The anterior paired lateral neuron normalizes odour-evoked activity at the mushroom body calyx

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Abstract

To identify and memorize discrete but similar environmental inputs, the brain needs to distinguish between subtle differences of activity patterns in defined neuronal populations. The Kenyon cells of the *Drosophila* adult mushroom body (MB) respond sparsely to complex olfactory input, a property that is thought to support stimuli discrimination in the MB. To understand how this property emerges, we investigated the role of the inhibitory anterior paired lateral neuron (APL) in the input circuit of the MB, the calyx. Within the calyx, presynaptic boutons of projection neurons (PNs) form large synaptic microglomeruli (MGs) with dendrites of postsynaptic Kenyon cells (KCs). Combining EM data analysis and *in vivo* calcium imaging, we show that APL, via inhibitory and reciprocal synapses targeting both PN boutons and KC dendrites, normalizes odour-evoked representations in MGs of the calyx. APL response scales with the PN input strength and is regionalized around PN input distribution. Our data indicate that the formation of a sparse code by the Kenyon cells requires APL-driven normalization of their MG postsynaptic responses. This work provides experimental insights on how inhibition shapes sensory information representation in a higher brain centre, thereby supporting stimuli discrimination and allowing for efficient associative memory formation.

1 Introduction

Every day we are challenged to navigate through a complex and variable environment, often characterized by similar stimuli combined in different ways. Yet, our brain excels in assessing if, and how, the current experience is

different or similar to a previously encountered one. The ability to discriminate across stimuli is achieved by minimizing the overlap between patterns of neuronal activity through a process defined as “pattern separation” (Santoro 2013). This conserved property is intrinsic to diverse circuits such as the mammalian cerebellum, the dentate gyrus and the *Drosophila* mushroom body (MB) (Cayco-Gajic and Silver 2019). In the current models, all the aforementioned circuits support pattern separation by utilizing different degree of inhibitory mechanisms. (Tyrrell and Willshaw 1992; Schweighofer, Doya, and Lay 2001; Sahay, Wilson, and Hen 2011; Cayco-Gajic, Clopath, and Silver 2017; Litwin-Kumar et al. 2017). Experimental evidence in support of these inhibitory circuits has been described over the years (Vos, Volny-Luraghi, and de Schutter 1999; Duguid et al. 2015; Inada, Tsuchimoto, and Kazama 2017; Parnas et al. 2013; Olsen, Bhandawat, and Wilson 2010; A. C. Lin et al. 2014), however, the mechanism by which inhibition contributes to pattern separation is not yet fully understood, often due to technical limitations.

With an extended genetic toolkit and a brain of only ~100,000 neurons (Raji and Potter 2021; Alivisatos et al. 2012) largely reconstructed at the EM Level (Zheng et al. 2018; F. Li et al. 2020a), *Drosophila* represents an attractive system to provide experimental evidence on the mechanisms behind pattern separation. The fly MB receives mainly olfactory input, though optical, temperature and humidity information is also represented (Marin et al. 2020; Frank et al. 2015; J. Li et al. 2020). The MB is required for memory formation and retrieval (Heisenberg et al. 1985; de Belle and Heisenberg 1994; Dubnau et al. 2001; S. E. McGuire, Le, and Davis 2001; Aso et al. 2014). Within the MB input region, in the main calyx, olfactory projection neurons (PNs) deliver

63 sensory information from 51 distinct olfactory glomeruli (Grabe et al. 2016;
64 Bates et al. 2020) to ~2,000 Kenyon cells (KCs) of the MB (Aso et al. 2009),
65 for an expansion ratio of 40 (Litwin-Kumar et al. 2017). In the calyx, PNs
66 synapse onto KCs via complex synaptic structures known as microglomeruli
67 (MGs) (Yasuyama, Meinertzhagen, and Schürmann 2002a; Leiss et al. 2009a).
68 At each MG, a single central PN bouton is enwrapped by, on average, 13 claw-
69 like dendritic terminals of as many different KCs (Davi D. Bock, personal
70 communication). KCs integrate inputs in a combinatorial manner, with each KC
71 receiving input from 6-8 PNs, on average (Butcher et al. 2012; Zheng et al.
72 2020; F. Li et al. 2020b; Turner, Bazhenov, and Laurent 2008), of which more
73 than half need to be coactive to elicit spikes (Gruntman and Turner 2013;
74 Inada, Tsuchimoto, and Kazama 2017). As a result, while PN odour-evoked
75 activity is broadly tuned (Perez-Orive et al. 2002; Bhandawat et al. 2007),
76 odour representation is sparse and decorrelated at the KCs layer (Honegger,
77 Campbell, and Turner 2011; Turner, Bazhenov, and Laurent 2008; Campbell et
78 al. 2013b; Perez-Orive et al. 2002), therefore reducing overlap between stimuli
79 representation and allowing for better discriminability (Kanerva 1988; Cayco-
80 Gajic, Clopath, and Silver 2017; Olshausen and Field 2004). In addition to
81 sparse PN:KC connectivity and KCs high input threshold, inhibition is required
82 to reduce the overlap among odour representations in the *Drosophila* MB (A. C.
83 Lin et al. 2014; Lei et al. 2013). At the MB, inhibition is provided by the
84 GABAergic anterior paired lateral (APL) neuron, which innervates both the
85 calyx and the lobes of the MB (Liu and Davis 2009; Pitman et al. 2011; Aso et
86 al. 2014). APL responds to odours with depolarization and calcium influx (Liu
87 and Davis 2009; Papadopoulou et al. 2011). Importantly, blocking APL output

88 disrupts the KCs sparse odour representation and impairs learned
 89 discrimination of similar odours, pointing to its critical role in the process (A. C.
 90 Lin et al. 2014; Lei et al. 2013). APL is suggested to regulate sparse coding by
 91 participating in a closed feedback loop with the MB, similarly to its homolog
 92 giant GABAergic neuron (GGN) in the locust (Papadopoulou et al. 2011; A. C.
 93 Lin et al. 2014; Litwin-Kumar et al. 2017). However, APL is both pre- and post-
 94 synaptic to PNs and KCs in the adult calyx (Yasuyama, Meinertzhagen, and
 95 Schürmann 2002b; Wu et al. 2013; Baltruschat et al. 2021). Additionally, APL
 96 response to localized stimuli is spatially restricted (Amin et al. 2020). In
 97 particular, APL branches at the MB lobes and the ones in the calyx appear to
 98 represent two separate compartments (Amin et al. 2020), suggesting a
 99 possible distinct role of APL inhibition in these two different compartments.
 100 Hence, the mechanisms by which APL modulates sparse coding and its
 101 involvement in the process of pattern separation are still unclear. In the
 102 present work, we challenge the concept of a broad feedback inhibition to the
 103 MB calyx by APL with primary experimental data. In particular, we focused on
 104 the APL processes within the MB calyx and set out to identify the role of
 105 GABAergic inhibition at the PN:KC synaptic layer. Taking advantage of recently
 106 released EM datasets (Scheffer et al. 2020; Zheng et al. 2018), we report the
 107 complex synaptic interaction of APL with PNs and KCs within the MGs of the MB
 108 calyx. Next, via *in vivo* calcium imaging in the calyx, we explored the role of
 109 APL inhibition onto MGs by recording the odour-evoked activity of APL, PN
 110 boutons and KC dendritic claws. Our results indicate that APL acts as a
 111 normalizer of postsynaptic responses to olfactory inputs in the MGs of the
 112 mushroom body calyx, an idea that we confirmed by blocking the output of

113 APL. Additionally, via volumetric calcium imaging, we addressed the locality of
114 APL activation in the calyx and found that it is odour-specific. We suggest that
115 the normalization of postsynaptic MG responses by APL is essential to
116 determine the key property of KCs to respond only to the coincident input of
117 PNs to multiple claws, allowing for an elevated stimulus discriminability.

118 **2 Results**

119 **2.1 APL is an integral part of the microcircuit** 120 **within microglomeruli in the MB calyx**

121 To better understand the role of GABAergic inhibition at the MB calyx, we
122 investigated APL involvement into the calycal microcircuits with the highest
123 resolution available. The APL of adult *Drosophila* innervates extensively all
124 compartments of the mushroom body, including calyx, lobes and pedunculus
125 (Liu and Davis 2009). Moreover, the neuron appears to be non-polarized in the
126 adult, with strong expression of both pre- and post-synaptic markers in all
127 compartments (Wu et al. 2013). However, little is known regarding the detailed
128 connectivity between APL and the cell types constituting the mushroom body.
129 Taking advantage of emerging electron microscopy (EM) datasets covering a
130 full adult fly brain (FAFB, (Zheng et al. 2018)) or a large fraction of it
131 (Hemibrain, (Scheffer et al. 2020)), we examined the distribution of synaptic
132 contacts between APL, PNs and KCs, the major cell types constituting the MGs
133 of the mushroom body calyx (Leiss et al. 2009b; Yasuyama, Meinertzhagen,
134 and Schürmann 2002a; Baltruschat et al. 2021) (Fig 1A). We recently
135 reconstructed an entire MG in the FAFB dataset, starting from a PN-bouton of

136 the DA1 glomerulus and tracing all its pre- and post-synaptic partners
 137 (Baltruschat et al. 2021). Here, we focused on the synaptic connections
 138 involving APL. We found APL to be highly involved in the MG structure, with
 139 pre- and post- synaptic contacts with both KC dendrites and PN boutons (Fig
 140 S1A) (Baltruschat et al. 2021). Many of those synapses were polyadic,
 141 displaying typical configurations within that specific MG (described in Fig S1B).
 142 To verify whether such features were specific to the DA1 MG reconstructed in
 143 Baltruschat et al. (2021) or common, we exploited the Hemibrain EM dataset
 144 (Scheffer et al. 2020) and extracted all calycal connections from and to APL
 145 with either KCs or PNs. Out of the 136 PNs reported innervating the main calyx
 146 (F. Li et al. 2020b), 126 made and received synapses with APL (full list of PNs
 147 and APL interactions available at: Mendeley data link will be available upon
 148 publication). To reveal the localization of these synapses, we rendered 3D
 149 graphs of single PNs derived from the Hemibrain dataset (Scheffer et al. 2020)
 150 and mapped the synapses that they receive from APL within the MB calyx (see
 151 Material and Methods for details). Most of the APL-to-PN connections were
 152 localized on PN boutons ($84 \pm 2\%$, mean $\pm$ SEM, of the total synapses received
 153 by each PN localised on boutons), demonstrating that the majority of APL-PN
 154 interactions happens at MGs (Fig 1B, all images available at: Mendeley data
 155 link will be available upon publication). Additionally, we found a positive
 156 correlation between the number of synapses made by the APL towards a
 157 specific PN and the reciprocal synapses formed by that PN onto APL (Fig 1D).
 158 Of notice, most of the PNs not connecting to the APL within the main calyx
 159 were already described as non-olfactory PNs (Marin et al. 2020), and they all
 160 seemed to extend most of their terminals elsewhere, with little to no branches

161 in the main calyx (Fig S1D). Similarly, of the 1919 KCs present in the dataset,
 162 1871 displayed interactions with APL. Mapping APL synapses onto single KC
 163 meshes (Fig 1C, all images available at: Mendeley data link will be available
 164 upon publication) showed a majority of connections on KC claws. However, we
 165 noticed inhibitory synapses along KC dendrites as well. The KCs constituting
 166 the MB are divided in 3 major classes based on their axonal projections: γ , α/β ,
 167 α'/β' (Crittenden et al. 1998; Lee, Lee, and Luo 1999). We found a difference in
 168 the spatial distribution of APL synapses depending on the KC type, suggesting
 169 that APL inhibition might have a different impact on different KC types. In
 170 particular, APL synapses onto α/β KCs were significantly less localised on claw-
 171 like dendritic terminals and more distributed along KC dendritic branches (Fig
 172 S1C, n=210 (70 per KC type, randomly selected), $p<0.0001$, Unpaired ANOVA
 173 with multiple comparisons). The KCs not interacting with APL displayed a
 174 rather atypical structure, with extensive dendritic arborization just outside of
 175 the main calyx rather than within (Fig S1E). As in the case of PNs, the number
 176 of KC-to-APL synapses positively correlated with the APL-to-KC synapse
 177 number (Fig 1E). In conclusion, EM dataset analysis revealed a large
 178 involvement of APL in the calycal circuitry, with reciprocal connections to the
 179 vast majority of PNs and KCs. APL involvement in the MG structure as reported
 180 in Baltruschat et al (2021) might be thus generalized to potentially all MGs of
 181 the main calyx.

182

Fig 1. APL participates in the MG microcircuit with reciprocal synapses.

(A) Left: example of a PN (red) sending collateral boutons into the MB calyx (grey volume), where it connects onto KCs claws via synaptic microglomeruli. For simplicity, only 1 KC is visualised here (green). Bottom-left box: magnification of a PN bouton interacting with a KC claw (black arrow). Right: APL (blue) innervates the entire MB including lobes, peduncle and the calyx. Asterisks indicate cell bodies. **(B)** Visualisation of APL synapses (blue dots) onto a PN 3D mesh within the MB calyx. Most connections are localised on PN boutons. **(C)** Localization of APL synapses (blue) on a KC 3D mesh within the MB calyx. While most are localised on dendritic claws, some connections along dendritic branches could be seen as well (see also S1C). The cell body is

195 marked by an asterisk. **(D)** Correlation between the number of PN-to-APL
 196 reciprocal synapses ($r^2=0.63$) and KC-to-APL ones **(E)** ($r^2=0.60$). The
 197 correlation was calculated among the entire synaptic weight that individual PNs
 198 or KCs had with APL. All 3D plots were created via the Neuprint-python
 199 package (see Materials & Methods).
 200

201
 202 **Fig S1 (Related to Fig 1). APL in the microglomerular circuit.**

203 **(A)** Schematized view of the connectivity patterns found in the DA1-PN MG
 204 reconstructed in Baltruschat et al. (2021). Green circles represent KC claws.
 205 Dark green circles represent presynaptic KCs. The red circle represents the
 206 reconstructed bouton of a DA1-PN. The blue circle represents APL. See
 207 Baltruschat et al., (2021) for further explanation, including the full connectome

208 of this DA1-PN MG. **(B)** Examples of connectivity patterns involving APL in the
 209 DA1-PN microglomerulus reconstructed in Baltruschat et al. (2021). Top left:
 210 EM image of APL (blue) presynaptic to the PN bouton (red) and the claw of a
 211 KC (green). Top right: PN bouton presynaptic to APL and three KC claws.
 212 Bottom-left: KC (dark green) presynaptic to PN bouton, APL and another claw.
 213 Bottom-right: APL and PN bouton presynaptic to the same KC claw. Scale bar
 214 = 250nm. **(C)** Spatial distribution of APL synapses among different KC types.
 215 The fraction of APL to KC $\alpha\beta$ synapses localised on KC claws was lower than in
 216 other KC types. n=210 (70 per KC type, randomly selected), p<0.0001,
 217 Unpaired ANOVA with multiple comparisons. Whiskers indicate SD. **(D)**
 218 Examples of PNs not interacting with APL at the MB calyx (grey volume). **(E)**
 219 Examples of KCs not interacting with APL at the MB calyx (grey volume). 3D
 220 neuronal meshes were created via the Neuprint-python package (see Materials
 221 & Methods).

222 **2.2 In the calyx, APL displays different response**

223 **levels to different odours**

224 The analysis of the EM data provided structural evidence for possible
 225 feedforward and feedback circuits between APL, PNs and KCs in the MB calyx
 226 (Fig S1A-B). To explore the functional role of APL in calycal MGs, we performed
 227 *in vivo* functional imaging experiments by expressing the calcium indicator
 228 GCaMP6m (Chen et al. 2013b) specifically in APL via the APL intersectional
 229 *NP2631-GAL4, GH146-FLP* (APLi) driver (A. C. Lin et al. 2014; Mayseless et al.
 230 2018) and recorded odour-evoked activity in the calyx (Fig 2A, see Materials
 231 and Methods). Flies were stimulated with 5 second puffs of odours diluted
 232 1:100 in mineral oil and exposed to sequences of 2 odours starting with 4-
 233 methylcyclohexanol (Mch) and 3-octanol (Oct), presented in a randomized
 234 fashion. Odour-elicited calcium transients in APL were detectable in the calyx

235 (Fig 2B,E). Interestingly, we observed a clear difference in the GCaMP
 236 fluorescence levels, measured as $\Delta F/F_0$ over the entire calycal region
 237 innervated by APL (see also Material and Methods) in response to the two
 238 odour stimulations, with Oct eliciting a stronger APL response (Fig 2C-D, n=10,
 239 p=0.002, Wilcoxon matched-pairs test). To extend this observation, we
 240 exposed flies also to δ -Decalactone (δ -DL), an odour reported to elicit the least
 241 overall activity in ORNs (Hallem and Carlson 2006a). Similarly, we measured a
 242 difference between the strength of the response to Oct compared to δ -DL (Fig
 243 2F-G, n=10, p<0.0001, paired t test). Moreover, the gap between the δ -DL
 244 signal peak and the Oct one was higher compared to the Mch vs Oct group
 245 ($\Delta(\text{Oct-Mch}) = 45 \pm 27\%$; $\Delta(\text{Oct-}\delta\text{-DL}) = 76 \pm 30\%$, n=10, p=0.0234,
 246 unpaired t-test with Welch's correction), suggesting that APL is able to provide
 247 a variable, odour-tuned inhibition to MGs of the MB calyx.

248

Figure 2. APL responds to odours with variable calcium transients.

(A) Schematic view of the two-photon *in vivo* imaging setup. Scale bar = 20 μ m. **(B)** Example of APL response to Mch or Oct in the calyx of *APLi-GAL4>UAS-GCaMP6m* flies. Scale bar = 10 μ m. **(C)** Fluorescence intensity over time for the fly showed in (B). **(D)** APL showed higher intracellular calcium transients in response to Oct compared to Mch. $n=10$, $p=0.002$, Wilcoxon matched-pairs test. **(E)** Example of APL GCaMP6m response to δ -DL or Oct. Scale bar = 10 μ m. **(F)** Fluorescence intensity over time for the fly showed in (E). **(G)** APL peak response comparison for the δ -DL vs Oct odours sequence. $n=10$, $p<0.0001$, paired t test. Odours were diluted 1:100, bars indicate means.

262

263 **2.3 The response to different odours is highly** 264 **variable in PNs, but more homogeneous in KC** 265 **dendrites**

266 To investigate the origin and the consequences of the observed difference in
267 APL response at the MB calyx, we performed functional imaging experiments
268 targeting the other two cell types participating in the microglomerular
269 structure: PNs and KCs (Leiss et al. 2009b; Yasuyama, Meinertzhagen, and
270 Schürmann 2002a). Odors are detected by a large set of olfactory receptor
271 neurons (ORNs) expressing chemically-tuned odorant receptors (Clyne et al.
272 1999; Hallem and Carlson 2006a). ORNs project to the 51 distinct olfactory
273 glomeruli in the adult antennal lobe (AL) in a stereotyped manner, with ORNs
274 expressing the same odorant receptor projecting to the same glomerulus (Q.
275 Gao, Yuan, and Chess 2000; Vosshall, Wong, and Axel 2000; Grabe et al.
276 2016). Within glomeruli, ORNs synapse onto second-order neurons, the PNs,
277 which deliver odour information to higher brain regions such as the MB and the
278 lateral horn (R. F. Stocker et al. 1990). To investigate whether odour-evoked
279 activity in PNs reflected the differences in strength observed in APL, we
280 expressed GCaMP6m in PNs via the generic PN-Gal4 driver *GH146* (Berdnik et
281 al. 2008), and imaged PN dendrites in the antennal lobe. Flies were exposed to
282 Mch or Oct as described for the APL imaging experiments, and the average
283 peak among the responding glomeruli per brain was used as a general
284 indicator of the total input transmitted by PNs. Imaging was performed on a

single optical section of the antennal lobe, and only the glomeruli that could be unequivocally identified among all tested animals were taken into consideration for the analysis. While the number of responding glomeruli was similar between the two tested odours (Fig S2, also shown on a larger number of AL glomeruli in (Barth et al. 2014)), the overall calcium transient was higher when flies were exposed to Oct (Fig S3A-B, $n=10$, $p=0.002$, Wilcoxon matched-pairs test), suggesting that the main source of difference was represented by the degree of PNs activation rather than an additional/decreased number of active neurons. Likewise, a strong difference could be measured when flies were exposed to the δ -DL/Oct odours sequence (Fig S3C-D, $n=10$, $p<0.0001$, paired t-test), resembling the differences in APL activation detected at the MB calyx.

To address whether this odour-dependent variability in PN dendrites activity is still detectable within the collateral boutons in the MB calyx, we expressed the presynaptically localized GCaMP3 transgene *UAS-Syp::GCaMP3* (Pech et al. 2015) in PNs and recorded odour-evoked activity in PN boutons of the MB calyx. We exposed flies to Mch or Oct, and calculated per calyx the average peak response among the boutons showing calcium transients. While the number of active boutons did not change between Mch and Oct stimulations (Fig 3D, $n=10$, $p=0.689$, paired t-test), the average boutons response was higher when flies were exposed to Oct (Fig 3B, $n=10$, $p=0.0002$, paired t-test). Furthermore, plotting the frequency distribution of all boutons activity peaks measured during these experiments showed a clear shift towards higher values of the entire Oct-responding population (Fig 3C, $n=10$, $p<0.0001$, Kolmogorov-Smirnov test). Hence, the difference shown in Fig 3B was not just due to a very high response of a few boutons, but rather to an overall increase in PN

310 boutons activation levels across stimuli . Taken together, these experimental
 311 data suggested that the APL neuron activation scales with PN inputs strength.
 312 To further extend the correlation between PNs and APL activity in a systematic
 313 way, we measured the APL calcium transient levels in response to odours
 314 contained in a ORNs response database (Hallem and Carlson 2006b) and
 315 plotted it against their PN spikes value obtained via the experimentally
 316 supported equation described in Olsen et al. (Olsen, Bhandawat, and Wilson
 317 2010; Parnas et al. 2013). We observed a positive linearity between the two
 318 variables (Fig S3E, Pearson $r=0.99$), hence supporting our experimental data
 319 described above. In conclusion, these experiments demonstrated that the
 320 overall level of PN activation varies with different odours and correlates with
 321 the APL response within the MB calyx.

322 To clarify the impact of the odour-tuned activation of APL on the response of
 323 KCs to odours, we next imaged the functional response of KC claws to odour
 324 stimulation. Flies expressing the postsynaptically-tagged calcium indicator
 325 *homer::GCaMP3* under the KCs promoter *MB247* (Pech et al. 2015) were
 326 prepared, stimulated and imaged as described before. Olfactory stimulation
 327 caused the activation of different patterns of MGs in an odour-dependent
 328 manner (Pech et al. 2015) (Fig 3E, S3F). The number of MGs responding to
 329 each odour was not significantly different between Mch and Oct exposure (Fig
 330 3H, $n=9$, $p=0.727$, Wilcoxon matched-pairs test), whereas it was lower when
 331 flies were stimulated with δ -DL (Fig S3I, $n=10$, $p=0.0059$, paired t-test), which
 332 elicits the least overall ORN activity (Hallem and Carlson 2006a) and induced a
 333 weak and restricted response in PN glomeruli at the AL (Fig S2). Importantly,
 334 the average microglomerular postsynaptic response to each odour was not

335 different when comparing the response to Mch vs Oct stimulation (Fig 3F, n=9,
 336 p=0.1648, paired t-test) or δ -DL vs Oct stimulation (Fig S3G, n=10, p=0.767,
 337 Wilcoxon matched-pairs test). Additionally, the frequency distributions of the
 338 odour-evoked activity peaks were overlapping (Fig 3G, S3H, n=9-10,
 339 p=0.0982 and p=0.9554 for Fig 3G and S3H, respectively, Kolmogorov-
 340 Smirnov test). Hence, the differences in activation strength described at the
 341 input population of MGs (the PN boutons) seemed to be normalized at the next
 342 neuronal layer, in the KC claw-like dendritic endings. Thus, the range of
 343 postsynaptic responses in MGs appears to be restrained.
 344

Fig 3. The strength of response to odour stimulation varies in an odour-dependent way in the PN boutons, but is homogenous at the postsynaptic KC claws.

(A) Example of PN boutons fluorescence increase in response to stimulation with Mch or Oct in *NP225-GAL4>UAS-Syp::GCaMP3* flies. Scale bar = 10 μ m.

(B) The average activity peak in PN boutons was higher when flies were exposed to Oct compared to Mch. n=10, p=0.0002, paired t-test. **(C)**

353 Frequency distribution of PN boutons activity peaks in the Mch vs Oct protocol.
 354 The Oct population was significantly shifted towards higher $\Delta F/F_0\%$ MAX values.
 355 $n=10$, $p<0.0001$, Kolmogorov-Smirnov test. **(D)** The number of ROIs showing
 356 odour-evoked activity did not change between the two odour exposures. $n=10$,
 357 $p=0.689$, paired t-test. **(E)** Example of KC claws fluorescence levels in
 358 response to Mch and Oct in *MB247-homer::GCaMP3* flies. Scale bar = $10\mu\text{m}$.
 359 **(F)** The average activity peak in KC claws was comparable between Mch and
 360 Oct exposures. $n=9$, $p=0.1648$, paired t-test. **(G)** Frequency distribution of KC
 361 claws activity peaks in the Mch vs Oct protocol. The two populations had a
 362 similar shape and spread among similar $\Delta F/F_0\%$ MAX values. $n=9$, $p=0.0982$,
 363 Kolmogorov-Smirnov test. **(H)** The number of ROIs showing odour-evoked
 364 activity did not change between the two odour exposures. $n=9$, $p=0.727$,
 365 Wilcoxon matched-pairs test. Odours were diluted 1:100, bars indicate means.
 366

Fig S2 (Related to Fig 3). Single PN glomeruli responses at the AL.

GH146-GAL4>UAS-GCaMP6m flies were stimulated with either Mch vs Oct or δ -DL vs Oct. The activity peak per each glomerulus was extracted and compared. $n=10$, paired t-test, p value > 0.05 (n.s.), ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***). Odours were diluted 1:100, bars indicate means.

375 **Fig S3 (Related to Fig 3). Additional data on PNs and KCs odour evoked** 376 **activity.**

377 Examples of PN dendritic calcium transients in response to either Mch and Oct
378 **(A)** or δ -DL and Oct **(C)** at the antennal lobe in *GH146-GAL4>UAS-GCaMP6m*
379 flies. Scale bar = 20 μ m. **(B)** The average activity peak among responding
380 glomeruli was higher when flies were exposed to Oct compared to Mch. n=10,
381 p=0.002, Wilcoxon matched-pairs test. **(D)** The average activity peak among
382 responding glomeruli was higher when flies were exposed to Oct compared to
383 δ -DL. n=10, p<0.0001, paired t-test. **(E)** The APL calcium transient levels in
384 response to 4 chosen odours (ethyl acetate (EA) 1:1000 dilution, pentyl
385 acetate (PA) 1:1000, δ -DL and EtOH 1:100) in *APLi-GAL4>UAS-GCaMP6m* flies
386 linearly correlated with the average PN spiking rate for the respective odour
387 stimulations. The PN spiking rate for 24 AL glomeruli were obtained by feeding
388 ORNs spike values from the Hallem and Carlson 2006 dataset into the ORN-to-
389 PN spike transformation equation described in Olsen et al. 2010 (see also
390 (Parnas et al. 2013)). Pearson r=0.99 **(F)** Example of KC claws fluorescence
391 levels in response to δ -DL and Oct in *MB247-homer::GCaMP3* flies. Scale bar =
392 10 μ m. **(F)** The average activity peak in KC claws was comparable between δ -
393 DL and Oct exposures. n=10, p=0.767, Wilcoxon matched-pairs test. **(G)**
394 Frequency distribution of KC claws activity peaks in the δ -DL vs Oct protocol.
395 The two populations had a similar shape and spread among similar $\Delta F/F_0\%$ MAX
396 values. n=10, p=0.9554, Kolmogorov-Smirnov test. **(H)** The number of ROIs
397 responding to Oct was higher compared to the δ -DL ones. n=10, p=0.0059,
398 paired t-test. Odours were diluted 1:100 unless stated otherwise, bars indicate
399 means.

400 **2.4 APL silencing leads to more variable odour** 401 **evoked activity at the MGs postsynapses**

402 Taken together, we showed that APL activation varies with different odours and
403 scales with PN boutons activation levels. Together with the higher similarity

among KC claws responses to different odours, this suggests a role of APL as normalizer of olfactory input-elicited response at the KC dendritic claws. If this is correct, blocking APL output would possibly lead to more variable activation of KC claws, mirroring PN bouton activation. We tested this hypothesis by expressing in APL tetanus toxin light chain (TNT), to block vesicle exocytosis and thus silence APL output (Sweeney et al. 1995). To suppress toxin expression during development, we co-expressed the temperature sensitive GAL4 inhibitor *tubP-GAL80^{ts}*. Flies were kept at 18 °C until eclosion, and then transferred at 31 °C for 24h to 48h prior to the experiments. KC responses in APL-silenced flies (APL OFF) were imaged with the postsynaptically-tagged *MB247-homer::GCaMP3* construct. Due to the stochastic nature of *APLi-GAL4* mediated expression (A. C. Lin et al. 2014), flies in which the flippase-dependent expression of TNT in APL did not happen were imaged as control (APL ON). Animals were stimulated with Mch or Oct. As expected, control animals neither show a difference in the average peak response to the two stimuli (Fig 4B, n=10, p=0.949, 2-way ANOVA with Tukey's multiple comparisons) nor in the number of responding units (Fig 4D, n=10, p=0.995, 2-way ANOVA with Tukey's multiple comparisons). Furthermore, the frequency distributions of the odour-evoked activity peaks overlapped with each other (Fig 4E, n=10, p=0.0533, Kolmogorov-Smirnov test; see also Fig 3G). In calyces where the APL output was blocked by TNT expression though, we measured a significant difference in the response to the two tested odours, with Oct causing a stronger average odour-evoked activity (Fig 4B, n=10, p=0.0003, 2-way ANOVA with Tukey's multiple comparisons) as well as a slight increase in the number of responding MGs (Fig 4D, n=10, p=0.047, 2-

way ANOVA with Tukey's multiple comparisons). Interestingly, small clusters of responding MGs were often found spatially close to each other in the APL OFF scenarios (e.g. compare spatial distribution of Oct responders in 4C vs 4A) suggesting a possible locality of APL-mediated inhibition. Finally, the frequency distribution plot for the APL OFF flies showed two shifted populations, with Oct responses skewed towards higher values (Fig 4F, $n=10$, $p<0.0001$, Kolmogorov-Smirnov test), resembling the distribution displayed by the PN boutons (Fig 3C). In summary, blocking APL output led to a more variable odour representation at the level of KC claws. This variable odour representation bore a higher similarity to the activity of the input population, hence supporting the hypothesis that APL acts as an input strength normalizer on MGs in the MB calyx.

Fig 4. APL silencing leads to more variable odour representations at the MB calyx.

Examples of KC claws fluorescence levels in response to Mch and Oct in control animals (**A**, APL ON) or in flies where the output from APL was blocked (**C**, APL OFF). The genotype used is *APLi-GAL4>UAS-TeTx, UAS-mCherry; MB247-homer::GCaMP3*. Scale bar = 10 μ m. (**B**) The average activity peak in KC claws was similar when APL was active (APL ON, $p=0.949$), but was highly variable in the absence of APL output (APL OFF, $p=0.0003$). $n=10$, 2-way ANOVA with Tukey's multiple comparisons. (**D**) The number of odour-responsive ROIs was

comparable in the presence of active APL (APL ON, $p=0.995$) and it was slightly increased in the absence of APL output (APL OFF, $p=0.047$). $n=10$, $p=0.047$, 2-way ANOVA with Tukey's multiple comparisons. **(E)** Frequency distribution of MGs activity peaks in the presence of APL activity. The two populations are highly overlapping, as in Fig 3G. $n=10$, $p=0.0533$, Kolmogorov-Smirnov test. **(F)** In the absence of APL inhibition, the distribution of MGs responding to Oct shifted towards higher values, resembling presynaptic PN boutons data shown in Fig 3C. $n=10$, $p<0.0001$, Kolmogorov-Smirnov test. Odours were diluted 1:100, bars indicate means.

2.5 APL inhibition onto MGs of the MB calyx is local

Our data indicate that APL contribution to the MG microcircuit yields a normalized postsynaptic response, independently of the variability of olfactory input strength. To understand how this input-tuned inhibition is achieved, we next asked whether the inhibition provided by APL is global and equally delivered to the entire calyx, or whether it might be more locally restricted to the sites of PN activation. Towards this aim, we selected 3 sets of PNs: DM3, VA1d and DC3, that appear to have distinctive bouton distributions in the calyx based on the Hemibrain dataset (F. Li et al. 2020b). We expect the bouton distribution of these PNs to be reproducible among animals (H.-H. Lin et al. 2007; Jefferis et al. 2007). Based on an available database of odorant representations (Münch and Galizia 2016), these PNs are activated by the following odours: Pentyl acetate (PA) activates the glomerulus DM3, whose PNs terminate in the posterior part of the calyx mainly; Methyl Palmitate (MP) activates the glomerulus VA1d, whose PN terminals populate the anterior part of the calyx; Farnesol (FA) activates glomerulus DC3, with PN terminals that populate the anterior part of the calyx, similarly to VA1d (Fig 5A-B). Flies were

477 exposed to random sequences of the 3 odours, and 3D image stacks were
 478 acquired over time to define the localization of the APL response within the
 479 entire calycal volume (Fig 5B). Interestingly, we found the ratio of the calcium
 480 transients to be highly inconstant throughout the volume of the calyx for the
 481 structurally more distant odours PA and MP (see also Material and Methods for
 482 details). In particular, the PA/MP fluorescence ratio was higher in the posterior
 483 sections of the calyx and reduced in the anterior ones (Fig 5C, black line),
 484 reflecting the bouton distribution of the PNs activated by these two odours. By
 485 contrast, the fluorescence ratio of FA to MP was more homogeneous across the
 486 calyx volume, reflecting the fact that the boutons of the PNs that respond to
 487 these odours are located in a similar region of the calyx (Fig 5C, blue line). In
 488 other words, not only APL calcium transients were different with different
 489 odours, as already shown (Fig 2), but these transients were also differently
 490 distributed in the calycal volume depending on the odour, strengthening the
 491 link between structural and functional data. Combined with the information
 492 about synapse distribution, these data suggest that APL is locally activated in
 493 the calyx at the MGs corresponding to activated PNs, and that the tone of APL
 494 inhibition in the calyx is a gradient that slowly degrades with increasing
 495 distance from the active boutons. Taken together, APL inhibition onto MGs of
 496 the MB calyx showed signs of locality. Of notice, a similar spreading
 497 mechanism has been observed in APL's parallel neurites at the MB lobes,
 498 where calcium transients failed to propagate over long distances (Amin et al.
 499 2020).

500

501 Fig 5. APL inhibition is local within the MB calyx.

502 **(A)** 3D skeletons of the PNs activated by the odours used in this experiment:
503 PA (orange), MP (magenta), FA (blue). Asterisk indicate PN cell bodies. **(B)**
504 Side view of the calyx showing the distribution of the PN terminals for the
505 odours used in this experiment. Note the higher spatial segregation between
506 PA and MP PN terminals compared to FA and MP ones. The ticked lines Z1-Z5
507 show an example of sectioning applied when acquiring image stacks over time.
508 **(C)** The calcium transient ratio of PA to MP (black line) was highly variable
509 throughout different section of the calyx, and was significantly different to the
510 FA to MP one (blue line). $n=7$, $p=0.007$ (**), Kolmogorov-Smirnov test.
511 Coloured areas represent SEM.

512 3 Discussion

513 While the importance of inhibition in reducing the overlap among stimuli
514 representation has been postulated many decades ago (Marr 1969; Albus
515 1971; Litwin-Kumar et al. 2017; Cayco-Gajic and Silver 2019) and supported
516 by more recent experimental evidence (Parnas et al. 2013; Olsen, Bhandawat,
517 and Wilson 2010), the complete mechanism by which inhibition supports
518 stimuli discrimination is not fully understood yet. Here, we show that the

519 inhibitory APL neuron, by participating in the structure of MGs of the
520 *Drosophila* MB calyx, provides inhibition scaled to the PNs excitatory inputs to
521 the calyx. As a result, the average strength of postsynaptic responses in KC
522 dendritic claws is homogenous among MGs and different odour
523 representations. We suggest that this normalization of postsynaptic responses
524 operated by APL is at the core of pattern separation in the MB.

525 Pattern separation is obtained in the MB through the formation of a sparse
526 response in the KC layer (Honegger, Campbell, and Turner 2011; Turner,
527 Bazhenov, and Laurent 2008; Campbell et al. 2013a; Perez-Orive et al. 2002).
528 The decoding of a sparse code, in general, increases the storage capacity of
529 associative networks, thereby supporting learning and classification tasks
530 (Olshausen and Field 2004; Kanerva 1988; Tsodyks and Feigel'man 1988;
531 Perez Vicente and Amit 1989; Huerta et al. 2004; García-Sánchez and Huerta
532 2003; Jortner, Farivar, and Laurent 2007). In fact, sparse neuronal
533 representations are described in several organisms including mammals,
534 songbirds, and insects (Rolls and Tovee 1995; Vinje and Gallant 2000;
535 Hahnloser, Kozhevnikov, and Fee 2002; Laurent 2002; Quiroga et al. 2005;
536 GoodSmith et al. 2017; Danielson et al. 2017). APL was reported to play a key
537 role in maintaining KCs responses sparse (A. C. Lin et al. 2014; Lei et al.
538 2013), but the underlying mechanism was far from understood. KCs receive
539 inputs from 6-8 PNs on average (Butcher et al. 2012; Zheng et al. 2020; F. Li
540 et al. 2020b) and, due to KCs high firing threshold (Turner, Bazhenov, and
541 Laurent 2008), require more than half of those inputs to be coactive to spike
542 (Gruntman and Turner 2013). Our data suggest that the APL neuron, by
543 confining KC claws responses within a certain range of activation, ensures that

KCs requirement of multiple coactive claws is respected even in the presence of highly variable input strengths. In other words, APL inhibition makes KC input integration dependent on the combinatorial pattern of inputs rather than on the strength of individual inputs. Of notice, odour discrimination is achieved at multiple levels of the *Drosophila* olfactory pathway by different means of inhibitory mechanisms: from the input gain control normalization executed by GABAergic interneurons in the AL (Olsen and Wilson 2008), to the high-pass filter function performed by inhibitory iPNs at the lateral horn (Parnas et al. 2013).

Interestingly, we found a slight increase in the number of MGs responding to an odour in the absence of APL inhibition (Fig 4D). This could have two possible explanations: i) the postsynaptic response of these additional MGs was so weak in the presence of APL that their activation was below the limit of detection; or ii) the increase in number is the consequence of an expanded number of KC axons spiking action potentials that back-propagate into their dendrites. We tend to exclude the second hypothesis as each MG is constituted, on average, by 13 claw-like dendritic endings from different KCs (Davi D. Bock, personal communication), and each KC connects with its other claws to random PNs (Caron et al. 2013). It seems unlikely that all or a large fraction of randomly assembled claws are all invested by back-propagating action potentials and therefore visible in our recordings.

Our structural and functional data point towards the involvement of APL in a feedforward loop from PN boutons to KC claws, as well as a closed feedback loop with PN boutons. An advantage of using recurrent circuits to provide

inhibition is that such a system can deal with a wide range of input strength, as inhibition and excitation strengths are proportional. Indeed, EM analysis revealed both pre- and postsynaptic connection between APL and PN boutons, linearly proportional to each other (Fig 1D), and the differences in the APL calcium influx in response to odours correlated to the variability measured in PNs (Fig 2 and 3). So far, APL has been mainly described as a feedback neuron for KCs (A. C. Lin et al. 2014; Lei et al. 2013; Amin et al. 2020). However, feedforward inhibitory neurons from the input population onto the next layer have been described in other neuronal networks performing pattern separation (Cayco-Gajic and Silver 2019). For example, granule cells receive both feedforward and feedback inhibition from Golgi cells at the cerebellar cortex (Vos, Volny-Luraghi, and de Schutter 1999; Duguid et al. 2015), which are driven by excitatory inputs from the mossy fibers (Kanichay and Silver 2008) and granule cells' axons, respectively (Cesana et al. 2013). Moreover, it has recently been demonstrated that Golgi cells recruitment scales with the mossy fibers input density (Tabuchi et al. 2019), similarly to what we observed in our functional imaging experiments. Additionally, adaptive regulation of KCs sparseness by feedforward inhibition has already been theorized in realistic computational models of insect's mushroom bodies (Assisi et al. 2007; Luo, Axel, and Abbott 2010). Regarding KCs-to-APL connections, we found a positive linearity among pre- and post-synapses between these two cells (Fig 1E), confirming the presence of a local feedback loop within KC dendrites and the APL at the calyx (Amin et al. 2020). Furthermore, we reported that α/β KCs receive more inhibitory synapses along their dendritic trees compared to γ and α'/β' , where the majority of synapses received from the APL is localised on KC

593 claws instead (Fig S1C). As the ability of inhibitory synapses to shunt current
 594 from excitatory synapses depends on the spatial arrangement of the two inputs
 595 (Spruston, Stuart, and Häusser 2016), we speculate that the difference in APL
 596 synapses localisation could contribute to some of the electrophysiological
 597 differences recorded among distinct KCs type. For example, α/β_c KCs were
 598 found to have a higher input resistances and longer membrane time constants
 599 compared to α'/β' KCs (Groschner et al. 2018), resulting in a sigmoidal
 600 current-spike frequency function rather than a linear one (Groschner et al.
 601 2018). Additionally, a difference in synapses distribution can also indicate that
 602 two inhibitory mechanisms coexist at the MB calyx, similarly to what has been
 603 shown in the cerebellum where Golgi cells are responsible for both tonic
 604 inhibition, controlling granule cells spike number (Brickley, Cull-Candy, and
 605 Farrant 1996), gain control (Mitchell and Silver 2003), and phasic inhibition,
 606 limiting the duration of granule cells responses (D'Angelo and de Zeeuw 2009).
 607 Finally, volumetric calcium imaging showed that the APL inhibition is local
 608 within the MB calyx. In particular, we found a difference in the APL calcium
 609 transients when flies were stimulated with odours that activate PN subsets with
 610 segregated bouton distribution in the calyx (Fig 5). These data suggest that
 611 APL inhibition onto MGs can be imagined as a gradient that peaks at the MGs
 612 active during a given stimulus and attenuates with distance. Non-spiking
 613 interneurons in insects are typically large and characterized by complex neurite
 614 branching, an ideal structure to support local microcircuits (Roberts, Bush, and
 615 Society for Experimental Biology (Great Britain). Neurobiology Group. 1981).
 616 As a matter of fact, similar examples of localized APL response as described
 617 here have been reported in the *Drosophila* MB (Amin et al. 2020; Wang et al.

2019) as well as in the APL's homolog GGN in the locust (Ray, Aldworth, and Stopfer 2020; Leitch and Laurent 1996). An advantage of having local microcircuits is that it allows a single neuron to mimic the activity of several inhibitory interneurons, as described in amacrine cells of both mammals (Grimes et al. 2010) and *Drosophila* (Meier and Borst 2019). Additionally, a parallel local-global inhibition is suggested to expand the dynamic range of inputs able to activate KCs (Ray, Aldworth, and Stopfer 2020).

An important open question is whether the APL inhibition onto MGs of the MB calyx is more of a presynaptic phenomenon, therefore acting on PN boutons output, or a postsynaptic one on KCs claws. Our functional data reveal a clear impact of APL on the postsynaptic response in MGs (Fig 3H, S3F), while the PN boutons display a broad range of activity levels (Fig 3A-D, Fig S2, Fig S3A-D). Accordingly, silencing of the GABA_A receptor *Rdl* on KCs increased calcium responses in the MB, including the calyx (Liu, Krause, and Davis 2007), and reduced sparseness of odour representations (Lei et al. 2013). However, due to the presence of presynapses from APL to PN boutons (Fig 1B, 1D), a presynaptic component of APL inhibition is certainly possible.

One possible caveat to our hypothesis is given by the fact that reducing GABA synthesis in APL by RNAi has been found to improve olfactory learning (Liu and Davis 2009). However, this could be explained by a low efficiency of RNAi in this case. Indeed, incomplete silencing might increase KCs' output without affecting sparseness. As a matter of fact, blockage of APL output via *shibire^{ts}* led to impaired olfactory discrimination (A. C. Lin et al. 2014).

641 Taken together, our study provides novel insights on how feed-forward
642 inhibition via APL shapes the postsynaptic response to olfactory inputs in the
643 MB calyx and contributes to maintaining odour evoked KC activity sparse. In
644 the future, it will be interesting to investigate the impact of APL on memory
645 consolidation, which has been associated with structural plasticity in the calyx
646 (Baltruschat et al. 2021) and with changes in the KC response (Delestro et al.
647 2020).

648

649 **4 Materials and Methods**

650 **4.1 Connectomics**

651 Connectomics data were obtained from the Hemibrain EM dataset (Scheffer et
652 al. 2020) via the Neuprint analysis Tool (Clements et al. 2020). In particular,
653 the Neuprint-python package ([https://github.com/connectome-](https://github.com/connectome-neuprint/neuprint-python)
654 [neuprint/neuprint-python](https://github.com/connectome-neuprint/neuprint-python)) was used to filter for annotated synapses made and
655 received by the APL only within the CA(R) ROI. The command
656 *fetch_adjacencies* was used to extract data regarding the connectivity among
657 cell types. To visualize neuron skeletons or filled renders in 3D, the commands
658 *fetch_skeletons* or *fetch_mesh_neuron* were used instead. To visualize APL
659 synapses onto PNs or KCs, the coordinates of those synapses were obtained
660 via *fetch_synapse_connections* and plotted together with the 3D neuronal
661 meshes. The localization of the synapses (e.g., on PN bouton or not) was
662 addressed manually by two separate users in a blind manner, and the average
663 counts were calculated. Detailed tables containing the list of all PNs and KCs

interconnected with the APL within the MB calyx, as well as the weight of those synapses, and 3D images of APL synapses mapped onto PNs and KCs meshes can be found at: Mendeley data link will be available upon publication.

4.2 Fly strains

The following lines were used for experiments: *GH146-Gal4* (Reinhard F. Stocker et al. 1997), *NP225-Gal4* (Hayashi et al. 2002), *NP2631-Gal4* (Hayashi et al. 2002), *GH146-Flp* (Hong et al. 2009), *tubP-GAL80^{ts}* (Sean E. McGuire et al. 2003), *tubP-FRT-GAL80-FRT* (S. Gao et al. 2008; Gordon and Scott 2009), *UAS-GCaMP6m* (Chen et al. 2013a), *MB247-homer::GCaMP3* (Pech et al. 2015), *UAS-Syp::GCaMP3* (Pech et al. 2015), *UAS-TeTx* (Sweeney et al. 1995), *UAS-mCherry::CAAX* (Kakihara et al. 2008). Flies were raised in a 12h/12h light-dark cycle on a standard cornmeal-based diet at 25 °C, 60% relative humidity unless they expressed the temperature-sensitive gene product Gal80^{ts}. Flies carrying *tubP-GAL80^{ts}* were raised at 18°C and placed at 31°C for 24 h-48 h <24 h after eclosion. 1-7 days old flies were used for experiments. All experiments were performed on mixed populations of males and females.

4.3 Two-photon *in vivo* calcium imaging

For *in vivo* imaging in the MB calyx, adult flies were briefly anaesthetized on ice, positioned in a polycarbonate imaging chamber (Louis et al. 2017), and immobilized using Myristic Acid (Sigma-Aldrich). To allow optical access to the Calyx, a small window was opened through the head capsule under Ringer's solution (5 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM

687 MgCl₂; pH adjusted to 7.2). To minimize movement, fly heads were stabilized
688 with 1,5% low melting agarose (Thermo Scientific) in Ringer's solution,
689 immediately before dissection. Flies were imaged with a two-photon laser-
690 scanning microscope (LaVision BioTec, TriM Scope II) equipped with an ultra-
691 fast z-motor (PIFOC® Objective Scanner Systems 100µm range) and a Nikon
692 25X CFI APO LWD Objective, 1.1 NA water-immersion objective. GCaMP
693 molecules were excited at 920 nm using a Ti:sapphire laser (Coherent
694 Chameleon). Odours were delivered to the *in vivo* preparation via a 220A
695 Olfactometer (Aurora Scientific) in a randomized fashion. Odours were loaded
696 into the respective odour vials with a dilution 10X higher than the desired one,
697 and further diluted 1:10 with clean air during odour stimulation. A constant
698 flow of clean air was provided by a Stimulus Controller CS 55 (Ockenfels
699 SYNTECH GbmH), equipped with two activated carbon inlet filters to avoid air
700 contamination. Animals were stimulated with 2 odour puffs of 5 s each,
701 separated by 20 s clean air intervals. Both clean air and odour flows were kept
702 around 0.5L/min for the entire experimental procedure. For imaging, a region
703 large enough to include an entire z-section of the mushroom body calyx was
704 chosen. The scanning frequency was set around 9 Hz. Single plane videos were
705 acquired unless stated otherwise.

706 For volumetric calcium imaging (Fig 5), flies were mounted and stimulated as
707 described above and 3D stacks of 5 images each were acquired over time for
708 the entire stimulation time. To compensate for the reduced speed caused by
709 the stack acquisition, the frame rate was adjusted to around 16 Hz.

710 For *in vivo* AL imaging, female adult flies were briefly anaesthetized on ice,
 711 positioned in a custom-built fly chamber (Hancock, Bilz, and Fiala 2019), and
 712 immobilized using UV-hardening dental glue (Kentoflow, Kent Dental). A small
 713 dissection was performed in Ringer's solution (5 mM HEPES, pH 7.4, 130 mM
 714 NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂; pH adjusted to 7.2). Flies were
 715 imaged with a two-photon laser-scanning microscope LSM 7MP (Zeiss) equipped
 716 with a 20X/1.0 DIC M27 75mm Plan-Apochromat objective (Zeiss). GCaMP
 717 molecules were excited at 920 nm using a Ti:sapphire laser (Coherent
 718 Chameleon). Odours were delivered to the *in vivo* preparation via a custom-built
 719 olfactometer. Odours were diluted in mineral oil (Sigma-Aldrich) at the required
 720 dilution. A constant flow of clean air was provided by a membrane pump
 721 "optimal" (SCHEGO). Animals were stimulated with 2 odour puffs of 5 s each,
 722 separated by 20 s clean air intervals. Both clean air and odour flows were kept
 723 around 1mL/s for the entire experimental procedure. For imaging, a region large
 724 enough to include an entire z-section of the mushroom body calyx was chosen.
 725 The scanning frequency was set around 5 Hz. Single plane videos were acquired
 726 unless stated otherwise.

727 As the PN Gal4 driver *GH146* used in AL imaging experiments drives expression
 728 also in APL (Liu and Davis 2009), the *NP225-Gal4* driver line (Hayashi et al.
 729 2002) was chosen for PN boutons imaging at the calyx, as it targets a similar
 730 amount of PNs as *GH146* without including the APL.

731

732 4.4 Data Analysis

733 Two-photon images were analysed using Fiji (Schindelin et al. 2012). Briefly,
734 raw videos were motion corrected via the “Template Matching and Slice
735 Alignment” ImageJ plugin (Tseng et al. 2012). Afterwards, ROIs of the single
736 MGs responding to a given odour were created via the “Cell Tracking by
737 Calcium” ImageJ Macro (designed and written by DZNE IDAF). The generated
738 mask was then applied to the previously registered video in order to extract
739 the average intensity value over time per each of the detected ROIs. Finally,
740 the $\Delta F/F_0\%$ and the $\Delta F/F_0\%_{MAX}$ of each ROI was calculated by using the
741 average intensity of the first 30 frames as F_0 . A detailed manual related to the
742 ImageJ Macro, as well as Python notebooks computing the $\Delta F/F_0\%$ and
743 $\Delta F/F_0\%_{MAX}$ given a dataframe of intensity values over time, is available at:
744 Mendeley data link will be available upon publication.

745 To measure calcium influx among the APL neurites branching in the mushroom
746 body calyx, a manual ROI was drawn around the entire calycal region
747 expressing the GCaMP and the average intensity value over time was
748 extracted. $F/F_0\%$ and $\Delta F/F_0\%_{MAX}$ were calculated as described above.

749 For the odours response ratio calculated in the APL volumetric calcium imaging
750 experiment (Fig 5), the $\Delta F/F_0\%_{MAX}$ per each odour was calculated as
751 described above. The $\Delta F/F_0\%_{MAX}$ values per each of the 5 frames contained in
752 an image-stack were obtained and averaged among all animals tested. Next, a
753 $\Delta F/F_0\%_{MAX}$ ratio between the odour pairs being compared was calculated and
754 analysed per each of the sections included in an image stack.

755 **4.5 Confocal imaging**

756 To address the presence or not of the *APLi.GAL4* driven *UAS-TNT* and *UAS-*
757 *mCherry* products, whole flies were fixed on Formaldehyde (FA) 4% in PBS
758 with 0.1% Triton X-100 (PBT 0.1%) immediately after *in vivo* imaging. Once all
759 animals sustained the *in vivo* imaging protocol, brains were dissected using a
760 pair of forceps in a small petri dish covered with a layer of silicon, fixed for
761 further 20 min on FA 4% in PBT 0.1%, washed 3 times for 5 min ca in PBT
762 0.1% and mounted with Vectashield® Plus Antifade Mounting Medium
763 (Vectorlabs) on 76 x 26 mm Microscope slides (Thermo scientific) with 1#
764 coverslips (Carl Roth). Brains were oriented with the dorsal part facing
765 upwards. Imaging was performed on a LSM 780 confocal microscope (Zeiss)
766 equipped with a Plan-Apochromat 63x/1.4 Oil DIC M27 objective (Zeiss).
767 512x512 pixels images were acquired, covering a region of the brain big
768 enough to include the APL soma and branches around the MB calyx and MB
769 lobes. Brains and their related *in vivo* imaging data were assigned to the
770 classes "APL OFF" or "APL ON" based on the presence or not of the TNT-co-
771 expressed mCherry fluorescence, respectively.

772 **4.6 Statistics**

773 Statistics were carried out in Prism 8 (GraphPad). Parametric (t-test, ANOVA)
774 or non-parametric tests (Wilcoxon, Mann-Whitney, Kruskal-Wallis, Kolmogorov-
775 Smirnov) were used depending on whether data passed the D'Agostino-
776 Pearson normality test. Statistical power analysis was conducted in G*Power
777 (Faul et al. 2007).

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787 **6 Financial interests or conflicts of interest**

788 The authors declare no competing interests.

789

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