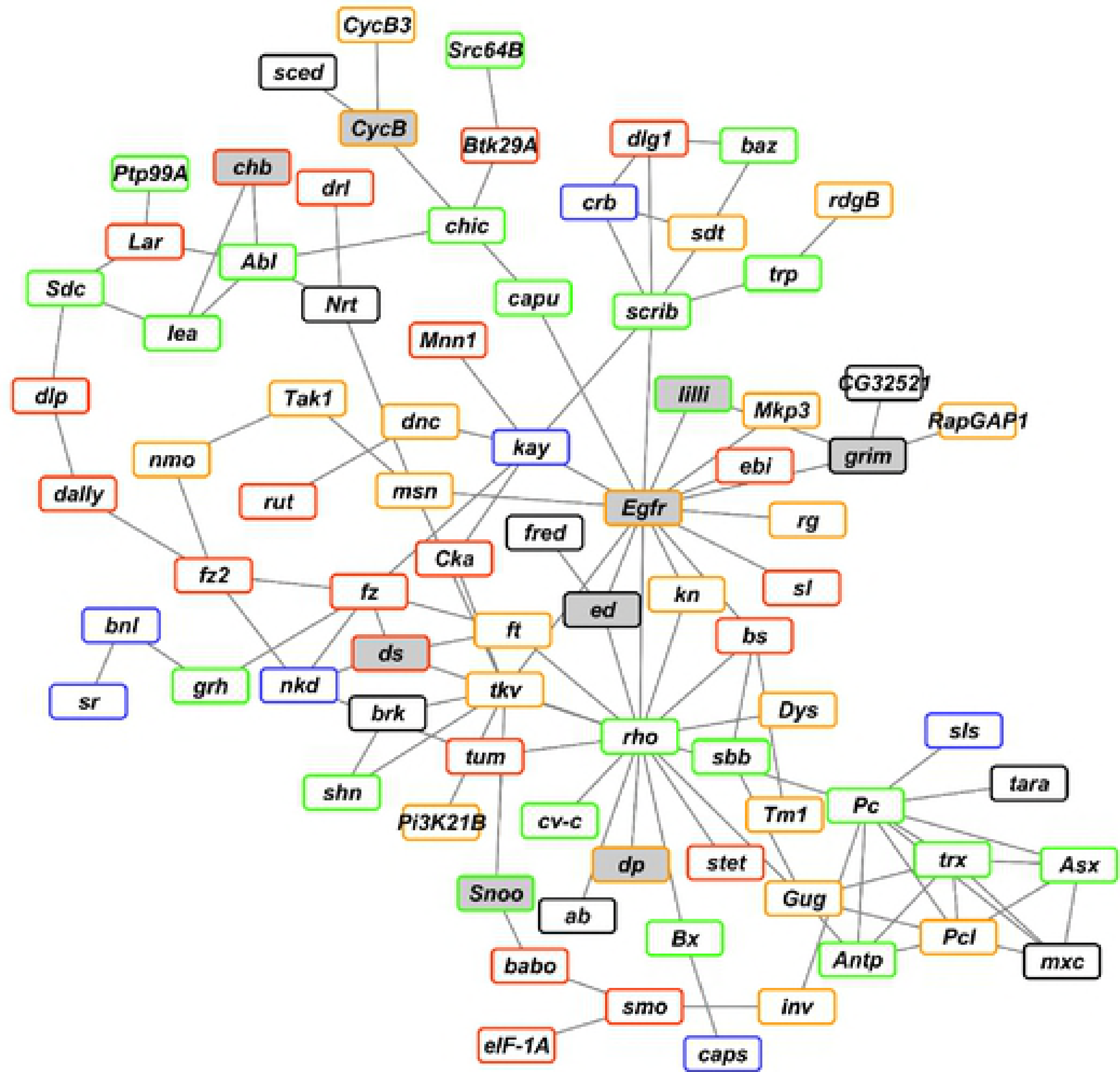

Figure 3

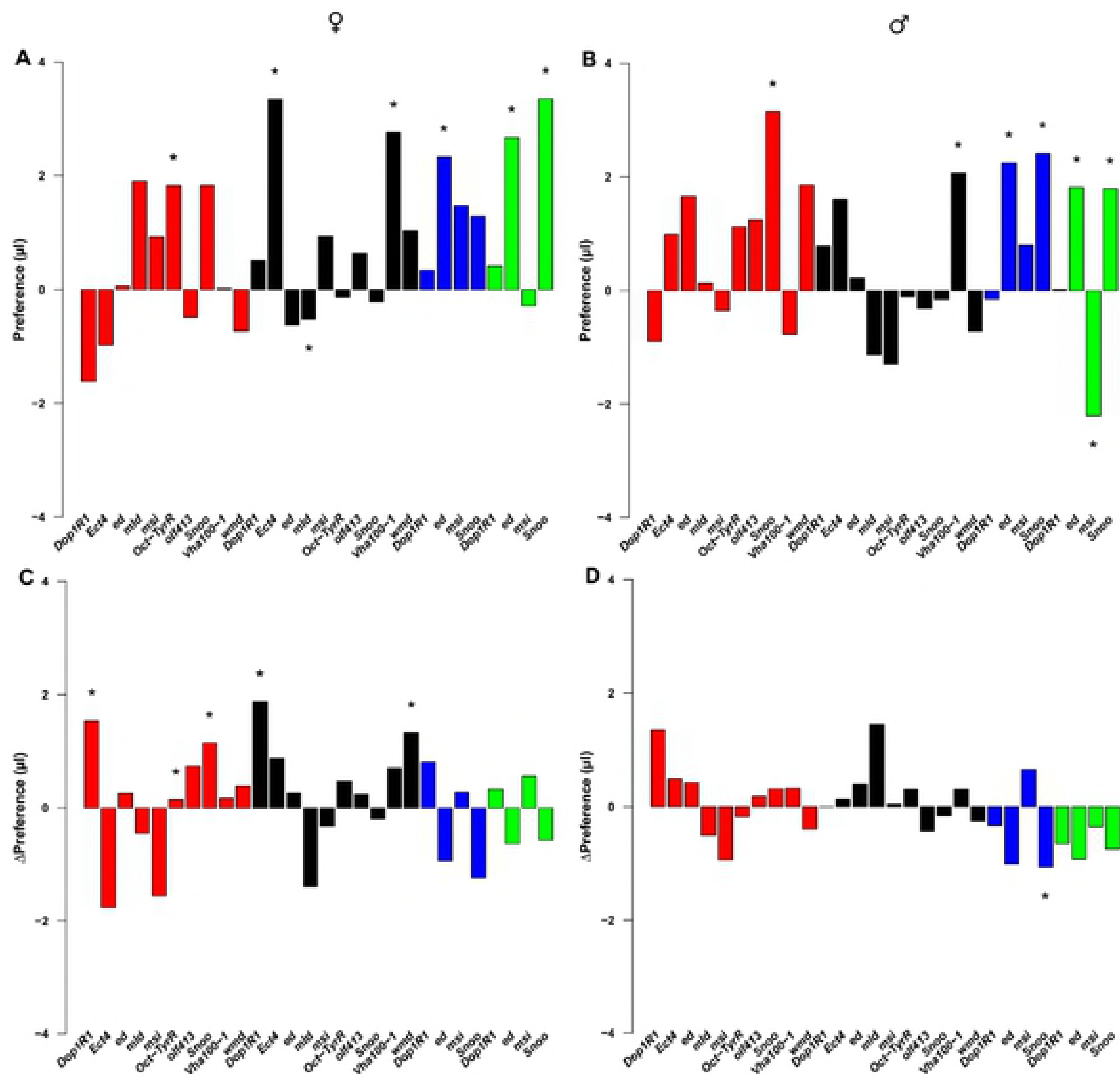

Figure 4

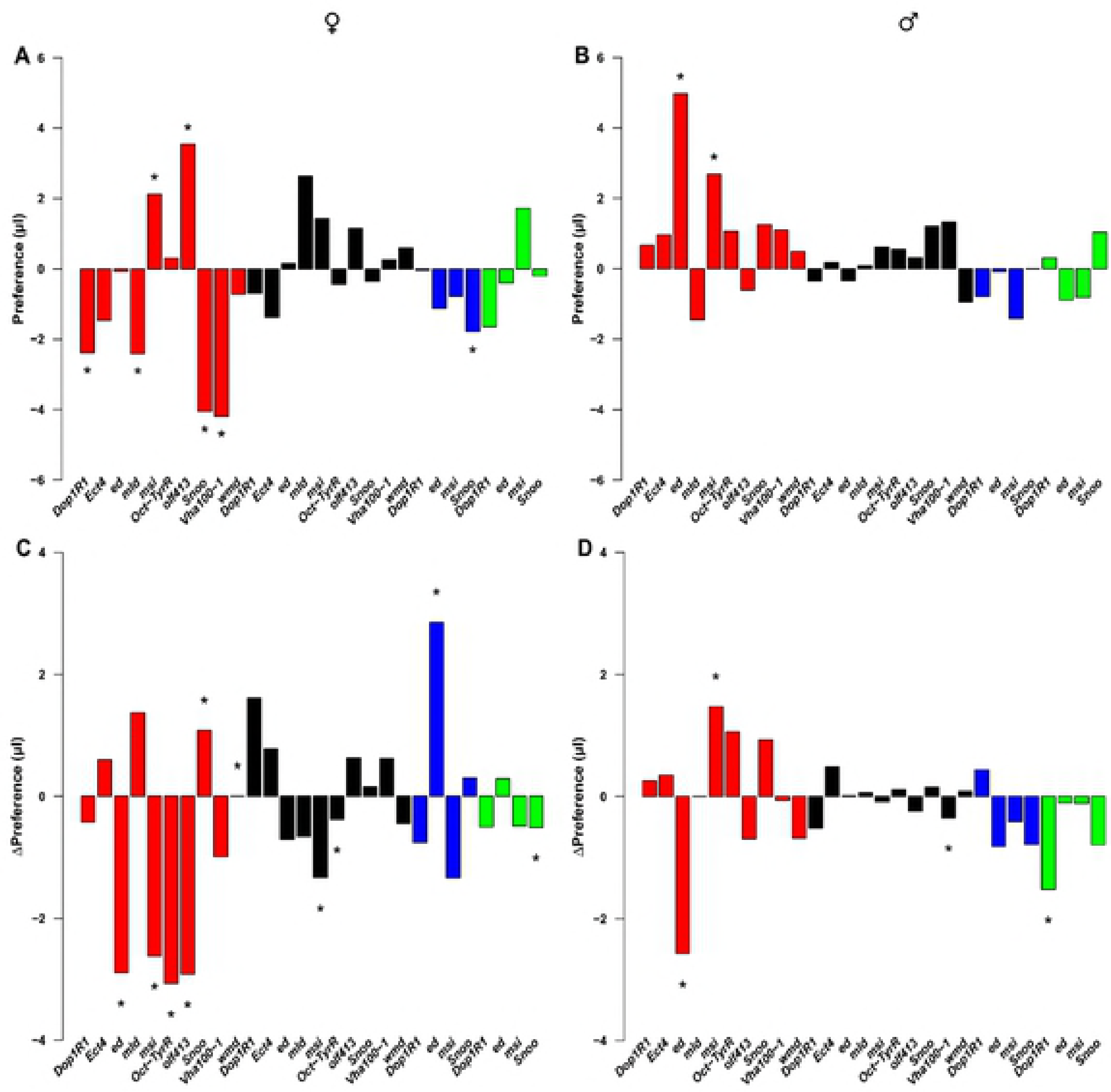

Figure 5