

1 **Higher-order olfactory neurons in the lateral horn supports odor valence and**
2 **odor identity coding in *Drosophila***

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17 **Abstract**

18 Understanding neuronal representations of odor-evoked activities and their progressive
19 transformation from the sensory level to higher brain centers features one of the major aims in
20 olfactory neuroscience. Here, we investigated how odor information is transformed and
21 represented in higher-order neurons of the lateral horn, one of the higher olfactory centers
22 implicated in determining innate behavior, using *Drosophila melanogaster*. We focused on a
23 subset of third-order glutamatergic lateral horn neurons (LHNs) and characterized their odor
24 coding properties in relation to their presynaptic partner neurons, the projection neurons (PNs) by
25 two-photon functional imaging. We found that odors evoke reproducible, stereotypic and odor-
26 specific response patterns in LHNs. Notably, odor-evoked responses in these neurons are valence-
27 specific in a way that their response amplitude is positively correlated with innate odor preferences.
28 We postulate that this valence-specific activity is the result of integrating inputs from multiple
29 olfactory channels through second-order neurons. GRASP and micro-lesioning experiments
30 provide evidence that glutamatergic LHNs obtain their major excitatory input from uniglomerular
31 PNs, while they receive an odor-specific inhibition through inhibitory multiglomerular PNs. In
32 summary, our study indicates that odor representations in glutamatergic LHNs encode hedonic
33 valence and odor identity and primarily retain the odor coding properties of second-order neurons.

34

35 **Introduction**

36 Insects are the most successful taxon among the whole animal kingdom in terms of their
37 distribution and ability to survive in a multitude of environmental conditions. Largely they rely on
38 their olfactory sense to carry out their fundamental goal directed behaviors, such as food
39 navigation, mating, ovipositing or escape from predators. The powerful ability to detect odor cues,
40 to evaluate the information efficiently with a relatively small number of neurons and to transform
41 the neuronal signal into an appropriate behavioral output, makes the insect olfactory system a
42 premier model system for olfactory research. Numerous studies have investigated the neuronal
43 representation of odors at successive neuronal layers from the periphery to higher brain levels
44 using *Drosophila melanogaster* as a model organism (Bhandawat et al., 2007; Ng et al., 2002;
45 Root et al., 2007; Schmuker et al., 2007; Schmuker & Schneider, 2007; Seki et al., 2017; Wilson

46 et al., 2004). Although much progress has been made in understanding odor coding at the antennal
47 lobe (AL) level (Bhandawat et al., 2007; Galizia, 2014; Ng et al., 2002; Wilson et al., 2004), the
48 coding strategies and processing mechanisms of higher brain centers still remain largely elusive.
49 In this regard, the lateral horn (LH) has recently gained attention as a crucial signal processing
50 center integrating both innate as well as learned behavioral information (Das Chakraborty &
51 Sachse, 2021). Several studies during recent years have advanced our understanding of the
52 anatomical and functional properties of higher-order lateral horn neurons (LHNs) regarding odor
53 processing (Das Chakraborty & Sachse, 2021; Dolan et al., 2019; Frechter et al., 2019; Jeanne et
54 al., 2018; Varela et al., 2019). The generation of several LH cell type specific lines,
55 characterization of polarity and neurotransmitter identity of LHNs as well as the establishment of
56 detailed EM connectomic data sets have led to a significant progress in the field to study the
57 function of specific LHN classes (Dolan et al., 2019; Frechter et al., 2019). The LH is comprised
58 of three categories of neurons, which include LH input neurons (LHINs, which are mainly
59 olfactory projection neurons (PNs) along with mechanosensory, thermosensory and gustatory
60 neurons), LH local neurons (LHLNs) and LH output neurons (LHONs) (Bates et al., 2020; Dolan
61 et al., 2019; Frechter et al., 2019). In terms of PN-LHN connectivity, the olfactory PNs deriving
62 from individual glomeruli of the AL form stereotyped and conserved connections with certain
63 LHNs (Fişek & Wilson, 2014; Jeanne et al., 2018; Jefferis et al., 2007; Marin et al., 2002; Wong
64 et al., 2002). Although all kind of connections are possible, PNs having similar odor tuning patterns
65 are prone to target similar LHN types (Jeanne et al., 2018). Certain pairs of narrowly tuned
66 glomeruli encoding ecologically relevant odors and eliciting specific kinds of behavior, such as
67 courtship, aggregation or food seeking, converge onto the same LHN types and have been shown
68 to be over-represented in the LH in terms of synaptic densities (Bates et al., 2020; Jeanne et al.,
69 2018). Furthermore, a high amount of divergence has also been described to occur at the level of
70 PN to LHN connectivity (Huoviala et al., 2018). Altogether these complex connectivity patterns
71 in addition to direct pooling of feed-forward inputs from PNs innervating different glomeruli
72 results in broader tuning patterns of LHNs compared to their presynaptic PNs (Bates et al., 2020;
73 Frechter et al., 2019). In addition to the observed broadly tuned LHNs, narrowly tuned LHNs also
74 exist, which receive input from a single type of PN and which are assumed to be further modulated
75 by odorant selective inhibition through inhibitory neurons (Fişek & Wilson, 2014). Although
76 several studies agree that odors are compartmentalized in the LH based on either their chemical

77 identity (Frechter et al., 2019), behavioral significance (Bates et al., 2020; Jeanne et al., 2018;
78 Jefferis et al., 2007) or hedonic valence (Strutz et al., 2014; Tanaka et al., 2004), it still remains
79 controversial how the odor information is transformed from the PN to the LHN level and which
80 odor features are coded by subtypes of LHNs.

81 In the present study, we aimed to elucidate the odor coding and processing strategies of
82 LHNs by investigating how a neuronal subset of particular neurotransmitter identity encodes
83 different odor features in the LH and how this representation is correlated to their presynaptic
84 partner neurons in the AL, the uni- and multiglomerular PNs (uPN, mPN). Using photoactivatable
85 GFP, we first identified diverse clusters of LHNs based on their different neurotransmitter
86 identities and further focused our detailed analysis exclusively on glutamatergic LHNs. Using *in*
87 *vivo* two-photon functional imaging, we characterized several aspects of odor-evoked activity in
88 these neurons, such as odor-specific response patterns, reproducibility of repeated stimulations as
89 well as stereotypy across different individuals. We could successfully demonstrate that attractive
90 and aversive odors are clearly segregated and that their response amplitudes are positively
91 correlated with the innate behavioral preference to an odor. We also dissected how excitatory input
92 from uPNs and odor-specific inhibition from mPNs contribute to the fine-tuning of odor-specific
93 response patterns of LHNs. Altogether this study demonstrates a significant role of glutamatergic
94 LHNs regarding olfactory processing and extends our knowledge about the transformation
95 processes of neuronal information taking place from the periphery to higher brain levels, such as
96 the LH.

97

98 **Results**

99 **Identification of higher-order neurons in the LH**

100 To unravel distinct neuronal circuits in the LH, we expressed photoactivatable GFP (*UAS-C3PA*
101 *GFP*) (Ruta et al., 2010) under different Gal4 driver lines with various neurotransmitter and
102 neuromodulator identities (i.e. *Cha-Gal4*, *GAD1-Gal4*, *dVGlut-Gal4*, *TH-Gal4* and *tdc2-Gal4*)
103 (**Figure 1A, B**). To precisely define the upper and lower limit of the LH, we labeled uPNs using
104 *GHI46-QF*, *QUAS-td tomato* in the background as a landmark in every experiment. Using two-
105 photon excitation we photoactivated *C3PA-GFP* throughout the LH neuropil with laser pulses of

106 760 nm in the *in vivo* fly brain. Photoactivation of the LH using driver lines for cholinergic (*Cha-*
107 *Gal4*) and GABAergic neurons (*GAD1-Gal4*) revealed clusters of LHNs with labeled somata
108 along with cholinergic and GABAergic PNs, respectively. The labeled cholinergic and
109 GABAergic LHN clusters were located ventrolaterally to the LH. Selective labeling of
110 glutamatergic neurons in the LH by photoactivation using the *dVGlut-Gal4* driver line revealed
111 several clusters of LHNs cell bodies positioned dorsomedial, ventrolateral and ventral to the LH
112 with major neurite tracts entering the LH at distinctive locations. Glutamatergic LHNs are
113 comprised of a large number of labeled somata (~ 40) and a few ventrolateral and ventral clusters
114 possessing long neuronal tracts (**Figure 1B**). The labelled glutamatergic LHNs may include both
115 LHLNs and LHONs (Frechter et al., 2019). Photoactivation of the LH using drivers for
116 dopaminergic (*TH-Gal4*) and octopaminergic neurons (*tdc2-Gal4*) revealed the presence of both
117 kinds of LHNs (i.e. LHLNs, LHONs) in the LH (**Figure 1A**). These neurons innervate the LH
118 sparsely with a very long neuronal tract and their somata positioned far away from the LH. Since
119 we observed a high abundance of glutamatergic neurons in the LH, which were non-overlapping
120 with second-order neurons (i.e. PNs) deriving from the AL, we confined our further experiments
121 on this subset of LHNs to elucidate their functional properties regarding odor coding.

122

123 **Glutamatergic LHNs reveal reproducible, stereotypic and odor-specific responses that** 124 **emerge from the PN level**

125 To elucidate how odors are represented in the LH, we monitored odor-evoked functional responses
126 of glutamatergic LHNs. To do so, we expressed the genetically encoded calcium sensor *UAS-*
127 *GCaMP6f* (Chen et al., 2013) that has a high sensitivity and low basal fluorescence under the
128 control of *dVGlut-Gal4*. In order to accurately and consistently identify a comparable focal plane
129 for functional imaging across different animals, we expressed *QUAS-td tomato* in uPNs using
130 *GHI46-QF* in the background as a landmark. We performed *in vivo* two-photon functional
131 imaging from the glutamatergic LHNs at three different focal planes to comprehensively monitor
132 odor responses throughout the LH (i.e. upper, middle and lower plane). We used a panel of 14
133 ecologically relevant odors at a concentration of 10^{-3} , which have been shown to be behaviorally
134 either attractive or aversive (Knaden et al., 2012). Our imaging data show that each odor evoked a
135 specific activity pattern across all three focal planes in the LH (**Figure 2A**). Since the LH appears

136 rather homogenous and lacks clear morphological landmarks, we used a grid approach for imaging
137 data analysis (**Figure 2B**) (see Methods for details) and determined the $\Delta F/F$ to analyze the
138 neuronal activity across odors for each animal. At first, we compared the reproducibility of the
139 evoked responses of these glutamatergic LHNs to two sequential presentations of each odor. This
140 analysis of neuronal activity for repeated odor stimulations clearly showed that the odor-evoked
141 responses were highly reproducible both qualitatively and quantitatively across trials (**Figure 2C**),
142 which is further depicted by a high correlation coefficient across repeated stimulations for all odors
143 (**Figure 2D**). Hence, although the odor responses appear generally broad, each odor induced an
144 unique response pattern in these glutamatergic LHNs (**Figure 2D**). Furthermore, a cluster analysis
145 based on the linkage distance between the spatial patterns of each odor response reveals that the
146 majority of the replicates for each odor are clustered together, confirming that individual odors are
147 represented in an odor-specific manner by glutamatergic LHNs (**Figure 2F**). To investigate
148 whether the observed odor response properties of glutamatergic LHNs derived from their
149 presynaptic partner neurons, we determined the odor representations of uPNs in the LH to the same
150 odor set. Previous studies based on the innervation pattern of PNs reported a compartmentalization
151 in the LH either based on the hedonic valence of an odor, i.e. attractive versus aversive odors (Seki
152 et al., 2017), the odor category, i.e. pheromones versus food odors (Jefferis et al., 2007) or with
153 regard to the behavioral ‘odor scene’ (Bates et al., 2020; Jeanne et al., 2018). However, whether
154 an odor-specific response map exists at the uPN level in the LH has so far not been unambiguously
155 shown with a functional approach. We therefore expressed *UAS-GCaMP6f* under control of
156 *GHI46-Gal4* and monitored odor-evoked responses at the two-photon microscope from the same
157 three focal planes as used for the LHN imaging experiments. Notably, when we compared the odor
158 response properties of uPNs with those of LHNs we observed that the PNs possess a significantly
159 reduced correlation coefficient as compared to LHNs, implying that odors are better segregated at
160 the uPN level in the LH than at the third-order neuron level (**Figure 2E**). In addition, a cluster
161 analysis based on the linkage distance between the spatial patterns of each odor response at the PN
162 level confirms that uPNs also reveal odor-specific response patterns in the LH (**Figure 2G**).
163 Altogether, these observations clearly demonstrate and signify that uPNs as well as glutamatergic
164 LHNs exhibit an odor-specific response map in the LH.

165

166 **Response amplitude of glutamatergic LHNs reflects odor valence**

167 Next, we investigated whether hedonic valence is still preserved in the LH level in this particular
168 subset of LHNs as it has been described for both types of PNs, i.e. uPNs and mPNs (Knaden et al.,
169 2012; Strutz et al., 2014). We therefore quantified and pooled the correlation coefficients of all
170 odor responses of glutamatergic LHNs to all attractive and aversive odors and compared those that
171 shared the same valence (i.e. within valence) as well as for odors having an opposing valence (i.e.
172 across valence) for all three focal planes measured (**Figure 3A**). Interestingly, we observed that
173 the responses to odors with the same valence were significantly more similar, depicted by a higher
174 correlation coefficient, compared to the odor responses across valence. In order to visualize the
175 odor representations in a multidimensional space, we employed a principal component analysis
176 (PCA) of the odor response profiles by taking into account two parameters, the spatial response
177 patterns as well as the intensity of odor-evoked responses by each odor. As expected, the attractive
178 and aversive odor responses were segregated significantly throughout the LH (* $p < 0.05$, one way
179 ANOSIM) confirming that odors are indeed categorized according to their behavioral value by
180 these third-order glutamatergic LHNs (**Figure 3B**). Notably, when only the response amplitude
181 was considered, it was obvious that attractive odors in general evoked a stronger activity in
182 glutamatergic LHNs compared to responses to behaviorally aversive odors across all three focal
183 planes (**Figure 3C and D**). Next, we examined whether odor responses of second-order uPNs also
184 reflect the hedonic valence of an odor within the LH as it has been shown for the AL level
185 previously (Knaden et al., 2012). A PCA of the odor response profile of uPNs revealed that the
186 attractive and aversive odors are clearly segregated (** $p < 0.005$, one way ANOSIM) at the PN
187 level also in the LH (**Figure 3E**). In order to investigate if, similar to LHNs, the response
188 magnitude in uPNs contributes to the observed clear segregation, we averaged the overall response
189 activities to attractive and aversive odors across all three planes. Indeed, we observed a similar
190 trend, meaning that uPNs responded stronger to attractive odors than to aversive ones in the LH
191 (**Figure 3D and F**). This observation was novel and unexpected and tempted us to postulate that
192 the flies' innate odor preference might be determined by the response amplitude of uPNs and
193 glutamatergic LHNs. To address whether the odor-evoked response strength is correlated with
194 behavioral preference, we performed T-maze assays to the same odor set as used for the functional
195 imaging experiments. As shown in previous studies, flies displayed different levels of attraction
196 and aversion to individual odors (Knaden et al., 2012; MacWilliam et al., 2018; Min et al., 2013;

197 Strutz et al., 2014). Plotting the olfactory preference indices of all tested odors with the response
198 strengths of glutamatergic LHNs indeed revealed a significant correlation (**Figure 3G**) showing
199 that odors eliciting stronger LHN activity induce stronger attraction, while odors that elicit lower
200 LHN responses induce a stronger aversion. Interestingly, the response strength of uPNs did not
201 correlate significantly with the olfactory preference indices (**Figure 3H**), although uPNs activity
202 displayed a valence-specific representation (**Figure 3C**). This observation suggests that the
203 activity in the subset of glutamatergic LHNs may contribute significantly to the observed
204 behavioral odor preferences. However, this correlation does not confirm any causality between the
205 response strength of glutamatergic LHNs and the behavioral output. To test a causal relationship,
206 it would be necessary to silence or artificially activate the glutamatergic LHNs and to determine
207 the functional consequences with regard to behavioral valence. However, since the line *dVGlut-*
208 *Gal4* is broadly expressed throughout the whole brain, genetic manipulations would not be
209 confined to the subset of glutamatergic LHNs and would therefore be difficult to evaluate. One
210 might argue that the observed differential LHN responses emerge already at the sensory neuron
211 level and are not the result of neuronal processing within the AL or LH. To examine this idea, we
212 obtained odor-evoked response of olfactory sensory neurons (OSNs) to individual odors from the
213 DoOR database (Münch & Galizia, 2016) and plotted the response strengths of glutamatergic
214 LHNs either against the overall OSN activity (**Figure 3I**) or the number of ORs (olfactory
215 receptors) activated by a specific odor (**Figure 3J**). Interestingly, we could not observe any
216 correlation in either case, indicating that the differential LHN activity observed here is not deriving
217 from the OSN level, but is the result of second-order neuronal processing. Notably, the comparison
218 between uPNs and LHNs responses also did not show any correlation (**Figure 3K**), further
219 suggesting that the differential activity observed in the glutamatergic LHNs is not a mere relay of
220 the AL input through uPNs, but rather processed by either mPNs or other LHNs to generate the
221 observed graded odor responses in higher-order glutamatergic LHNs.

222

223 **Polarity and connectivity of glutamatergic LHNs with uPNs and mPNs**

224 To determine the neuronal polarity of the glutamatergic LHNs, we expressed pre- and postsynaptic
225 markers (*UAS-sypGC3*, *UAS-homerGC3*) under the control of *dVGlut-Gal4*.
226 Immunohistochemistry followed by confocal scanning demonstrated that these neurons possess

227 both, presynaptic dendrites and postsynaptic axon terminals in the LH (**Figure 4A**, left and right
228 panel), indicating that the cell cluster of glutamatergic LHNs probably comprises both LHLNs as
229 well as LHONs (Dolan et al., 2019). The presence of presynaptic terminals suggests that these
230 neurons provide input to other LHONs, LHLNs or even perform a feedback inhibition onto PNs.
231 To assess the neuronal connectivity of these neurons with excitatory and inhibitory PNs at the
232 morphological level in detail, we employed the GRASP technique (Feinberg et al., 2008). Initially,
233 we employed two split-GFP fragments, tagged to the extracellular domain of the CD4
234 transmembrane protein (hereafter referred to as *CD4-GRASP*). To determine the connectivity
235 between uPNs and glutamatergic LHNs, we expressed one fragment of GFP in glutamatergic
236 LHNs, using *dVGlut-Gal4* and the other fragment in uPNs, using *GH146-LexA* (**Figure 4B**, left
237 panel). Next, to examine whether mPNs and glutamatergic LHNs are synaptic partner neurons, we
238 employed a similar approach by using *dVGlut-LexA* and *MZ699-Gal4* (**Figure 4B**, right panel). In
239 both cases we observed a clear neuronal connectivity to the glutamatergic LHNs. However, since
240 *CD4-GRASP* is not synaptically targeted and can potentially lead to false positive signals at non-
241 synaptic locations, we employed in addition a GFP 1-10 fragment, which is tagged to the synaptic
242 protein neurexin, resulting in an enhancement of synaptic specificity (Fan et al., 2013; Shearin et
243 al., 2018). Neurexin is present predominantly at the presynaptic site (Fan et al., 2013; Xing et al.,
244 2018), but also occurs along with postsynaptic terminals (Taniguchi et al., 2007). When we
245 performed GRASP by expressing neurexin in glutamatergic LHNs, we observed a clear GFP signal
246 in the LH (**Figure 4C**, left panel) implying that glutamatergic LHNs provide presynaptic output
247 onto uPNs and therefore convey a feedback inhibition to the AL. Such a feedback connection needs
248 to be verified in future experiments. Next, in order to determine the synaptic connectivity between
249 glutamatergic LHNs and mPNs, we expressed neurexin in mPNs and observed distinct GFP puncta
250 in the LH implying that also mPNs form presynaptic and probably postsynaptic connections with
251 glutamatergic LHNs (**Figure 4C**, right panel).

252

253 **Glutamatergic LHNs receive major excitatory input from uPNs and an odor-specific** 254 **inhibition from mPNs**

255 In order to further elucidate the neural connectivity and to verify our GRASP experiments, we
256 determined the functional connectivity of glutamatergic LHNs with uPNs and mPNs by transection

257 experiments using two-photon laser-mediated micro-dissection. To manipulate the input from
258 uPNs, we micro-lesioned the iACT tract entering the LH and compared the odor-evoked responses
259 to vinegar and benzaldehyde from the glutamatergic LHNs before and after laser ablation (**Figure**
260 **5**, schematic upper panel). After transecting the iACT tract we observed that the odor-induced
261 responses to both, vinegar and benzaldehyde were completely abolished across all three focal
262 planes of the LH (**Figure 5 A-A'', B-B''**) This finding implies that glutamatergic LHNs receive
263 their major excitatory input from the AL through uPNs. To verify whether this complete
264 elimination of the odor-evoked activity is not just a technical side effect of the laser ablation
265 procedure, we also monitored odor responses from the glutamatergic LHNs in the other, untreated
266 brain hemisphere before and after micro-lesioning, which proved not to be affected (**Figure 5-**
267 **figure supplement 1**). In order to examine the functional connectivity between mPNs and
268 glutamatergic LHNs, we also transected the mACT tract entering the LH and monitored odor
269 responses from glutamatergic LHNs before and after laser transection. Notably, transecting the
270 mACT led to a significant increase in the odor-evoked responses in the middle and upper plane of
271 the glutamatergic LHNs (**Figure 5 C-C'', D-D''**). We further observed that the mACT transection
272 affected the odor-evoked responses differentially in a way that the response increase was higher
273 in the case of vinegar than for benzaldehyde. This observation indicates that the glutamatergic
274 LHNs receive an odor selective inhibition from mPNs which is stronger for the food odor vinegar
275 than for the repellent odor benzaldehyde.

276

277 **mPN- mediated inhibition onto glutamatergic LHNs contributes to odor specificity**

278 Next, we aimed to understand the impact of the observed odor-specific inhibition of mPNs with
279 regard to the odor-specific responses of glutamatergic LHNs in the LH. To address this issue, we
280 monitored odor-evoked responses in the glutamatergic LHNs to two repeated stimulations of
281 vinegar and benzaldehyde (depicted as 1 and 2 in the schematic in **Figure 6A, A'**), followed by
282 measuring LHN activity after micro-lesioning the mACT tract (depicted as 3 in **Figure 6A, A'**).
283 Since, we observed a significant response increase following mPN-mediated inhibition only in the
284 middle and upper plane (**Figure 5C', C'', D' and D''**), we confined our analysis to these two areas.
285 In order to determine whether the laser ablation of mACT alters also the odor-evoked spatial
286 pattern in addition to an increase in response amplitude, we compared the correlation coefficients

287 between the repeated responses before laser ablation to those after the transection. Our results
288 revealed that removal of the inhibitory input from mPNs leads to a reduced correlation coefficient
289 of the odor response patterns evoked by vinegar in LHNs (**Figure 6B, B'**). Interestingly, such an
290 effect was not observed in the case of benzaldehyde although we observed a weak mPN-mediated
291 inhibition for this odor before (**Figure 6B, B'**). Since the odor-evoked spatial patterns were
292 modified after silencing the mPN input into the LH, we next wondered whether this also affected
293 the odor specificity of the LHN responses. We therefore compared the correlation coefficients
294 'across odors' (i.e. between vinegar and benzaldehyde) before and after microlesion (**Figure 6C**).
295 Strikingly, we observed that the odor-evoked response patterns of vinegar and benzaldehyde
296 became increasingly similar after removal of the mPN-mediated inhibition depicted as an
297 increased correlation coefficient after laser ablation. This finding suggests that the inhibitory input
298 from mPNs onto glutamatergic LHNs is crucial to generate a distinct and odor-specific response
299 pattern for individual odors at the LH level.

300

301 **Discussion**

302 Our study functionally characterizes a subset of glutamatergic higher-order neurons in the LH
303 regarding odor coding and processing. We demonstrate that glutamatergic LHNs respond in a
304 reproducible, stereotypic and odor-specific manner and these response properties emerge at the
305 level of presynaptic uPNs (**Figure 6D**). Notably, the differential activity levels of glutamatergic
306 LHNs to attractive and aversive odors are positively correlated to the olfactory behavioral
307 preference indicating that these neurons are mainly tuned to attractive odors. The response features
308 do not arise from the OSN level, but rather derive from local processing within the LH by
309 integrating inputs from multiple olfactory channels through uPNs, which also show valence
310 specific odor representation. Furthermore, laser transection experiments demonstrate that these
311 higher-order neurons receive their major excitatory input from uPNs and an odor-specific
312 inhibitory input from mPNs. Lastly, our data show that the observed mPN-mediated inhibition
313 seems to be required for generating an odor-specific response map in the LH (**Figure 6D**).

314

315

316 **Glutamatergic LHNs respond in a stereotypic and odor-specific manner**

317 A growing body of evidence suggests the existence of an anatomical and functional stereotypy in
318 early processing centers of the insect olfactory pathway (Hildebrand & Shepherd, 1997;
319 Korsching, 2002). This stereotypy becomes obvious first at the sensory neuron (OSN) level, where
320 OSNs expressing a certain OR target and converge on a stereotypic glomerulus resulting in a
321 conserved spatial map in the AL between different individuals (Wang et al., 2003; Wilson et al.,
322 2004). This anatomical stereotypy was shown to be retained at the postsynaptic PN level (Jefferis
323 et al., 2007; Tanaka et al., 2004).

324 Several studies support the notion that an anatomical stereotypy might also be present at
325 the level of the LH, in particular shown for the PN to LHN connectivity (Bates et al., 2020; Fişek
326 & Wilson, 2014; Jeanne et al., 2018; Jefferis et al., 2007). Along this line, functional studies have
327 demonstrated that LHNs respond in a reproducible and stereotyped manner to odors and this
328 stereotypy is a general feature of the LH (Fişek & Wilson, 2014; Frechter et al., 2019). However,
329 how an ensemble of LHNs integrates inputs from several olfactory channels, i.e. the presynaptic
330 excitatory and inhibitory PNs, and whether each odor induces a specific and stereotyped response
331 pattern in the LH was not clearly addressed before. In our study we demonstrate that each odor is
332 represented by an odor-specific activity pattern in the LH, while the examined glutamatergic LHNs
333 display broader tuning patterns than their presynaptic partner neurons. Although it has been
334 assumed previously that odor specificity may not be encoded in higher-order brain centers (Grabe
335 & Sachse, 2017; Strutz et al., 2014), our findings are in accordance with a recent study by Frechter
336 et al. (2019) who demonstrate the existence of 33 different LH cell types exhibiting stereotypic
337 odor response properties with increased tuning breadth than PNs. The observed broader tuning
338 breadth of LHNs could be due to their property of integrating various inputs from multiple odor
339 channels, e.g. one LHN receives on average excitatory input from ~ 5.2- 6.2 glomeruli (Frechter
340 et al., 2019; Jeanne et al., 2018). Here, we observed that uPNs are more efficient in encoding odor
341 identity than the glutamatergic LHNs in the LH (**Figure 6D**), whereas LHNs reveal an improved
342 categorization of odors either based on behavioral significance, ‘odor scene’ or chemical group
343 (Bates et al., 2020; Frechter et al., 2019; Jeanne et al., 2018). However, the distinct odor-specific
344 response map by glutamatergic LHNs observed in our study suggests that the dimensionality of

345 odor features might not get reduced but still retains information about the odor specificity at the
346 third-order processing stage.

347

348 **Coding of hedonic odor valence**

349 Our functional imaging recordings revealed that odor valence is encoded by glutamatergic LHNs
350 leading to different activation strengths and patterns for attractive and aversive odors in the LH.
351 This observation is well in line with previous studies that have revealed that odor-evoked responses
352 in higher brain centers are generally categorized according to certain odor features as already
353 mentioned above. For example, using functional imaging or patch-clamp recordings of second-
354 order olfactory neurons revealed the existence of distinct attractive and aversive odor response
355 domains in the LH formed by uPNs and mPNs (Seki et al., 2017; Strutz et al., 2014). Such a
356 categorization according to hedonic valence is also visible in our study, when the odor-evoked
357 responses of glutamatergic LHNs were plotted in a PCA, taking into account the spatial response
358 patterns as well as the intensity of activity. Although no prominent spatial domain of attractive or
359 aversive odors was evident in our recordings, we observed that attractive odors evoked a generally
360 stronger activity when compared to aversive odors in this subset of LHNs. We noted a similar
361 trend in second-order uPNs. However, their response strength was neither correlated with the
362 olfactory preference determined in behavior nor the odor response properties of LHNs. The
363 observed significant correlation between the amount of odor-evoked activity in glutamatergic
364 LHNs to the behavioral valence of an odor leads us to postulate that the activity strengths of higher-
365 order olfactory neurons to odor stimulation might determine the behavioral response – an
366 assumption that needs to be tested in future studies.

367 Our study suggests that glutamatergic LHNs use different strategies to extract different
368 features of odor information, 1) conserving the identity of an olfactory stimulus by forming an
369 odor-specific activity map and, 2) encoding the valence of an odor by integrating information from
370 multiple olfactory channels. It is an ongoing debate regarding how neurons in the LH evaluate an
371 odor stimulus. It can be noted that irrespective of how an animal detects, encodes and categorizes
372 an odor at different olfactory processing levels, the behavioral output is in any case a binary choice,
373 reflected in either to approach (positive) or to leave (negative), to copulate (positive) or to reject

374 (negative), to oviposit (positive) or to find another suitable oviposition site (negative) (Galizia,
375 2014). Hence, based on the context or ecological relevance, an odor can just be evaluated either as
376 ‘pleasant’ or ‘unpleasant’, which is well reflected by the response properties of glutamatergic
377 LHNs regarding their valence-specificity and their correlation between response strength and
378 behavioral odor preference.

379 Notably, such a correlation between response intensity and behavioral preference has also
380 been observed in previous studies, where the amplitude of food odor-evoked activity in
381 neuropeptide F (dNPF) neurons was found to strongly correlate with food odor attractiveness
382 (Beshel & Zhong, 2013). Another study that combined functional imaging with tracking of innate
383 behavioral responses revealed that the behavioral output could be accurately predicted by a model
384 summing up the normalized glomerular responses, in which each glomerulus contributes a small
385 but specific part to the resulting odor preference (Badel et al., 2016). At the level of the LH, LHNs
386 then integrate the olfactory information from the glomerular responses conveyed via uPNs and
387 mPNs. In one of our previous studies we demonstrated that mPNs respond differently to attractive
388 and aversive odors, and mediate behavioral attraction (Strutz et al., 2014). In this context our study
389 complements this previous finding by showing that also uPNs display distinct valence-specific
390 responses in the LH to attractive and aversive odors. Information from these two PN pathways
391 become integrated and processed in the LH resulting in valence-specific activities in glutamatergic
392 LHNs, which may in turn determine the relative behavioral preference.

393

394 **mPN-mediated inhibition generates odor-specific response patterns in the LH**

395 We demonstrate that mPNs inhibit the glutamatergic LHNs in an odor-selective manner leading to
396 an odor-specific response pattern. According to our observations, glutamatergic LHNs receive a
397 stronger inhibition from mPNs in response to the odor vinegar than to benzaldehyde. In the absence
398 of this inhibition, we noted that in addition to an increased response amplitude and altered odor
399 representation, the activity patterns of different odors became more strongly correlated and hence
400 more similar. We therefore conclude that the mPN-mediated selective inhibition on this
401 glutamatergic subset of LHNs is necessary to maintain odor specificity. Along this line, a previous
402 study has reported that mPNs provide an odor-selective input to vlpr neurons, another class of

403 third-order LHNs (Liang et al., 2013). According to these authors, this odor-specific modulation
404 depends on the nature of the odor and results from the stereotyped connectivity of mPNs in the AL
405 as well as in the LH. Although this study provides evidence that uPNs are not presynaptically
406 inhibited by mPNs, another study established that mPNs indeed inhibit uPNs in the LH, facilitating
407 odor discrimination (Parnas et al., 2013). In addition to uPNs and vlpr neurons, our study identifies
408 another class of recipient neurons (glutamatergic LHNs) that receives mPN-mediated odorant-
409 selective inhibition. The glutamatergic LHNs population in our study comprises glutamatergic
410 LHONs as well as LHLNs (Dolan et al., 2019). Although we were not able to clearly differentiate
411 the functional properties between different populations of LHONs and LHLNs, since we used a
412 general marker for glutamatergic neurons, our study provides the first understanding of how odors
413 are integrated, transformed and finally represented in the LH by an ensemble of glutamatergic
414 LHNs.

415 Intriguingly, the neurotransmitter identity of this class of LHNs opens up another
416 interesting aspect: Knowing that glutamate can act as an excitatory (Aungst et al., 2003; Das et al.,
417 2011a) or inhibitory neurotransmitter (Liu & Wilson, 2013), as well as a coincident detector (Das
418 et al., 2011b), depending upon the receptors present in the postsynaptic neurons, further
419 experiments are needed to reveal the consequences of glutamatergic LHN input onto their
420 postsynaptic partner neurons. Certainly, the presence of an impressive amount of vesicular
421 glutamate in the LH points towards a significant role of glutamatergic LHNs with regard to odor
422 coding and processing at this higher brain center.

423

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429

430

431 **Author contributions**

432 S.D.C and S.S designed research; S.D.C and H.C performed research; S.D.C, H.C and S.S
433 analyzed data; B.S.H. provided intellectual and financial support, S.D.C, B.S.H. and S.S. wrote
434 the paper.

435 **Declaration of Interests**

436 The authors declare no conflict of interest.

437

438 **Materials and methods:**

439 **Fly stocks**

440 Flies were raised on autoclaved cornmeal-yeast-sucrose-agar food in a 12-h light/dark cycle at
441 25°C incubator. The following lines have been used for functional imaging: *dVGlut-Gal4* (II)
442 (Mahr & Aberle, 2006) (Bloomington 26160), *GHI46-Gal4* (II) (Stocker et al., 1997) (from Leslie
443 Vosshall's lab.), *GHI46-QF*, *QUAS mtd Tomato* (BDSC 30037) and *UAS-GCaMP6f* (III) (Chen
444 et al., 2013) (Bloomington 52869). For photoactivation experiments *Cha-Gal4* (II) (Bloomington
445 6798), *GAD1-Gal4* (II) (Ng et al., 2002) (Bloomington 51630), *dVGlut-Gal4*, *TH-Gal4* (III)
446 (Zhang et al., 2007) (from Mani Ramaswami's lab), *tdc2-Gal4* (II) (Koon et al., 2010) (NCBS,
447 India) and *UAS-C3PA* (Ruta et al., 2010) (gift from Sandeep Datta) flies have been used. Wild
448 type *Canton-S* flies have been used for behavioral experiments.

449

450 **Photoactivation**

451 *UAS-C3PA* was expressed under *Cha-Gal4*, *GAD1-Gal4*, *dVGlut-GAL4*, *TH-Gal4* and *tdc2-Gal4*
452 in the background of *GHI46-QF*, *QUAS mtd Tomato* for the photoactivation experiment. An initial
453 pre-photoactivation scan of the whole LH area was taken at 925 nm with a 40× water immersion
454 objective. The extent of the LH in z section was identified based on the innervation pattern of uPNs
455 (visualized by *GHI46-QF*, *QUAS mtd Tomato*). A region of interest in different focal planes
456 covering the entire LH area was photoactivated for ~1-2 min followed by 2-min rest using ~7mW

457 of 760 nm of laser. We allowed 10–15 min for photoactivated GFP to diffuse in more distal neural
458 processes. The post-photoactivation scan was taken using the same set-up as used for the pre-
459 photoactivation scan.

460

461 **Functional imaging**

462 All functional imaging experiments were performed on 4-7 day old mated female flies. Flies were
463 dissected for optical imaging according to the protocol by Strutz et al. (2014). Flies were briefly
464 immobilized on ice and then mounted onto a custom-made Plexiglas stage with a copper plate
465 (Athene Grids, Plano). A needle before the head was placed to stabilize the proboscis and to align
466 the head properly. Protomp II composite (3M ESPE) was used to fix the head with the copper
467 plate. We bent the anterior part of the fly's head with fine gold wire, and a small plastic plate
468 having a round window was placed on top. We sealed the head with that plate using two-
469 component silicone (Kwik Sil) and leaving the center part open to make a cut. The cuticle between
470 the eyes and the ocelli was gently cut under saline (130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2
471 mM CaCl₂, 36 mM saccharose, 5 mM Hepes, 1 M NaOH, pH 7.3). The cuticle was either bent
472 forward and fixed to the silicon or removed. All fat, trachea, and air sacs were removed carefully.

473 Functional imaging was performed using a 2-photon laser scanning microscope (2PCLSM,
474 Zeiss LSM 710 meta NLO) equipped with an infrared Chameleon Ultra™ diode-pumped laser
475 (Coherent, Santa Clara, CA, USA) and a 40× water immersion objective lens (W Plan-Apochromat
476 40×/1.0 DIC M27). The microscope and the laser were placed on a smart table UT2 (New
477 Corporation, Irvine, CA, USA). The fluorophore of GCaMP6f was excited with 925 nm. For each
478 individual measurement, a series of 40 frames (corresponding to 10 s) acquired at a resolution of
479 256 × 256 pixels was taken with a frequency of 4 Hz. During the entire 10 s of recording each odor
480 was delivered after 2 s (8 frames) for 2 s (8 frames). To cover the entire LH, images were acquired
481 from three different focal planes (i.e. upper, middle and lower), each 15µm apart in z direction.
482 *GHI46-QF*, *QUAS-td tomato* was always used in the background to precisely locate the planes for
483 all imaging experiments. A set of 14 odors was used including phenylacetaldehyde (PHA), vinegar
484 (VIN), 2,3 butanedione (BUD), acetoin acetate (AAC), ethyl acetate (ETA), propionic acid (PAC)
485 and gamma-butyrolactone (G-BUT) as attractive odors, 1-octanol (OCT), benzaldehyde (BEA),

486 acetophenone (ACP), methyl salicylate (MSC), geosmin (GEO) and linalool (LIN) as aversive
487 odors and cis-vaccenyl acetate (cVA) as a pheromone. All odors have been used at a concentration
488 of 10^{-3} diluted in mineral oil. Vinegar was diluted in double distilled water. Flies were imaged for
489 up to 1 hr, with a minimum inter-stimulus interval of one minute. For odor delivery we used a
490 computer-controlled odor delivery system (described in (Mohamed et al., 2019)).

491

492 **Data and statistical analysis for functional imaging**

493 The LSM file obtained from the imaging software ZEN, was processed and analyzed in Fiji. 40
494 frames for each odor (40 frames X 14 odors) were opened and stitched together using
495 'Image>Stacks>Tools>Concatenate' command. The file combining all the frames for all odors
496 were movement corrected using 'Plug in>Registration>Stackreg>Rigid body' command. The
497 entire LH area was encircled and cropped and divided into 65 pixels, each having $30 \times 30 \mu\text{m}^2$
498 dimension. Using 'Analyze>Tools>ROI manager>more>multi measure' command fluorescence
499 intensity for every pixel for every frame for every odor was obtained. This huge data set was
500 further processed using Excel software. Average $\Delta F/F$ was calculated for 10-18 frames for each
501 pixel for each odor.

502 The $\Delta F/F$ for 65 pixels for 14 odors for one replicate was aligned in table format and
503 processed using 'past' statistical software. The correlation coefficient for each odor pair using
504 Euclidean distance was analyzed.

505

506 **2-Photon-mediated laser transection**

507 Transection of either the iACT or mACT tract of PNs was performed using *GHI46-QF*, *QUAS-td*
508 *tomato* as a guiding landmark. Transection of the PN tracts was done in one brain hemisphere of
509 each fly only. The chosen area for transection was located using 925 nm of laser, close to the LH
510 but far enough not to damage the neurites branching in the LH neuropil. ~60 mW of 760 nm laser
511 was provided at the chosen area at a pulse for 2-3 s for 2-3 times with 1 s time interval (Strutz et
512 al., 2014). Successful transection was verified after visualization of a small bubble. As a control,
513 the LH from the other, untreated brain hemisphere was imaged to stimulation with benzaldehyde

514 before and after laser ablation to rule out that the laser transection of the specific targeted area
515 caused any non-specific damage to other brain areas.

516

517 **Behavioral assay**

518 T-maze experiments with 4-7 days old flies were carried out for the olfactory preference assay.
519 ~30 flies were starved for 24 hrs in each vial containing wet filter paper. Mated males and females
520 were used for the behavioral assay. All odors were used at a concentration of 10^{-3} , which is the
521 same as used for the functional imaging experiments. At each arm of the T-maze small filter papers
522 containing either 6 μ l of mineral oil (control) or an odor (odorant) were placed. Briefly cold
523 anaesthetized flies were released at the junction of horizontally placed T maze. After ~30 min flies
524 in each side of the arm were counted. The preference index was calculated as (odorant-control)/
525 total number of flies (i.e., 30). Each odor has ~20 replicates.

526

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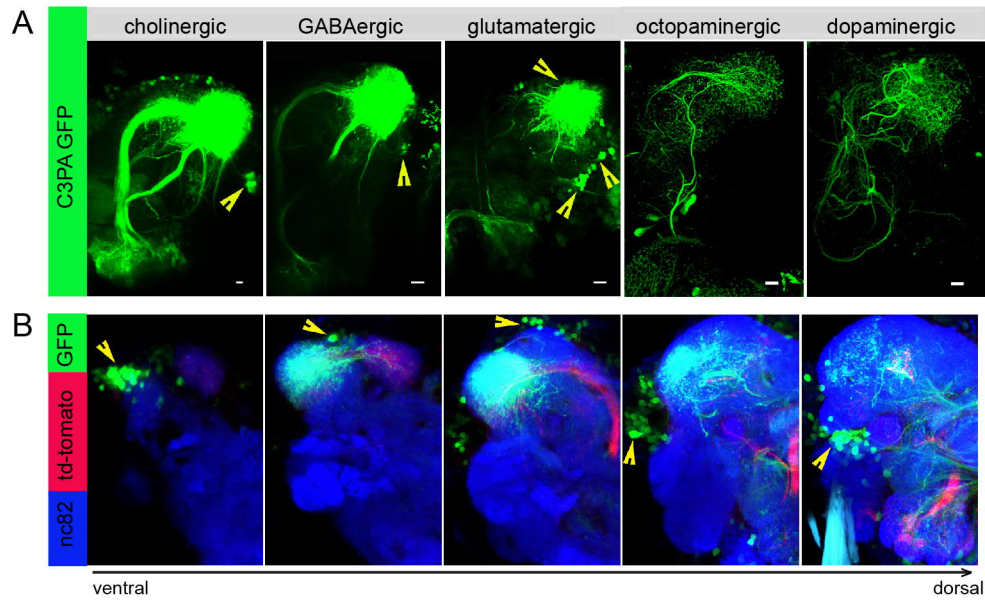
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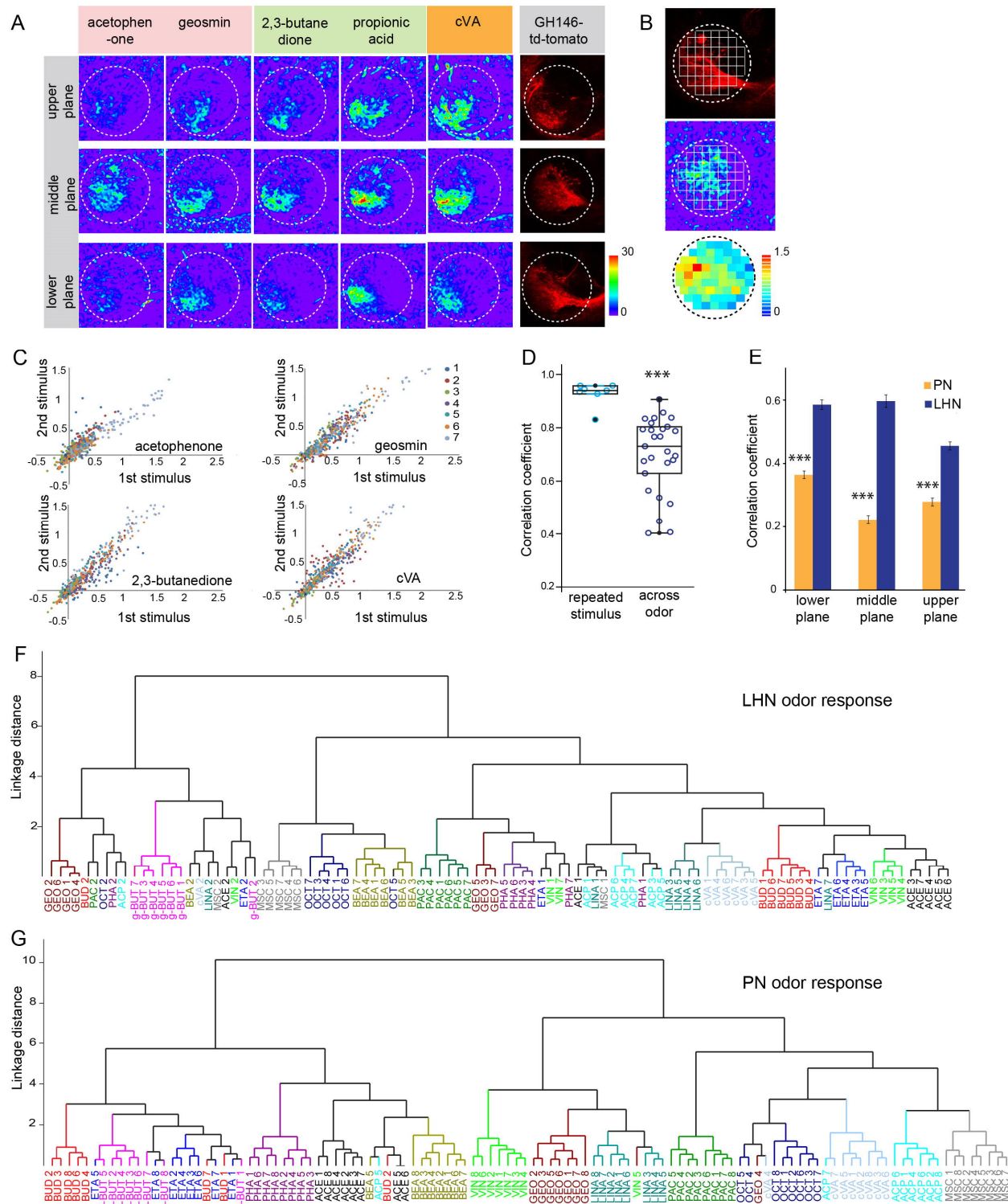
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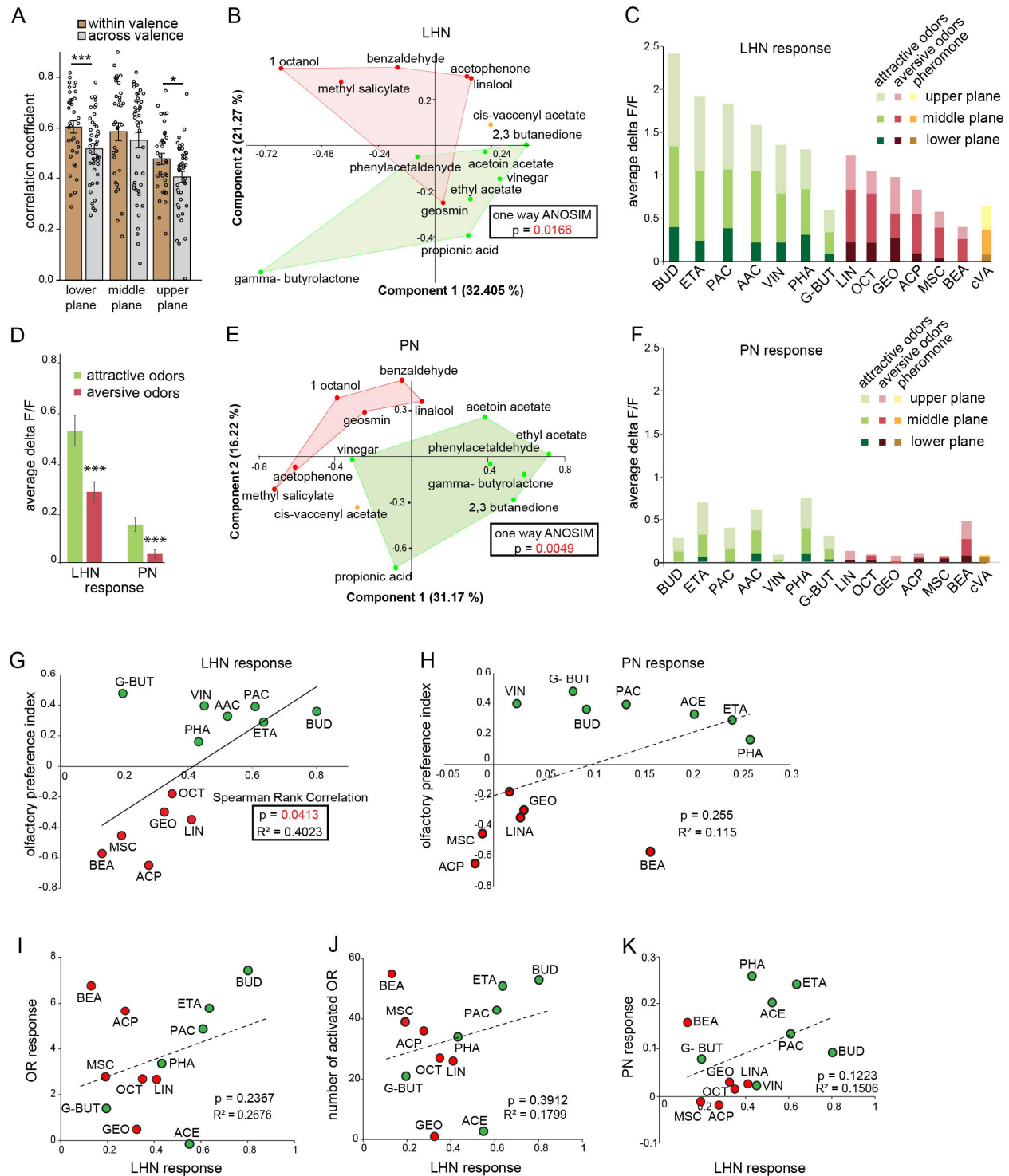


698 **Figure 1: Selective labeling of LHNs based on their neurotransmitter identities.** (A)
699 Photoactivation of *UAS-C3PA-GFP* expressed under the control of different Gal4 lines with
700 various neurotransmitter and neuromodulator identities (*Cha-Gal4*, *GAD1-Gal4*, *dVGlut-Gal4*,
701 *TH-Gal4* and *tdc2-Gal4*) reveals subsets of cholinergic, GABAergic, glutamatergic,
702 octopaminergic and dopaminergic LHNs (left to right). Position of the cell bodies are demarcated
703 with yellow arrowheads. (B) Immunohistochemistry of photoactivated brains with *dVGlut-Gal4*
704 driving *UAS-C3PA-GFP* reveals different cell body clusters of glutamatergic LHNs found at
705 various locations in different focal planes. *GH146-QF* driving *QUAS-td tomato* in the background
706 are depicted in red and *nc82* in blue. Position of the cell bodies are shown with yellow arrowheads.
707 Scale bars = 10 μm.



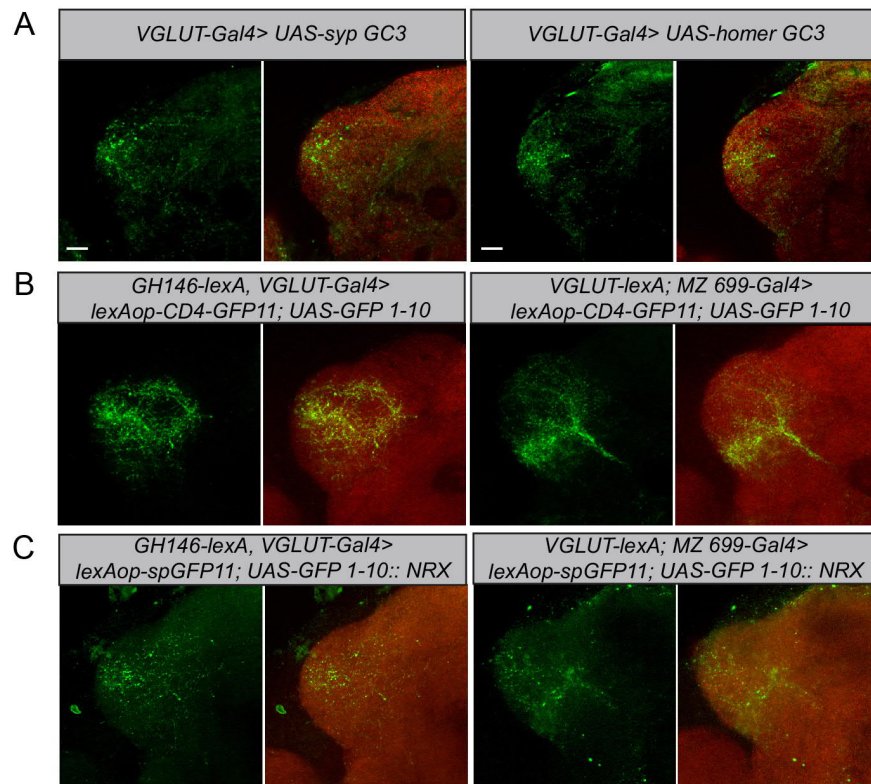
708 **Figure 2: Odor response properties of glutamatergic LHNs and uPNs.** (A) Representative
 709 images of calcium responses of glutamatergic LHNs in the LH brain area in three focal planes,
 710 evoked by various odors (repulsive odors: acetophenone, geosmin; attractive odors: 2,3

711 butanedione, propionic acid; pheromone: cVA). Right panel shows the innervation pattern of uPNs
712 labeled by GH146 in three different focal planes, used as a landmark to maintain comparable focal
713 planes for functional imaging across different animals. (B) Grid approach to analyze odor-evoked
714 responses of glutamatergic LHNs. Representative images of the grid onto uPN labeling (upper
715 panel), used as a background landmark, odor-evoked responses of glutamatergic LHNs (middle
716 panel) and analyzed $\Delta F/F$ of each pixels from glutamatergic LHNs in the LH area (lower panel).
717 (C) Scatter plot showing calcium responses of each pixel of glutamatergic LHNs at middle plane
718 to repeated presentation of an odor stimulus. Different colored dots depict responses obtained from
719 different animals. (D) Box plot represents comparison of odor-evoked responses of glutamatergic
720 LHNs at middle plane, between repeated and across odor stimuli (student t-test, *** $p < 0.001$). (E)
721 Comparison of correlation coefficients of uPN and LHN responses at three different focal planes.
722 Different odors seem to be better segregated at the uPN level than at the level of LHNs (student t-
723 test, *** $p < 0.001$). (F) Cluster analysis of LHN odor responses based on linkage distance ($n = 7$,
724 ANOSIM, sequential Bonferroni significance, $p = 0.0001$). (G) Cluster analysis of uPN odor
725 responses based on linkage distance ($n = 8$; ANOSIM, sequential Bonferroni significance, $p =$
726 0.0001). Both cluster analyses show that the majority of replicates for each odor are grouped
727 together.

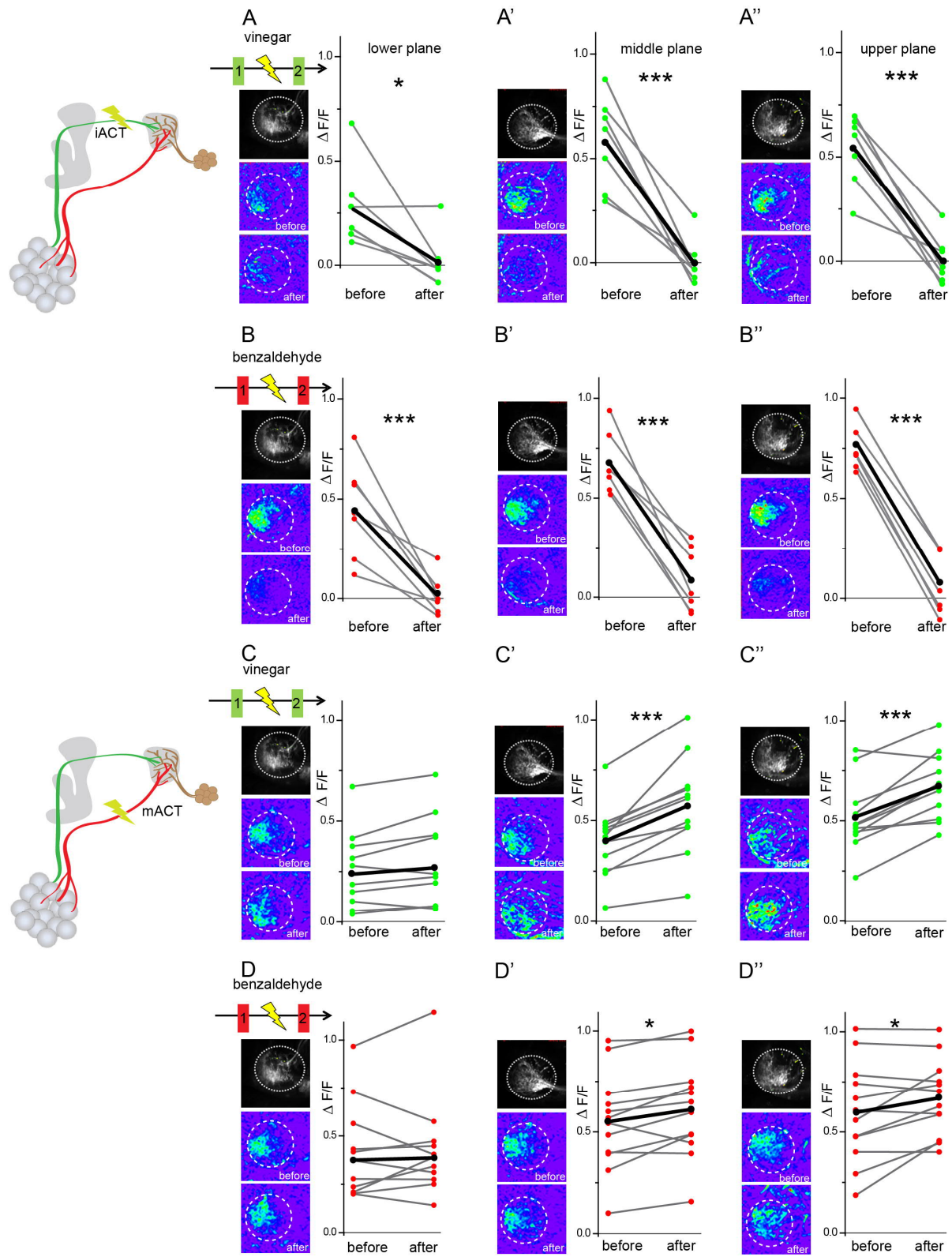


728 **Figure 3: Segregation of hedonic valence in the LH.** (A) Comparison of the correlation
 729 coefficients of odor responses of glutamatergic LHNs between the odors that share the same
 730 valence (i.e. within valence) and odors having an opposing valence (i.e. across valence). Responses
 731 to odors sharing the same valence are significantly more similar than those across valence (student

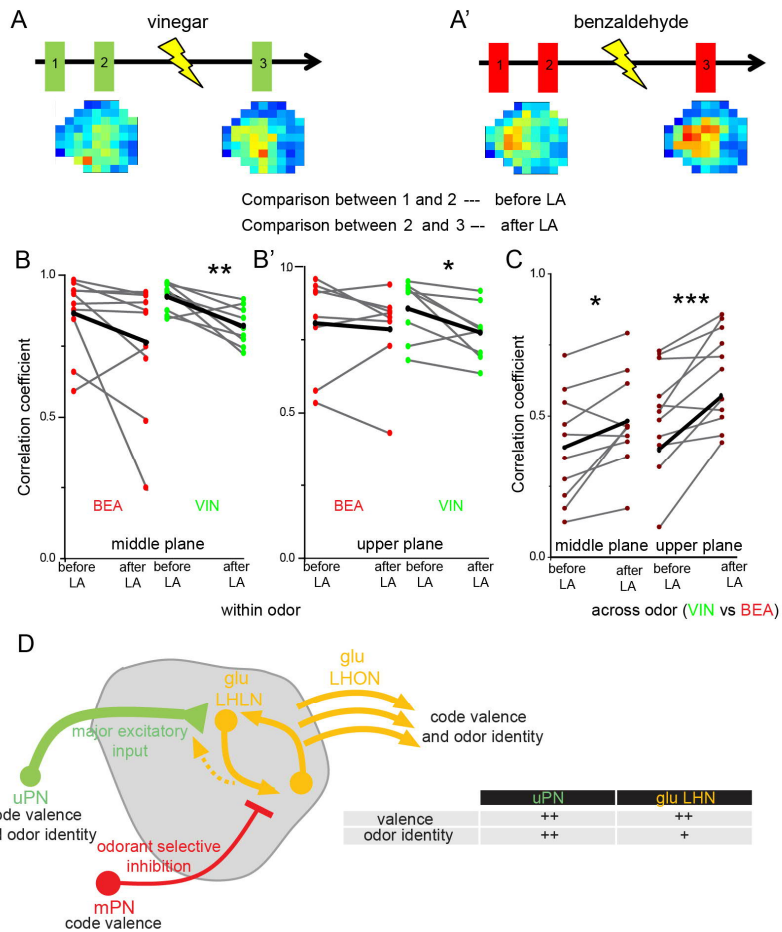
732 t-test, *** $p < 0.001$, * $p < 0.05$) (B) PCA analysis of odor responses of glutamatergic LHNs shows
733 that attractive (green) and aversive (red) odors are significantly segregated in the LH (one way
734 ANOSIM, $p = 0.0166$). (C) Response amplitudes of glutamatergic LHNs to individual attractive
735 and aversive odors. (D) Comparison of the overall response strength of glutamatergic LHNs and
736 uPNs to all attractive and aversive odors (student t-test, *** $p < 0.001$) (E) PCA analysis of odor
737 responses of uPNs shows that attractive (green) and aversive (red) odors are significantly
738 segregated in the LH (one way ANOSIM, $p = 0.0049$). (F) Response amplitudes of uPNs to
739 individual attractive and aversive odors. (G) Scatter plot of olfactory preference indices of all
740 tested odors to the response strengths of glutamatergic LHNs. The solid trend line demarcates
741 significant correlation between the two parameters (Spearman Rank Correlation, $p = 0.04$). (H)
742 Scatter plot of olfactory preference indices of all tested odors to the response strengths of uPNs,
743 which does not show a significant correlation (dotted trend line). (I) Scatter plot of the response
744 strength of glutamatergic LHNs for individual odors to the overall OSN activity. (J) Scatter plot
745 of the response strength of glutamatergic LHNs for individual odors to the number of ORs
746 activated. (K) Scatter plot of the response strength of glutamatergic LHNs and the response
747 strengths of uPNs for individual odors.



748 **Figure 4: Polarity and morphological connectivity of glutamatergic LHNs with uPNs and**
749 **mPNs.** (A) Expression of syp GC3 (left panel) and homer GC3 (right panel) in glutamatergic
750 LHNs suggesting the presence of pre- as well as postsynapses in the LH. (B) Membrane-targeted
751 CD4-GRASP between glutamatergic LHNs and uPNs (left panel) or mPNs (right panel). (C) The
752 synaptic protein neurexin tagged to GRASP (Nrx-GRASP) was employed to confirm synaptic
753 connectivity of glutamatergic LHNs with uPNs (left panel) or mPNs (right panel) in the LH. Scale
754 bars = 10 μm.

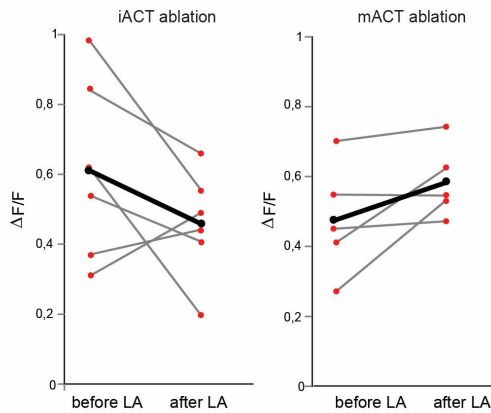


755 **Figure 5: Glutamatergic LHNs receive major excitatory input from uPNs and an odorant**
756 **selective inhibition from mPNs.** Upper panel schematic represents the experimental approach:
757 the iACT tract was laser transected while odor-evoked responses were monitored from
758 glutamatergic LHNs. (A, A' and A'') Representative images and graphical comparison of
759 response evoked by vinegar in glutamatergic LHNs before and after laser transection of the
760 iACT, across three different planes. (B, B' and B'') Representative images and graphical
761 comparison of response evoked by benzaldehyde in glutamatergic LHNs before and after laser
762 transection of the iACT. Lower panel schematic represents the experimental approach: the mACT
763 tract was laser transected while odor-evoked responses were measured from glutamatergic
764 LHNs. (C, C' and C'') Representative images and graphical comparison of vinegar-evoked
765 responses of glutamatergic LHNs before and after laser transection of the mACT. (D, D' and
766 D''). Representative images and graphical comparison of benzaldehyde-evoked response of
767 glutamatergic LHNs before and after laser transection of the mACT (paired t-test *** $p < 0.001$,
768 * $p < 0.05$).



769 **Figure 6: mPN-mediated inhibition facilitates odor specificity in glutamatergic LHNs.** (A and
 770 A') Schematic represents the experimental approach: odor-evoked responses of glutamatergic
 771 LHNs were monitored to repeated odor presentations (1 and 2) before and after laser transection
 772 of the mACT (3) for the odors vinegar and benzaldehyde. (B and B') Comparison of the correlation
 773 coefficients between the repeated odor responses before laser ablation to those after the transection
 774 for vinegar and benzaldehyde (within odor comparison). (C) Comparison of the correlation
 775 coefficients across vinegar and benzaldehyde before and after microlesion (across odor
 776 comparison; paired t-test, *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$). (D) Schematic summarizing the
 777 observed connectivity between PNs and glutamatergic LHNs in the LH: Glutamatergic LHNs
 778 consists of both, LHLNs and LHONs. uPNs (green) provide the major excitatory input to
 779 glutamatergic LHNs (yellow) while they might also receive feedback input from LHNs. mPNs
 780 provide an odorant selective inhibition to glutamatergic LHNs. mPNs are known to encode odor
 781 valence in the LH (Strutz et al., 2014), uPNs and glutamatergic LHNs encode odor identity as well

782 as odor valence. The table illustrates that uPNs encode an improved odor identity than postsynaptic
783 glutamatergic LHNs whereas hedonic valence is equally maintained at the level of uPNs and
784 glutamatergic LHNs in the LH.



785 **Figure 5- figure supplement 1: Laser transaction does not affect activity of glutamatergic**
786 **LHNs in the intact brain hemisphere.** Left panel shows the comparison of benzaldehyde-evoked
787 activity in glutamatergic LHNs in the intact hemisphere, before and after laser ablation of the iACT
788 in the other, treated brain hemisphere (paired t-test, $p=0.19$). Right panel shows the comparison
789 of benzaldehyde-evoked activities in glutamatergic LHNs in the intact hemisphere, before and
790 after laser ablation of the mACT in the other hemisphere (paired t-test, $p=0.25$).