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1 **Inhibitory muscarinic acetylcholine receptors enhance**  
2 **aversive olfactory learning in adult *Drosophila***

3  
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15

16 **Abstract**

17           Olfactory associative learning in *Drosophila* is mediated by synaptic plasticity  
18 between the Kenyon cells of the mushroom body and their output neurons. Both  
19 Kenyon cells and their inputs from projection neurons are cholinergic, yet little is known  
20 about the physiological function of muscarinic acetylcholine receptors in learning in  
21 adult flies. Here we show that aversive olfactory learning in adult flies requires type A  
22 muscarinic acetylcholine receptors (mAChR-A), particularly in the gamma subtype of  
23 Kenyon cells. mAChR-A inhibits odor responses and is localized in Kenyon cell  
24 dendrites. Moreover, mAChR-A knockdown impairs the learning-associated depression  
25 of odor responses in a mushroom body output neuron. Our results suggest that  
26 mAChR-A function in Kenyon cell dendrites is required for synaptic plasticity between  
27 Kenyon cells and their output neurons.

28

## 29 **Introduction**

30           Animals learn to modify their behavior based on past experience by changing  
31 connection strengths between neurons, and this synaptic plasticity is often regulated by  
32 metabotropic receptors. In particular, neurons commonly express both ionotropic and  
33 metabotropic receptors for the same neurotransmitter, where the two may mediate  
34 different functions (e.g., direct excitation/inhibition vs. synaptic plasticity). In mammals,  
35 where glutamate is the principal excitatory neurotransmitter, metabotropic glutamate  
36 receptors (mGluRs) have been widely implicated in synaptic plasticity and memory  
37 (Jörntell and Hansel, 2006; Lüscher and Huber, 2010). Given the complexity of linking  
38 behavior to artificially induced plasticity in brain slices (Schonewille et al., 2011;  
39 Yamaguchi et al., 2016), it would be useful to study the role of metabotropic receptors in  
40 learning in a simpler genetic model system with a clearer behavioral readout of synaptic  
41 plasticity. One such system is *Drosophila*, where powerful genetic tools and well-defined  
42 anatomy have yielded a detailed understanding of the circuit and molecular  
43 mechanisms underlying associative memory (Busto et al., 2010; Cognigni et al., 2017;  
44 Hige, 2018). The principal excitatory neurotransmitter in *Drosophila* is acetylcholine, but,  
45 surprisingly, little is known about the function of metabotropic acetylcholine signaling in  
46 synaptic plasticity or neuromodulation in *Drosophila*. Here we address this question  
47 using olfactory associative memory.

48           Flies can learn to associate an odor (conditioned stimulus, CS) with a positive  
49 (sugar) or a negative (electric shock) unconditioned stimulus (US), so that they later  
50 approach 'rewarded' odors and avoid 'punished' odors. This association is thought to be  
51 formed in the presynaptic terminals of the ~2,000 Kenyon cells (KCs) that make up the  
52 mushroom body (MB), the fly's olfactory memory center (Busto et al., 2010; Cognigni et  
53 al., 2017; Hige, 2018). These KCs are activated by odors via second-order olfactory  
54 neurons called projection neurons (PNs). Each odor elicits responses in a sparse  
55 subset of KCs (Campbell et al., 2013; Lin et al., 2014) so that odor identity is encoded in  
56 which KCs respond to each odor. When an odor (CS) is paired with reward/punishment  
57 (US), an odor-specific set of KCs is activated at the same time that dopaminergic  
58 neurons (DANs) release dopamine onto KC presynaptic terminals. The coincident

59 activation causes long-term depression (LTD) of synapses from the odor-activated KCs  
60 onto mushroom body output neurons (MBONs) that lead to approach or avoidance  
61 behavior (Aso and Rubin, 2016; Aso et al., 2014b; Cohn et al., 2015; Hige et al., 2015;  
62 Oswald et al., 2015; Perisse et al., 2016; Séjourné et al., 2011). In particular, training  
63 specifically depresses KC-MBON synapses of the 'wrong' valence (e.g., odor-  
64 punishment pairing depresses odor responses of MBONs that lead to approach  
65 behavior), because different pairs of 'matching' DANs/MBONs (e.g.  
66 punishment/approach, reward/avoidance) innervate distinct regions along KC axons  
67 (Aso et al., 2014a).

68 Both MB input (PNs) and output (KCs) are cholinergic (Barnstedt et al., 2016;  
69 Yasuyama and Salvaterra, 1999), and KCs express both ionotropic (nicotinic) and  
70 metabotropic (muscarinic) acetylcholine receptors (Crocker et al., 2016; Croset et al.,  
71 2018; Davie et al., 2018; Shih et al., 2019). The nicotinic receptors mediate fast  
72 excitatory synaptic currents (Su and O'Dowd, 2003), while the physiological function of  
73 the muscarinic receptors is unknown. Muscarinic acetylcholine receptors (mAChRs) are  
74 G-protein coupled receptors; out of the three mAChRs in *Drosophila* (mAChR-A,  
75 mAChR-B and mAChR-C), mAChR-A (also called Dm1, mAChR-60C or mAChR) is the  
76 most closely homologous to mammalian mAChRs (Collin et al., 2013). Mammalian  
77 mAChRs are typically divided between 'M<sub>1</sub>-type' (M<sub>1</sub>/M<sub>3</sub>/M<sub>5</sub>), which signal via G<sub>q</sub> and are  
78 generally excitatory, and 'M<sub>2</sub>-type' (M<sub>2</sub>/M<sub>4</sub>), which signal via G<sub>i/o</sub> and are generally  
79 inhibitory (Caulfield and Birdsall, 1998). *Drosophila* mAChR-A seems to use 'M<sub>1</sub>-type'  
80 signaling: when heterologously expressed in Chinese hamster ovary (CHO) cells, it  
81 signals via G<sub>q</sub> protein (Collin et al., 2013; Ren et al., 2015) to activate phospholipase C,  
82 which produces inositol trisphosphate to release Ca<sup>2+</sup> from internal stores.

83 Recent work indicates that mAChR-A is required for aversive olfactory learning in  
84 *Drosophila* larvae, as knocking down mAChR-A expression in KCs impairs learning  
85 (Silva et al., 2015). However, it is unclear whether mAChR-A is involved in olfactory  
86 learning in adult *Drosophila*, given that mAChR-A is thought to signal through G<sub>q</sub>, and in  
87 adult flies G<sub>q</sub> signaling downstream of the dopamine receptor Damb promotes  
88 forgetting, not learning (Berry et al., 2012; Himmelreich et al., 2017). Moreover, it is

89 unknown how mAChR-A affects the activity or physiology of KCs, where it acts (at KC  
90 axons or dendrites or both), and how these effects contribute to olfactory learning.

91 Here we show that mAChR-A is required in KCs for aversive olfactory learning in  
92 adult *Drosophila*. Surprisingly, genetic and pharmacological manipulations of mAChR-A  
93 suggest that mAChR-A is inhibitory and acts on KC dendrites. Moreover, mAChR-A  
94 knockdown impairs the learning-associated depression of odor responses in an MB  
95 output neuron, MB-MVP2, that is required for aversive memory retrieval. We suggest  
96 that dendritically-acting mAChR-A is required for synaptic depression between KCs and  
97 their outputs.

98

## 99 **Results**

### 100 **mAChR-A expression in KCs is required for aversive olfactory learning in adult** 101 **flies**

102 *Drosophila* larvae with reduced mAChR-A expression in KCs show impaired  
103 aversive olfactory learning (Silva et al., 2015), but it remains unknown whether mAChR-  
104 A in KCs also functions in learning in adult flies. We addressed this question by  
105 knocking down mAChR-A expression in KCs using two UAS-RNAi lines, “RNAi 1” and  
106 “RNAi 2” (see Methods). Only RNAi 2 requires co-expression of Dicer-2 (Dcr-2) for  
107 optimal knockdown. To test the efficiency of these RNAi constructs, we expressed them  
108 pan-neuronally using elav-GAL4 and measured their effects on mAChR-A expression  
109 levels using quantitative real time polymerase chain reaction (qRT-PCR). Both RNAi  
110 lines strongly reduce mAChR-A levels (RNAi 1:  $39\pm 8\%$  of elav-GAL4 control, i.e.,  
111  $61\pm 8\%$  below normal; RNAi 2:  $43\pm 10\%$  of normal; mean $\pm$ s.e.m.; see **Figure 1A**). We  
112 then examined whether knocking down mAChR-A in KCs using the pan-KC driver  
113 OK107-GAL4 affects short term aversive learning in adult flies. We used the standard  
114 odors used in the field (i.e. 3-octanol, OCT, and 4-methylcyclohexanol, MCH; see  
115 Methods). Under these conditions both UAS-RNAi transgenes significantly reduced  
116 aversive learning, whether training against MCH or OCT (**Figure 1B,C** and **Figure 1—**  
117 **figure supplement 1**). Interestingly, knocking down mAChR-A did not affect learning

118 when we trained flies with a more intense shock (90 V instead of 50 V, **Figure 1—**  
119 **figure supplement 1**), suggesting that mAChR-A may only be required for learning with  
120 moderate intensity reinforcement, not severe reinforcement. Consistent with this,  
121 knocking down mAChR-A had no effect on naive avoidance of MCH and OCT (**Figure**  
122 **1D**; see Methods) or flies' reaction to electric shock (**Figure 1—figure supplement 1**),  
123 showing that the defect was specific to learning, rather than reflecting a failure to detect  
124 odors or shock.

125         Given that mAChR-A is expressed in the larval MB and indeed contributes to  
126 aversive learning in larvae, it is possible that developmental effects underlie the reduced  
127 learning observed in mAChR-A KD flies. To test this, we used tub-GAL80<sup>ts</sup> to suppress  
128 RNAi 1 expression during development. Flies were grown at 23°C until 3 days after  
129 eclosion and were then transferred to 31°C for 7 days. Adult-only knockdown of  
130 mAChR-A in KCs reduced learning (**Figure 1E**), just as constitutive knockdown did,  
131 indicating that mAChR-A plays a physiological, not purely developmental, role in  
132 aversive learning. To further verify that GAL80<sup>ts</sup> efficiently blocks RNAi expression (i.e.,  
133 that GAL80<sup>ts</sup> is not leaky), flies were grown at 23°C without transferring them to 31°C,  
134 thus blocking RNAi expression also in adults. When tested for learning at 10 days old,  
135 these flies showed normal learning (**Figure 1E**).

136

### 137 **mAChR-A is required for olfactory learning in $\gamma$ KCs, not $\alpha\beta$ or $\alpha'\beta'$ KCs**

138         Kenyon cells are subdivided into three main classes according to their  
139 innervation of the horizontal and vertical lobes of the MB:  $\gamma$  neurons send axons only to  
140 the  $\gamma$  lobe of the horizontal lobes, while the axons of  $\alpha\beta$  and  $\alpha'\beta'$  neurons bifurcate and  
141 go to both the vertical and horizontal lobes ( $\alpha\beta$  axons make up the  $\alpha$  lobe of the vertical  
142 lobe and  $\beta$  lobe of the horizontal lobe, while  $\alpha'\beta'$  axons make up the  $\alpha'$  lobe of the  
143 vertical lobe and  $\beta'$  portion of the horizontal lobe). These different classes play different  
144 roles in olfactory learning (Güven-Ozkan and Davis, 2014; Krashes et al., 2007). To  
145 unravel in which class(es) mAChR-A functions, we used a Minos-mediated integration  
146 cassette (MiMIC) line to investigate where mAChR-A is expressed (Venken et al.,  
147 2011). The MiMIC insertion in mAChR-A lies in the first 5' non-coding intron, creating a

148 gene trap where GFP in the MiMIC cassette should be expressed in whichever cells  
149 endogenously express mAChR-A. Because the GFP in the original mAChR-A MiMIC  
150 cassette produced very little fluorescent signal (data not shown), we used recombinase-  
151 mediated cassette exchange (RMCE) to replace the original MiMIC cassette with a  
152 MiMIC cassette containing GAL4 (Venken et al., 2011). These new mAChR-A-MiMIC-  
153 GAL4 flies should express GAL4 wherever mAChR-A is endogenously expressed. To  
154 reveal the expression pattern of mAChR-A, we crossed mAChR-A-MiMIC-GAL4 and  
155 20xUAS-eGFP flies. mAChR-A-MiMIC-GAL4 drove GFP expression throughout the  
156 brain, consistent with previous reports (Blake et al., 1993; Croset et al., 2018; Davie et  
157 al., 2018; Hannan and Hall, 1996) and with the fact that the *Drosophila* brain is mostly  
158 cholinergic. In the mushroom bodies, GFP was expressed in the  $\alpha\beta$  and  $\gamma$  lobes, but not  
159 the  $\alpha'\beta'$  lobes (**Figure 2A**). No GFP signal was observed with an inverted insertion  
160 where GAL4 is inserted in the MiMIC locus in the wrong direction (data not shown).  
161 Consistent with these MiMIC results, two recently reported databases of single-cell  
162 transcriptomic analysis of the *Drosophila* brain (Croset et al., 2018; Davie et al., 2018)  
163 confirm that mAChR-A is more highly expressed in  $\alpha\beta$  and  $\gamma$  KCs than in  $\alpha'\beta'$  KCs  
164 (**Figure 2—figure supplement 1**). However, mAChR-A is still clearly present in  $\alpha'\beta'$   
165 KCs' transcriptomes, suggesting that mAChR-A-MiMIC-GAL4 may not reveal all  
166 neurons that express mAChR-A.

167 The higher expression of mAChR-A in  $\alpha\beta$  and  $\gamma$  KCs compared to  $\alpha'\beta'$  KCs  
168 suggests that learning would be impaired by mAChR-A knockdown in  $\alpha\beta$  or  $\gamma$ , but not  
169  $\alpha'\beta'$ , KCs. To test this, we expressed mAChR-A RNAi in different KC classes. As  
170 expected, aversive olfactory learning was reduced by knocking down mAChR-A in  $\alpha\beta$   
171 and  $\gamma$  KCs together using MB247-GAL4, but not by knockdown in  $\alpha'\beta'$  KCs using c305a-  
172 GAL4. To examine if  $\alpha\beta$  and  $\gamma$  KCs both participate in the reduced learning observed in  
173 mAChR-A knockdown flies, we sought to limit mAChR-A RNAi expression to either  $\alpha\beta$   
174 or  $\gamma$  neurons. While strong driver lines exist for  $\alpha\beta$  neurons, the  $\gamma$  GAL4 drivers we  
175 tested were fairly weak (H24-GAL4, MB131B, R45H04-GAL4, data not shown), perhaps  
176 too weak to drive mAChR-A-RNAi enough to knock down mAChR-A efficiently.  
177 Therefore, we used MB247-GAL4, which was strong enough to affect behavior, and  
178 blocked GAL4 activity in either  $\alpha\beta$  or  $\gamma$  KCs by expressing the GAL80 repressor under

179 the control of R44E04-LexA ( $\alpha\beta$  KCs) or R45H04-LexA ( $\gamma$  KCs) (Bräcker et al., 2013).  
180 These combinations drove strong, specific expression in  $\alpha\beta$  or  $\gamma$  KCs (**Figure 2—figure**  
181 **supplement 2**). Learning was reduced by mAChR-A RNAi expression in  $\gamma$ , but not  $\alpha\beta$ ,  
182 KCs (**Figure 2B**). These results suggest that mAChR-A is specifically required in  $\gamma$  KCs  
183 for aversive olfactory learning and short-term memory.

184

### 185 **mAChR-A suppresses odor responses in $\gamma$ KCs**

186 We next asked what effect mAChR-A knockdown has on the physiology of KCs,  
187 by expressing GCaMP6f and mAChR-A RNAi 2 together in KCs using OK107-GAL4  
188 (this driver and RNAi combination was also used for behavior in **Figure 1C**). Knocking  
189 down mAChR-A in KCs increased odor-evoked  $\text{Ca}^{2+}$  influx in the mushroom body calyx,  
190 where KC dendrites reside (**Figure 3**). This result is somewhat surprising because  
191 mAChR-A is a  $G_q$  coupled receptor whose activation leads to  $\text{Ca}^{2+}$  release from internal  
192 stores (Ren et al., 2015), which predicts that mAChR-A knockdown should decrease,  
193 not increase, odor-evoked  $\text{Ca}^{2+}$  influx in KCs. However, some examples have been  
194 reported of inhibitory signaling through  $G_q$  by  $M_1$ -type mAChRs (see Discussion), and  
195 *Drosophila* mAChR-A may join these as another example of an inhibitory mAChR  
196 signaling through  $G_q$ .

197 Because mAChR-A is required for aversive learning in  $\gamma$  KCs, not  $\alpha\beta$  or  $\alpha'\beta'$  KCs  
198 (**Figure 2**), we next asked how odor responses in  $\alpha\beta$ ,  $\alpha'\beta'$  and  $\gamma$  KCs are affected by  
199 mAChR-A knockdown.  $\alpha\beta$ ,  $\alpha'\beta'$  and  $\gamma$  KC dendrites are not clearly segregated in the  
200 calyx, so we examined odor responses in the axonal lobes. Indeed, although odor  
201 responses in all lobes were increased by mAChR-A knockdown, only in the  $\gamma$  lobe was  
202 the effect statistically significant for both MCH and OCT (**Figure 3**). This result is  
203 consistent with the behavioral requirement for mAChR-A only in  $\gamma$  KCs. However, we do  
204 not rule out the possibility that mAChR-A knockdown also affects  $\alpha\beta$  and  $\alpha'\beta'$  odor  
205 responses in a way that does not affect short-term memory, especially as  $\alpha\beta$  and  $\alpha'\beta'$   
206 odor responses were somewhat, though not consistently significantly, increased.  
207 Although the  $\Delta F/F$  traces from the  $\gamma$  lobe had higher signal-to-noise ratio (SNR) than  
208 some other lobes (**Figure 3—figure supplement 1**) due to its larger size (averaging

209 over more pixels) or shallower z-depth (less light scattering), a power analysis revealed  
210 that all lobes had SNRs high enough to detect an effect as large as that observed in the  
211  $\gamma$  lobe (**Figure 3—figure supplement 1**). However, note that we do not exclude the  
212 possibility that  $\alpha\beta$ - or  $\alpha'\beta'$ -specific (as opposed to pan-KC) knockdown of mAChR-A  
213 might significantly increase  $\alpha\beta$  or  $\alpha'\beta'$  KC odor responses.

214 Do increased odor responses in  $\gamma$  KCs prevent learning by increasing the overlap  
215 between the  $\gamma$  KC population representations of the two odors used in our task (Lin et  
216 al., 2014)? When GCaMP6f and mAChR-A-RNAi 2 were expressed in all KCs, mAChR-  
217 A knockdown did not affect the sparseness or inter-odor correlation of KC population  
218 odor responses (**Figure 4A-C**) even though it increased overall calyx responses. To  
219 focus specifically on  $\gamma$  KCs, we expressed GCaMP6f and mAChR-A-RNAi 1 only in  $\gamma$   
220 KCs, using mb247-Gal4, R44E04-LexA and lexAop-GAL80, the same driver and RNAi  
221 combination used in the behavioral experiments in **Figure 2B**. GCaMP6f was visible  
222 mainly in the  $\gamma$  lobe (**Figure 4D**).  $\gamma$ -only expression of mAChR-A-RNAi 1 increased odor  
223 responses in the calyx (here, dendrites of  $\gamma$  KCs only) and, in the case of OCT, in the  $\gamma$   
224 lobe (**Figure 4E,F**). Note that  $\gamma$  KC odor responses are increased by both RNAi 1  
225 (**Figure 3A,B**) and RNAi 2 (**Figure 4E,F**). As with pan-KC expression,  $\gamma$ -only expression  
226 of mAChR-A-RNAi 1 did not affect the sparseness or inter-odor correlation of  $\gamma$  KCs  
227 (**Figure 4G-I**). Thus, mAChR-A knockdown does not impair learning through increased  
228 overlap in KC population odor representations.

229

### 230 **KC odor responses are decreased by an mAChR agonist**

231 RNAi-based knockdown of mAChR-A might induce homeostatic compensation  
232 that obscures or even reverses the primary effect of reduced mAChR-A expression. To  
233 test the acute role of mAChR-A in regulating KC activity, we took the complementary  
234 approach of pharmacologically activating mAChR-A. Initially we bath-applied 10  $\mu$ M  
235 muscarine, an mAChR-A agonist (*Drosophila* mAChR-B is 1000-fold less sensitive to  
236 muscarine than mAChR-A is (Collin et al., 2013), and mAChR-C is not expressed in the  
237 brain (Davie et al., 2018)). Muscarine strongly decreased odor responses in all subtypes  
238 of KCs (**Figure 5A,B, Figure 5—figure supplement 1**). However, muscarine did not

239 significantly affect the amplitude of odor responses in PN axons in the calyx (**Figure**  
240 **5C**), suggesting that the effect of muscarine on KCs arose in KCs, not earlier in the  
241 olfactory pathway. KCs can be silenced by an inhibitory GABAergic neuron called the  
242 anterior paired lateral (APL) neuron (Lin et al., 2014; Masuda-Nakagawa et al., 2014;  
243 Papadopoulou et al., 2011), so we asked whether muscarine reduces KC odor  
244 responses indirectly by activating APL, rather than directly inhibiting KCs. We applied  
245 muscarine to flies with APL-specific expression of tetanus toxin (TNT), which blocks  
246 inhibition from APL and thereby greatly increases KC odor responses. In these flies,  
247 APL is labeled stochastically, so hemispheres where APL was unlabeled served as  
248 controls (Lin et al., 2014) (see Methods). Muscarine decreased KC odor responses both  
249 in control hemispheres and hemispheres where APL synaptic output was blocked by  
250 tetanus toxin (**Figure 5D**), and the effect of muscarine was not significantly different  
251 between the two cases (**Figure 5E**). This result indicates that muscarine does not act  
252 solely by activating APL or by enhancing inhibition on KCs (e.g., increasing membrane  
253 localization of GABA<sub>A</sub> receptors).

254 To test mAChR-A function even more acutely, we locally applied muscarine to  
255 the MB calyx by pressure ejection (**Figure 6, Figure 6—figure supplement 1**). Red  
256 dye included in the ejected solution confirmed that the muscarine remained in the calyx  
257 for several seconds but did not spread to the MB lobes (**Figure 6B**). Surprisingly,  
258 applying muscarine to the calyx in the absence of odor stimuli increased GCaMP signal  
259 in the calyx and  $\alpha$  lobe, with small increases in the  $\beta$  and  $\gamma$  lobe that were not  
260 statistically significant (**Figure 6A,C**). It also decreased GCaMP signal in the  $\alpha'$  and  $\beta'$   
261 lobes around 1–2 s after application (**Figure 6A**), although this effect was also not  
262 statistically significant. The increased Ca<sup>2+</sup> in the calyx most likely did not reflect  
263 increased excitability, as applying muscarine to the calyx did not increase the calyx odor  
264 response (**Figure 6D,E**). If anything, it likely *decreased* the calyx odor response,  
265 because the Ca<sup>2+</sup> increase induced by muscarine alone (no odor) lasted ~6–7 s and  
266 thus would have continued into the odor pulse in the muscarine + odor condition. If the  
267 odor response was unaffected by muscarine, the muscarine-evoked and odor-evoked  
268 increases in GCaMP6f signal should have summed. Instead, the peak calyx  $\Delta F/F$  during

269 the odor pulse was the same before and after locally applying muscarine, suggesting  
270 that the specifically odor-evoked increase in GCaMP6f was decreased by muscarine.

271 Indeed, applying muscarine to the calyx suppressed odor responses in KC axons  
272 (**Figure 6D,E**). Although muscarine did not significantly affect peak  $\Delta F/F$  during the odor  
273 in the  $\alpha$  lobe, muscarine most likely did decrease  $\alpha$  lobe odor responses, by the same  
274 logic as for calyx odor responses (see above). Given that calyx muscarine suppresses  
275  $\alpha'\beta'$  axonal odor responses, the decrease in  $\alpha'\beta'$  KC GCaMP signal in the absence of  
276 odor likely reflects suppression of spontaneous action potentials (**Figure 6A,C**), as  $\alpha'\beta'$   
277 KCs have the highest spontaneous spike rate out of the three subtypes (Groschner et  
278 al., 2018; Turner et al., 2008). The effect of muscarine on  $\alpha'\beta'$  KCs is consistent with  
279 single-cell transcriptome analyses showing that  $\alpha'\beta'$  KCs express mAChR-A, albeit at a  
280 lower level than  $\alpha\beta$  or  $\gamma$  KCs (**Figure 2—figure supplement 1**) (Croset et al., 2018;  
281 Davie et al., 2018). The increase in calyx  $\text{Ca}^{2+}$  induced by muscarine alone (without  
282 odor) might reflect  $\text{Ca}^{2+}$  release from internal stores triggered by  $G_q$  signaling, which  
283 then inhibits KC excitability (thus smaller odor responses). Note that muscarine on the  
284 calyx is unlikely to reduce KC odor responses via presynaptic inhibition of PNs, because  
285 bath muscarine does not affect odor-evoked  $\text{Ca}^{2+}$  influx in PNs in the calyx (**Figure 5C**),  
286 although we cannot rule out  $\text{Ca}^{2+}$ -independent inhibition.

287

## 288 **mAChR-A localized to the MB calyx can rescue learning in a mAChR-A** 289 **hypomorphic mutant**

290 We next asked where mAChR-A exerts its effect. To visualize the localization of  
291 mAChR-A, we created a new construct with mAChR-A tagged with FLAG on the C-  
292 terminus under UAS control. When we overexpressed FLAG-tagged mAChR-A in KCs  
293 using OK107-GAL4, we only observed anti-FLAG staining in the calyx (**Figure 7A**),  
294 suggesting that mAChR-A is localized to the calyx. To test whether the FLAG tag or  
295 overexpression might cause the mAChR-A to be mis-localized, we tested whether  
296 mb247-GAL4>mAChR-A-FLAG overexpression could rescue learning in a mAChR-A  
297 mutant background. The original MiMIC allele with a GFP insertion in the 5' UTR intron  
298 of mAChR-A contains a stop cassette and polyadenylation signal, and indeed, it is a

299 strongly hypomorphic allele: qPCR shows almost total lack of mAChR-A mRNA in the  
300 'MiMIC-stop' allele (**Figure 7B**). Flies homozygous for the 'MiMIC-stop' allele are viable  
301 but show impaired learning, while learning is significantly improved by using mb247-  
302 GAL4 to overexpress mAChR-A-FLAG in  $\alpha\beta$  and  $\gamma$  KCs (**Figure 7C**), indicating that  
303 overexpressed mAChR-A-FLAG can support learning. These flies ('MiMIC-stop',  
304 mb247>mAChR-A-FLAG) also show anti-FLAG staining only in the calyx (**Figure 7—**  
305 **figure supplement 1**). These results suggest that mAChR-A exerts its effect on  
306 learning in KC dendrites, consistent with the effect of locally applying muscarine to KC  
307 dendrites.

308

### 309 **mAChR-A knockdown prevents training-induced depression of MBON odor** 310 **responses**

311 The finding that mAChR-A functions in KC dendrites raises the question of how  
312 mAChR-A can affect learning. While learning-associated plasticity in KC dendrites has  
313 been observed in honeybees, In *Drosophila*, olfactory associative memories are stored  
314 by weakening the synapses between KCs and output neurons that lead to the “wrong”  
315 behavior. For example, aversive memory requires an output neuron downstream of  $\gamma$   
316 KCs, called MBON- $\gamma$ 1pedc> $\alpha/\beta$  or MB-MVP2. MB-MVP2 leads to approach behavior  
317 (Aso et al., 2014b), and aversive conditioning reduces MB-MVP2's responses to the  
318 aversively-trained odor (Hige et al., 2015; Perisse et al., 2016). We tested whether  
319 knocking down mAChR-A would prevent this depression. We knocked down mAChR-A  
320 in KCs using OK107-GAL4 and UAS-mAChR-A-RNAi 1, and expressed GCaMP6f in  
321 MB-MVP2 using R12G04-LexA and lexAop-GCaMP6f (**Figure 8A**). We trained flies in  
322 the behavior apparatus and then imaged MB-MVP2 odor responses (3 h after training to  
323 avoid cold-shock-sensitive memory). Because overall response amplitudes were  
324 variable across flies, for each fly we measured the ratio of the response to MCH (the  
325 trained odor) over the response to OCT (the untrained odor). Consistent with previous  
326 published results (Hige et al., 2015; Perisse et al., 2016), in control flies not expressing  
327 mAChR-A RNAi, the MCH/OCT ratio was substantially reduced in trained flies relative  
328 to mock-trained flies (**Figure 8B**). This was not because the OCT response increased,

329 because there was no difference between trained and mock-trained flies in the ratio of  
330 the response to OCT over the response to isoamyl acetate, a ‘reference’ odor that was  
331 absent in the training protocol. This was also not because of any general decrease in  
332 odor responses, as shown by analyzing absolute response amplitudes to MCH, OCT  
333 and isoamyl acetate (**Figure 8—figure supplement 1**). In contrast, in flies expressing  
334 mAChR-A RNAi in KCs, the MCH/OCT ratio was the same between trained and mock-  
335 trained flies (**Figure 8B**), indicating that the mAChR-A knockdown impaired the  
336 learning-related depression of the KC to MB-MVP2 synapse. This result suggests that  
337 mAChR-A function in KC dendrites is necessary for learning-related synaptic plasticity  
338 in KC axons.

339

## 340 **Discussion**

341 Here we show that mAChR-A is required in  $\gamma$  KCs for aversive olfactory learning  
342 and short-term memory in adult *Drosophila*. Knocking down mAChR-A increases KC  
343 odor responses, while the mAChR-A agonist muscarine suppresses KC activity.  
344 Knocking down mAChR-A prevents aversive learning from reducing responses of the  
345 MB output neuron MB-MVP2 to the conditioned odor, suggesting that mAChR-A is  
346 required for the learning-related depression of KC->MBON synapses.

347 Why is mAChR-A only required for aversive learning in  $\gamma$  KCs, not  $\alpha\beta$  or  $\alpha'\beta'$   
348 KCs? Although our mAChR-A MiMIC gene trap agrees with single-cell transcriptome  
349 analysis that  $\alpha'\beta'$  KCs express less mAChR-A than do  $\gamma$  and  $\alpha\beta$  KCs (Croset et al.,  
350 2018; Davie et al., 2018), transcriptome analysis indicates that  $\alpha'\beta'$  KCs do express  
351 some mAChR-A (**Figure 2—figure supplement 1**). Moreover,  $\gamma$  and  $\alpha\beta$  KCs express  
352 similar levels of mAChR-A (Crocker et al., 2016). It may be that the RNAi knockdown is  
353 less efficient at affecting the physiology of  $\alpha\beta$  and  $\alpha'\beta'$  KCs than  $\gamma$  KCs, whether  
354 because the knockdown is less efficient at reducing protein levels, or because  $\alpha\beta$  and  
355  $\alpha'\beta'$  KCs have different intrinsic properties or a different function of mAChR-A such that  
356 30% of normal mAChR-A levels is sufficient in  $\alpha\beta$  and  $\alpha'\beta'$  KCs but not  $\gamma$  KCs. This  
357 interpretation is supported by our finding that mAChR-A RNAi knockdown significantly  
358 increases odor responses only in the  $\gamma$  lobe, not the  $\alpha\beta$  or  $\alpha'\beta'$  lobes. Alternatively,  $\gamma$ ,  $\alpha\beta$

359 and  $\alpha'\beta'$  KCs are thought to be important mainly for short-term memory, long-term  
360 memory, and memory consolidation, respectively (Güven-Ozkan and Davis, 2014;  
361 Krashes et al., 2007); as we only tested short-term memory, mAChR-A may carry out  
362 the same function in all KCs, but only its role in  $\gamma$  KCs is required for short-term (as  
363 opposed to long-term) memory. Indeed, the key plasticity gene DopR1 is required in  $\gamma$ ,  
364 not  $\alpha\beta$  or  $\alpha'\beta'$  KCs, for short-term memory (Qin et al., 2012). It may be that mAChR-A is  
365 required in non- $\gamma$  KC types for other forms of memory besides short-term aversive  
366 memory, e.g., appetitive conditioning or other phases of memory like long-term memory.  
367 Our finding that mAChR-A is required in  $\gamma$  KCs for aversive short-term memory is  
368 consistent with our finding that mAChR-A knockdown in KCs disrupts training-induced  
369 depression of odor responses in MB-MVP2, an MBON postsynaptic to  $\gamma$  KCs required  
370 for aversive short-term memory (Perisse et al., 2016). However, the latter finding does  
371 not rule out the possibility that other MBONs postsynaptic to non- $\gamma$  KCs may also be  
372 affected by mAChR-A knockdown in KCs.

373         mAChR-A seems to inhibit KC odor responses, because knocking down mAChR-  
374 A increases odor responses in the calyx and  $\gamma$  lobe, while activating mAChR-A with bath  
375 or local application of muscarine decreases KC odor responses. Some details differ  
376 between the genetic and pharmacological results. In particular, while mAChR-A  
377 knockdown mainly affects  $\gamma$  KCs, with other subtypes inconsistently affected, muscarine  
378 reduces responses in all KC subtypes. What explains these differences? mAChR-A  
379 might be weakly activated in physiological conditions, in which case gain of function  
380 would cause a stronger effect than loss of function. Similarly, pharmacological activation  
381 of mAChR-A is likely a more drastic manipulation than a 60% reduction of mAChR-A  
382 mRNA levels. Although we cannot entirely rule out network effects from muscarine  
383 application, the effect of muscarine does not stem from PNs or APL (**Figure 5C,D**) and  
384 locally applied muscarine would have little effect on neurons outside the mushroom  
385 body.

386         How does mAChR-A inhibit odor-evoked  $\text{Ca}^{2+}$  influx in KCs? Given that mAChR-  
387 A signals through  $G_q$  when expressed in CHO cells (Ren et al., 2015), that muscarinic  
388  $G_q$  signaling normally increases excitability in mammals (Caulfield and Birdsall, 1998),

389 and that pan-neuronal artificial activation of  $G_q$  signaling in *Drosophila* larvae increases  
390 overall excitability (Becnel et al., 2013), it may be surprising that mAChR-A inhibits KCs.  
391 However,  $G_q$  signaling may exert different effects on different neurons in the fly brain,  
392 and some examples exist of inhibitory  $G_q$  signaling by mammalian mAChRs.  $M_1/M_3/M_5$   
393 receptors acting via  $G_q$  can inhibit voltage-dependent  $Ca^{2+}$  channels (Gamper et al.,  
394 2004; Kammermeier et al., 2000; Keum et al., 2014; Suh et al., 2010), reduce voltage-  
395 gated  $Na^+$  currents (Cantrell et al., 1996), or trigger surface transport of KCNQ  
396 channels (Jiang et al., 2015), thus increasing inhibitory  $K^+$  currents. *Drosophila* mAChR-  
397 A may inhibit KCs through similar mechanisms.

398         What is the source of ACh which activates mAChR-A and modulates odor  
399 responses? In the calyx, cholinergic PNs are certainly a major source of ACh. However,  
400 KCs themselves are cholinergic (Barnstedt et al., 2016) and release neurotransmitter in  
401 both the calyx and lobes (Christiansen et al., 2011). KCs form synapses on each other  
402 in the calyx (Zheng et al., 2018), possibly allowing mAChR-A to mediate lateral  
403 inhibition, in conjunction with the lateral inhibition provided by the GABAergic APL  
404 neuron (Lin et al., 2014).

405         What function does mAChR-A serve in learning and memory? Our results  
406 indicate that mAChR-A knockdown prevents the learning-associated weakening of KC-  
407 MBON synapses, in particular for MBON- $\gamma 1pedc > \alpha/\beta$ , aka MB-MVP2 (**Figure 7**). One  
408 potential explanation is that the increased odor-evoked  $Ca^{2+}$  influx observed in  
409 knockdown flies increases synaptic release, which overrides the learning-associated  
410 synaptic depression. However, increased odor-evoked  $Ca^{2+}$  influx *per se* is unlikely on  
411 its own to straightforwardly explain a learning defect, because other genetic  
412 manipulations that increase odor-evoked  $Ca^{2+}$  influx in KCs either have no effect on, or  
413 even improve, olfactory learning. For example, knocking down GABA synthesis in the  
414 inhibitory APL neuron increases odor-evoked  $Ca^{2+}$  influx in KCs (Lei et al., 2013; Lin et  
415 al., 2014) and improves olfactory learning (Liu and Davis, 2008).

416         The most intuitive explanation would be that mAChR-A acts at KC synaptic  
417 terminals in KC axons to help depress KC-MBON synapses. Yet overexpressed  
418 mAChR-A localizes to KC dendrites, not axons, and functionally rescues mAChR-A

419 hypomorphic mutants, showing that dendritic mAChR-A suffices for its function in  
420 learning and memory. Does this show that mAChR-A has no role in KC axons? Our  
421 inability to detect GFP expressed from the mAChR-A MiMIC gene trap suggests that  
422 normally there may only be a small amount of mAChR-A in KCs. It may be that with  
423 mAChR-A-FLAG overexpression, the correct (undetected) amount of mAChR-A is  
424 trafficked to and functions in axons, but due to a bottleneck in axonal transport, the  
425 excess tagged mAChR-A is trapped in KC dendrites. While our results do not rule out  
426 this possibility, a general bottleneck in axonal transport seems unlikely as many  
427 overexpressed proteins are localized to KC axons (Trunova et al., 2011). We feel it is  
428 more parsimonious to take the dendritic localization of mAChR-A-FLAG at face value  
429 and infer that mAChR-A functions in KC dendrites.

430         How can mAChR-A in KC dendrites affect synaptic plasticity in KC axons?  
431 mAChR-A signaling might change the shape or duration of KC action potentials (Allen  
432 and Burnstock, 1990; Ghamari-Langroudi and Bourque, 2004), an effect that could  
433 potentially propagate to KC axon terminals (Juusola et al., 2007; Shu et al., 2006). Such  
434 changes in the action potential waveform may not be detected by calcium imaging, but  
435 could potentially affect a 'coincidence detector' in KC axons that detects when odor (i.e.,  
436 KC activity) coincides with reward/punishment (i.e., dopamine). This coincidence  
437 detector is generally believed to be the  $Ca^{2+}$ -dependent adenylyl cyclase *rutabaga*  
438 (Levin et al., 1992). Changing the waveform of KC action potentials could potentially  
439 affect local dynamics of  $Ca^{2+}$  influx near *rutabaga* molecules. In addition, *rutabaga*  
440 mutations do not abolish learning (mutants have ~40-50% of normal learning scores)  
441 (Yildizoglu et al., 2015), so there may be additional coincidence detection mechanisms  
442 affected by action potential waveforms. Testing this idea would require a better  
443 understanding of biochemical events underlying learning at KC synaptic terminals.

444         Alternatively, mAChR-A's effects on synaptic plasticity may not occur acutely.  
445 Although we ruled out purely developmental effects of mAChR-A through adult-only  
446 RNAi expression (**Figure 1E**), knocking out mAChR-A for several days in adulthood  
447 might still affect KC physiology in a not-entirely-acute way. For example, as with other  
448 G-protein coupled receptors (Wang and Zhuo, 2012), muscarinic receptors can affect

449 gene expression (Kammer et al., 1998), which could have wide-ranging effects on KC  
450 physiology, e.g. action potential waveform, expression of key genes required for  
451 synaptic plasticity, etc. Another intriguing possibility is suggested by an apparent  
452 paradox: both mAChR-A and the dopamine receptor Damb signal through G<sub>q</sub>  
453 (Himmelreich et al., 2017), but mAChR-A promotes learning while Damb promotes  
454 forgetting (Berry et al., 2012). How can G<sub>q</sub> mediate apparently opposite effects?  
455 Perhaps G<sub>q</sub> signaling aids both learning and forgetting by generally rendering synapses  
456 more labile. Indeed, although *damb* mutants retain memories for longer than wildtype,  
457 their initial learning is slightly impaired (Berry et al., 2012); *damb* mutant larvae are also  
458 impaired in aversive olfactory learning (Selcho et al., 2009). Although one study reports  
459 that knocking down G<sub>q</sub> in KCs did not impair initial memory (Himmelreich et al., 2017),  
460 the G<sub>q</sub> knockdown may not have been strong enough; also, that study shocked flies with  
461 90 V shocks, which also gives normal learning in mAChR-A knockdown flies (**Figure**  
462 **1—figure supplement 1**).

463         Such hypotheses posit that mAChR-A regulates synaptic plasticity ‘competence’  
464 rather than participating directly in the plasticity mechanism itself. Why should synaptic  
465 plasticity competence be controlled by an activity-dependent mechanism? It is tempting  
466 to speculate that mAChR-A may allow a kind of metaplasticity (Abraham, 2008) in which  
467 exposure to odors (hence activation of mAChR-A in KCs) makes flies’ learning  
468 mechanisms more sensitive. Indeed, mAChR-A is required for learning with moderate  
469 (50 V) shocks, not severe (90 V) shocks. Future studies may further clarify how  
470 muscarinic signaling contributes to olfactory learning.

471

472

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
gene ( <i>Drosophila melanogaster</i> )	mAChR-A		FLYB: FBgn0000037	Also known as: mAChR, mAChR-60C, DM1, Acr60C, CG4356
genetic reagent ( <i>D. melanogaster</i> )	<i>MiMIC mAChR-A-stop</i>	(Venken et al., 2011) PMID 21985007	BDSC:59216	<i>mAChR-A<sup>MI13848</sup></i>
genetic reagent ( <i>D. melanogaster</i> )	<i>UAS-GCaMP6f (attP40)</i>	(Chen et al., 2013) PMID 23868258	BDSC:42747	
genetic reagent ( <i>D. melanogaster</i> )	<i>UAS-GCaMP6f (VK00005)</i>	(Chen et al., 2013) PMID 23868258	BDSC:52869	
genetic reagent ( <i>D. melanogaster</i> )	<i>lexAop-GCaMP6f</i>	(Barnstedt et al., 2016) PMID 26948892		Gift from S. Waddell
genetic reagent ( <i>D. melanogaster</i> )	<i>UAS-mAChR-A RNAi 1</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC:27571	TRiP.JF02725
genetic reagent ( <i>D. melanogaster</i> )	<i>UAS-mAChR-A RNAi 2</i>	Vienna <i>Drosophila</i> Resource Center	VDRRC:101407	
genetic reagent ( <i>D. melanogaster</i> )	<i>UAS-Dcr-2</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC:24651	
genetic reagent ( <i>D. melanogaster</i> )	<i>lexAop-GAL80</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC:32216	
genetic reagent ( <i>D. melanogaster</i> )	<i>tub-GAL80<sup>ts</sup></i>	(McGuire et al., 2003) PMID 14657498	BDSC:7108	
genetic reagent ( <i>D. melanogaster</i> )	<i>mb247-dsRed</i>	(Riemensperger et al., 2005) PMID 16271874	FLYB:FBtp0022384	
genetic reagent ( <i>D. melanogaster</i> )	<i>GH146-GAL4</i>	(Stocker et al., 1997) PMID 9110257	BDSC:30026	
genetic	<i>OK107-</i>	(Connolly et al.,	BDSC:854	

reagent ( <i>D. melanogaster</i> )	<i>GAL4</i>	1996) PMID 8953046		
genetic reagent ( <i>D. melanogaster</i> )	<i>c305a-GAL4</i>	(Krashes et al., 2007) PMID 17196534	BDSC:30829	
genetic reagent ( <i>D. melanogaster</i> )	<i>mb247-GAL4</i>	(Zars, 2000) PMID 10784450	BDSC:50742	
genetic reagent ( <i>D. melanogaster</i> )	<i>R44E04-LexA</i>	(Jenett et al., 2012) PMID 23063364	BDSC:52736	Gift from A. Thum
genetic reagent ( <i>D. melanogaster</i> )	<i>R45H04-LexA</i>	(Bräcker et al., 2013) PMID 23770186	FLYB:FBti0155893	Gift from A. Thum
genetic reagent ( <i>D. melanogaster</i> )	<i>R12G04-LexA</i>	(Jenett et al., 2012) PMID 23063364	BDSC:52448	
genetic reagent ( <i>D. melanogaster</i> )	<i>elav-GAL4</i>	(Lin and Goodman, 1994) PMID 7917288	BDSC:458	
genetic reagent ( <i>D. melanogaster</i> )	<i>NP2631-GAL4</i>	(Lin et al., 2014; Tanaka et al., 2008) PMID 24561998, 18395827	Kyoto Stock Center 104266	
genetic reagent ( <i>D. melanogaster</i> )	<i>GH146-FLP</i>	(Hong et al., 2009; Lin et al., 2014) PMID 19915565, 24561998	FLYB:FBtp0053491	
genetic reagent ( <i>D. melanogaster</i> )	<i>tub-FRT-GAL80-FRT</i>	(Gordon and Scott, 2009; Lin et al., 2014) PMID 19217375, 24561998	BDSC:38880	
genetic reagent ( <i>D. melanogaster</i> )	<i>UAS-TNT</i>	(Lin et al., 2014; Sweeney et al., 1995) PMID 24561998, 7857643	FLYB:FBtp0001264	
genetic reagent ( <i>D. melanogaster</i> )	<i>UAS-mCherry-CAAX</i>	(Kakihara et al., 2008; Lin et al., 2014) PMID 18083504, 24561998	FLYB:FBtp0041366	
genetic reagent ( <i>D. melanogaster</i> )	<i>mb247-LexA</i>	(Lin et al., 2014; Pitman et al.,	FLYB:FBtp0070099	

<i>melanogaster</i> )		2011) PMID 24561998		
genetic reagent ( <i>D. melanogaster</i> )	20xUAS-6xGFP	(Shearin et al., 2014) PMID 24451596	BDSC:52266	
genetic reagent ( <i>D. melanogaster</i> )	UAS-mCD8-GFP	(Lee et al., 1999) PMID 10457015	BDSC:5130	
Antibody	nc82 (mouse monoclonal)	Developmental Studies Hybridoma Bank	nc82	(1:50, supernatant or 1:200, concentrate)
Antibody	FLAG (mouse monoclonal M2)	Sigma-Aldrich	F3165	(1:250)
Antibody	Goat anti-mouse secondary Alexa 647	Abcam	ab150115	(1:500)
Antibody	Goat anti-mouse secondary Alexa 546	Thermo Fisher	A11018	(1:1000)

474

## 475 **Fly Strains**

476 Fly strains (see below) were raised on cornmeal agar under a 12 h light/12 h dark  
477 cycle and studied 1–10 days post-eclosion. Strains were cultivated at 25 °C unless they  
478 expressed temperature-sensitive gene products (GAL80<sup>ts</sup>); in these cases the  
479 experimental animals and all relevant controls were grown at 23 °C. To de-repress the  
480 expression of RNAi with GAL80<sup>ts</sup>, experimental and control animals were incubated at  
481 31 °C for 7 days. Subsequent behavioral experiments were performed at 25 °C.

482 Experimental animals carried transgenes over Canton-S chromosomes where  
483 possible to minimize genetic differences between strains. Details of fly strains are given  
484 in the Key Resources Table.

485 UAS-mAChR-A-FLAG plasmid was generated by Gibson assembly of fragments  
486 using the NEBuilder HiFi Master Mix (NEB). Fragments were created by PCR using

487 Phusion® High-Fidelity DNA Polymerase (NEB). The full-length mAChR-A cDNA was  
488 purchased from GenScript (clone ID OFa11160). The vector was pTWF-attB, a gift from  
489 Prof. Oren Schuldiner (Yaniv et al., 2012). This vector consists of a FLAG tag in the C-  
490 terminal of the inserted gene and an attB site for site-specific integration of the  
491 transgene. PCR and Gibson assembly were carried out following the manufacturer's  
492 recommendations with the following primers:

493 For mAChR-A: tggaattatcgacaagttgtacaaaaaagcaggctATGGAGCCGGTCATGAGTC  
494 and cactttgtacaagaaagctgggtaATTGTAGACGCCGCGTAC

495 For pTWF-AttB : aaagctgggtaCTTGTACAAAGTGGTGAGCTCC and  
496 agcctgctttttgtacAAACTTGTGCGATAATTCCC

497 Transgenes were injected into the attP2 landing site using  $\phi$ C31 integration (by  
498 BestGene).

#### 499 ***Quantitative Real-time PCR***

500 Total RNA was extracted by EZ-RNA II Total RNA Isolation kit (Biological  
501 Industries, Israel) from 30 adult heads for each biological replicate. cDNA was  
502 generated from 1  $\mu$ g total RNA with the High-Capacity cDNA Reverse Transcription Kit  
503 with RNase Inhibitor (Applied Biosystems). Real-time quantitative PCR was carried with  
504 TaqMan™ Fast Advanced Master Mix (Applied Biosystems) and run in technical  
505 triplicates on a StepOne Plus Real-Time PCR System (Applied Biosystems). Taqman  
506 assays were Dm01820303\_g1 for mAChR-A and Dm02151962\_g1 for EF1  
507 (Ef1alpha100E, ThermoFisher). The expression levels obtained for mAChR-A were  
508 normalized to those of the housekeeping gene EF1. The fold change for mAChR-A was  
509 subsequently calculated by comparing to the normalized value of either ELAV-gal4  
510 parent (for RNAi experiments) or W1118 flies (for MIMiC experiments).

#### 511 ***Behavioral Analysis***

512 Behavioral experiments were performed in a custom-built, fully automated  
513 apparatus (Claridge-Chang et al., 2009; Lin et al., 2014; Parnas et al., 2013). Single  
514 flies were housed in clear polycarbonate chambers (length 50 mm, width 5 mm, height

515 1.3 mm) with printed circuit boards (PCBs) at both floors and ceilings. Solid-state relays  
516 (Panasonic AQV253) connected the PCBs to a 50 V source.

517 Air flow was controlled with mass flow controllers (CMOSens PerformanceLine,  
518 Sensirion). A carrier flow (2.7 l/min) was combined with an odor stream (0.3 l/min)  
519 obtained by circulating the air flow through vials filled with a liquid odorant. Odors were  
520 prepared at 10 fold dilution in mineral oil. Therefore, liquid dilution and mixing carrier  
521 and odor stimulus stream resulted in a final 100 fold dilution of odors. Fresh odors were  
522 prepared daily.

523 The 3 liter/min total flow (carrier and odor stimulus) was split between 20  
524 chambers resulting in a flow rate of 0.15 l/min per half chamber. Two identical odor  
525 delivery systems delivered odors independently to each half of the chamber. Air or odor  
526 streams from the two halves of the chamber converged at a central choice zone. The 20  
527 chambers were stacked in two columns each containing 10 chambers and were backlit  
528 by 940 nm LEDs (Vishay TSAL6400). Images were obtained by a MAKO CMOS  
529 camera (Allied Vision Technologies) equipped with a Computar M0814-MP2 lens. The  
530 apparatus was operated in a temperature-controlled incubator (Panasonic MIR-154)  
531 maintained at 25 °C.

532 A virtual instrument written in LabVIEW 7.1 (National Instruments) extracted fly  
533 position data from video images and controlled the delivery of odors and electric  
534 shocks. Data were analyzed in MATLAB 2015b (The MathWorks) and Prism 6  
535 (GraphPad).

536 A fly's preference was calculated as the percentage of time that it spent on one  
537 side of the chamber. Training and odor avoidance protocols were as depicted in **Figure**  
538 **1**. The naïve avoidance index was calculated as (preference for left side when it  
539 contains air) – (preference for left side when it contains odor). During training, MCH was  
540 paired with 12 equally spaced 1.25 s electric shocks at 50 V (Tully and Quinn, 1985).  
541 The learning index was calculated as (preference for MCH before training) –  
542 (preference for MCH after training). Flies were excluded from analysis if they entered  
543 the choice zone fewer than 4 times during odor presentation.

544 **Functional Imaging**

545 Brains were imaged by two-photon laser-scanning microscopy (Ng et al., 2002;  
546 Wang et al., 2003). Cuticle and trachea in a window overlying the required area were  
547 removed, and the exposed brain was superfused with carbogenated solution (95% O<sub>2</sub>,  
548 5% CO<sub>2</sub>) containing 103 mM NaCl, 3 mM KCl, 5 mM trehalose, 10 mM glucose, 26 mM  
549 NaHCO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 3 mM CaCl<sub>2</sub>, 4 mM MgCl<sub>2</sub>, 5 mM N-Tris (TES), pH 7.3.  
550 Odors at 10<sup>-1</sup> dilution were delivered by switching mass-flow controlled carrier and  
551 stimulus streams (Sensirion) via software controlled solenoid valves (The Lee  
552 Company). Flow rates at the exit port of the odor tube were 0.5 or 0.8 l/min.

553 Fluorescence was excited by a Ti-Sapphire laser centered at 910 nm, attenuated  
554 by a Pockels cell (Conoptics) and coupled to a galvo-resonant scanner. Excitation light  
555 was focussed by a 20X, 1.0 NA objective (Olympus XLUMPLFLN20XW), and emitted  
556 photons were detected by GaAsP photomultiplier tubes (Hamamatsu Photonics,  
557 H10770PA-40SEL), whose currents were amplified and transferred to the imaging  
558 computer. Two imaging systems were used, #1 for **Figures 3-6** except **5C**, and #2 for  
559 **Figure 5C** and **Figure 7**, which differed in the following components: laser (1: Mai Tai  
560 eHP DS, 70 fs pulses; 2: Mai Tai HP DS, 100 fs pulses; both from Spectra-Physics);  
561 microscope (1: Movable Objective Microscope; 2: DF-Scope installed on an Olympus  
562 BX51WI microscope; both from Sutter); amplifier for PMT currents (1: Thorlabs TIA-60;  
563 2: Hamamatsu HC-130-INV); software (1: ScanImage 5; 2: MScan 2.3.01). Volume  
564 imaging on System 1 was performed using a piezo objective stage (nPFocus400,  
565 nPoint). Muscarine was applied locally by pressure ejection from borosilicate patch  
566 pipettes (resistance ~10 MΩ; capillary inner diameter 0.86 mm, outer diameter 1.5  
567 mm; concentration in pipette 20 mM; pressure 12.5 psi) using a Picospritzer III (Parker).  
568 A red dye was added to the pipette to visualize the ejected fluid (SeTau-647, SETA  
569 BioMedicals) (Podgorski et al., 2012).

570 Movies were motion-corrected in X-Y using the moco ImageJ plugin (Dubbs et  
571 al., 2016), with pre-processing to collapse volume movies in Z and to smooth the image  
572 with a Gaussian filter (standard deviation = 4 pixels; the displacements generated from  
573 the smoothed movie were then applied to the original, unsmoothed movie), and motion-

574 corrected in Z by maximizing the pixel-by-pixel correlation between each volume and  
575 the average volume across time points.  $\Delta F/F$ , activity maps, sparseness and inter-odor  
576 correlation were calculated as in (Lin et al., 2014). Briefly, movies were smoothed with a  
577 5-pixel-square Gaussian filter (standard deviation 2). Baseline fluorescence was taken  
578 as the average fluorescence during the pre-stimulus period. Frames with sudden, large  
579 axial movements were discarded by correlating each frame to the baseline image and  
580 discarding it if the correlation fell below a threshold value, which was manually selected  
581 for each brain by noting the constant high correlation value when the brain was  
582 stationary and sudden drops in correlation when the brain moved.  $\Delta F/F$  was calculated  
583 for each pixel as the difference between mean fluorescence during the stimulus period  
584 vs. the baseline fluorescence ( $\Delta F$ ), divided by the baseline fluorescence. For pixels  
585 where  $\Delta F$  did not exceed 2 times the standard deviation over time of that pixel's  
586 intensity during the pre-stimulus period, the pixel was considered non-responsive. We  
587 excluded non-responsive flies and flies whose motion could not be corrected.

588 Inter-odor correlations were calculated by first aligning the activity maps of each  
589 odor response by maximizing the inter-odor correlations of baseline fluorescence, and  
590 then converting image matrices of the activity maps of each odor response into linear  
591 vectors and calculating the Pearson correlation coefficients between each “odor vector”.  
592 A threshold for baseline fluorescence was applied as a mask to the activity map to  
593 exclude pixels with no baseline GCaMP6f signal. Population sparseness was calculated  
594 for activity maps using the following equation (Vinje and Gallant, 2000; Willmore and  
595 Tolhurst, 2001):

$$S_p = \frac{1}{1 - \frac{1}{N}} \left( 1 - \frac{\left( \sum_{i=1}^N \frac{r_i}{N} \right)^2}{\sum_{i=1}^N \frac{r_i^2}{N}} \right)$$

596

## 597 ***Structural Imaging***

598 Brain dissections, fixation, and immunostaining were performed as described  
599 (Pitman et al., 2011; Wu and Luo, 2006). To visualize native GFP fluorescence,  
600 dissected brains were fixed in 4% (w/v) paraformaldehyde in PBS (1.86 mM  $\text{NaH}_2\text{PO}_4$ ,

601 8.41 mM Na<sub>2</sub>HPO<sub>4</sub>, 175 mM NaCl) and fixed for 20 min at room temperature. Samples  
602 were washed for 3×20 min in PBS containing 0.3% (v/v) Triton-X-100 (PBT). The  
603 neuropil was counterstained with nc82 (DSHB) or monoclonal anti-FLAG M2 antibody  
604 (F3165, Sigma) and goat anti-mouse Alexa 647 or Alexa 546. Primary antisera were  
605 applied for 1-2 days and secondary antisera for 1-2 days in PBT at 4 °C, followed by  
606 embedding in Vectashield. Images were collected on a Leica TCS SP5, SP8, or Nikon  
607 A1 confocal microscope and processed in ImageJ.

608 APL expression of tetanus toxin was scored by widefield imaging of mCherry.  
609 mCherry expression in APL was distinguished from 3XP3-driven dsRed from the  
610 GH146-FLP transgene by using separate filter cubes for dsRed (49004, Chroma:  
611 545/25 excitation; 565 dichroic; 605/70 emission) and mCherry (LED-mCherry-A-000,  
612 Semrock: 578/21 excitation; 596 dichroic; 641/75 emission).

### 613 **Statistics**

614 Statistical analyses were carried out in GraphPad Prism as described in figure  
615 legends and **Supplementary File 1**. In general, no statistical methods were used to  
616 predetermine sample sizes, but where conclusions were drawn from the absence of a  
617 statistically significant difference, a power analysis was carried out in G\*Power to  
618 confirm that the sample size provided sufficient power to detect an effect of the  
619 expected size. The experimenter was blind to which hemispheres had APL neurons  
620 expressing tetanus toxin before post-experiment dissection (**Figure 5**) but not  
621 otherwise.

622

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631

632

633 **Figure legends**

634 **Figure 1: mAChR-A is required in the MB for short term aversive olfactory**  
635 **learning and memory but not for naïve behavior**

636 **(A)** qRT-PCR of mAChR-A with mAChR-A RNAi driven by elav-GAL4. The  
637 housekeeping gene eEF1 $\alpha$ 2 (eukaryotic translation elongation factor 1 alpha 2,  
638 CG1873) was used for normalization. Knockdown flies have ~40% of the control levels  
639 of mAChR-A mRNA (mean  $\pm$  SEM; number of biological replicates (left to right): 6, 7, 7,  
640 4, 4, each with 3 technical replicates; \*  $p < 0.05$ ; Kruskal-Wallis test with Dunn's multiple  
641 comparisons test and Welch ANOVA test with Dunnett's T3 multiple comparisons test).  
642 For detailed statistical analysis see **Supplementary File 1**.

643 **(B)** Each trace shows the movement of an individual fly during the training protocol, with  
644 fly position in the chamber (horizontal dimension) plotted against time (vertical  
645 dimension). Colored rectangles illustrate which odor is presented on each side of the  
646 chamber during training and testing. Flies were conditioned against MCH (blue  
647 rectangles; see Methods).

648 **(C)** Learning scores in flies with mAChR-A RNAi driven by OK107-GAL4. mAChR-A  
649 knockdown reduced learning scores compared to controls (mean  $\pm$  SEM, n (left to right):  
650 69, 69, 70, 71, 71, 47, 48, 53, 58, 51 \*  $p < 0.05$ ; Kruskal-Wallis test with Dunn's multiple  
651 comparisons test).

652 **(D)** mAChR-A KD flies show normal olfactory avoidance to OCT and MCH compared to  
653 their genotypic controls (mean  $\pm$  SEM, n (left to right): 68, 67, 58, 63, 91, 67,  $p = 0.82$   
654 for OCT,  $p = 0.64$  for MCH; Kruskal-Wallis test). Colored rectangles show stimulus  
655 protocol as in **(B)**; red for odor (MCH or OCT), white for air.

656 **(E)** Learning scores in flies with mAChR-A RNAi 1 driven by OK107-GAL4 with GAL80<sup>ts</sup>  
657 repression. Flies raised at 23 °C and heated to 31 °C as adults (red outlines) had  
658 impaired learning compared to controls. Control flies kept at 23 °C throughout (blue  
659 outline), thus blocking mAChR-A RNAi expression, showed no learning defects (mean  $\pm$   
660 SEM, n (left to right): 51, 41, 58, 51, \*\*  $p < 0.05$ , Kruskal-Wallis test with Dunn's multiple  
661 comparisons test). For detailed statistical analysis see **Supplementary File 1**.

662 **Figure 1—figure supplement 1: Controls and additional learning data**

663 **(A)** Flies were subjected to the same protocol as in Figure 1 but no, or stronger, electric  
664 shock. With no electric shock, the flies do not change their odor preference and have a  
665 learning index which is not statistically different from 0 (n (left to right): 79, 73, 71;  $p >$   
666 0.3, one-sample t-test). When flies were conditioned against MCH using 90 V electric  
667 shock instead of 50 V (as in the main Figures; see Methods), driving mAChR-A RNAi in  
668 KCs using OK107-GAL4 did not affect learning compared to controls (mean  $\pm$  SEM, n  
669 (left to right): 52, 46, 51,  $p > 0.13$ , Kruskal-Wallis test). For detailed statistical analysis  
670 see **Supplementary File 1**.

671 **(B)** Sensitivity to shock (extent to which flies walk faster while being shocked) is not  
672 affected by knocking down mAChR-A in KCs. Shown here is walking speed during  
673 training (time = 5-6 and 7-8 min in Figure 1B), taking the difference between speed  
674 during MCH (CS+) and speed during OCT (CS-). In mock training, the difference is  
675 close to zero, but during training, when MCH is paired with shock, flies walk much faster  
676 in MCH (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , Mann-Whitney test with Bonferroni  
677 correction, comparing training vs. mock training). The effect of shock is not significantly  
678 different between OK107 alone and OK107>mAChR-A-RNAi flies (n.s.:  $p = 0.44$  for  
679 interaction between genotype and training vs. mock training, 2-way ANOVA). n (left to  
680 right): 72, 100, 80, 80, 140, 160.

681 **Figure 1—source data 1: Source data for Figure 1A**

682 **Figure 1—source data 2: Source data for Figure 1C-E**

683 **Figure 1—source data 3: Source data for Figure 1—figure supplement 1.**

684

685 **Figure 2: mAChR-A is required for short term aversive olfactory learning and**  
686 **memory in  $\gamma$  KCs**

687 **(A)** Maximum intensity projection of 70 confocal sections (2  $\mu$ m) through the central  
688 brain of a fly carrying MiMIC-mAChR-A-GAL4 and 20xUAS-6xGFP transgenes. MB  $\alpha\beta$   
689 and  $\gamma$  lobes are clearly observed. No GFP expression is observed in  $\alpha'\beta'$  lobes.

690 **(B)** mAChR-A RNAi 1 was targeted to different subpopulations of KCs. Learning scores  
691 were reduced compared to controls when mAChR-A RNAi 1 was expressed in  $\alpha\beta$  and  $\gamma$   
692 KCs or  $\gamma$  KCs alone, but not when mAChR-A RNAi 1 was expressed in  $\alpha\beta$  or  $\alpha'\beta'$  KCs.  
693 (mean  $\pm$  SEM, n (left to right): 69, 41, 70, 76, 69, 66, 71, 50, 68, \*\*  $p < 0.01$ , \*\*\*  $p <$   
694 0.001, Kruskal-Wallis test with Dunn's multiple comparisons test). For detailed statistical  
695 analysis see **Supplementary File 1**. The data for the UAS-mAChR-A RNAi 1 control  
696 are duplicated from **Figure 1**.

697 **Figure 2—figure supplement 1: Expression of mAChR-A from single-cell**  
698 **transcriptome profiling.**

699 **(A)** Data from Davie et al., 2018. 56,902 *Drosophila* brain cells arranged according to  
700 their single-cell transcriptome profiles, along the top 2 principal components using t-  
701 SNE. Red coloring indicates expression of mAChR-A. KC subtype clusters are labeled  
702 as identified in Davie et al., 2018.

703 **(B)** Expression of *DAT* (marker for  $\alpha'\beta'$  KCs), *trio* (marker for  $\alpha'\beta'$  and  $\gamma$  KCs), and  
704 *mAChR-A* for cells identified as  $\alpha'\beta'$ ,  $\alpha\beta$  and  $\gamma$  KCs in Davie et al., 2018. mAChR-A  
705 expression is higher in  $\alpha\beta$  and  $\gamma$  KCs compared to  $\alpha'\beta'$  KCs.

706 **(C)** As in A but with data from Croset et al., 2018 (10,286 *Drosophila* brain cells).

707 **(D)** As in B but with data from Croset et al., 2018.

708 Images screenshotted and raw data downloaded from SCoPe (<http://scope.aertslab.org>)  
709 on 24 June 2018.

710 **Figure 2—figure supplement 2: Expression patterns of GAL4 and LexA driver**  
711 **lines used in this study.**

712 GFP expression was driven by the named GAL4 or LexA driver lines and the general  
713 neuropil was stained with an antibody to NC82 (magenta). Images are maximum-  
714 intensity Z-projections of confocal stacks. Panels A-D, G show only the planes of the  
715 mushroom body lobes and peduncle to more clearly show which lobes are labeled.

716 **(A)** OK107-GAL4 labels all KCs.

717 **(B)** MB247-GAL4 labels  $\alpha\beta$  and  $\gamma$  KCs.

718 **(C)** c305a-GAL4 labels  $\alpha'\beta'$  KCs.

719 **(D)** R44E04-LexA labels  $\alpha\beta$  KCs.

720 **(E)** R45H04-LexA strongly labels  $\gamma$  KCs.

721 **(F)** Silencing MB247-GAL4 expression in  $\gamma$  KCs by using R45H04-LexA to drive *lexAop-*  
722 *GAL80* in  $\gamma$  KCs results in fairly specific expression in  $\alpha\beta$  KCs.

723 **(G)** Silencing MB247-GAL4 expression in  $\alpha\beta$  KCs by using R44E04 to drive *lexAop-*  
724 *GAL80* in  $\alpha\beta$  KCs results in fairly specific expression in  $\gamma$  KCs.

725 **(H)** R12G04-GAL4 labels MBON- $\gamma$ 1pedc> $\alpha/\beta$ , aka MB-MVP2.

726 **Figure 2—source data 1: Source data for Figure 2.**

727

728 **Figure 3: mAChR-A knockdown increases odor responses in  $\gamma$  KCs.**

729 Odor responses to MCH and OCT were measured in control (OK107-GAL4>GCaMP6f,  
730 *Dcr-2*) and knockdown (OK107-GAL4>GCaMP6f, *Dcr-2*, mAChR-A-RNAi 2) flies.

731 **(A)**  $\Delta F/F$  of GCaMP6f signal in different areas of the MB in control (black) and  
732 knockdown (red) flies, during presentation of odor pulses (horizontal lines). Data are  
733 mean (solid line)  $\pm$  SEM (shaded area). Diagrams illustrate which region of the MB was  
734 analyzed.

735 **(B)** Peak response of the traces presented in A (mean  $\pm$  SEM.) *n* given as number of  
736 hemispheres (number of flies) for control and knockdown flies, respectively: calyx, 23  
737 (13), 17 (10);  $\alpha$  and  $\alpha'$ , 24 (13), 20 (10);  $\beta$ ,  $\beta'$  and  $\gamma$ , 27 (14), 22 (11). \*  $p < 0.05$ , \*\*\*  $p <$   
738  $0.001$ , 2-way ANOVA with Holm-Sidak multiple comparisons test). For detailed  
739 statistical analysis see **Supplementary File 1**.

740 **Figure 3—figure supplement 1: Statistical power is not affected by inter-lobe**  
741 **differences in signal-to-noise ratio (SNR)**

742 **(A)** SNR in the baseline GCaMP6f signal differs among regions of the mushroom body.  
743 SNR was measured as the reciprocal of the standard deviation of  $\Delta F/F$  during the 2 s  
744 immediately preceding odor onset (the period used to calculate baseline fluorescence,  
745 or F0). SNR is mean signal divided by standard deviation; here the standard deviation  
746 of  $\Delta F/F$  equals (the standard deviation of F) divided by F0, which is the mean signal  
747 during the pre-stimulus period.

748 **(B)** Statistical power to detect the effect size of the difference in  $\gamma$  lobe odor response  
749 between control and mAChR-A knockdown flies (Cohen's  $d = 1.3$  for OCT, **Figure 3B**),  
750 for different SNRs. Statistical power did not differ for SNRs in the range observed in **(A)**  
751 (SNR = 20–50). Method: We simulated 2 groups of 20 random samples ( $n=20$  was the  
752 smallest sample size out of the  $\alpha\beta$  and  $\alpha'\beta'$  lobes) where the effect size of the difference  
753 between the 2 groups was 1.3. Each sample had a 'ground truth' value, from which we  
754 sampled 3 'time points' that were subject to noise with SNR from 1–50 (we sampled 3  
755 time points because the peak of the odor response almost always occurred between 1–  
756 2 s after odor onset, and our frame rate was  $\sim 3$  Hz). The maximum of these 3 time  
757 points was taken as the measured 'peak odor response'. We ran 1000 simulations, ran  
758 t-tests on the simulated data, and counted how many gave a p-value  $< 0.0125$  (a Holm-  
759 Bonferroni correction for the 4 mushroom body regions that did not consistently show  
760 significant differences between control and mAChR-A knockdown flies) – this fraction is  
761 the statistical power for detecting a difference in the non- $\gamma$  lobes with effect size 1.3.

762 **Figure 3—source data 1: Source data for Figure 3.**

763

764 **Figure 4: mAChR-A knockdown does not affect KC odor identity coding.**

765 **(A)** Example activity maps (single optical sections from a z-stack) of KC odor responses  
766 to MCH and OCT in control (OK107-GAL4>GCaMP6f, Dcr-2) and mAChR-A knockdown  
767 (OK107-GAL4>GCaMP6f, Dcr-2, mAChR-A-RNAi 2) flies where all KCs are imaged.  
768 False-coloring indicates  $\Delta F/F$  of the odor response, overlaid on grayscale baseline  
769 GCaMP6f signal. Scale bar, 10  $\mu\text{m}$ . For detailed statistical analysis see **Supplementary**  
770 **File 1.**

771 **(B)** Sparseness of pan-KC population responses is not affected by mAChR-A  
772 knockdown ( $p = 0.38$ , 2-way repeated-measures ANOVA).

773 **(C)** Correlation between pan-KC population responses to MCH and OCT is not affected  
774 by mAChR-A knockdown ( $p = 0.75$ , t-test).

775 **(D)** Upper: diagram of  $\gamma$  KCs (green). Lower: False-colored average-intensity Z-  
776 projection of the horizontal lobe in a control fly imaged from a dorsal view in panel E  
777 (mb247-GAL4>GCaMP6f, R44E04-LexA>GAL80), averaged over 10 s before the odor  
778 stimulus. R44E04-LexA>GAL80 almost completely suppresses  $\beta$  lobe expression.  
779 Scale bar, 20  $\mu\text{m}$ .

780 **(E)** Knocking down mAChR-A only in  $\gamma$  KCs increases  $\gamma$  KC odor responses. Shown  
781 here are odor responses in the calyx and  $\gamma$  lobe of control (mb247-GAL4>GCaMP6f,  
782 R44E04-LexA>GAL80) and knockdown (mb247-GAL4>GCaMP6f, mAChR-A-RNAi 1,  
783 R44E04-LexA>GAL80) flies.

784 **(F)** Peak response of the traces presented in D (mean  $\pm$  SEM.) n given as number of  
785 hemispheres (number of flies): 11 (6) for control, 12 (6) for knockdown. \*  $p < 0.05$ , \*\*  $p <$   
786  $0.01$ , 2-way repeated-measures ANOVA with Holm-Sidak multiple comparisons test.

787 **(G)** Example activity maps (single optical sections from a z-stack) of  $\gamma$  KC odor  
788 responses to MCH and OCT in control (mb247-GAL4>GCaMP6f, R44E04-  
789 LexA>GAL80) and knockdown (mb247-GAL4>GCaMP6f, mAChR-A-RNAi 1, R44E04-  
790 LexA>GAL80) flies. Note the gaps in baseline GCaMP6f signal due to lack of  $\alpha\beta$  and  
791  $\alpha'\beta'$  KCs labeled. Scale bar, 10  $\mu\text{m}$

792 **(H)** Sparseness of  $\gamma$  KC population responses is not affected by mAChR-A knockdown  
793 ( $p = 0.76$ , 2-way repeated-measures ANOVA).

794 **(I)** Correlation between  $\gamma$  KC population responses to MCH and OCT is not affected by  
795 mAChR-A knockdown ( $p = 0.32$ , t-test).

796 **Figure 4—source data 1: Source data for Figure 4.**

797

798 **Figure 5: KC odor responses are decreased by muscarine.**

799 **(A)** Odor responses in the calyx and  $\gamma$  lobe of OK107-GAL4>GCaMP6f flies, before  
800 (black) and after (red) adding 10  $\mu$ M muscarine in the bath. Data are mean (solid line)  $\pm$   
801 SEM (shaded area); horizontal lines indicate the odor pulse. Traces for all lobes are  
802 shown in **Figure S5**. For detailed statistical analysis see **Supplementary File 1**.

803 **(B)** Peak  $\Delta F/F$  during the odor pulse before and after muscarine.  $n = 11$  hemispheres  
804 from 6 flies. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  by 2-way repeated measures ANOVA  
805 with Holm-Sidak multiple comparisons test.

806 **(C)** Odor responses in PN axons in the calyx are not affected by 10  $\mu$ M muscarine, in  
807 GH146-GAL4>GCaMP6f flies ( $p > 0.49$ , 2-way repeated measures ANOVA,  $n = 5$  flies).

808 **(D)** Peak  $\Delta F/F$  during the odor pulse before and after muscarine in control hemispheres  
809 where APL was unlabeled (left,  $n = 6$  hemispheres from 6 flies) and hemispheres where  
810 APL expressed tetanus toxin (TNT) (right,  $n = 6$  hemispheres from 5 flies). \*  $p < 0.05$ , \*\*  
811  $p < 0.01$ , \*\*\*  $p < 0.001$  by 2-way repeated measures ANOVA with Holm-Sidak multiple  
812 comparisons test.

813 **(E)** (Response (peak  $\Delta F/F$  during the odor pulse) after muscarine) / (response before  
814 muscarine), using data from **(D)**. No significant differences were observed ( $p > 0.05$ , 2-  
815 way repeated measures ANOVA with Holm-Sidak multiple comparisons test).

816 **Figure 5—figure supplement 1: KC odor responses are decreased by**  
817 **muscarine.** Extended data for **Figure 5**. Odor responses in OK107-GAL4>GCaMP6f  
818 flies **(A)**, control APL unlabeled hemispheres **(B)**, and APL>TNT hemispheres **(C)**,  
819 before (black) and after (red) adding 10  $\mu$ M muscarine in the bath. Data are mean (solid  
820 line)  $\pm$  SEM (shaded area); diagrams illustrate which region of the MB was analyzed;  
821 horizontal lines indicate the odor pulse. These are the traces for the summary data  
822 shown in **Figure 5B,D**.

823 **Figure 5—source data 1: Source data for Figure 5.**

824

825 **Figure 6: Local muscarine application to the calyx inhibits KC odor responses**

826 **(A)** Left: Schematic of MB, showing color scheme for the different regions where  
827 responses are quantified. Right: Average  $\Delta F/F$  GCaMP6f signal in different areas of the  
828 MB of OK107>GCaMP6f flies in response to a 10 ms pulse of 20 mM muscarine on the  
829 calyx. Data are mean (solid line)  $\pm$  SEM (shaded area). Dashed vertical line shows the  
830 timing of muscarine application. Shaded bar indicates time window used to quantify  
831 responses in panel **C**. n = 7 hemispheres (5 flies).

832 **(B)**  $\Delta F/F$  traces of red dye indicator, showing which MB regions the muscarine spread  
833 to. The traces follow the same color scheme and visuals as shown in panel A.

834 **(C)** Scatter plot showing average  $\Delta F/F$  of GCaMP6f signal of the different MB regions at  
835 time 0–1 s 10 ms pulse of 20 mM muscarine on the calyx, quantified from traces shown  
836 in **(A)**. n as in **(A)**. \*  $p < 0.05$ , one-sample t-test (different from 0), Bonferroni correction  
837 for multiple comparisons.

838 **(D)** Average  $\Delta F/F$  GCaMP6f signal of different areas of the MB during odor pulses of  
839 OCT (horizontal bar), before (black) and after (red) muscarine application on the calyx,  
840 1 s before the odor pulse (vertical bar). Data are mean (solid line)  $\pm$  SEM (shaded area).  
841 n: 7 hemispheres (5 flies). See **Figure S6** for all traces.

842 **(E)** Line-bar plots showing paired peak  $\Delta F/F$  GCaMP6f responses of the different MB  
843 regions during 5 s odor pulses of MCH or OCT, before (gray) and after (pink) muscarine  
844 application to the calyx, in the hemisphere where the muscarine was applied (same  
845 side, right) or the opposite (opposite side, left). Muscarine was applied 1 s before the  
846 odor pulse. Bars show mean value. n given as number of hemispheres (number of  
847 flies): Same side MCH 7 (6), OCT 9 (8), opposite side MCH 7 (5), OCT 8 (5). \*  $p < 0.05$ ,  
848 \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  by 2-way repeated measures ANOVA with Holm-Sidak  
849 multiple comparisons test.

850 **Figure 6—figure supplement 1: Local muscarine application to the calyx inhibits**  
851 **KC odor responses.**

852 Average  $\Delta F/F$  GCaMP6f traces of the different MB regions of OK107>GCaMP6f flies  
853 that only received the muscarine pulse **(A)** or received an odor pulse (MCH or OCT)  
854 before (black) or after (red) 10 ms pulse of 20 mM muscarine **(B,C)**. Panel **A** is

855 duplicated from **Figure 6A**; panel **B** is the traces corresponding to the **Figure 6E**.  
856 Muscarine was applied in the calyx, 1 s before the odor pulse where applicable. Traces  
857 are from the same side or the opposite side that muscarine was applied. Data are mean  
858 (solid line)  $\pm$  SEM (shaded area). Horizontal bars indicate odor pulse timing and  
859 duration. Vertical bars indicate timing of muscarine pulse. n, by number of hemispheres  
860 (number of flies): same side MCH 6 (4), OCT 7 (5), opposite side MCH 5 (3), OCT 5 (3),  
861 muscarine alone 7 (5).

862 **Figure 6—source data 1: Source data for Figure 6.**

863

864 **Figure 7: Dendritic function of mAChR-A suffices to rescue learning in mAChR-A**  
865 **mutants.**

866 **(A)** mAChR-A-FLAG overexpressed in KCs by OK107-GAL4 appears in the calyx but  
867 not the lobes of the mushroom body.

868 **(B)** Flies homozygous for the MiMIC mAChR-A-stop allele (which contains a stop  
869 cassette as part of the Minos gene-trap cassette in the 5'UTR) have virtually no  
870 mAChR-A mRNA. In contrast, flies with the MiMIC mAChR-A-GAL4 allele do not have  
871 reduced mAChR-A mRNA levels, because the stop cassette was replaced with GAL4  
872 (indeed, their mAChR-A levels are slightly higher than the control). (mean  $\pm$  SEM; n=4  
873 each with 3 technical replicates; \*\* p = 0.0001; Welch ANOVA test with Dunnett's T3  
874 multiple comparisons test). For detailed statistical analysis see **Supplementary File 1**.

875 **(C)** Homozygous MiMIC mAChR-A-stop flies are defective in olfactory aversive learning,  
876 but learning is rescued by driving mAChR-A-FLAG in  $\alpha\beta$  and  $\gamma$  KCs by mb247-GAL4. n  
877 (left to right): 49, 70, 56, 47, \* p < 0.05, Kruskal-Wallis test with Dunn's multiple  
878 comparisons test). For detailed statistical analysis see **Supplementary File 1**.

879 **Figure 7—figure supplement 1. Localization of mb247-GAL4>mAChR-A-FLAG**

880 Anti-FLAG immunostaining shows signal only in the calyx in flies expressing mAChR-A-  
881 FLAG under the control of mb247-GAL4 in a homozygous MiMIC mAChR-A-stop

882 hypomorphic background. The signal is less clear than in **Figure 7A** most likely  
883 because OK107-GAL4 is a stronger driver than mb247-GAL4.

884 **Figure 7—source data 1: Source data for Figure 7B.**

885 **Figure 7—source data 2: Source data for Figure 7C.**

886

887 **Figure 8: mAChR-A KD prevents aversive conditioning from decreasing the**  
888 **response to the trained odor in MB-MVP2**

889 **(A)** Odor responses in MB-MVP2 to isoamyl acetate (IAA, not presented during  
890 training), OCT (not shocked during training) and MCH (shocked during training), in  
891 control (OK107-GAL4, R12G04-LexA>GCaMP6f, mb247-dsRed) and knockdown  
892 (OK107-GAL4>mAChR-A-RNAi 1, R12G04-LexA>GCaMP6f, mb247-dsRed) flies, with  
893 mock training (no shock) or training against MCH. Traces show mean (solid line)  $\pm$  SEM  
894 (shaded area).

895 **(B)** MCH:OCT or OCT:IAA ratios of peak  $\Delta F/F$  values from **(A)**.  $n = 5$ . \*  $p < 0.05$ , Mann-  
896 Whitney test. Power analysis shows that  $n = 5$  would suffice to detect an effect as  
897 strong as the difference between training and mock training in the MCH:OCT ratio, with  
898 power 0.9. See **Figure S8** for absolute  $\Delta F/F$  values.

899 **Figure 8—figure supplement 1. Diagram and additional data for Figure 8 (mAChR-**  
900 **A knockdown prevents learning-associated depression of odor responses in**  
901 **MVP2)**

902 **(A)** Diagram of genotype: mAChR-A RNAi 1 was expressed in KCs with OK107 (gray),  
903 while GCaMP6f was expressed in MB-MVP2 with R12G04-LexA (green). The imaging  
904 plane is shown in blue.

905 **(B)** Absolute  $\Delta F/F$  values from MB-MVP2 corresponding to the ratios shown in **Figure**  
906 **8B**. Odors and genotypes as in **Figure 8B**. No general depression was observed  
907 following RNAi expression. (mean  $\pm$  SEM;  $n=5$ ,  $p > 0.05$  for all mock vs. trained  
908 comparisons, Mann-Whitney tests). The difference between mock vs. trained for MCH  
909 in control flies is not statistically significant because of variability in overall

910 responsiveness to odors between flies. When MCH responses are normalized to OCT  
911 responses as in **Figure 8B**, the difference is statistically significant.

912 **Figure 8—source data 1: Source data for Figure 8 and Figure 8—figure**  
913 **supplement 1.**

914

915 **Supplementary File 1. Details of statistical analysis.**

916

917 **Supplementary File 2. Detailed genotypes used in this study.**

918

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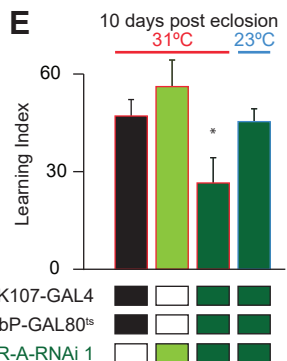
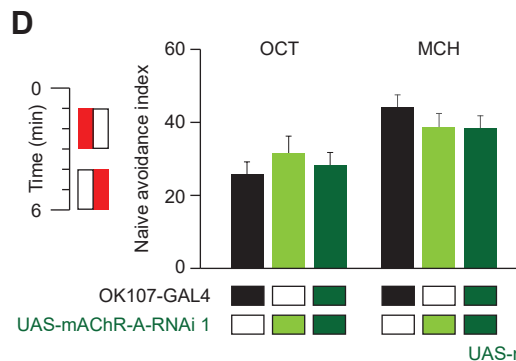
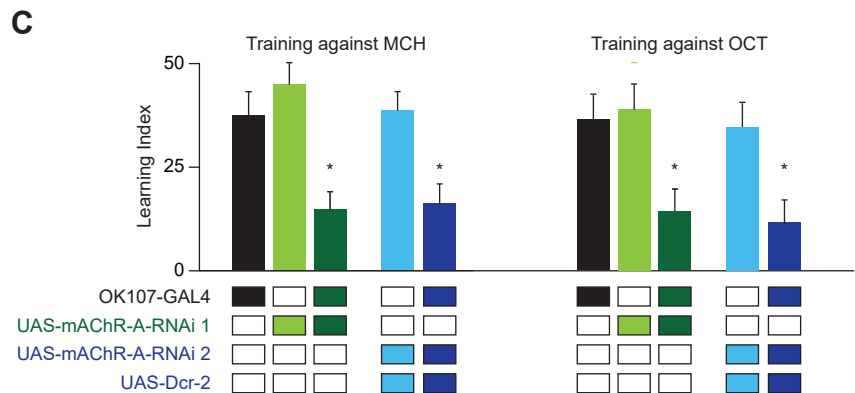
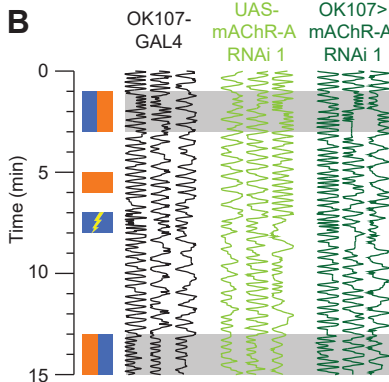
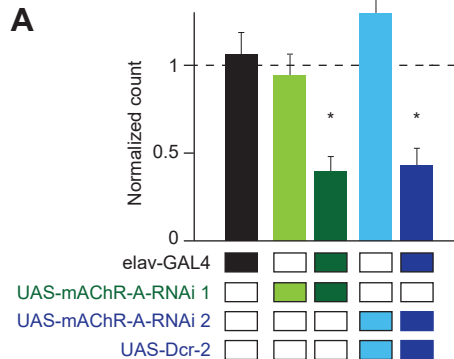
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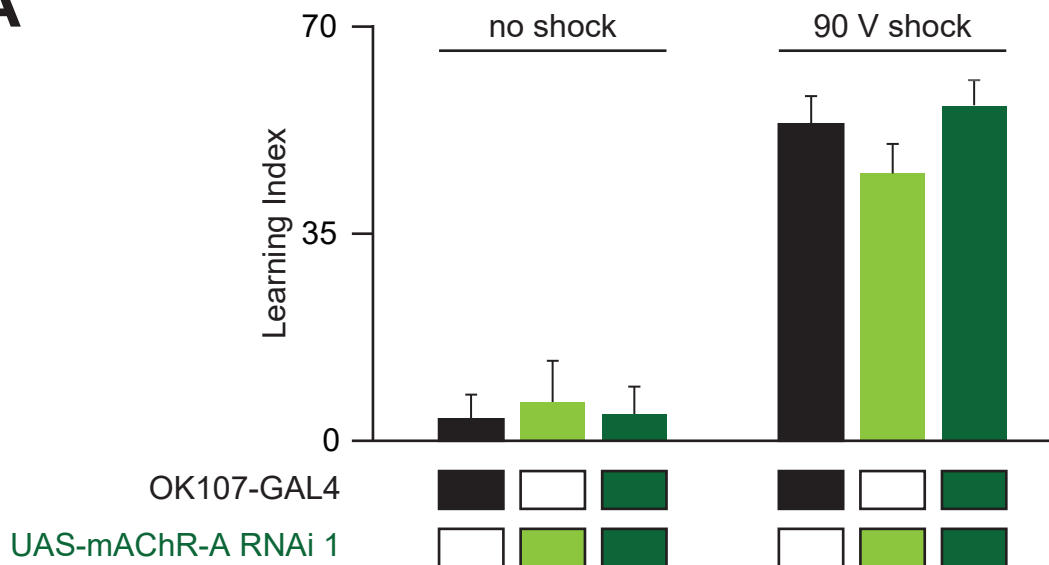
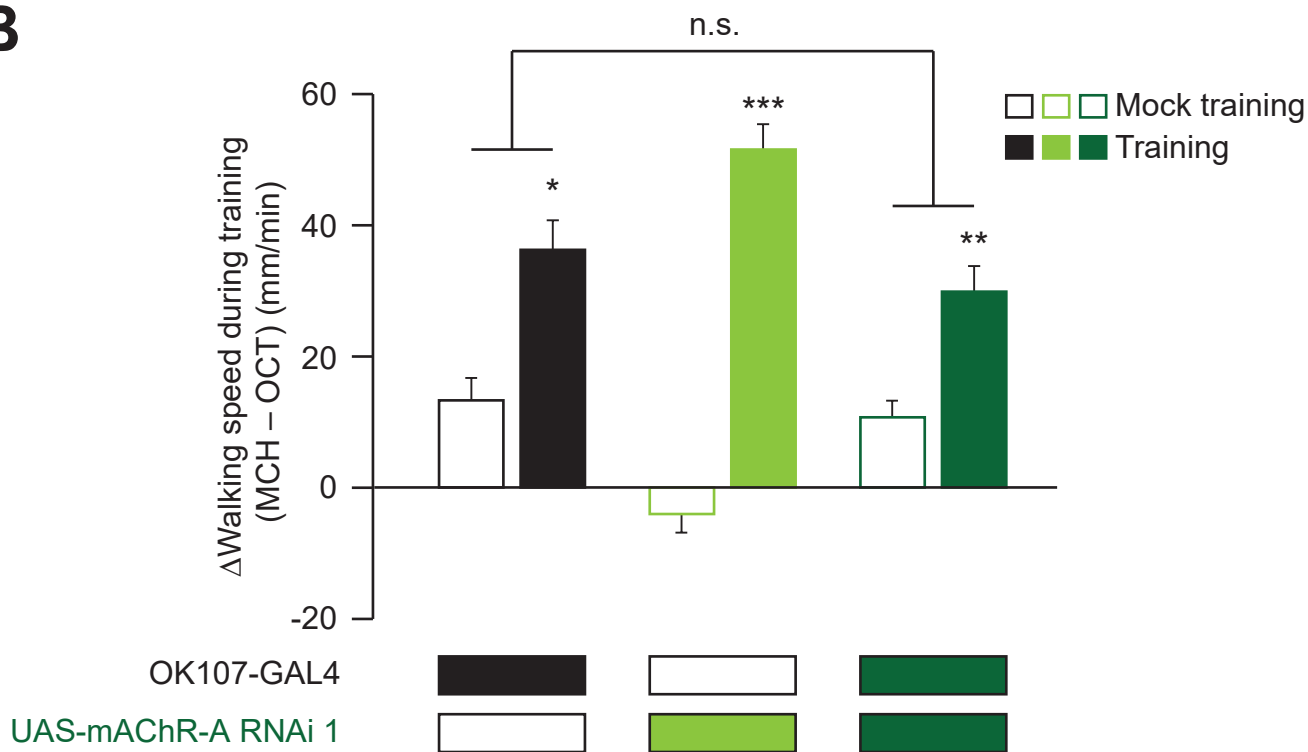
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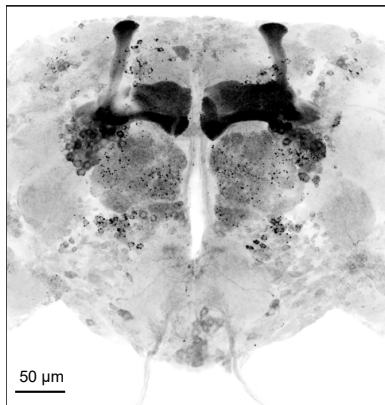
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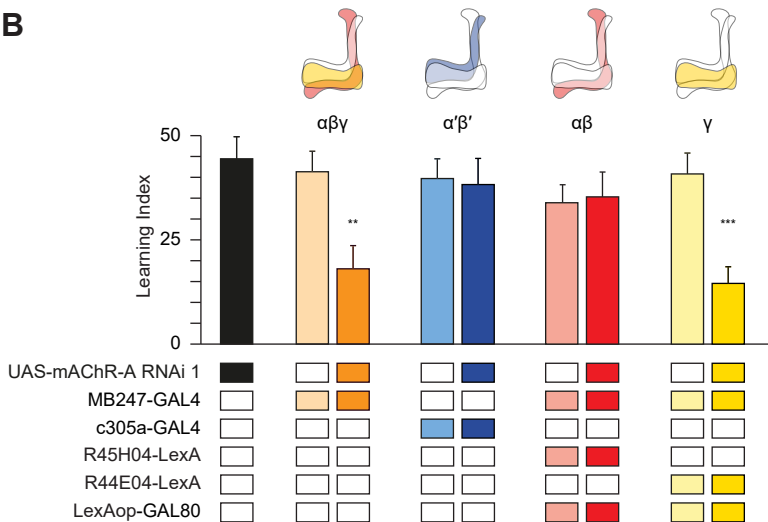
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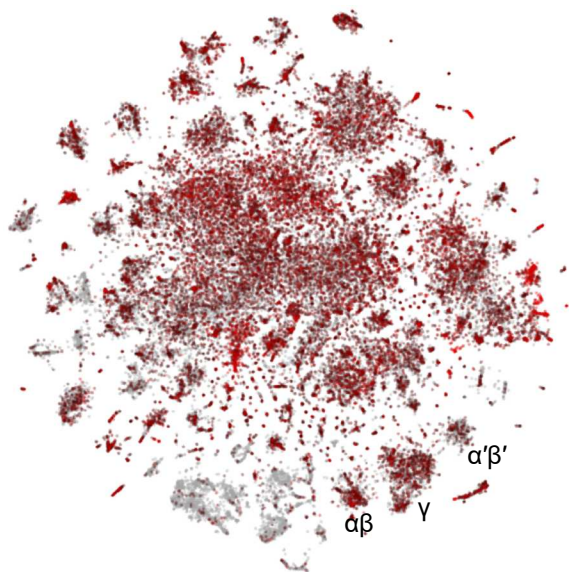
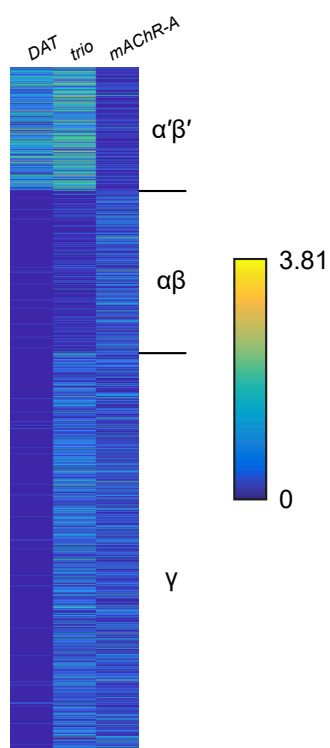
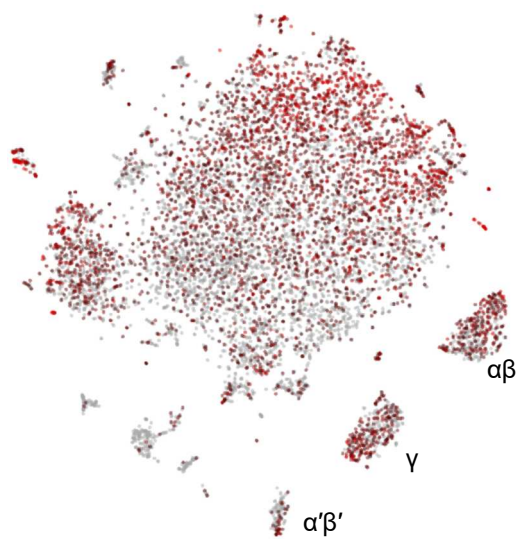
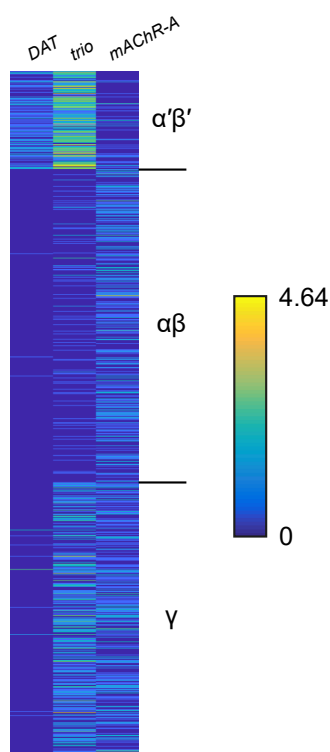


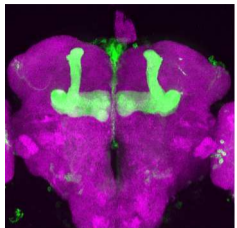
**A****B**

**A**

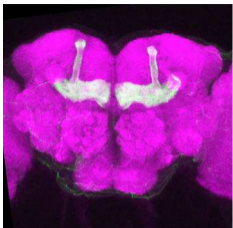
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**B**

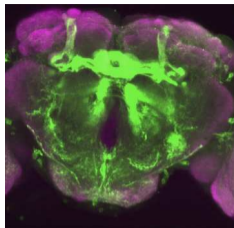
**A****B****C****D**

**A**

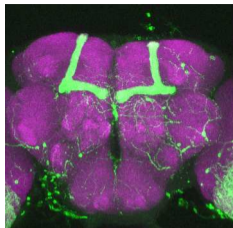
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**B**

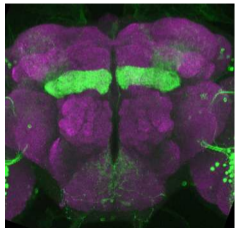
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**C**

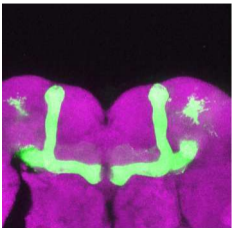
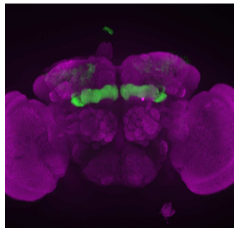
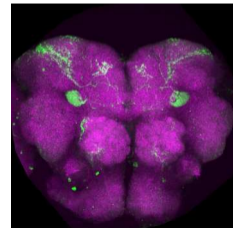
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**D**

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**E**

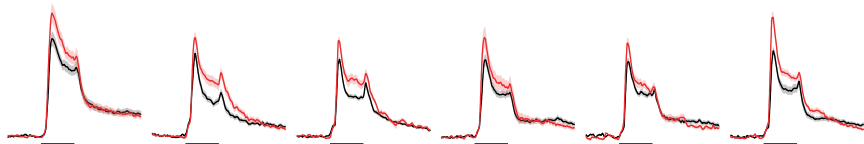
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R44E04-LexA>LexAop-GAL80**H**

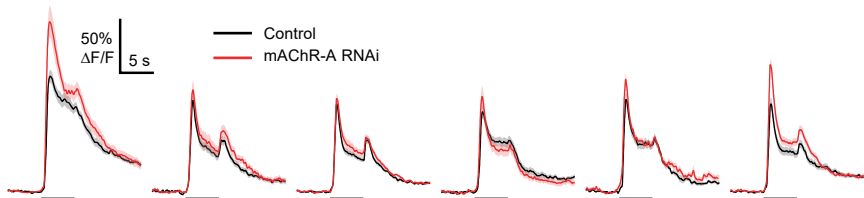
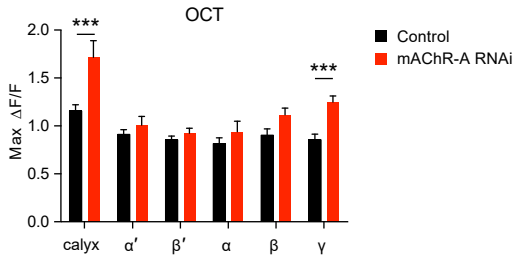
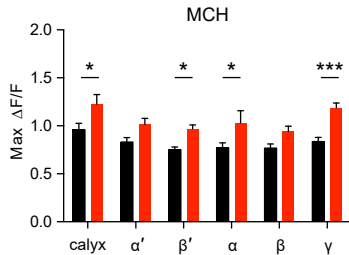
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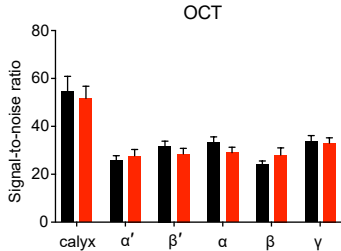
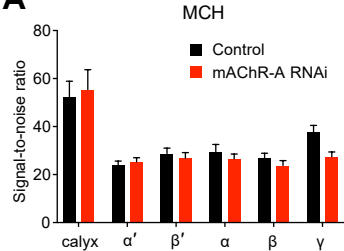
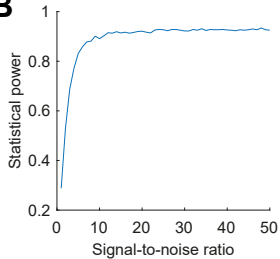
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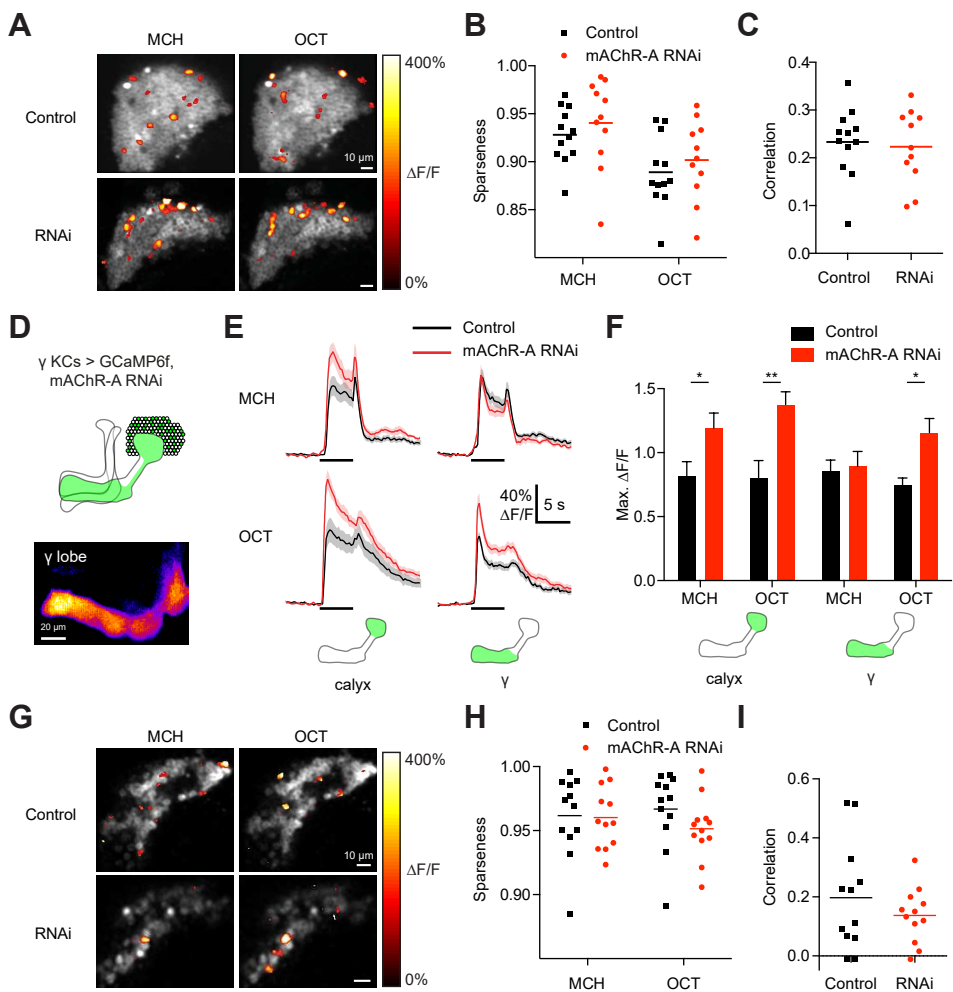
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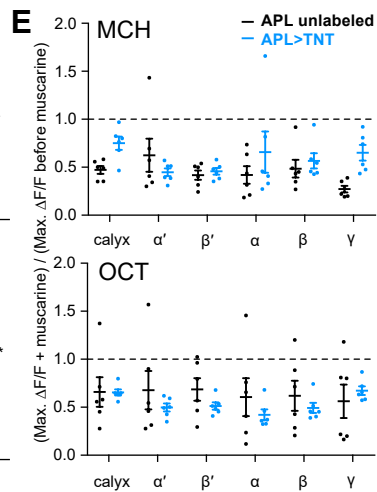
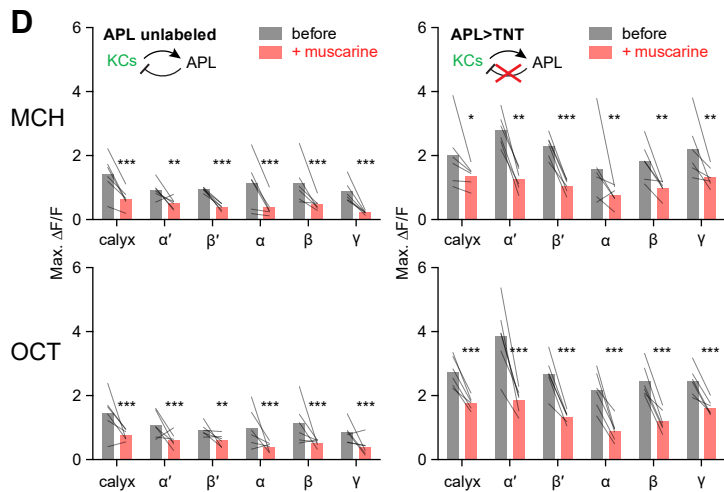
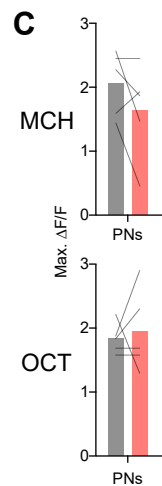
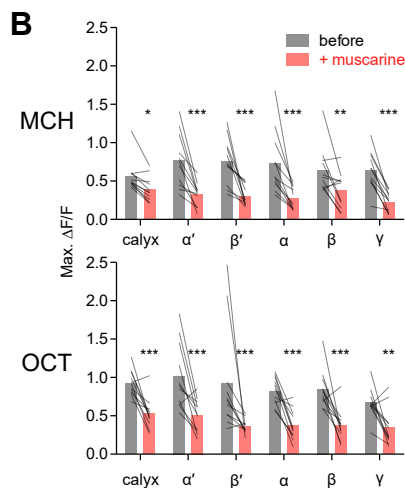
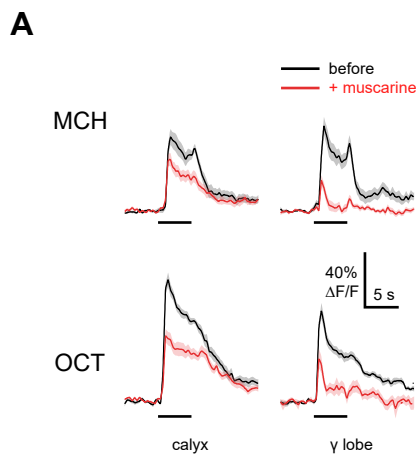


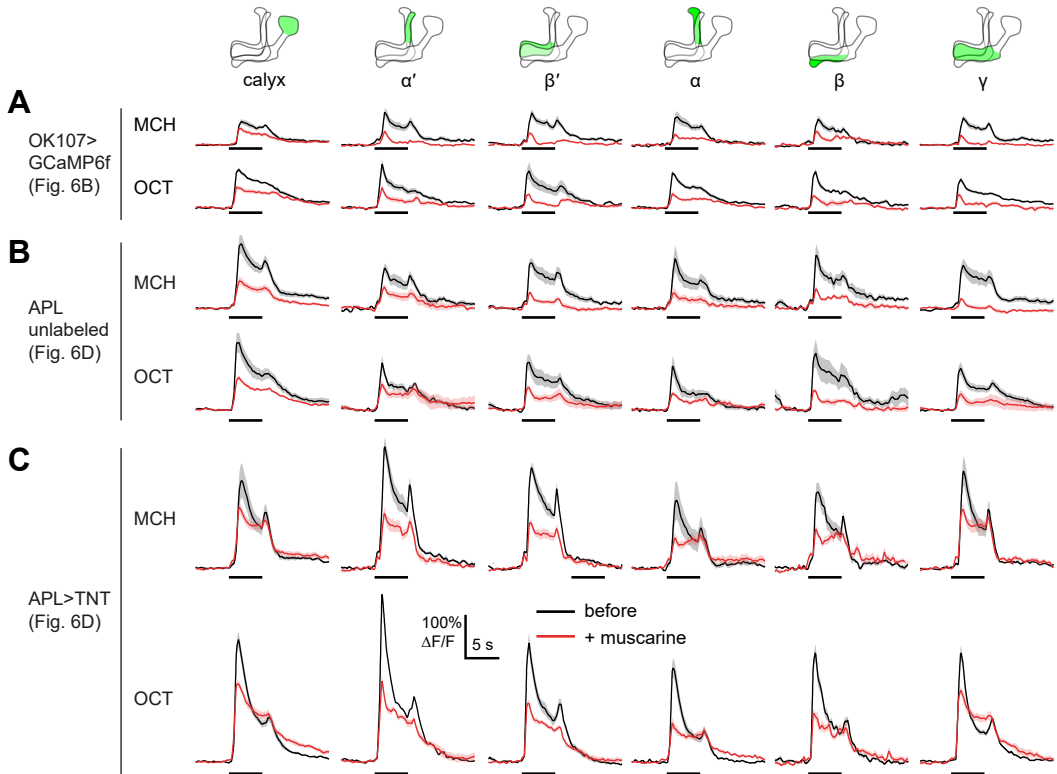
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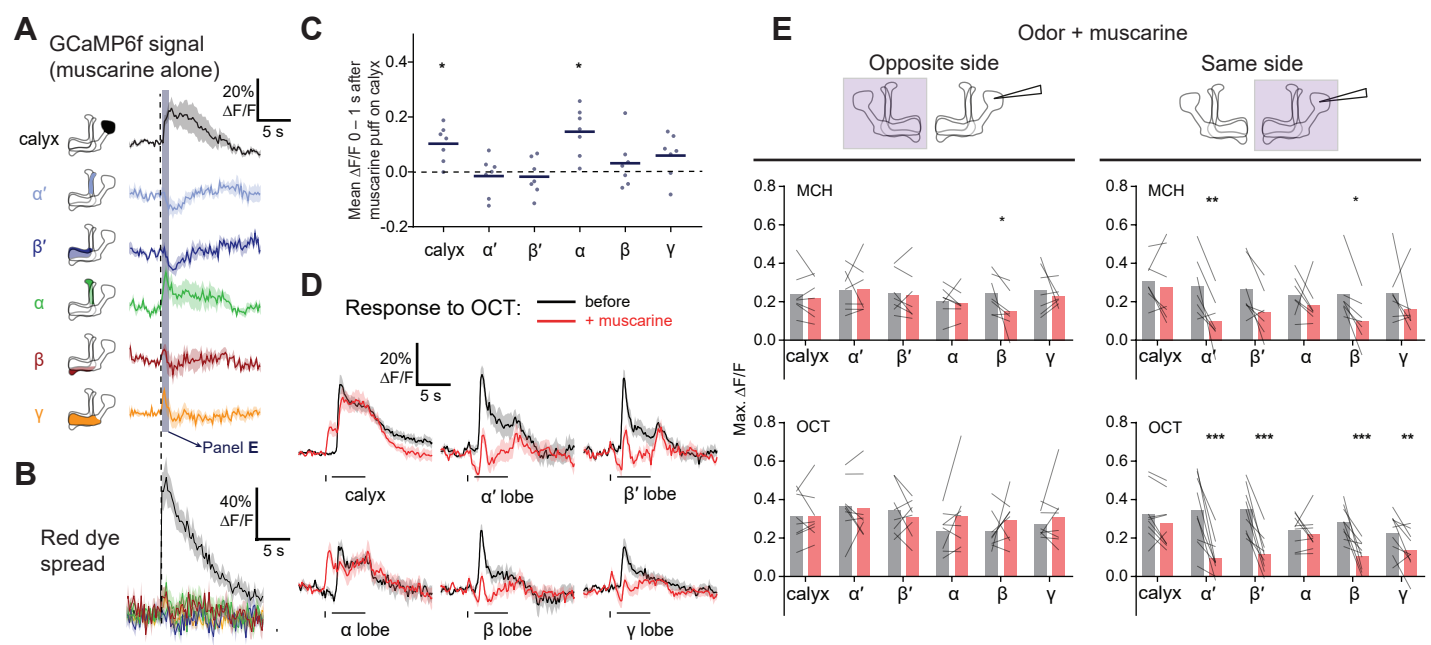
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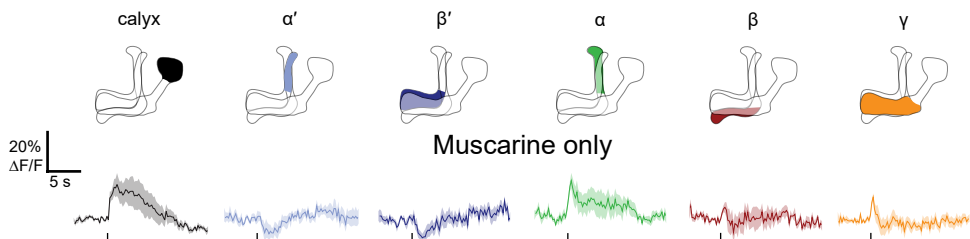
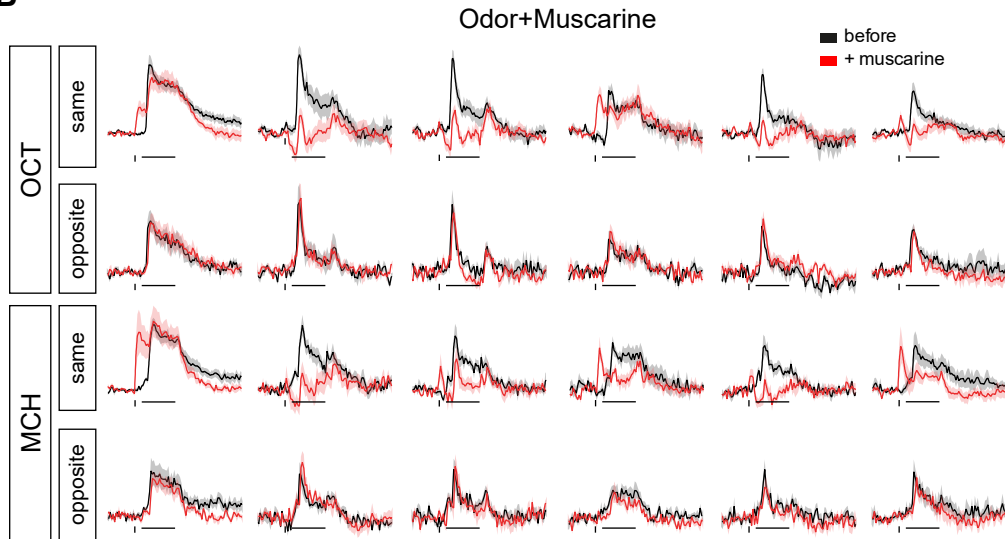
**A****B**

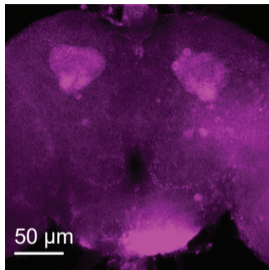




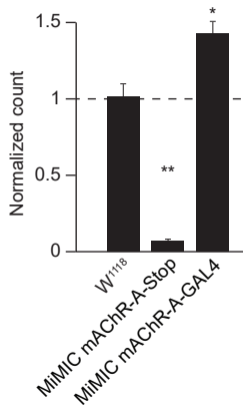
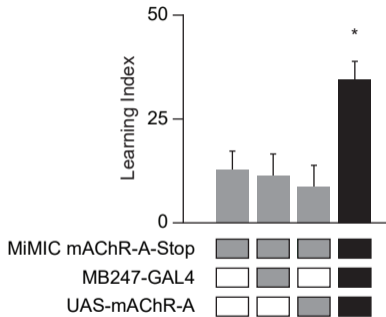


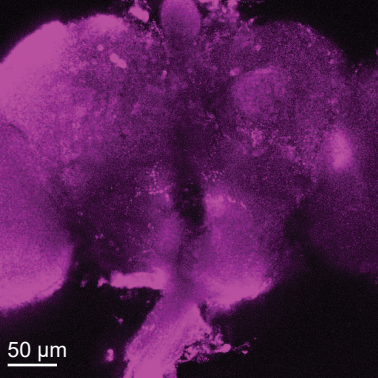


**A****B**

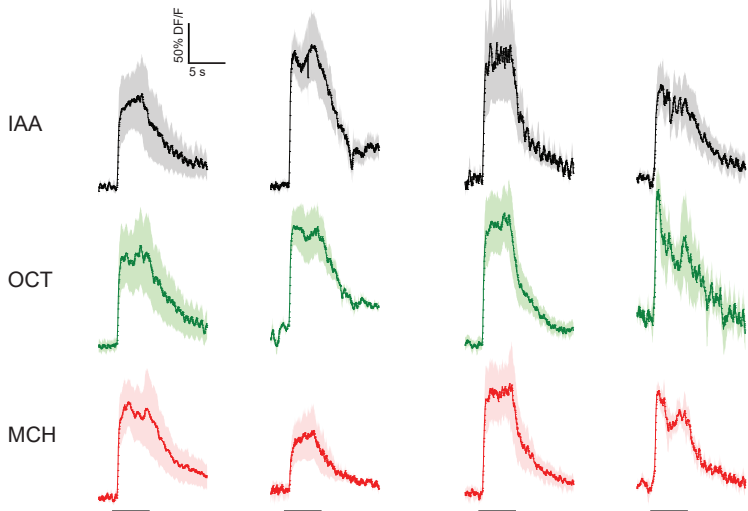
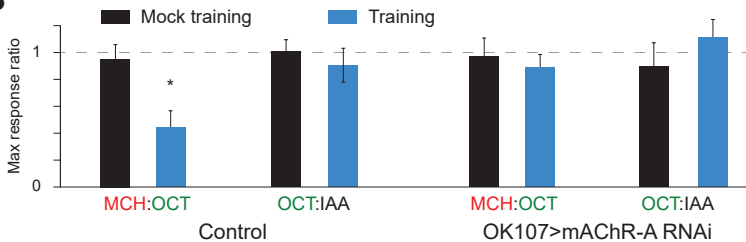
**A**

OK107&gt;UAS-mAChR-A

**B****C**



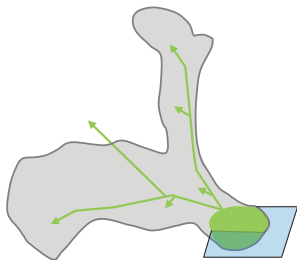
50  $\mu\text{m}$

**A**OK107,  
R12G04 GCaMPOK107 mAChR-A RNAi,  
R12G04 GCaMPMock  
trainingTraining  
against MCHMock  
trainingTraining  
against MCH**B**

**A**

OK107, R12G04 GCaMP

OK107 mAChR-A RNAi, R12G04 GCaMP

**B**

■ ■ ■ OK107, R12G04 GCaMP  
■ ■ ■ OK107 mAChR-A RNAi, R12G04 GCaMP

