

1 **Title: “Carbon monoxide, a retrograde messenger generated in post-synaptic**  
2 **mushroom body neurons evokes non-canonical dopamine release.”**

3 Abbreviated title “Carbon monoxide evokes dopamine release”

4

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37

## 38 **AUTHOR CONTRIBUTION**

39 K.U. and M.S. designed the experiments. K.U. performed most imaging experiments  
40 with K.O., S.N., and E.S. CO scavenger was synthesized by S.M., and H.K. CO probe  
41 was synthesized by J.M., and C.J.C. The limit of detection of COP-1 is measured by S.P.R.  
42 and B.W.M. The manuscript was written by M.S. and J.H. with K.U.

43

44 **ABSTRACT**

45 **Dopaminergic neurons innervate extensive areas of the brain and release dopamine**  
46 **(DA) onto a wide range of target neurons. However, DA release is also precisely**  
47 **regulated, and in *Drosophila*, DA is released specifically onto mushroom body (MB)**  
48 **neurons, which have been coincidentally activated by cholinergic and glutamatergic**  
49 **inputs. The mechanism for this precise release has been unclear. Here we found that**  
50 **coincidentally activated MB neurons generate carbon monoxide (CO) which**  
51 **functions as a retrograde signal evoking local DA release from presynaptic**  
52 **terminals. CO production depends on activity of heme oxygenase in post-synaptic**  
53 **MB neurons, and CO-evoked DA release requires  $Ca^{2+}$  efflux through ryanodine**  
54 **receptors in DA terminals. CO is only produced in MB areas receiving coincident**  
55 **activation, and removal of CO using scavengers blocks DA release. We propose that**  
56 **DA neurons utilize two distinct modes of transmission to produce global and local**  
57 **DA signaling.**

58

59 **SIGNIFICANCE STATEMENT**

60 Dopamine (DA) is needed for various higher brain functions including memory  
61 formation. However, DA neurons form extensive synaptic connections, while memory  
62 formation requires highly specific and localized DA release. Here we identify a  
63 mechanism through which DA release from presynaptic terminals is controlled by  
64 postsynaptic activity. Postsynaptic neurons activated by cholinergic and glutamatergic  
65 inputs generate carbon monoxide, which acts as a retrograde messenger inducing  
66 presynaptic DA release. Released DA is required for memory-associated plasticity. Our

67 work identifies a novel mechanism that restricts DA release to the specific postsynaptic  
68 sites that require DA during memory formation.

69

70

## 71 **INTRODUCTION**

72 Dopamine (DA) is required for various brain functions including the regulation of global  
73 brain states such as arousal and moods(Huang and Kandel, 1995; Molina-Luna et al.,  
74 2009; Yagishita et al., 2014). To perform these functions, individual DA neurons  
75 innervate extensive areas of the brain and release DA onto a wide range of target neurons  
76 through a processes known as volume transmission(Agnati et al., 1995; Rice and Cragg,  
77 2008; Matsuda et al., 2009). However, this extensive innervation is not suitable for  
78 precise, localized release of DA, and it has been unclear how widely innervating  
79 dopaminergic neurons can also direct DA-release onto specific target neurons.

80 In *Drosophila*, olfactory associative memories are formed and stored in the mushroom  
81 bodies (MBs) where Kenyon cells, MB intrinsic neurons which are activated by different  
82 odors, form synaptic connections with various MB output neurons (MBONs) which  
83 regulate approach and avoidance behaviors(Gerber et al., 2004; Aso et al., 2014).  
84 Dopaminergic neurons modulate plasticity of Kenyon cell MBON  
85 synapses(Claridge-Chang et al., 2009; Aso et al., 2010; Aso et al., 2012; Liu et al., 2012).  
86 However, while there are approximately 2000 to 2500 Kenyon cells that form thousands  
87 of synapses with MBONs, plasticity at these synapses is regulated by relatively few DA  
88 neurons(Mao and Davis, 2009). This indicates that canonical action potential-dependent  
89 release cannot fully explain DA release and plasticity. We recently determined that in  
90 *Drosophila*, synaptic vesicular (SV) exocytosis from DA terminals is restricted to

91 mushroom body (MB) neurons that have been activated by coincident inputs from  
92 odor-activated cholinergic pathways, and glutamatergic pathways, which convey  
93 somatosensory (pain) information(Ueno et al., 2017). Odor information is transmitted to  
94 the MB by projection neurons (PNs) from the antennal lobe (AL)(Marin et al., 2002;  
95 Wong et al., 2002), while somatosensory information is transmitted to the brain via  
96 ascending fibers of the ventral nerve cord (AFV). AL stimulation evokes  $Ca^{2+}$  responses  
97 in the MB by activating nicotinic acetylcholine receptors (nAChRs), and AFV  
98 stimulation evokes  $Ca^{2+}$  responses in the MBs by activating NMDA receptors (NRs) in  
99 the MBs(Ueno et al., 2013). Significantly, when the AL and AFV are stimulated  
100 simultaneously (AL + AFV) or the AL and NRs are stimulated simultaneously (AL +  
101 NMDA), plasticity occurs such that subsequent AL stimulations causes increased  $Ca^{2+}$   
102 responses in the  $\alpha 3/\alpha'3$  compartments of the vertical MB lobes(Wang et al., 2008; Ueno  
103 et al., 2013). This plasticity is known as long-term enhancement (LTE) of MB responses  
104 and requires activation of D1 type DA receptors (D1Rs) in the MBs. Furthermore, while  
105 activation of D1Rs alone is sufficient to produce LTE, neither AL nor AFV stimulation  
106 alone is able to cause SV exocytosis from presynaptic DA terminals projecting onto the  
107  $\alpha 3/\alpha'3$  compartments of the vertical MB lobes. Instead, exocytosis from DA terminals  
108 occurs only when postsynaptic Kenyon cells are activated by coincident AL + AFV or AL  
109 + NMDA stimulation. Strikingly, while MBs are bilateral structures and DA neurons  
110 project terminals onto both sides of MBs(Mao and Davis, 2009), SV exocytosis occurs  
111 specifically in MB areas that have been coincidentally activated. Based on these results, we  
112 proposed that coincident inputs specify the location where DA is released, while DA  
113 induces plastic changes needed to encode associations. However, it has been unclear how  
114 activated Kenyon cells induce SV exocytosis from presynaptic DA terminals. Locally

115 restricted SV exocytosis upon coincident activation of Kenyon cells requires activity of  
116 the *rutabaga* type of adenylyl cyclase, which is proposed to be a coincident detector in  
117 the MBs. Thus Kenyon cells may sense coincident activation and send a retrograde  
118 “demand” signal to presynaptic terminals to evoke local DA release(Ueno et al., 2017).

119 In this study, we used a *Drosophila* dissected brain system to examine synaptic  
120 plasticity and DA release, and found that coincidentally activated post-synaptic Kenyon  
121 cells generate the retrograde messenger, carbon monoxide (CO). CO is generated by  
122 heme oxygenase (HO) in post-synaptic MB neurons, and induces DA release from  
123 pre-synaptic terminals by evoking  $Ca^{2+}$  release from internal stores via ryanodine  
124 receptors (RyRs). Thus, while individual DA neurons extensively innervate the MBs,  
125 on-demand SV exocytosis allows DA neurons to induce plasticity in specific target  
126 neurons.

127

## 128 **MATERIALS AND METHODS**

### 129 **Fly Stock maintenance**

130 All fly stocks were raised on standard cornmeal medium at  $25 \pm 2^\circ\text{C}$  and  $60 \pm 10\%$   
131 humidity under a 12/12 h light–dark cycle. Flies were used for experiments 1-3 d after  
132 eclosion.

133

### 134 **Transgenic and mutant lines**

135 All transgenic and mutant lines used in this study are listed in supplemental Table S1.  
136 *UAS-G-CaMP3* (BDSC\_32234, Bloomington Stock Center, Indiana), *LexAop-G-CaMP2*  
137 (Ueno et al., 2013) and *LexAop-R-GECO1* lines were used for measuring  $Ca^{2+}$  responses  
138 as described previously(Ueno et al., 2013). *UAS-synapto-pHluorin (UAS-spH)*(Ng et al.,

139 2002) and *LexAop-synapto-pHluorin* (*LexAop-spH*) lines were used for measuring  
140 vesicle release(Ueno et al., 2013). *MB-LexA::GAD* (Ueno et al., 2013) was used for the  
141 *LexA* MB driver, *c747* (Aso et al., 2009) was used as the *GAL4* MB driver, and  
142 (Friggi-Grelin et al., 2003) and *TH-LexAp65* (Ueno et al., 2013) were used for *TH-DA*  
143 drivers. *UAS-shi<sup>ts</sup>* (Kitamoto, 2001) and  
144 *pJFRC104-13XLexAop2-IVS-Syn21-Shibire-ts1-p10* (*LexAop-shi<sup>ts</sup>*)(Pfeiffer et al., 2012)  
145 lines were used for inhibition of synaptic transmission. *MB247-Switch* (*MBsw*) was  
146 to express a *UAS* transgene in the MBs upon RU486 (RU+) feeding for 3-5 days(Mao et  
147 al., 2004). *UAS-dHO IR* was used to knockdown of *dHO* expression(Cui et al., 2008).  
148 *dHO<sup>d</sup>* is a deficiency line *Df(3R)Exel7309* (BDSC 7960), lacking 65 Kbp including  
149 *dHO* (Flybase; <http://flybase.org>) in the third chromosome. *P{KK101716}VIE-260B*  
150 (VDRC ID 109631, Vienna Drosophila Resource Center, Vienna, Austria) (*UAS-RyR*  
151 *RNAi*) was used to knock down *RyR*. *Mi{Trojan-GAL4.0}RyR[MI08146-TG4.0]* (BDSC  
152 67480) carries a *GAL4* sequence between 18 and 19 exon of *RyR* and was used to  
153 monitor *RyR* gene expression.

154

### 155 **Isolated whole brain preparation**

156 Brains were prepared for imaging as previously described(Ueno et al., 2013). Briefly,  
157 brains were dissected in ice cold 0 mM Ca<sup>2+</sup> HL3 medium (in mM, NaCl, 70; sucrose,  
158 115; KCl, 5; MgCl<sub>2</sub>, 20; NaHCO<sub>3</sub>, 10; trehalose, 5; Hepes, 5; pH 7.3 and 359  
159 mOsm)(Stewart et al., 1994), and placed in a recording chamber filled with normal,  
160 room temperature HL3 medium (the same recipe as above, containing 1.8 mM CaCl<sub>2</sub>).  
161 To deliver hemoCD through the blood brain barrier, brains were treated with papain (10  
162 U/ml) for 15 min at room temperature, and washed several times with 0 mM Ca<sup>2+</sup> HL3

163 medium prior to use(Gu and O'Dowd, 2007; Ueno et al., 2017).

164

### 165 **Imaging analysis**

166 Imaging analysis was performed in HL3 solution as described previously (Ueno et al.,  
167 2013; Ueno et al., 2017). Briefly, fluorescent images were captured at 15 Hz using a  
168 confocal microscope system (A1R, Nikon Corp., Tokyo, Japan) with a 20x  
169 water-immersion lens (numerical aperture 0.5; Nikon Corp). We obtained  $F_0$  by  
170 averaging the 5 sequential frames before stimulus onset and calculated  $\Delta F/F_0$ . To  
171 evaluate stimulation-induced fluorescent changes of spH,  $\Delta F/F_0$  calculated in the  
172 absence of stimulation or pharmacological agents was subtracted from stimulus or drug  
173 induced  $\Delta F/F_0$ . To quantitatively evaluate the spH fluorescent changes, the average  
174 values of fluorescent changes at indicated time points during and after stimulation were  
175 statistically compared.

176 The AL was stimulated (30 pulses, 100 Hz, 1.0 ms pulse duration) using glass  
177 micro-electrodes. For NMDA stimulation, 200  $\mu\text{M}$  NMDA, diluted in HL3 containing 4  
178 mM  $\text{Mg}^{2+}$  (Miyashita et al., 2012), was applied by micro pipette.

179 For application of CO-saturated HL3, CO or control  $\text{N}_2$  gas was dissolved in HL3  
180 saline by bubbling. CO or  $\text{N}_2$  saturated solutions were immediately placed in glass  
181 pipettes and puffed onto the MB lobes for 1 min (pressure = 6 psi) using a Picospritzer  
182 III system (Parker Hannifin Corp., USA). While we first used thin tip micropipettes,  
183 approximately 5 micro m diameter (Fig. 2A), we also used larger tip micropipettes,  
184 approximately 15 micro m (Fig. 5E), to avoid clogging.

185 To measure SV exocytosis using FFN511, *MB-LexA:GAD*, *LexAop-R-GECO1* brains  
186 were incubated in 10  $\mu\text{M}$  FFN511/HL3 for 30 min. To remove non-specific binding of



187 the dye, FFN511 loaded brains were washed in 200  $\mu$ M ADVASEP-7/HL3 for 15 min  
188 two times(Kay et al., 1999; Gubernator et al., 2009). To evaluate stimulation-induced  
189 release of FFN511,  $\Delta F/F_0$  in the absence of stimulation was subtracted from  $\Delta F/F_0$

190

## 191 **Behaviors**

192 Olfactory aversive memory: The procedure for measuring olfactory memory has been  
193 previously described(Tully and Quinn, 1985; Tamura et al., 2003). Briefly, two mildly  
194 aversive odors (3-octanol [OCT]) or 4-methylcyclohexanol [MCH]) were sequentially  
195 delivered to approximately 100 flies for 1 min with a 45 sec interval between each odor  
196 presentation. When flies were exposed to the first, CS<sup>+</sup> odor (either OCT or MCH), they  
197 were also subjected to 1.5 sec pulses of 60 V DC electric shocks every five sec. To test  
198 olfactory memory, flies were placed at the choice point of a T-maze where they were  
199 allowed to choose either the CS<sup>+</sup> or CS<sup>-</sup> odor for 1.5 min. Memory was calculated as a  
200 performance index (PI), such that a 50:50 distribution (no memory) yielded a  
201 performance index of zero and a 0:100 distribution away from the CS<sup>+</sup> yielded a  
202 performance index of 100.

203

204 Odor and Shock avoidance. Peripheral control experiments including odor and shock  
205 reactivity assays were performed as previously described(Tully and Quinn, 1985) to  
206 measure sensitivity to odors and electrical shocks. Approximately 100 flies were placed  
207 at the choice point of a T maze where they had to choose between an odor (OCT or  
208 MCH) and mineral oil or between electrical shocks and non-shocked conditions. A  
209 performance index was calculated as described above.

210

211 **Identification of dHO localization**

212 To detect of dHO protein in fly brains, wild-type, w(CS)(Dura et al., 1993), and *dHO<sup>d</sup>*  
213 flies were dissected and fixed in 4 % paraformaldehyde for 20 min at 4°C. Brains were  
214 incubated in PBS with 5 % FBS and 0.1 % Triton-X for 30 min at 4°C, and then in  
215 primary antibodies, 1:50 anti-HO (Cui et al., 2008) and 1:20 anti-Fas2 (1D4,  
216 Developmental Studies Hybridoma Bank, Iowa, USA) for 3 days at 4°C. After washing,  
217 brains were incubated with secondary antibodies, Alexa488-conjugated donkey anti-rat  
218 antibody (1:200) (Invitrogen, Carlsbad, USA) and Alexa555-conjugated donkey  
219 anti-mouse antibody (1:200) (Invitrogen) for 2 days at 4°C. Images were captured using  
220 an A1R confocal microscope (Nikon, Tokyo, Japan).

221

222 **Identification of RyR positive neurons (Trojan)**

223 For *Mi{Trojan-GAL4.0}RyR[MI08146-TG4.0]/UAS-mCD8::GFP* imaging, heads were  
224 dissected in 4 % paraformaldehyde for 30 min at 4°C. Brains were incubated with  
225 primary antibodies, anti-GFP (1:400) (ab13970, Abcam, Cambridge, UK) and  
226 anti-tyrosine hydroxylase (#22941, Immunostar, Hudson, USA) in PBS with 10 %  
227 ImmunoBlock (DS Pharma Biomedical Co., Osaka, Japan) and 0.1 % Triton-X  
228 overnight at 4°C. After washing, brains were incubated with secondary antibodies,  
229 Alexa488-conjugated donkey anti-chick antibody (1:400) (Jackson ImmunoResearch,  
230 West Grove, USA) and Alexa555-conjugated donkey anti-mouse antibody (1:400)  
231 (Invitrogen) overnight at 4°C. Images were captured using an A1R confocal microscope  
232 (Nikon, Tokyo, Japan).

233

234 **Chemicals and treatments**

235 RU486 (mifepristone), NMDA (N-methyl-D-aspartate), L-NAME (N<sup>ω</sup>-L-nitro arginine  
236 methyl ester), 1-octanol, 2-arachidonil glycerol, arachidonic acid and CrMP (chromium  
237 mesoporphyrin) and ADVASEP-7 were purchased from Sigma-Aldrich (Missouri,  
238 USA). Thapsigargin and tetrodotoxin (TTX) were purchased from Wako Pure Chemical  
239 Industries (Osaka, Japan). Dantrolen was purchased from Alomone labs (Jerusalem,  
240 Israel). BAPTA-AM and O,O'-Bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic  
241 acid (EGTA) were purchased from Dojindo lab (Kumamoto, Japan). FFN511 was  
242 purchased from Abcam (Cambridge, England). Papain was purchased from Worthington  
243 Biochemical Corporation (New Jersey, USA). RU486 was dissolved in ethanol,  
244 butaclamol was dissolved in DMSO, L-NAME and SCH23390 were dissolved in water,  
245 CrMP was dissolved in 0.5% 2-aminoethanol and 2 mM HCl. Oxy-hemoCD was  
246 reduced in sodium dithionite and purified using a HiTrap Desalting column (GE  
247 Healthcare Japan, Tokyo, Japan) and eluted in PBS. The concentration of purified  
248 oxy-hemoCD is estimated by absorbance at 422 nm([Kitagishi et al., 2010](#)). COP-1 and  
249 CORM-3 were prepared according to previous publications([Clark et al., 2003](#); [Michel et  
250 al., 2012](#)). Both reagents were stored at -20 °C and dissolved in DMSO before use. For  
251 RU486 treatment, dissolved RU486 was mixed in fly food. Flies were fed RU486 for 5  
252 days prior to brain preparation. Other chemicals were treated as described in the main  
253 text and figure legends.

254

## 255 **Statistics**

256 Statistical analyses were performed using Prism software (GraphPad Software, Inc., La  
257 Jolla, CA, USA). All data in bar and line graphs are expressed as means  $\pm$  SEMs.  
258 Student's t-test and Mann Whitney test was used to evaluate the statistical significance

259 between two data sets. For multiple comparisons, one-way or two-way ANOVA  
260 followed by Bonferroni post hoc analyses were employed. Statistical significances are  
261 shown as  $*P < 0.05$ ,  $**P < 0.01$ .  $P$  values greater than 0.05 were considered not  
262 statistically significant,  $NS > 0.05$ .

263

## 264 **RESULTS**

265

### 266 **CO synthesis in the MB neurons is required for DA release upon coincident** 267 **stimulation**

268 Previously, we used an *ex vivo* dissected brain system to examine SV exocytosis from  
269 DA terminals projecting onto the  $\alpha 3/\alpha'3$  compartments of the vertical MB lobes. We  
270 measured SV exocytosis from DA terminals using a vesicular exocytosis sensor,  
271 synapto-pHluorin (spH)(Miesenbock et al., 1998), expressed in dopaminergic neurons  
272 using a tyrosine hydroxylase (TH) driver, and found that release occurred only upon  
273 coincident activation of post-synaptic MB neurons by cholinergic inputs from the ALs  
274 and glutamatergic inputs from the AFV(Ueno et al., 2017).

275 If postsynaptic MB activity evokes SV exocytosis from presynaptic DA terminals,  
276 vesicular output from MB neurons may be needed to activate DA neurons that loop  
277 back to the MBs, as has been previously suggested(Ichinose et al., 2015;  
278 Cervantes-Sandoval et al., 2017; Takemura et al., 2017; Horiuchi, 2019). To test this  
279 possibility, we inhibited synaptic transmission from MB neurons by expressing  
280 temperature-sensitive *shi<sup>ts</sup>* from a pan-MB driver, *MB-LexA*. We confirmed that MB  
281 synaptic output is blocked at restrictive temperature in *MB-LexA>LexAop-shi<sup>ts</sup>* flies by  
282 demonstrating that memory recall, which requires MB output(Dubnau et al., 2001;

283 [McGuire et al., 2001](#)), is defective in these flies ([Supplemental Fig. S1A](#)).

284 Interestingly, SV exocytosis from TH-DA terminals occurs normally at restrictive

285 temperature in these flies upon coincident AL + NMDA stimulation ([Fig. 1A](#)),

286 suggesting while looping activity may be necessary for memory, it is not required for

287 DA release. SV exocytosis from TH-DA terminals also occurred normally when *shi<sup>ts</sup>*

288 was expressed using a different MB driver (*c747-GAL4>UAS-shi<sup>ts</sup>*) ([Supplemental Fig.](#)

289 [S1B](#)), even though memory recall was also disrupted at restrictive temperature in this

290 line([Dubnau et al., 2001](#)). 1 mM 1-octanol, a blocker of gap junctional

291 communication([Rorig et al., 1996](#); [Goncharenko et al., 2014](#)), also did not inhibit SV

292 exocytosis in TH-DA terminals ([Fig. 1B](#)). These results suggest that output from

293 chemical and electrical synapses is not required for post-synaptic MB neurons to induce

294 pre-synaptic DA-release from DA neurons.

295 We next examined whether a retrograde messenger, such as nitric oxide (NO), may

296 be released from MB neurons to regulate pre-synaptic DA release. However, 100  $\mu$ M

297 L-NAME (N $\omega$ -L-nitro arginine methyl ester), a NO synthetase blocker([Boultadakis and](#)

298 [Pitsikas, 2010](#)) had no effect on AL + NMDA stimulation-induced SV exocytosis from

299 TH-DA terminals ([Fig. 1C](#)).

300 Olfactory memory is disrupted by mutations in *nemy*, a gene that encodes a

301 *Drosophila* homolog of cytochrome B561 (CytB561)([Iliadi et al., 2008](#)), which is

302 involved in metabolism of carbon monoxide (CO)([Sugimura et al., 1980](#); [Cypionka and](#)

303 [Meyer, 1983](#); [Jacobitz and Meyer, 1989](#)), a diffusible gas similar to NO, that also been

304 proposed to act as a retrograde messenger during synaptic plasticity([Alkadhi et al.,](#)

305 [2001](#); [Shibuki et al., 2001](#)). Thus, we next examined whether CO may be required for

306 DA release. CO is synthesized by heme oxygenase (HO), and we found that exocytosis

307 from TH-DA terminals upon coincident activation of MB neurons is abolished upon  
308 application of chromium mesoporphyrin (CrMP), a HO blocker(Vreman et al., 1993)  
309 (Fig. 1D). LTE was also significantly inhibited by CrMP (Suppelemental Fig. S2A),  
310 demonstrating the importance of DA release in plasticity. To verify that the *Drosophila*  
311 homologue of HO (dHO)(Cui et al., 2008) is present in the MBs, we used anti-dHO  
312 antibodies and found strong expression in the MBs and in insulin producing cells  
313 (Suppelemental Fig. S2B). We next inhibited *dHO* expression in the MBs using  
314 *MB<sub>sw</sub>>UAS-dHO-IR* flies, which express a *dHO-RNAi* construct from an  
315 RU486-inducible *MB247-switch* (*MB<sub>sw</sub>*) driver(Mao et al., 2004). We found that both  
316 SV exocytosis from TH-DA terminals (Fig. 1E and Suppelemental Movie S1A, B) as  
317 well as LTE (Suppelemental Fig. S2C) were impaired when these flies were fed RU486.  
318 Furthermore, acute knock down of dHO in the MBs in *MB<sub>sw</sub>>UAS-dHO-IR* flies  
319 disrupted olfactory memory (Suppelemental Fig. S2D) without affecting task-related  
320 responses (Suppelemental Fig. S2E). Altogether, these results indicate that dHO in the  
321 MBs is required for olfactory memory, MB plasticity, and DA release onto MBs.

322

### 323 **CO generated from coincidentally activated MB neurons evokes DA release**

324 If CO functions as a retrograde messenger inducing DA release, direct application of  
325 CO to DA terminals should induce release. Thus we next applied CO-saturated saline  
326 from micropipettes to the vertical lobes of the MBs, and observed robust SV exocytosis  
327 from TH-DA terminals (Fig. 2B and Suppelemental Movie S2). Further, we found that  
328 application of a CO-releasing molecule-3 (CORM-3), a water-soluble  
329 CO-donor(Tinajero-Trejo et al., 2014; Aki et al., 2018), also evokes SV exocytosis from  
330 TH-DA terminals (Fig. 2B). In contrast, application of other retrograde messengers,

331 including 200  $\mu$ M arachidonic acid and 200  $\mu$ M 2-arachidonylglycerol, an  
332 endocannabinoid receptor agonist, had no effects on release (Fig. 2C and 2D). To further  
333 examine whether endogenously generated CO is required for DA release, we used a CO  
334 selective scavenger, hemoCD(Kitagishi et al., 2010) and found that hemoCD  
335 significantly inhibited vesicular exocytosis from TH-DA terminals upon AL + NMDA  
336 stimulation (Fig. 2E).

337 We next visualized the generation and release of CO from MB neurons using a CO  
338 selective fluorescent probe, CO Probe 1 (COP-1)(Michel et al., 2012). While COP-1  
339 fluorescence increased immediately after coincident AL + NMDA stimulation,  
340 fluorescence remained unchanged after AL stimulation or NMDA application alone  
341 (Fig. 3A). Thus, changes in COP-1 fluorescence parallel changes in DA release.  
342 Furthermore, the fluorescence increase in COP-1 occurred on the lobes of the MBs  
343 ipsilateral, but not contralateral, to the stimulated AL (Fig. 3B and Supplemental  
344 Movie S3). Since each AL innervates its ipsilateral, but not contralateral MB, this  
345 suggests that CO production occurs in areas of coincident AL and NMDA activation.  
346 Again this result parallels that of DA release(Ueno et al., 2017). Significantly, increased  
347 COP-1 fluorescence was attenuated by knocking down dHO in the MBs (Fig. 3C),  
348 indicating that COP-1 fluorescence detects dHO-dependent CO production.  
349 Collectively, these results suggest that coincident stimulation of MB neurons induces  
350 dHO to generate the retrograde messenger, CO, which then evokes SV exocytosis from  
351 presynaptic DA terminals.

352

### 353 **CO evokes non-canonical SV exocytosis**

354 SV exocytosis requires an increase in  $Ca^{2+}$  concentration in presynaptic terminals(Katz

355 and Miledi, 1967; Augustine et al., 1985; Sabatini and Regehr, 1996; Meinrenken et al.,  
356 2003). Consistent with this, we observed a robust  $\text{Ca}^{2+}$  increase in TH-DA terminals that  
357 project onto the MB lobes receiving coincident AL + NMDA stimulation (Fig. 4A and  
358 Supplemental Movie S4), but not in terminals that project to the contralateral side (Fig.  
359 4B).  $\text{Ca}^{2+}$  increases in DA terminals were also observed upon application of CORM-3  
360 (Fig. 4C), and this increase was abolished by addition of the membrane-permeable  $\text{Ca}^{2+}$   
361 chelator BAPTA-AM (Fig. 4D). In canonical SV exocytosis, neuronal activity opens  
362 voltage-gated calcium channels, allowing influx of extracellular  $\text{Ca}^{2+}$  (Katz and Miledi,  
363 1967; Augustine et al., 1985). However, we found that CORM-3 is able to induce SV  
364 exocytosis from TH-DA terminals even in  $\text{Ca}^{2+}$  free saline in the presence of the  $\text{Ca}^{2+}$   
365 chelator, EGTA, and the sodium channel blocker, TTX (Fig. 4E). This suggests that  
366 CO-evoked DA release is action potential independent and does not require  $\text{Ca}^{2+}$  influx  
367 from extracellular sources.

368 Since extracellular  $\text{Ca}^{2+}$  is not responsible for CO-dependent DA release, we next  
369 examined whether  $\text{Ca}^{2+}$  efflux from internal stores may be required. Significantly,  
370 CORM-3 failed to increase  $\text{Ca}^{2+}$  in TH-DA terminals in the presence of EGTA and  
371 thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase  
372 (SERCA), which depletes internal  $\text{Ca}^{2+}$  stores (Kijima et al., 1991; Sagara and Inesi,  
373 1991) (Fig. 4F). Thus, CO-evoked DA release occurs through a non-canonical  
374 mechanism that depends on  $\text{Ca}^{2+}$  efflux from internal stores rather than from  
375 extracellular sources.

376

### 377 **Ryanodine receptors mediate $\text{Ca}^{2+}$ efflux for CO-evoked DA release.**

378 What mediates  $\text{Ca}^{2+}$  efflux from internal stores in DA terminals? Inositol



379 1,4,5-trisphosphate receptors (IP<sub>3</sub>Rs) and ryanodine receptors (RyRs) are the major  
380 channels mediating Ca<sup>2+</sup> release from internal stores(Bardo et al., 2006). While SV  
381 exocytosis evoked by coincident MB stimulation was not suppressed by  
382 2-Aminoethoxydiphenyl borate (2-APB), an IP<sub>3</sub>R antagonist(Maruyama et al., 1997)  
383 (Fig. 5A), exocytosis was significantly inhibited by dantrolene, a RyR antagonist(Zhao  
384 et al., 2001) (Fig. 5B). Conversely, application of a RyR agonist,  
385 4-chloro-3-methylphenol (4C3MP)(Zorzato et al., 1993) was sufficient to evoke  
386 exocytosis (Fig. 5C). These data suggest that RyRs in DA neurons are required for SV  
387 exocytosis upon coincident activation of MB neurons.

388 To address whether RyRs are expressed in DA terminals, we examined expression of  
389 mCD8::GFP in *Mi{Trojan-GAL4.0}RyR<sup>MI08146-TG4.0</sup>; P{UAS-mCD8::GFP}* flies. In  
390 *Mi{Trojan-GAL4.0}RyR<sup>MI08146-TG4.0</sup>*, a Trojan GAL4 exon is inserted between exons 18  
391 and 19 in the same orientation as the RyR gene(Diao et al., 2015). Thus GAL4, and  
392 mCD8::GFP, expression should reflect RyR expression. In these flies, mCD8::GFP  
393 signals overlapped with anti-TH antibody signals, indicating that RyRs are expressed in  
394 DA neurons (Suppelemental Fig. 3A). To determine whether RyRs in the DA terminals  
395 are required for DA release, we used the TARGET system(McGuire et al., 2003) to  
396 acutely knock down RyRs in adult TH-DA neurons, and found that this significantly  
397 suppressed SV exocytosis from DA terminals upon AL + NMDA stimulation (Fig. 5D).  
398 Furthermore, acute knock down RyRs also suppressed SV exocytosis induced by direct  
399 CO application to TH-DA terminals (Fig. 5E), and also suppressed LTE upon coincident  
400 AL + NMDA stimulation (Suppelemental Fig. S3B). Thus, pre-synaptic RyRs are  
401 required for both activation-dependent and CO-dependent DA release, MB plasticity,  
402 and olfactory memory.

403

404 **DISCUSSION**

405

406 **CO functions as a retrograde on-demand messenger for SV exocytosis in**  
407 **presynaptic DA terminals**

408 A central tenet of neurobiology is that action potentials, propagating from the cell bodies,  
409 induce  $\text{Ca}^{2+}$  influx in presynaptic terminals to evoke SV exocytosis. However, recent  
410 mammalian studies have shown that only a certain fraction of a large number of  
411 pre-synaptic release sites is involved in canonical SV exocytosis([Pereira et al., 2016](#); [Liu](#)  
412 [et al., 2018](#)). In this study we identify a novel mechanism of SV exocytosis in which  
413 activity in post-synaptic neurons evokes pre-synaptic release to induce plastic changes.  
414 This mechanism allows the timing and location of DA release to be strictly defined by  
415 activity of postsynaptic neurons.

416 On-demand SV exocytosis utilizes CO as a retrograde signal from postsynaptic MB  
417 neurons to presynaptic DA terminals. We demonstrate that CO fulfills the criteria that  
418 have been proposed for a retrograde messenger([Regehr et al., 2009](#)). First, we  
419 demonstrate that HO, which catalyzes CO production, is highly expressed in postsynaptic  
420 MB neurons, indicating that MB neurons have the capacity to synthesize the messenger.  
421 Second, we show that pharmacological and genetic suppression of HO activity in the  
422 MBs inhibits CO production, pre-synaptic DA release, and LTE. Third, using a CO  
423 fluorescent probe, COP-1([Michel et al., 2012](#)), we demonstrate that CO is generated in  
424 the MBs following coincident stimulation of the MBs, and CO generation is restricted to  
425 lobes of MB neurons that receive coincident stimulation. We further show that direct  
426 application of CO, or a CO donor, induces DA release from presynaptic terminals, while

427 addition of a CO scavenger, HemoCD, suppresses release. Fourth, we demonstrate that  
428 CO activates RyRs in presynaptic terminals to induce SV exocytosis. Strikingly,  
429 CO-dependent SV exocytosis does not depend on influx of extracellular  $\text{Ca}^{2+}$ , but instead  
430 requires efflux of  $\text{Ca}^{2+}$  from internal stores via RyRs. Finally, we show that  
431 pharmacological inhibition and genetic suppression of RyRs in DA neurons impairs DA  
432 release after coincident stimulation and CO application.

433 Other retrograde signals, such as NO and endo cannabinoids enhance or suppress  
434 canonical SV exocytosis, we find that CO-dependent DA release occurs even in  
435 conditions which block neuronal activity and  $\text{Ca}^{2+}$  influx in presynaptic DA terminals.  
436 This suggests that CO does not function to modulate canonical SV exocytosis, but may  
437 instead evoke exocytosis through a novel mechanism. Several previous studies have  
438 indicated that CO and RyR-dependent DA release also occurs in mammals. A  
439 microdialysis study has shown that CO increases the extracellular DA concentration in  
440 the rat striatum and hippocampus(Hiramatsu et al., 1994), either through increased DA  
441 release, or inhibition of DA reuptake(Taskiran et al., 2003). Also, pharmacological  
442 stimulation of RyRs has been reported to induce DA release in the mice  
443 striatum(Oyamada et al., 1998; Wan et al., 1999). This release is attenuated in RyR3  
444 deficient mice, while KCl-induced DA release, which requires influx of extracellular  $\text{Ca}^{2+}$ ,  
445 is unaffected, suggesting that RyR-dependent release is distinct from canonical DA  
446 release. However, it has been unknown whether and how CO is generated endogenously.  
447 Also physiological conditions that activate RyRs to evoke DA release have also been  
448 unclear.

449 While our results demonstrate that CO signaling is necessary and sufficient for DA  
450 release, we note that our studies use fluorescent reporters which are not optimal for

451 detailed kinetic analysis of release and reuptake. For example, increases in spH  
452 fluorescence can be used to determine vesicular release from DA neurons, but the  
453 decrease in spH fluorescence after release does not reflect the kinetics of clearance of  
454 DA from synaptic sites. Similarly, increases in COP-1 fluorescence reflect increases in  
455 CO production and release, but the kinetics of this increase depends on CO binding  
456 affinities and limit of detection ([Suppelemental Fig. S4](#)) as well as CO production, and  
457 the gradual increase in COP-1 fluorescence following coincident activation does not  
458 indicate that CO production is similarly gradual. In addition, since COP-1 binding to  
459 CO is irreversible, we do not see a decrease in COP-1 fluorescence after the end of  
460 stimulation. Thus, although our functional imaging studies reliably measure significant  
461 changes in synaptic release, calcium signaling, and CO production, they are not precise  
462 enough to accurately measure the fast dynamics of these changes.

463

#### 464 **Signaling pathway for CO-dependent on-demand release of DA**

465 While most neurotransmitters are stored in synaptic vesicles and released upon neuronal  
466 depolarization, the release of gaseous retrograde messengers such as NO and CO is likely  
467 coupled to activation of their biosynthetic enzymes, NOS and HO. Previously, we  
468 demonstrated that activity-dependent DA release onto the MBs requires *rutabaga*  
469 adenylyl cyclase (*rut-AC*) in the MBs([Ueno et al., 2017](#)). *rut-AC* is proposed to function  
470 as a neuronal coincidence detector that senses coincident sensory inputs and activates the  
471 PKA pathway by increasing production of cAMP. In mammals, activation of the  
472 cAMP/PKA pathway increases expression of an HO isoform, HO-1, through  
473 transcriptional activation of the transcription factor CREB([Durante et al., 1997](#); [Park et](#)  
474 [al., 2013](#); [Astort et al., 2016](#)). However, gene expression changes are not fast enough to

475 explain CO-dependent DA release. A second mammalian HO isoform, HO-2 is  
476 selectively enriched in neurons, and HO-2-derived CO is reported to function in plasticity.  
477 HO-2 is activated by  $\text{Ca}^{2+}$ /calmodulin (CaM) binding(Boehning et al., 2004), and by  
478 casein kinase II (CKII) phosphorylation(Boehning et al., 2003), two mechanisms that can  
479 generate CO with sufficient speed to account for LTE. Currently, it is unclear whether  
480 *rut*-AC and the cAMP/PKA pathway functions in parallel with  $\text{Ca}^{2+}$ /CaM and CKII, or in  
481 concert with these pathways (ie functions as a priming kinase for CKII(Huang et al.,  
482 2007)) to activate HO. Supporting the concept that PKA regulates HO, in the golden  
483 hamster retina, PKA has been shown to increase HO activity without affecting HO gene  
484 expression(Sacca et al., 2003).

485 While *Drosophila* has a single isoform of RyR, mammals have three isoforms, RyR1  
486 to RyR3. Skeletal muscle and cardiac muscle primarily express RyR1 and RyR2, and the  
487 brain, including the striatum, hippocampus and cortex, expresses all three  
488 isoforms(Giannini et al., 1995). RyRs are known to be activated by  $\text{Ca}^{2+}$  to mediate  $\text{Ca}^{2+}$   
489 induced  $\text{Ca}^{2+}$  release (CICR)(Endo, 2009). However, CO-evoked DA release occurs even  
490 in the presence of  $\text{Ca}^{2+}$ -free extracellular solutions containing TTX and EGTA,  
491 suggesting that CO activates RyRs through a different mechanism. Besides  $\text{Ca}^{2+}$ , RyRs  
492 can be activated by calmodulin, ATP, PKA, PKG, cADP-ribose, and NO(Takasago et al.,  
493 1991; Xu et al., 1998; Verkhatsky, 2005; Zalk et al., 2007; Lanner et al., 2010; Kakizawa,  
494 2013). NO can directly stimulate RyR1 non-enzymatically by S-nitrosylating a histidine  
495 residue to induce  $\text{Ca}^{2+}$  efflux(Xu et al., 1998; Kakizawa, 2013). Similarly, CO has been  
496 reported to activate  $\text{Ca}^{2+}$ -activated potassium channels ( $\text{K}_{\text{Ca}}$ ) through a non-enzymatic  
497 reaction in rat artery smooth muscle(Wang and Wu, 2003). Alternatively, both NO and  
498 CO can bind to the heme moiety of soluble guanylate cyclase (sGC) leading to its

499 activation(Stone and Marletta, 1994). Activated sGC produces cGMP, and  
500 cGMP-dependent protein kinase (PKG) rapidly phosphorylates and activates  
501 RyRs(Takasago et al., 1991). Interestingly, NO increases DA in the mammalian striatum  
502 in a neural activity independent manner(Hanbauer et al., 1992; Zhu and Luo, 1992;  
503 Lonart et al., 1993). Since activation of RyRs also increases extracellular DA in the  
504 striatum, hippocampus and cortex(Oyamada et al., 1998; Wan et al., 1999), NO may play  
505 a pivotal role in RyR activation and DA release in mammals. However, NOS expression  
506 has not been detected in the MBs(Muller, 1994; Regulski and Tully, 1995), suggesting  
507 that in *Drosophila*, CO rather than NO may function in this process.

508

### 509 **Biological significance of on-demand DA release**

510 DA plays a critical role in associative learning and synaptic plasticity(Huang and Kandel,  
511 1995; Jay, 2003; Puig et al., 2014; Lee et al., 2016; Yamasaki and Takeuchi, 2017). In  
512 flies, neutral odors induce MB responses by activating sparse subsets of MB neurons.  
513 After being paired with electrical shocks during aversive olfactory conditioning, odors  
514 induce larger MB responses in certain areas of the MBs(Yu et al., 2006; Wang et al., 2008;  
515 Akalal et al., 2011; Davis, 2011). We modeled this plastic change in *ex vivo* brains as LTE,  
516 and showed that DA application alone is sufficient to induce this larger response(Ueno et  
517 al., 2017). However, in the *Drosophila* brain, only a small number of DA neurons (~12 for  
518 aversive and ~100 for appetitive) regulate plasticity in ~2000 MB Kenyon cells(Mao and  
519 Davis, 2009). Thus to form odor-specific associations, there should be a mechanism  
520 regulating release at individual synapses. CO-dependent on-demand DA release provides  
521 this type of control. If on-demand release is involved in plasticity and associative learning,  
522 knockdown of genes associated with release should affect learning. Indeed, we show that

523 knocking down either dHO in the MBs or RyRs in DA neurons impairs olfactory  
524 conditioning. While these knockdowns did not completely abolish olfactory conditioning,  
525 this may be due to inefficiency of our knockdown lines. Alternatively, on-demand release  
526 may not be the only mechanism responsible for memory formation, but may instead be  
527 required for a specific phase of olfactory memory.

528 In olfactory aversive conditioning, CS+ odor is paired with US electrical shock.  
529 Given that AFV conveys US information, it seems strange that DA is not released by  
530 AFV stimulation alone in our *ex vivo* imaging, while previous *in vivo* imaging study  
531 demonstrated that DA is released by electrical shock presentation alone (Sun et al.,  
532 2018). Notably, projection of DA terminals are compartmentalized on the MB lobes and  
533 show distinct responses and DA release during sensory processing (Cohn et al., 2015;  
534 Sun et al., 2018). In our *ex vivo* imaging, we looked at DA release onto the  $\alpha 3/\alpha'3$   
535 compartments of the MB vertical lobes, while the previous *in vivo* imaging study found  
536 DA release onto the  $\gamma 2$  and  $\gamma 3$  compartments of the MB horizontal lobes upon electrical  
537 shock (Sun et al., 2018). Therefore, AFV stimulation may also induce DA release onto  
538 these horizontal compartments although it does not induce DA release onto  $\alpha 3/\alpha'3$   
539 compartments. However, due the location of the microelectrode for AL stimulation, we  
540 did not look at the horizontal lobes in this study. Another possibility is that arousal state  
541 of the flies in *in vivo imaging* may be essential for DA release upon shock presentation.  
542 While our *ex vivo* imaging study supposes that glutamatergic neurons transmit AFV  
543 information to the MBs (Ueno et al., 2017), aversive US information has been proposed  
544 to be transmitted by DA neurons (Claridge-Chang et al., 2009; Aso et al., 2010; Aso et  
545 al., 2012; Burke et al., 2012; Liu et al., 2012). Synaptic terminals immunoreactive for  
546 the vesicular glutamate transporter, VGLUT, have been identified at the  $\alpha 3$

547 compartment of the MB vertical lobes (Daniels et al., 2008). However, recent  
548 connectome study demonstrates that they are presynaptic terminals of the MB output  
549 neurons (Takemura et al., 2017). Nevertheless, it is noteworthy that these  
550 immunohistochemical and connectome data do not correlate with expression of NMDA  
551 receptors in the MBs (Miyashita et al., 2012). These mismatch observations implicate  
552 that AFV mediated shock information may be transmitted to the MBs by another type of  
553 glutamatergic neurons.

554 In mammals, the role of CO in synaptic plasticity is unclear. Application of CO paired  
555 with low frequency stimulation induces LTP, while inhibiting HO blocks LTP in the CA1  
556 region of the hippocampus (Zhuo et al., 1993). However, HO-2 deficient mice have been  
557 reported to have normal hippocampal CA1 LTP (Poss et al., 1995). In contrast to CO, a  
558 role for NO in synaptic plasticity and learning has been previously reported (Muller, 1996;  
559 Balaban et al., 2014; Korshunova and Balaban, 2014). Thus, at this point it is an open  
560 question whether CO or NO evokes DA release in mammals. Downstream from CO or  
561 NO, RyRs have been shown to be required for hippocampal and cerebellum synaptic  
562 plasticity (Wang et al., 1996; Balschun et al., 1999; Lu and Hawkins, 2002; Kakizawa et  
563 al., 2012).

564 Our current results suggest that DA neurons release DA via two distinct mechanisms:  
565 canonical exocytosis and on-demand release. Canonical exocytosis is evoked by  
566 electrical activity of presynaptic DA neurons, requires  $Ca^{2+}$  influx, and may be involved  
567 in volume transmission. This mode of release can activate widespread targets over time,  
568 and is suited for regulating global brain functions. In contrast, on-demand release is  
569 evoked by activity of postsynaptic neurons, requires  $Ca^{2+}$  efflux via RyRs, and can  
570 regulate function of specific targets at precise times. DA neurons may differentially



571 utilize these two modes of SV exocytosis in a context dependent manner. Understanding  
572 how DA neurons differentially utilize these modes of transmission will provide new  
573 insights into how a relatively small number of DA neurons can control numerous  
574 different brain functions.

575

576

577 **REFERENCES**

- 578 Agnati LF, Zoli M, Stromberg I, Fuxe K (1995) Intercellular communication in the  
579 brain: wiring versus volume transmission. *Neuroscience* 69:711-726.
- 580 Akalal DB, Yu D, Davis RL (2011) The long-term memory trace formed in the  
581 *Drosophila* alpha/beta mushroom body neurons is abolished in long-term  
582 memory mutants. *The Journal of neuroscience : the official journal of the*  
583 *Society for Neuroscience* 31:5643-5647.
- 584 Aki T, Unuma K, Noritake K, Kurahashi H, Funakoshi T, Uemura K (2018) Interaction  
585 of carbon monoxide-releasing ruthenium carbonyl CORM-3 with plasma  
586 fibronectin. *Toxicol In Vitro* 50:201-209.
- 587 Alkadhi KA, Al-Hijailan RS, Malik K, Hogan YH (2001) Retrograde carbon monoxide  
588 is required for induction of long-term potentiation in rat superior cervical  
589 ganglion. *The Journal of neuroscience : the official journal of the Society for*  
590 *Neuroscience* 21:3515-3520.
- 591 Aso Y, Grubel K, Busch S, Friedrich AB, Siwanowicz I, Tanimoto H (2009) The  
592 mushroom body of adult *Drosophila* characterized by GAL4 drivers. *J*  
593 *Neurogenet* 23:156-172.
- 594 Aso Y, Siwanowicz I, Bracker L, Ito K, Kitamoto T, Tanimoto H (2010) Specific  
595 dopaminergic neurons for the formation of labile aversive memory. *Curr Biol*  
596 20:1445-1451.
- 597 Aso Y, Herb A, Ogueta M, Siwanowicz I, Templier T, Friedrich AB, Ito K, Scholz H,  
598 Tanimoto H (2012) Three dopamine pathways induce aversive odor memories  
599 with different stability. *PLoS genetics* 8:e1002768.
- 600 Aso Y et al. (2014) Mushroom body output neurons encode valence and guide  
601 memory-based action selection in *Drosophila*. *Elife* 3:e04580.
- 602 Astort F, Repetto EM, Rocha-Viegas L, Mercau ME, Puch SS, Finkielstein CV, Pecci A,  
603 Cymeryng CB (2016) Role of CREB on heme oxygenase-1 induction in adrenal

- 604 cells: involvement of the PI3K pathway. *J Mol Endocrinol* 57:113-124.
- 605 Augustine GJ, Charlton MP, Smith SJ (1985) Calcium entry and transmitter release at  
606 voltage-clamped nerve terminals of squid. *The Journal of physiology*  
607 367:163-181.
- 608 Balaban PM, Roshchin M, Timoshenko AK, Gainutdinov KL, Bogodvid TK, Muranova  
609 LN, Zuzina AB, Korshunova TA (2014) Nitric oxide is necessary for labilization  
610 of a consolidated context memory during reconsolidation in terrestrial snails.  
611 *The European journal of neuroscience* 40:2963-2970.
- 612 Balschun D, Wolfer DP, Bertocchini F, Barone V, Conti A, Zuschratter W, Missiaen L,  
613 Lipp HP, Frey JU, Sorrentino V (1999) Deletion of the ryanodine receptor type 3  
614 (RyR3) impairs forms of synaptic plasticity and spatial learning. *EMBO J*  
615 18:5264-5273.
- 616 Bardo S, Cavazzini MG, Emptage N (2006) The role of the endoplasmic reticulum  
617 Ca<sup>2+</sup> store in the plasticity of central neurons. *Trends Pharmacol Sci* 27:78-84.
- 618 Boehning D, Sedaghat L, Sedlak TW, Snyder SH (2004) Heme oxygenase-2 is activated  
619 by calcium-calmodulin. *J Biol Chem* 279:30927-30930.
- 620 Boehning D, Moon C, Sharma S, Hurt KJ, Hester LD, Ronnett GV, Shugar D, Snyder  
621 SH (2003) Carbon monoxide neurotransmission activated by CK2  
622 phosphorylation of heme oxygenase-2. *Neuron* 40:129-137.
- 623 Boulதாகிs A, Pitsikas N (2010) Effects of the nitric oxide synthase inhibitor L-NAME  
624 on recognition and spatial memory deficits produced by different NMDA  
625 receptor antagonists in the rat. *Neuropsychopharmacology : official publication*  
626 *of the American College of Neuropsychopharmacology* 35:2357-2366.
- 627 Burke CJ, Huetteroth W, Oswald D, Perisse E, Krashes MJ, Das G, Gohl D, Silies M,  
628 Certel S, Waddell S (2012) Layered reward signalling through octopamine and  
629 dopamine in *Drosophila*. *Nature* 492:433-437.
- 630 Cervantes-Sandoval I, Phan A, Chakraborty M, Davis RL (2017) Reciprocal synapses

- 631 between mushroom body and dopamine neurons form a positive feedback loop  
632 required for learning. *Elife* 6.
- 633 Claridge-Chang A, Roorda RD, Vrontou E, Sjulson L, Li H, Hirsh J, Miesenbock G  
634 (2009) Writing memories with light-addressable reinforcement circuitry. *Cell*  
635 139:405-415.
- 636 Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R, Motterlini  
637 R (2003) Cardioprotective actions by a water-soluble carbon  
638 monoxide-releasing molecule. *Circ Res* 93:e2-8.
- 639 Cohn R, Morante I, Ruta V (2015) Coordinated and Compartmentalized  
640 Neuromodulation Shapes Sensory Processing in *Drosophila*. *Cell*  
641 163:1742-1755.
- 642 Cui L, Yoshioka Y, Suyari O, Kohno Y, Zhang X, Adachi Y, Ikehara S, Yoshida T,  
643 Yamaguchi M, Taketani S (2008) Relevant expression of *Drosophila* heme  
644 oxygenase is necessary for the normal development of insect tissues.  
645 *Biochemical and biophysical research communications* 377:1156-1161.
- 646 Cypionka H, Meyer O (1983) Carbon monoxide-insensitive respiratory chain of  
647 *Pseudomonas carboxydovorans*. *J Bacteriol* 156:1178-1187.
- 648 Daniels RW, Gelfand MV, Collins CA, DiAntonio A (2008) Visualizing glutamatergic  
649 cell bodies and synapses in *Drosophila* larval and adult CNS. *J Comp Neurol*  
650 508:131-152.
- 651 Davis RL (2011) Traces of *Drosophila* memory. *Neuron* 70:8-19.
- 652 Diao F, Ironfield H, Luan H, Diao F, Shropshire WC, Ewer J, Marr E, Potter CJ,  
653 Landgraf M, White BH (2015) Plug-and-play genetic access to *drosophila* cell  
654 types using exchangeable exon cassettes. *Cell Rep* 10:1410-1421.
- 655 Dubnau J, Grady L, Kitamoto T, Tully T (2001) Disruption of neurotransmission in  
656 *Drosophila* mushroom body blocks retrieval but not acquisition of memory.  
657 *Nature* 411:476-480.

- 658 Dura JM, Preat T, Tully T (1993) Identification of linotte, a new gene affecting learning  
659 and memory in *Drosophila melanogaster*. *J Neurogenet* 9:1-14.
- 660 Durante W, Christodoulides N, Cheng K, Peyton KJ, Sunahara RK, Schafer AI (1997)  
661 cAMP induces heme oxygenase-1 gene expression and carbon monoxide  
662 production in vascular smooth muscle. *Am J Physiol* 273:H317-323.
- 663 Endo M (2009) Calcium-induced calcium release in skeletal muscle. *Physiol Rev*  
664 89:1153-1176.
- 665 Friggi-Grelin F, Coulom H, Meller M, Gomez D, Hirsh J, Birman S (2003) Targeted  
666 gene expression in *Drosophila* dopaminergic cells using regulatory sequences  
667 from tyrosine hydroxylase. *J Neurobiol* 54:618-627.
- 668 Gerber B, Tanimoto H, Heisenberg M (2004) An engram found? Evaluating the  
669 evidence from fruit flies. *Curr Opin Neurobiol* 14:737-744.
- 670 Giannini G, Conti A, Mammarella S, Scrobogna M, Sorrentino V (1995) The ryanodine  
671 receptor/calcium channel genes are widely and differentially expressed in  
672 murine brain and peripheral tissues. *J Cell Biol* 128:893-904.
- 673 Goncharenko K, Eftekharpour E, Velumian AA, Carlen PL, Fehlings MG (2014)  
674 Changes in gap junction expression and function following ischemic injury of  
675 spinal cord white matter. *Journal of neurophysiology* 112:2067-2075.
- 676 Gu H, O'Dowd DK (2007) Whole cell recordings from brain of adult *Drosophila*. *J Vis*  
677 *Exp*:248.
- 678 Gubernator NG, Zhang H, Staal RG, Mosharov EV, Pereira DB, Yue M, Balsanek V,  
679 Vadola PA, Mukherjee B, Edwards RH, Sulzer D, Sames D (2009) Fluorescent  
680 false neurotransmitters visualize dopamine release from individual presynaptic  
681 terminals. *Science* 324:1441-1444.
- 682 Hanbauer I, Wink D, Osawa Y, Edelman GM, Gally JA (1992) Role of nitric oxide in  
683 NMDA-evoked release of [3H]-dopamine from striatal slices. *Neuroreport*  
684 3:409-412.

- 685 Hiramatsu M, Yokoyama S, Nabeshima T, Kameyama T (1994) Changes in  
686 concentrations of dopamine, serotonin, and their metabolites induced by carbon  
687 monoxide (CO) in the rat striatum as determined by in vivo microdialysis.  
688 *Pharmacol Biochem Behav* 48:9-15.
- 689 Horiuchi J (2019) Recurrent loops: Incorporating prediction error and semantic/episodic  
690 theories into *Drosophila* associative memory models. *Genes Brain*  
691 *Behav*:e12567.
- 692 Huang G, Chen S, Li S, Cha J, Long C, Li L, He Q, Liu Y (2007) Protein kinase A and  
693 casein kinases mediate sequential phosphorylation events in the circadian  
694 negative feedback loop. *Genes Dev* 21:3283-3295.
- 695 Huang YY, Kandel ER (1995) D1/D5 receptor agonists induce a protein  
696 synthesis-dependent late potentiation in the CA1 region of the hippocampus.  
697 *Proc Natl Acad Sci U S A* 92:2446-2450.
- 698 Ichinose T, Aso Y, Yamagata N, Abe A, Rubin GM, Tanimoto H (2015) Reward signal  
699 in a recurrent circuit drives appetitive long-term memory formation. *Elife*  
700 4:e10719.
- 701 Iliadi KG, Avivi A, Iliadi NN, Knight D, Korol AB, Nevo E, Taylor P, Moran MF,  
702 Kamyshev NG, Boulianne GL (2008) *nemy* encodes a cytochrome b561 that is  
703 required for *Drosophila* learning and memory. *Proc Natl Acad Sci U S A*  
704 105:19986-19991.
- 705 Jacobitz S, Meyer O (1989) Removal of CO dehydrogenase from *Pseudomonas*  
706 *carboxydovorans* cytoplasmic membranes, rebinding of CO dehydrogenase to  
707 depleted membranes, and restoration of respiratory activities. *J Bacteriol*  
708 171:6294-6299.
- 709 Jay TM (2003) Dopamine: a potential substrate for synaptic plasticity and memory  
710 mechanisms. *Progress in neurobiology* 69:375-390.
- 711 Kakizawa S (2013) Nitric Oxide-Induced Calcium Release: Activation of Type 1  
712 Ryanodine Receptor, a Calcium Release Channel, through Non-Enzymatic

- 713 Post-Translational Modification by Nitric Oxide. *Front Endocrinol (Lausanne)*  
714 4:142.
- 715 Kakizawa S, Yamazawa T, Chen Y, Ito A, Murayama T, Oyamada H, Kurebayashi N,  
716 Sato O, Watanabe M, Mori N, Oguchi K, Sakurai T, Takeshima H, Saito N, Iino  
717 M (2012) Nitric oxide-induced calcium release via ryanodine receptors regulates  
718 neuronal function. *EMBO J* 31:417-428.
- 719 Katz B, Miledi R (1967) Ionic requirements of synaptic transmitter release. *Nature*  
720 215:651.
- 721 Kay AR, Alfonso A, Alford S, Cline HT, Holgado AM, Sakmann B, Snitsarev VA,  
722 Stricker TP, Takahashi M, Wu LG (1999) Imaging synaptic activity in intact  
723 brain and slices with FM1-43 in *C. elegans*, lamprey, and rat. *Neuron*  
724 24:809-817.
- 725 Kijima Y, Ogunbunmi E, Fleischer S (1991) Drug action of thapsigargin on the Ca<sup>2+</sup>  
726 pump protein of sarcoplasmic reticulum. *J Biol Chem* 266:22912-22918.
- 727 Kitagishi H, Negi S, Kiriyama A, Honbo A, Sugiura Y, Kawaguchi AT, Kano K (2010)  
728 A diatomic molecule receptor that removes CO in a living organism. *Angew*  
729 *Chem Int Ed Engl* 49:1312-1315.
- 730 Kitamoto T (2001) Conditional modification of behavior in *Drosophila* by targeted  
731 expression of a temperature-sensitive shibire allele in defined neurons. *J*  
732 *Neurobiol* 47:81-92.
- 733 Korshunova TA, Balaban PM (2014) Nitric oxide is necessary for long-term facilitation  
734 of synaptic responses and for development of context memory in terrestrial  
735 snails. *Neuroscience* 266:127-135.
- 736 Lanner JT, Georgiou DK, Joshi AD, Hamilton SL (2010) Ryanodine receptors: structure,  
737 expression, molecular details, and function in calcium release. *Cold Spring Harb*  
738 *Perspect Biol* 2:a003996.
- 739 Lee JH, Lee S, Kim JH (2016) Amygdala Circuits for Fear Memory: A Key Role for

- 740 Dopamine Regulation. *Neuroscientist*.
- 741 Liu C, Kershberg L, Wang J, Schneeberger S, Kaeser PS (2018) Dopamine Secretion Is  
742 Mediated by Sparse Active Zone-like Release Sites. *Cell* 172:706-718 e715.
- 743 Liu C, Placais PY, Yamagata N, Pfeiffer BD, Aso Y, Friedrich AB, Siwanowicz I, Rubin  
744 GM, Preat T, Tanimoto H (2012) A subset of dopamine neurons signals reward  
745 for odour memory in *Drosophila*. *Nature* 488:512-516.
- 746 Lonart G, Cassels KL, Johnson KM (1993) Nitric oxide induces calcium-dependent  
747 [3H]dopamine release from striatal slices. *J Neurosci Res* 35:192-198.
- 748 Lu YF, Hawkins RD (2002) Ryanodine receptors contribute to cGMP-induced  
749 late-phase LTP and CREB phosphorylation in the hippocampus. *Journal of*  
750 *neurophysiology* 88:1270-1278.
- 751 Mao Z, Davis RL (2009) Eight different types of dopaminergic neurons innervate the  
752 *Drosophila* mushroom body neuropil: anatomical and physiological  
753 heterogeneity. *Frontiers in neural circuits* 3:5.
- 754 Mao Z, Roman G, Zong L, Davis RL (2004) Pharmacogenetic rescue in time and space  
755 of the rutabaga memory impairment by using Gene-Switch. *Proc Natl Acad Sci*  
756 *U S A* 101:198-203.
- 757 Marin EC, Jefferis GS, Komiyama T, Zhu H, Luo L (2002) Representation of the  
758 glomerular olfactory map in the *Drosophila* brain. *Cell* 109:243-255.
- 759 Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K (1997) 2APB,  
760 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of  
761 Ins(1,4,5)P<sub>3</sub>-induced Ca<sup>2+</sup> release. *Journal of biochemistry* 122:498-505.
- 762 Matsuda W, Furuta T, Nakamura KC, Hioki H, Fujiyama F, Arai R, Kaneko T (2009)  
763 Single nigrostriatal dopaminergic neurons form widely spread and highly dense  
764 axonal arborizations in the neostriatum. *The Journal of neuroscience : the*  
765 *official journal of the Society for Neuroscience* 29:444-453.
- 766 McGuire SE, Le PT, Davis RL (2001) The role of *Drosophila* mushroom body signaling



- 767 in olfactory memory. *Science* 293:1330-1333.
- 768 McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL (2003) Spatiotemporal rescue  
769 of memory dysfunction in *Drosophila*. *Science* 302:1765-1768.
- 770 Meinrenken CJ, Borst JG, Sakmann B (2003) Local routes revisited: the space and time  
771 dependence of the Ca<sup>2+</sup> signal for phasic transmitter release at the rat calyx of  
772 Held. *The Journal of physiology* 547:665-689.
- 773 Michel BW, Lippert AR, Chang CJ (2012) A reaction-based fluorescent probe for  
774 selective imaging of carbon monoxide in living cells using a palladium-mediated  
775 carbonylation. *J Am Chem Soc* 134:15668-15671.
- 776 Miesenbock G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic  
777 transmission with pH-sensitive green fluorescent proteins. *Nature* 394:192-195.
- 778 Miyashita T, Oda Y, Horiuchi J, Yin JC, Morimoto T, Saitoe M (2012) Mg(2+) block of  
779 *Drosophila* NMDA receptors is required for long-term memory formation and  
780 CREB-dependent gene expression. *Neuron* 74:887-898.
- 781 Molina-Luna K, Pekanovic A, Rohrich S, Hertler B, Schubring-Giese M, Rioult-Pedotti  
782 MS, Luft AR (2009) Dopamine in motor cortex is necessary for skill learning  
783 and synaptic plasticity. *PLoS One* 4:e7082.
- 784 Muller U (1994) Ca<sup>2+</sup>/calmodulin-dependent nitric oxide synthase in *Apis mellifera*  
785 and *Drosophila melanogaster*. *The European journal of neuroscience*  
786 6:1362-1370.
- 787 Muller U (1996) Inhibition of nitric oxide synthase impairs a distinct form of long-term  
788 memory in the honeybee, *Apis mellifera*. *Neuron* 16:541-549.
- 789 Ng M, Roorda RD, Lima SQ, Zemelman BV, Morcillo P, Miesenbock G (2002)  
790 Transmission of olfactory information between three populations of neurons in  
791 the antennal lobe of the fly. *Neuron* 36:463-474.
- 792 Oyamada T, Hayashi T, Kagaya A, Yokota N, Yamawaki S (1998) Effect of dantrolene  
793 on K(+)- and caffeine-induced dopamine release in rat striatum assessed by in

- 794 vivo microdialysis. *Neurochem Int* 32:171-176.
- 795 Park SY, Kim JH, Lee SJ, Kim Y (2013) Involvement of PKA and HO-1 signaling in  
796 anti-inflammatory effects of surfactin in BV-2 microglial cells. *Toxicol Appl*  
797 *Pharmacol* 268:68-78.
- 798 Pereira DB, Schmitz Y, Meszaros J, Merchant P, Hu G, Li S, Henke A, Lizardi-Ortiz JE,  
799 Karpowicz RJ, Jr., Morgenstern TJ, Sonders MS, Kanter E, Rodriguez PC,  
800 Mosharov EV, Sames D, Sulzer D (2016) Fluorescent false neurotransmitter  
801 reveals functionally silent dopamine vesicle clusters in the striatum. *Nature*  
802 *neuroscience* 19:578-586.
- 803 Pfeiffer BD, Truman JW, Rubin GM (2012) Using translational enhancers to increase  
804 transgene expression in *Drosophila*. *Proc Natl Acad Sci U S A* 109:6626-6631.
- 805 Poss KD, Thomas MJ, Ebralidze AK, O'Dell TJ, Tonegawa S (1995) Hippocampal  
806 long-term potentiation is normal in heme oxygenase-2 mutant mice. *Neuron*  
807 15:867-873.
- 808 Puig MV, Antzoulatos EG, Miller EK (2014) Prefrontal dopamine in associative  
809 learning and memory. *Neuroscience* 282:217-229.
- 810 Regehr WG, Carey MR, Best AR (2009) Activity-dependent regulation of synapses by  
811 retrograde messengers. *Neuron* 63:154-170.
- 812 Regulski M, Tully T (1995) Molecular and biochemical characterization of dNOS: a  
813 *Drosophila* Ca<sup>2+</sup>/calmodulin-dependent nitric oxide synthase. *Proc Natl Acad*  
814 *Sci U S A* 92:9072-9076.
- 815 Rice ME, Cragg SJ (2008) Dopamine spillover after quantal release: rethinking  
816 dopamine transmission in the nigrostriatal pathway. *Brain Res Rev* 58:303-313.
- 817 Rorig B, Klaus G, Sutor B (1996) Intracellular acidification reduced gap junction  
818 coupling between immature rat neocortical pyramidal neurones. *The Journal of*  
819 *physiology* 490 ( Pt 1):31-49.
- 820 Sabatini BL, Regehr WG (1996) Timing of neurotransmission at fast synapses in the

- 821 mammalian brain. *Nature* 384:170-172.
- 822 Sacca GB, Saenz DA, Jaliffa CO, Minces L, Keller Sarmiento MI, Rosenstein RE  
823 (2003) Photic regulation of heme oxygenase activity in the golden hamster  
824 retina: involvement of dopamine. *Journal of neurochemistry* 85:534-542.
- 825 Sagara Y, Inesi G (1991) Inhibition of the sarcoplasmic reticulum Ca<sup>2+</sup> transport  
826 ATPase by thapsigargin at subnanomolar concentrations. *J Biol Chem*  
827 266:13503-13506.
- 828 Shibuki K, Kimura S, Wakatsuki H (2001) Suppression of the induction of long-term  
829 depression by carbon monoxide in rat cerebellar slices. *The European journal of*  
830 *neuroscience* 13:609-616.
- 831 Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF (1994) Improved stability of  
832 *Drosophila* larval neuromuscular preparations in haemolymph-like physiological  
833 solutions. *J Comp Physiol A* 175:179-191.
- 834 Stone JR, Marletta MA (1994) Soluble guanylate cyclase from bovine lung: activation  
835 with nitric oxide and carbon monoxide and spectral characterization of the  
836 ferrous and ferric states. *Biochemistry* 33:5636-5640.
- 837 Sugimura Y, Yoshizaki F, Katagiri J, Horiuchi C (1980) Studies on algal cytochromes. I.  
838 Purification and properties of cytochrome b-561 from *Enteromorpha prolifera*.  
839 *Journal of biochemistry* 87:541-547.
- 840 Sun F et al. (2018) A Genetically Encoded Fluorescent Sensor Enables Rapid and  
841 Specific Detection of Dopamine in Flies, Fish, and Mice. *Cell* 174:481-496  
842 e419.
- 843 Takasago T, Imagawa T, Furukawa K, Ogurusu T, Shigekawa M (1991) Regulation of  
844 the cardiac ryanodine receptor by protein kinase-dependent phosphorylation.  
845 *Journal of biochemistry* 109:163-170.
- 846 Takemura SY et al. (2017) A connectome of a learning and memory center in the adult  
847 *Drosophila* brain. *Elife* 6.

- 848 Tamura T, Chiang AS, Ito N, Liu HP, Horiuchi J, Tully T, Saitoe M (2003) Aging  
849 specifically impairs amnesiac-dependent memory in *Drosophila*. *Neuron*  
850 40:1003-1011.
- 851 Taskiran D, Kutay FZ, Pogun S (2003) Effect of carbon monoxide on dopamine and  
852 glutamate uptake and cGMP levels in rat brain. *Neuropsychopharmacology* :  
853 official publication of the American College of Neuropsychopharmacology  
854 28:1176-1181.
- 855 Tinajero-Trejo M, Denby KJ, Sedelnikova SE, Hassoubah SA, Mann BE, Poole RK  
856 (2014) Carbon monoxide-releasing molecule-3 (CORM-3;  
857 Ru(CO)<sub>3</sub>Cl(glycinate)) as a tool to study the concerted effects of carbon  
858 monoxide and nitric oxide on bacterial flavohemoglobin Hmp: applications and  
859 pitfalls. *J Biol Chem* 289:29471-29482.
- 860 Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant  
861 *Drosophila melanogaster*. *J Comp Physiol A* 157:263-277.
- 862 Ueno K, Naganos S, Hirano Y, Horiuchi J, Saitoe M (2013) Long-term enhancement of  
863 synaptic transmission between antennal lobe and mushroom body in cultured  
864 *Drosophila* brain. *The Journal of physiology* 591:287-302.
- 865 Ueno K, Suzuki E, Naganos S, Ofusa K, Horiuchi J, Saitoe M (2017) Coincident  
866 postsynaptic activity gates presynaptic dopamine release to induce plasticity in  
867 *Drosophila* mushroom bodies. *Elife* 6.
- 868 Verkhatsky A (2005) Physiology and pathophysiology of the calcium store in the  
869 endoplasmic reticulum of neurons. *Physiol Rev* 85:201-279.
- 870 Vreman HJ, Ekstrand BC, Stevenson DK (1993) Selection of metalloporphyrin heme  
871 oxygenase inhibitors based on potency and photoreactivity. *Pediatric research*  
872 33:195-200.
- 873 Wan K, Moriya T, Akiyama M, Takeshima H, Shibata S (1999) Involvement of  
874 ryanodine receptor type 3 in dopamine release from the striatum: evidence from  
875 mutant mice lacking this receptor. *Biochemical and biophysical research*

- 876           communications 266:588-592.
- 877   Wang R, Wu L (2003) Interaction of selective amino acid residues of K(ca) channels  
878           with carbon monoxide. *Exp Biol Med (Maywood)* 228:474-480.
- 879   Wang Y, Wu J, Rowan MJ, Anwyl R (1996) Ryanodine produces a low frequency  
880           stimulation-induced NMDA receptor-independent long-term potentiation in the  
881           rat dentate gyrus in vitro. *The Journal of physiology* 495 ( Pt 3):755-767.
- 882   Wang Y, Mamiya A, Chiang AS, Zhong Y (2008) Imaging of an early memory trace in  
883           the *Drosophila* mushroom body. *The Journal of neuroscience : the official*  
884           journal of the Society for Neuroscience 28:4368-4376.
- 885   Wong AM, Wang JW, Axel R (2002) Spatial representation of the glomerular map in the  
886           *Drosophila* protocerebrum. *Cell* 109:229-241.
- 887   Xu L, Eu JP, Meissner G, Stamler JS (1998) Activation of the cardiac calcium release  
888           channel (ryanodine receptor) by poly-S-nitrosylation. *Science* 279:234-237.
- 889   Yagishita S, Hayashi-Takagi A, Ellis-Davies GC, Urakubo H, Ishii S, Kasai H (2014) A  
890           critical time window for dopamine actions on the structural plasticity of  
891           dendritic spines. *Science* 345:1616-1620.
- 892   Yamasaki M, Takeuchi T (2017) Locus Coeruleus and Dopamine-Dependent Memory  
893           Consolidation. *Neural Plast* 2017:8602690.
- 894   Yu D, Akalal DB, Davis RL (2006) *Drosophila* alpha/beta mushroom body neurons  
895           form a branch-specific, long-term cellular memory trace after spaced olfactory  
896           conditioning. *Neuron* 52:845-855.
- 897   Zalk R, Lehnart SE, Marks AR (2007) Modulation of the ryanodine receptor and  
898           intracellular calcium. *Annu Rev Biochem* 76:367-385.
- 899   Zhao F, Li P, Chen SR, Louis CF, Fruen BR (2001) Dantrolene inhibition of ryanodine  
900           receptor Ca<sup>2+</sup> release channels. Molecular mechanism and isoform selectivity. *J*  
901           *Biol Chem* 276:13810-13816.

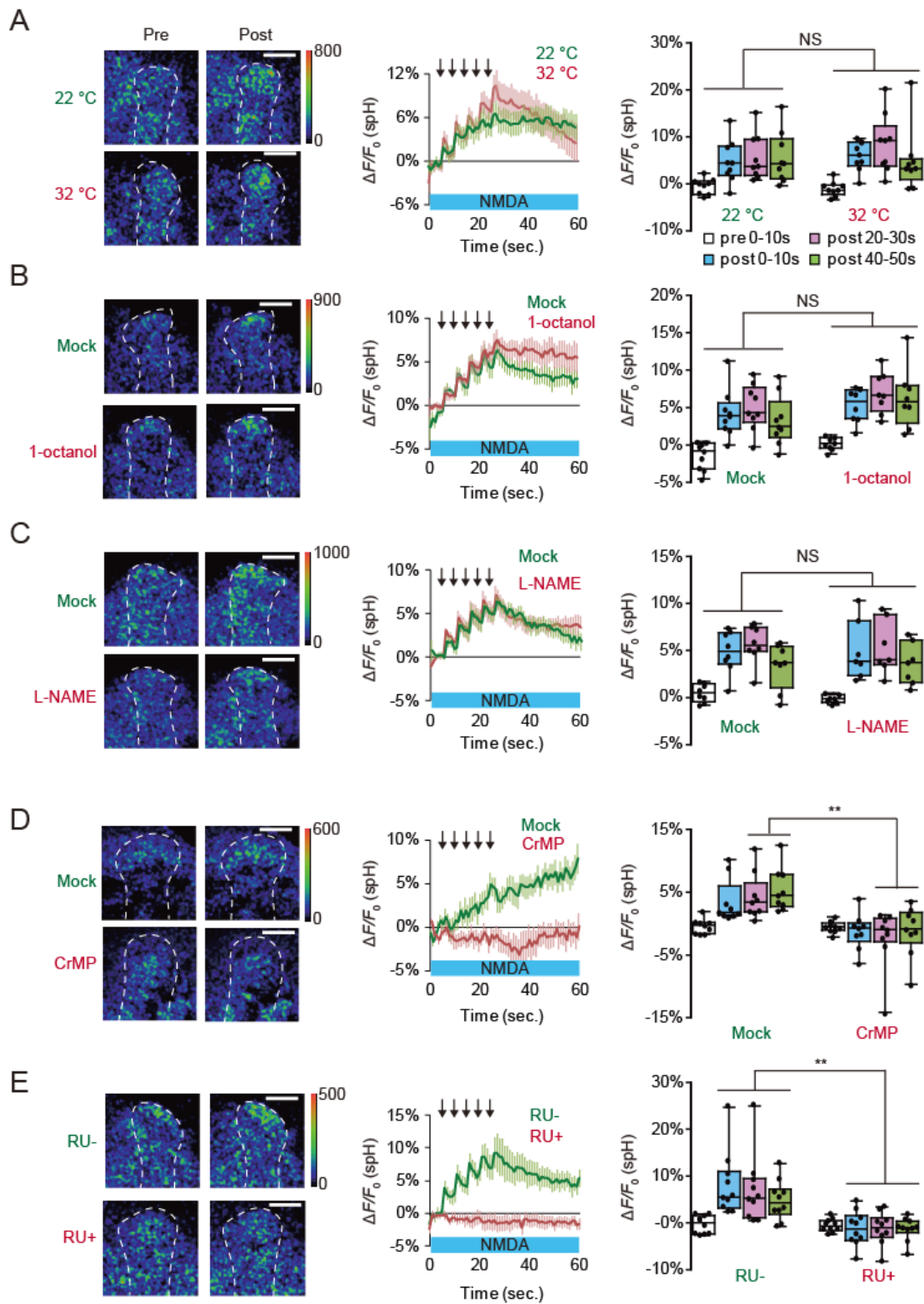
902 Zhu XZ, Luo LG (1992) Effect of nitroprusside (nitric oxide) on endogenous dopamine  
903 release from rat striatal slices. *Journal of neurochemistry* 59:932-935.

904 Zhuo M, Small SA, Kandel ER, Hawkins RD (1993) Nitric oxide and carbon monoxide  
905 produce activity-dependent long-term synaptic enhancement in hippocampus.  
906 *Science* 260:1946-1950.

907 Zorzato F, Scutari E, Tegazzin V, Clementi E, Treves S (1993) Chlorocresol: an  
908 activator of ryanodine receptor-mediated Ca<sup>2+</sup> release. *Mol Pharmacol*  
909 44:1192-1201.

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911 **Figures and legends**



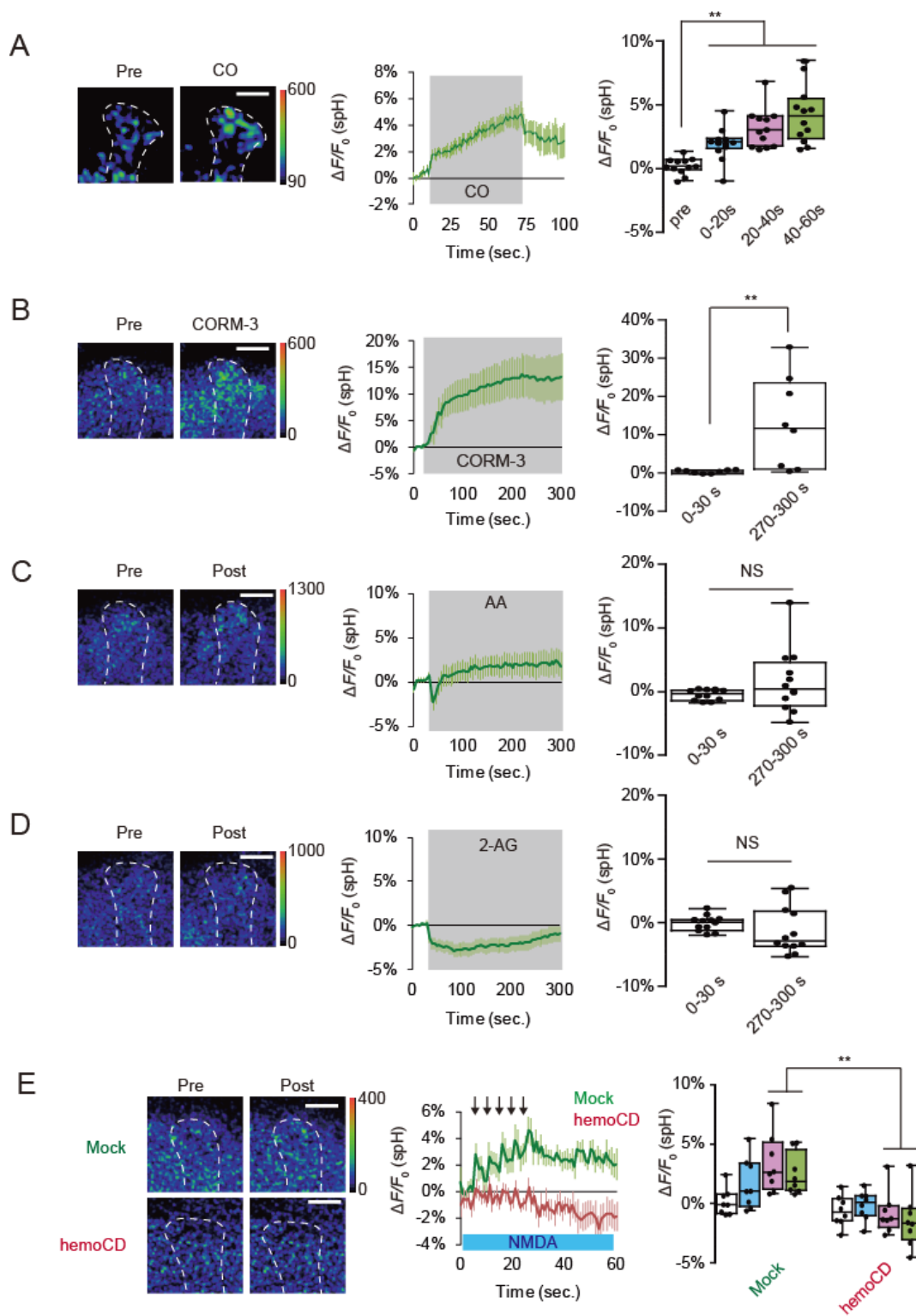
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913 **Fig. 1. Inhibiting heme oxygenase (HO) activity in the MBs blocks SV exocytosis**

914 **from pre-synaptic DA terminals**

915 **A**, Blocking SV exocytosis from the MBs does not affect DA release. spH fluorescence  
916 was measured at TH-DA terminals innervating the  $\alpha 3/\alpha'3$  compartments of the MB  
917 vertical lobes in *MB-LexA>LexAop-shi<sup>ΔS</sup>* brains. (Left) typical pseudocolor images 5 s  
918 before and 30 s after coincident AL + NMDA activation. (Middle) time course of  
919 fluorescent changes, and (right) summary graphs. Two-way ANOVA indicates no  
920 significant differences in spH fluorescence between restrictive (32 °C) and permissive  
921 (22 °C) temperatures. N = 9 for all data. Scale bars = 20 μm. **B**, The gap junction  
922 inhibitor, 1-octanol, does not affect DA release. Two-way ANOVA indicates no  
923 significant differences in spH fluorescence between mock and 1-octanol conditions. N =  
924 8 for all data. **C**, The NOS inhibitor, L-NAME, does not affect SV exocytosis from  
925 TH-DA terminals. Two-way ANOVA indicates no significant differences in spH  
926 fluorescence due to drug treatment. N=8 for all data. **D**, The HO inhibitor, CrMP,  
927 prevents DA release. Two-way ANOVA indicates significant differences in spH  
928 fluorescence due to drug treatment ( $F_{3,64} = 3.268$ ,  $P = 0.0268$ , N = 9 for mock and N =  
929 10 for 10 μM CrMP). \*  $P < 0.05$  and \*\*  $P < 0.01$  by Bonferroni *post hoc* tests. **E**, *dHO*  
930 knockdown in the MBs prevents DA release. Two-way ANOVA indicates significant  
931 differences in spH fluorescence due to RU treatment ( $F_{3,72} = 4.265$ ,  $P = 0.0079$ , N = 10  
932 for all data). \*\*  $P < 0.01$  by Bonferroni *post hoc* tests.





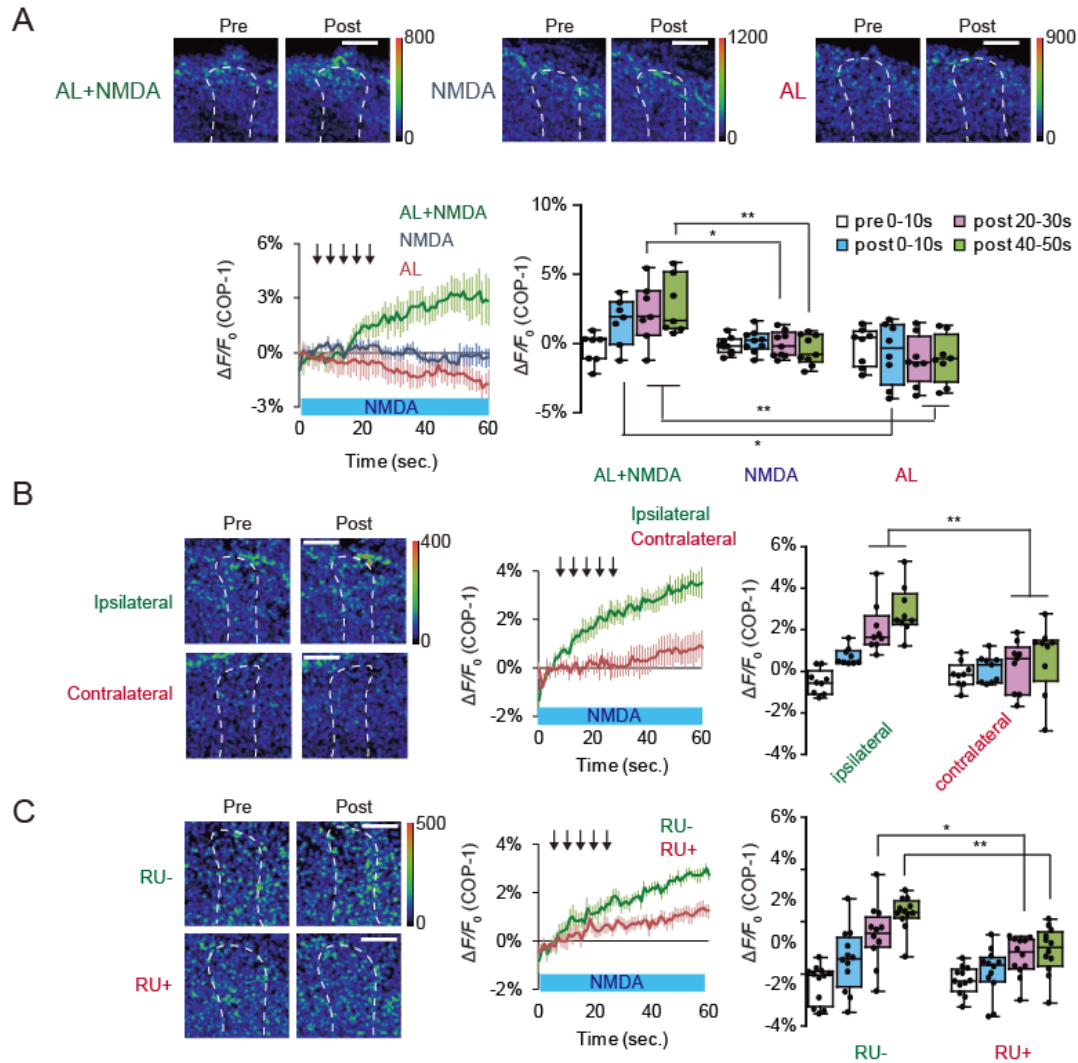
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934 **Fig. 2. CO evokes SV exocytosis from DA terminals**

935 **A**, CO induces DA release. CO-saturated saline was puffed onto the vertical lobes of the  
936 MB during the indicated time period (gray-shaded area in the middle panel).  
937 CO-induced spH responses were obtained by subtracting spH fluorescent changes  
938 induced by control N<sub>2</sub>-saturated saline from fluorescence changes induced by  
939 CO-saturated saline. One-way ANOVA and post hoc tests indicate significant changes  
940 in spH fluorescence upon CO application ( $F_{3,44} = 14.994$ ,  $P < 0.001$ ). N = 12 for all data.  
941 **B**, CORM-3 induces DA release. 20  $\mu$ M CORM-3 (dissolved in 0.2% DMSO) was bath  
942 applied and spH fluorescence was measured at DA terminals at the tip of the MB  
943 vertical lobes. CORM-3-induced spH responses were obtained by subtracting  
944 fluorescent changes induced by control 0.2% DMSO saline from fluorescence changes  
945 induced by CORM-3. \*\*  $P < 0.01$  determined by Student's t-test, comparing spH  
946 responses before (0-30s) and after application of CO (270-300s). N = 8 for all data. **C**,  
947 Arachidonic acid (AA) does not induce DA release. 200  $\mu$ M AA (dissolved in 0.4 %  
948 ethanol) was bath applied. AA-induced spH responses were obtained by subtracting  
949 fluorescence changes induced by 0.4% ethanol saline from fluorescence changes  
950 induced by AA. NS  $P > 0.05$  by Mann Whitney test and N = 16 for all data. **D**, Effects  
951 of 2-arachidonylglycerol (2-AG) on DA release. Bath application of 200  $\mu$ M AG  
952 (dissolved in 0.2 % DMSO) did not induce SV exocytosis in TH-DA terminals.  
953 AG-induced spH responses were obtained by subtracting fluorescence changes induced  
954 by 0.2% DMSO saline from fluorescence changes induced by AG. NS  $P > 0.05$   
955 determined by Student's t-test. N = 12 for all data. **E**, The CO scavenger, hemoCD,  
956 prevents SV exocytosis from DA terminals. AL + NMDA-dependent changes in spH  
957 fluorescence were measured in the presence and absence of 50  $\mu$ M hemoCD. Two-way  
958 ANOVA indicates significant decreases in spH fluorescence due to hemoCD treatment

959 ( $F_{3,56} = 2.845$ ,  $P = 0.0458$ ,  $N = 8$  for all data). \*\*  $P < 0.01$  by Bonferroni *post hoc* tests.

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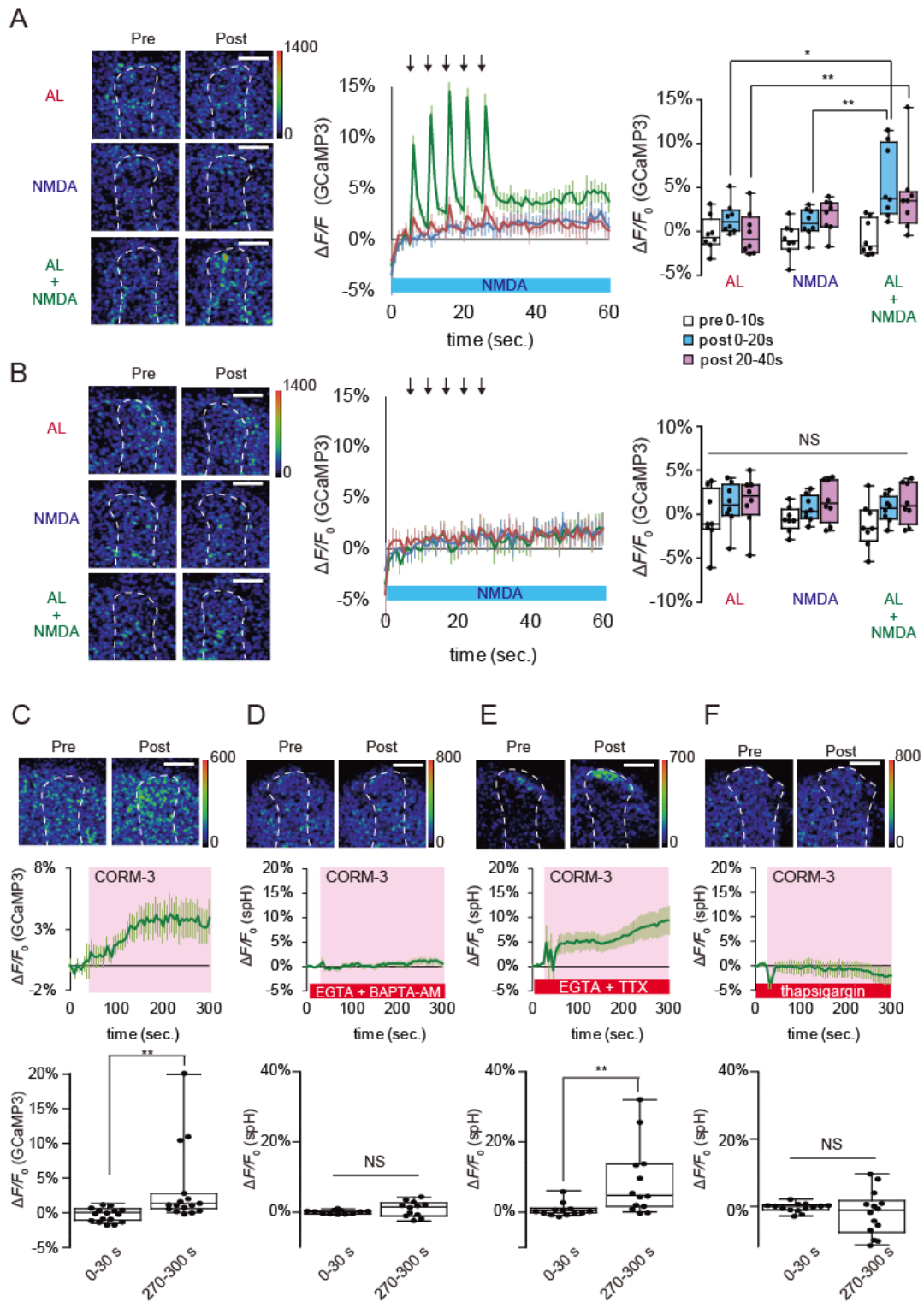


961

962 **Fig. 3. Endogenous CO released from MBs induces DA release**

963 **A**, Top panels, typical images of COP-1 fluorescence observed at the  $\alpha 3/\alpha'3$   
 964 compartments of the MB vertical lobes. Pre refers to images taken prior to stimulation,  
 965 while Post refers to images taken 30 secs after onset of indicated stimuli. Lower left,  
 966 time course of COP-1 fluorescence upon indicated stimulation protocols, and lower  
 967 right, summary of COP-1 fluorescence at different time intervals upon indicated

968 stimulation. Isolated brains were incubated in 4  $\mu$ M COP-1, and COP-1 responses were  
969 calculated by subtracting non-stimulated fluorescence changes from stimulated  
970 fluorescence changes. Two-way ANOVA indicates significant differences in COP-1  
971 fluorescence due to treatment, time, and interaction between treatment and time ( $F_{6, 84} =$   
972 3.094,  $P = 0.0087$ ,  $N = 8$  for AL or NMDA stimulation alone and  $N = 7$  for AL +  
973 NMDA stimulation). \*  $P < 0.05$  and \*\*  $P < 0.01$  by Bonferroni *post hoc* tests. Scale  
974 bars = 20  $\mu$ m. **B**, CO is released from the MB lobe ipsilateral to AL stimulation.  
975 Two-way ANOVA indicates significant differences in fluorescence between MB lobes  
976 ( $F_{3, 64} = 5.491$ ,  $P = 0.020$ ,  $N = 9$  for all data). \*\*  $P < 0.01$  by Bonferroni *post hoc* tests.  
977 **C**, Knocking down *dHO* expression in the MBs impairs CO production. Two-way  
978 ANOVA indicates significant differences in COP-1 fluorescence due to RU treatment  
979 ( $F_{3, 88} = 6.25$ ,  $P = 0.0086$ ,  $N = 12$  for all data). \*\*  $P < 0.01$  by Bonferroni *post hoc* tests.



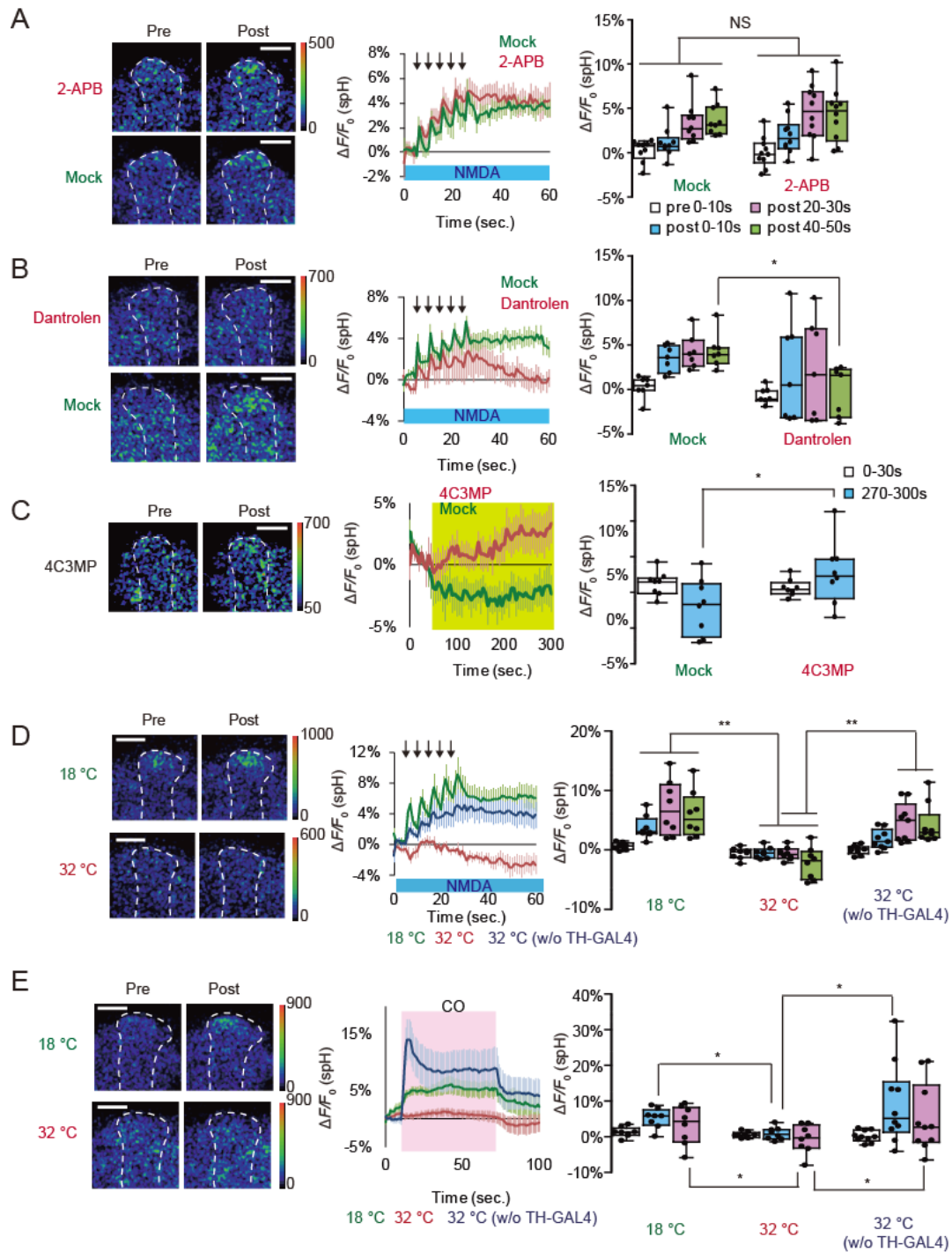
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981 **Fig. 4. CO induced DA release requires  $\text{Ca}^{2+}$  efflux from internal  $\text{Ca}^{2+}$  stores.**

982 **A**, Left panel, typical pseudo color images of G-CaMP3 fluorescence in TH-DA  
983 terminals innervating the  $\alpha 3/\alpha'3$  compartments of the vertical MB lobes ipsilateral to  
984 AL stimulation. The type of stimulation applied is indicated on the left, and responses  
985 before (pre) and after (post) stimulation are shown. Middle panel, time course of  $\text{Ca}^{2+}$   
986 responses in TH-DA terminals upon indicated stimulation. Right panel, summary of  
987 responses. Two-way ANOVA indicates significant differences in G-CaMP3 fluorescence  
988 due to stimulation type, time, and interaction between stimulation type and time ( $F_{4, 63} =$   
989  $2.610, P = 0.0437, N = 8$  for all data). \*  $P < 0.05$  and \*\*  $P < 0.01$  by Bonferroni *post*  
990 *hoc* tests. **B**, G-CaMP3 fluorescence changes in TH-DA terminals innervating the MB  
991 contralateral to AL stimulation. Panels are similar to those shown in (A). Two-way  
992 ANOVA indicates no significant differences in G-CaMP3 fluorescence due to time or  
993 stimulation type.  $N = 9$  for all data. **C**, CORM-3 induces  $\text{Ca}^{2+}$  increases in TH-DA  
994 terminals. CORM-3 induced  $\text{Ca}^{2+}$  changes were calculated by subtracting fluorescence  
995 changes under mock conditions from changes induced by CORM-3. 20  $\mu\text{M}$  CORM-3  
996 (dissolved in 0.2% DMSO saline) was puffed onto the  $\alpha 3/\alpha'3$  compartments of the  
997 vertical MB lobes during the indicated time period (shown in pink in the middle panel).  
998 \*\* $P < 0.01$  by Mann Whitney test.  $N = 16$  for all data. **D**, CORM-3-induced DA release  
999 depends on increases in intracellular  $\text{Ca}^{2+}$ . Brains were incubated in  $\text{Ca}^{2+}$ -free external  
1000 solution containing 2 mM EGTA and 10  $\mu\text{M}$  BAPTA-AM for 10 min prior to CORM-3  
1001 application.  $P = 0.166$  by Student's t-test.  $N = 12$ . **E**, CORM-3-induced DA release does  
1002 not require influx of external  $\text{Ca}^{2+}$  or activity of voltage-gated  $\text{Na}^+$  channels. Brains  
1003 were placed in  $\text{Ca}^{2+}$ -free external solution containing 5 mM EGTA and 10  $\mu\text{M}$  TTX 10  
1004 min prior to CORM-3 application. \*\* $P < 0.01$  by Mann Whitney test.  $N = 13$ . **F**,  
1005 CORM-3-induced DA release requires efflux from internal  $\text{Ca}^{2+}$  stores. Brains were

1006 incubated in  $\text{Ca}^{2+}$ -free external solution containing 10  $\mu\text{M}$  thapsigargin for 10 min prior  
 1007 to CORM-3 application.  $P = 0.364$  by Student's t-test.  $N = 14$ .

1008



1009

1010 **Fig. 5. CO induced DA release is mediated by ryanodine receptors.**

1011 **A**, An IP<sub>3</sub>R inhibitor does not affect DA release. 100 μM 2-aminoethoxydiphenylborane  
1012 (2-APB) was dissolved in 0.1 % DMSO saline. Two-way ANOVA indicates no  
1013 significant differences in spH fluorescence due to 2-APB treatment. N = 10 for 2-APB  
1014 treatment and N = 9 for mock control. **B**, The RyR inhibitor, dantrolen, inhibits DA  
1015 release. 10 μM dantrolen was dissolved in 0.1% DMSO saline. Two-way ANOVA  
1016 indicates significant effects of drug treatment on spH fluorescence ( $F_{1,48} = 6.781$ ,  $P =$   
1017  $0.0122$ , N = 8 for all data). \* $P < 0.05$  compared with mock treated samples assayed by  
1018 Bonferroni *post hoc* tests. **C**, Application of a RyR agonist induces DA release. 1 mM  
1019 4-chloro-3-methylphenol (4C3MP) was dissolved in 0.2% ethanol saline containing 1  
1020 μM TTX and applied for the indicated period of time (shown in yellow). \*\*  $P < 0.001$   
1021 as assayed by Student's t-test comparing before (0-30 s) and after (280-300 s) 4C3MP  
1022 treatment. N = 8 for all data. **D**, Temporal RyR knockdown at 30 °C in TH-DA neurons  
1023 prevents DA release evoked by AL + NMDA stimulation. Two-way ANOVA indicates  
1024 significant differences in spH fluorescence between restrictive and permissive  
1025 temperatures ( $F_{3,56} = 6.625$ ,  $P = 0.0007$ , N = 8 for all data). \*\*  $P < 0.01$  by Bonferroni  
1026 *post hoc* tests. **E**, Temporal knock down of RyR in TH-DA neurons prevents DA release  
1027 evoked by CO application. CO saturated saline was applied during the indicated time  
1028 period (shown in pink) from a micropipet. Two-way ANOVA indicates significant  
1029 interaction differences in spH fluorescence due to time, temperature, and interaction  
1030 between time and temperature ( $F_{2,42} = 12.6$ ,  $P < 0.0001$ , N = 8 for all data). \*\*  $P < 0.01$   
1031 by Bonferroni *post hoc* tests.

1032

1033



1034 **SUPPLEMENTAL INFORMATION FOR**

1035 **“Carbon monoxide, a retrograde messenger generated in post-synaptic mushroom**  
1036 **body neurons evokes non-canonical dopamine release.”**

1037

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1041

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1050

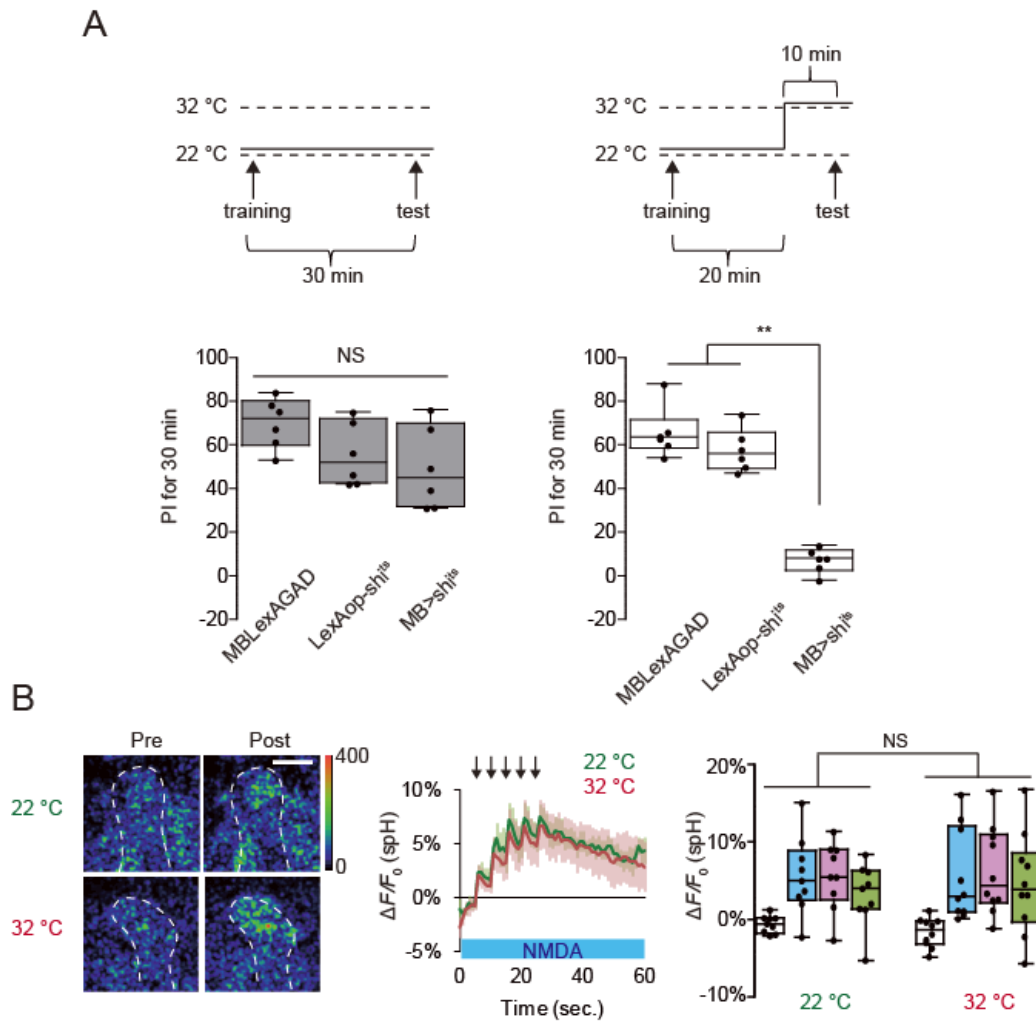
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1053

1054 **THIS SUPPLEMENTAL INFORMATION INCLUDES:**

1055 Figures S1 to S4

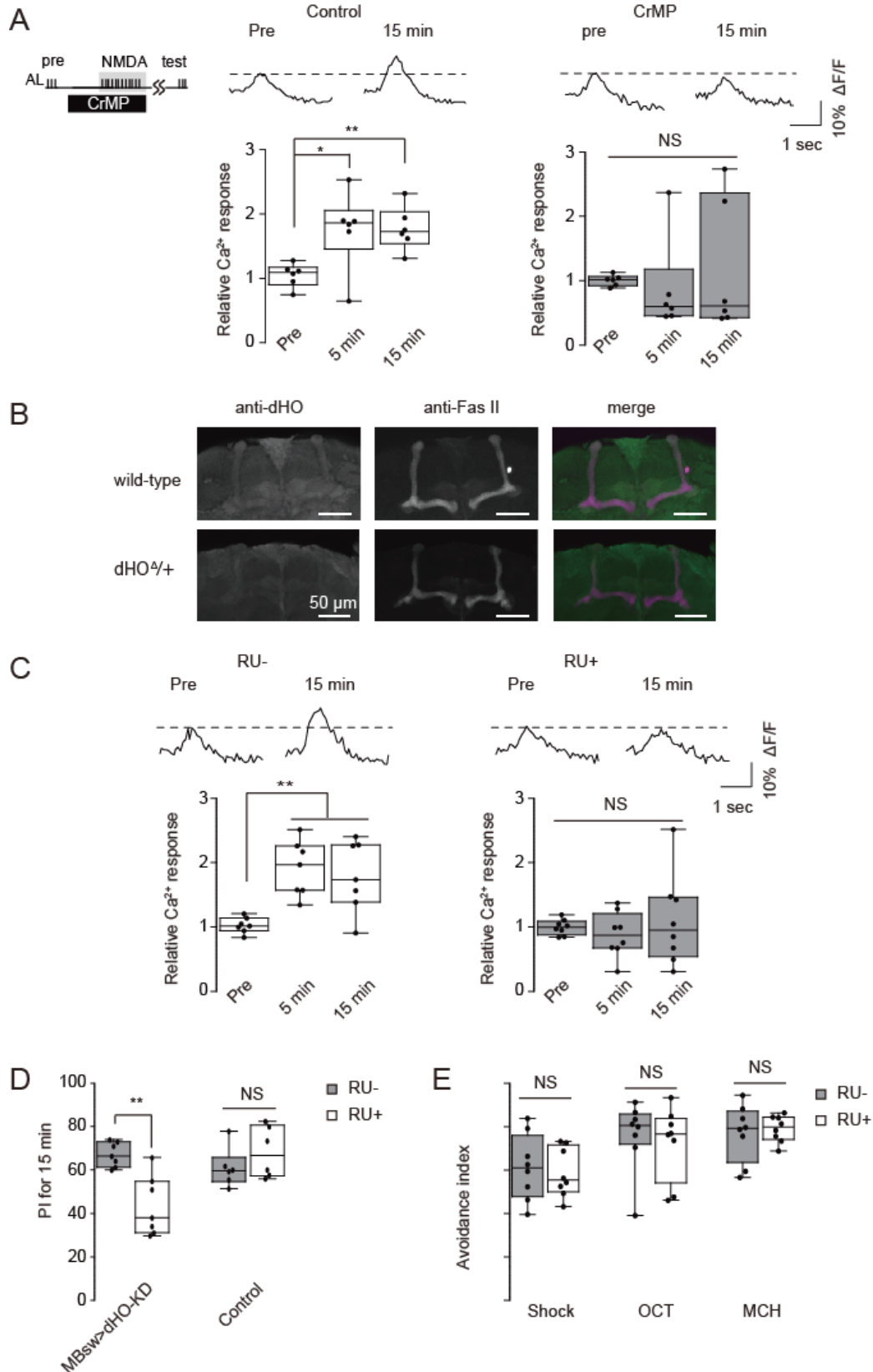
1056 Table S1



1057

1058 **Figure S1 Coincident MB stimulation induces SV exocytosis from DA neurons. MB**  
 1059 **output is not required for DA release.**

1060 **A**, Inhibiting MB output impairs recall of olfactory memory. One-way ANOVA and  
 1061 Bonferroni post hoc tests indicate significant impairment in memory recall in *MB > shi<sup>ts</sup>*  
 1062 flies at restrictive temperature (32°C) ( $F_{2,15} = 71.05$ ,  $P < 0.001$ ) but not at permissive  
 1063 temperature (22°C) ( $F_{2,15} = 2.854$ ,  $P = 2.854$ ). \*\* $P < 0.01$  and NS  $P > 0.05$ . N = 6 for  
 1064 all data. **B**, Inhibiting MB output did not affect DA release. Two-way ANOVA identified  
 1065 no significant changes in spH fluorescence between restrictive (32 °C) and permissive  
 1066 temperatures (22 °C). N = 9 for 32 °C and N = 10 for 22°C.



1068 **Figure S2 dHO in the MBs is required LTE and olfactory learning.**

1069 **A**, Effects of HO inhibitor, CrMP, on LTE induced by AL+ NMDA stimulation.

1070 One-way ANOVA and Bonferroni post hoc tests indicate significant changes in

1071 AL-evoked  $\text{Ca}^{2+}$  responses in the MB after AL + NMDA stimulation in control

1072 conditions ( $F_{2,15} = 5.836$ ,  $P = 0.013$ ,  $N = 6$ ) and but not in 10  $\mu\text{M}$  CrMP treated

1073 conditions ( $F_{2,18} = 0.339$ ,  $P = 0.717$ ,  $N = 7$ ). **B**, dHO antibody labels the MB lobes and

1074 midline cells in the *Drosophila* brain. Fas II antibody staining is included to identify

1075 subsets of the MB lobes. dHO signals are reduced in  $dHO^d$  hemizygotes ( $dHO^d/+$ )

1076 demonstrating the specificity of the antibody. **C**, Knockdown of  $dHO$  in the MBs

1077 impairs LTE induced by AL + NMDA stimulation. One-way ANOVA and Bonferroni

1078 post hoc tests indicate significant LTE in the MB after AL + NMDA stimulation in

1079 control (RU-) conditions where  $dHO$  is not knocked down (Left panel,  $F_{2,18} = 9.630$ ,  $P =$

1080  $0.001$ ,  $N = 7$ ). LTE is not observed when  $dHO$  is knocked down (RU+) (Right panel,

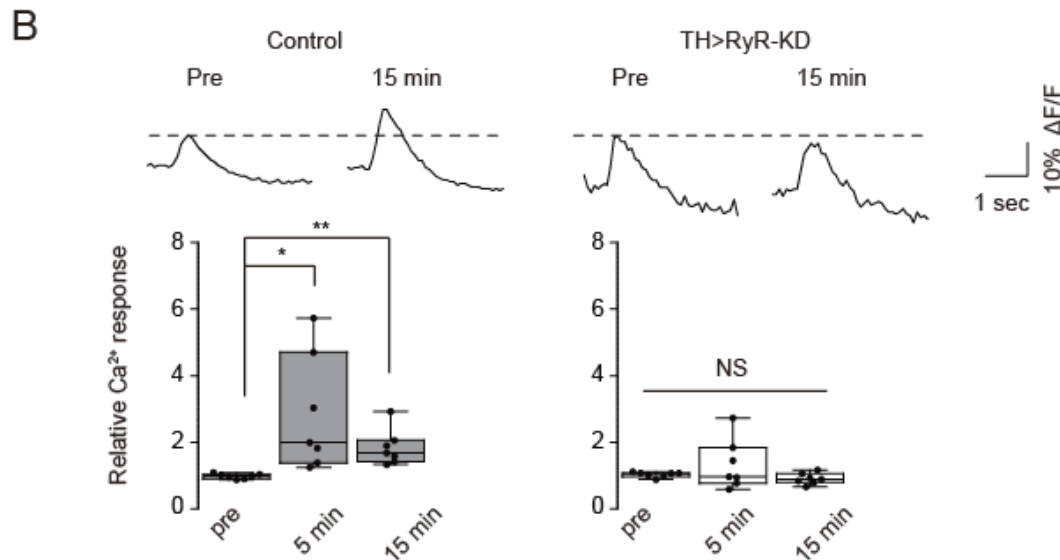
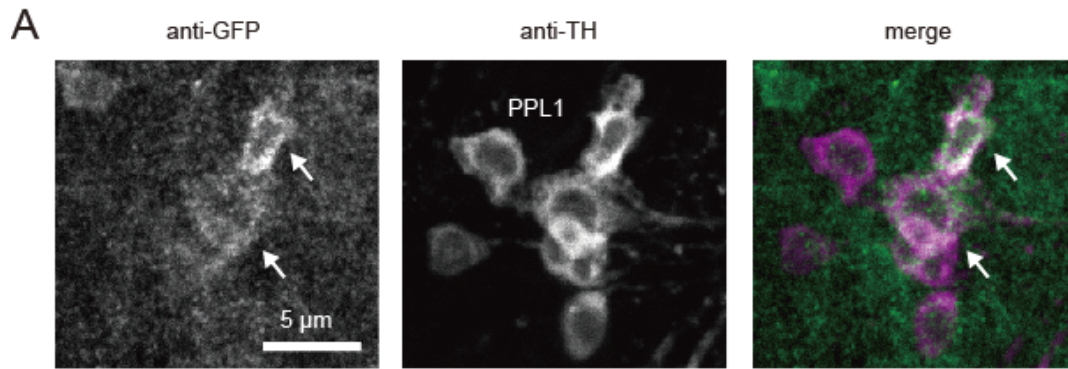
1081  $F_{2,21} = 0.444$ ,  $P = 0.647$ ,  $N = 8$ ). **D**, Knocking down  $dHO$  in the MBs impairs olfactory

1082 learning.  $**P < 0.01$  determined by Student's t-test.  $N = 7$  for all data. **e**, Naïve

1083 responses to odors and electrical shock are not affected by knocking down  $dHO$  in the

1084 MBs.  $N = 8$  for all experiments.

1085



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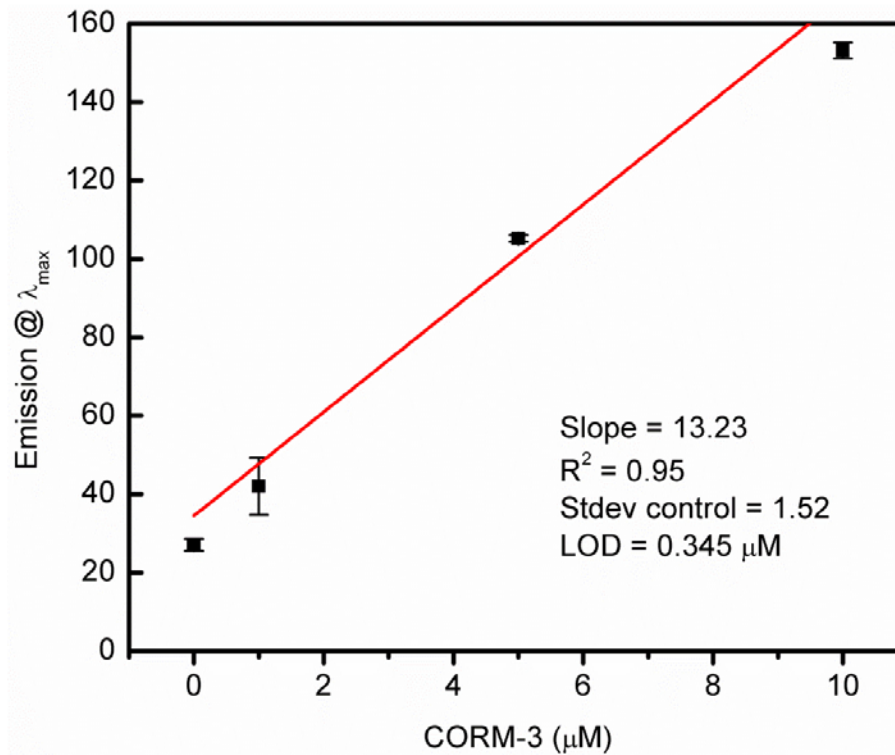
1087 **Figure S3 RyRs in TH-DA terminals are required for LTE.**

1088 **A**, RyR localization was examined in *UAS-mCD8::GFP/Mi{Trojan-GAL4.0}*  
1089 *RyR[MI08146-TG4.0]* flies in which expression of mCD8::GFP is driven by  
1090 Trojan-GAL4 inserted in the endogenous *RyR* gene. GFP expression overlapped with  
1091 expression of tyrosine hydroxylase (TH) in PPL1 DA neurons (arrows) that innervate  
1092 the vertical lobes of the MBs. **B**, Knocking down RyRs in TH-DA neurons abolishes  
1093 LTE induced by AL + NMDA stimulation. One-way ANOVA and Bonferroni post hoc  
1094 tests indicate significant increases in AL-evoked  $\text{Ca}^{2+}$  responses after AL + NMDA

1095 stimulation in control brains ( $F_{2,18} = 5.455$ ,  $P = 0.014$ ) but not in TH>RyR-KD brains

1096 ( $F_{2,18} = 1.666$ ,  $P = 0.217$ ).  $N = 7$  for all data.

1097



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1099 **Figure S4 Limit of detection determination for COP-1.**

1100 Limit of detection (LOD) of COP-1 at 90 min determined by fluorescence intensity at

1101 508 nm as a function of CORM-3 concentration. While LOD of COP-1 is 0.345  $\mu\text{M}$  at

1102 90 min, a similar range of LOD's were found at other timepoints (LOD = 0.660  $\mu\text{M}$  at t

1103 = 30 min, 0.673  $\mu\text{M}$  at t = 45 min, 0.556  $\mu\text{M}$  at 60 min). Each concentration was run in

1104 triplicate and control was run five times. All data are shown as mean  $\pm$  SD.

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Table S1 Genotypes used in each experiment

Fig. #	genotype
1A	<i>UAS-spH/MB-LexA:GAD;TH-GAL4/LexAop-shi<sup>Δ5</sup></i>
1B	<i>UAS-spH; TH-GAL4</i>
1C	<i>UAS-spH; TH-GAL4</i>
1D	<i>UAS-spH; TH-GAL4</i>
1E	<i>UAS-dicer/LexAop-spH;UAS-dHO-IR, MBsw/TH-LexAGAD</i>
2A	<i>UAS-spH; TH-GAL4</i>
2B	<i>UAS-spH; TH-GAL4</i>
2C	<i>UAS-spH; TH-GAL4</i>
2D	<i>UAS-spH; TH-GAL4</i>
3A	<i>MB-LexA:GAD, LexAop-R-GECO1</i>
3B	<i>MB-LexA:GAD, LexAop-R-GECO1</i>
3C	<i>MB-LexA:GAD, LexAop-R-GECO1/UAS-dicer; UAS-dHOIR, MBsw</i>
3D	<i>MB-LexA:GAD, LexAop-R-GECO1/UAS-spH; TH-GAL4</i>
4A	<i>MB-LexA:GAD, LexAop-R-GECO1/UAS-G-CaMP3; TH-GAL4</i>
4B	<i>MB-LexA:GAD, LexAop-R-GECO1/UAS-G-CaMP3; TH-GAL4</i>
4C	<i>UAS-G-CaMP3; TH-GAL4</i>
4D	<i>UAS-spH; TH-GAL4</i>
4E	<i>UAS-spH; TH-GAL4</i>
4F	<i>UAS-spH; TH-GAL4</i>
5A	<i>UAS-spH; TH-GAL4</i>
5B	<i>UAS-spH; TH-GAL4</i>
5C	<i>UAS-spH; TH-GAL4</i>
5D	<i>UAS-RyR RNAi, tubp-GAL80(ts)/LexAop-spH; TH-GAL4/TH-LexAp65</i>
5E	<i>UAS-RyR RNAi, tubp-GAL80(ts)/LexAop-spH; TH-GAL4/TH-LexAp65</i>
Supplementary Fig.	
#	
S1A	<i>UAS-spH; TH-GAL4</i>
S1B	<i>MB-LexA:GAD, LexAop-G-CaMP2</i>
S1C	<i>MB-LexA:GAD, LexAop-G-CaMP2</i>

S2A	<i>MB-LexA:GAD; LexAop-shi<sup>ts</sup> as MB &gt; shi<sup>ts</sup></i>
	<i>MB-LexA:GAD</i>
	<i>LexAop-shi<sup>ts</sup></i>
S2B	<i>c747-GAL4/LexAop-spH;UAS-shi<sup>ts</sup>/TH-LexAp65</i>
S3A	<i>MB-LexA:GAD, LexAop-G-CaMP2</i>
S3B	<i>CS(w) as wild-type</i>
	<i>Df(3R)Exel7309/+ as dHO<sup>Δ</sup>/+</i>
S3C	<i>UAS-dicer/MBLexA:GAD, LexAop-G-CaMP2;UAS-dHO-IR, MBsw/+</i>
S3D	<i>UAS-dicer/+; MBsw, UAS-dHO-IR/Df(3R)Exel7309 as MBsw &gt; UAS-dHO IR</i>
	<i>UAS-dicer/+; Df(3R)Exel7309/+ as control</i>
S4A	<i>Mi{Trojan-GAL4.0}RyR[MI08146-TG4.0]/UAS-mCD8::<i>GFP</i></i>
S4B	<i>UAS-G-CaMP2/+; TH-GAL4 as control</i>
	<i>UAS-RyR RNAi/UAS-G-CaMP2/+; TH-GAL4 as TH&gt;RyR-KD</i>

1108