# The unique synaptic circuitry of specialized olfactory glomeruli in *Drosophila melanogaster*

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## ABSTRACT

In the *Drosophila* olfactory system most odorants are encoded in the antennal lobe in a combinatory way, activating several glomerular circuits. However, odorants of particular ecological role for the fly are encoded through activation of a single specialized olfactory pathway. Comparative analyses of densely reconstructed connectomes of one broadly tuned glomerulus (DL5) and one narrowly tuned glomerulus (DA2) gained detailed insight into the variations of synaptic circuitries of glomeruli with different computational tasks. Our approach combined laser-branding of glomeruli of interest with volume based focused ion beam-scanning electron microscopy (FIB-SEM) to enable precise targeting and analysis of the two glomeruli. We discovered differences in their neuronal innervation, synaptic composition and specific circuit diagrams of their major cell types: olfactory sensory neurons (OSNs), uniglomerular projection neurons (uPNs) and multiglomerular neurons (MGNs). By comparing our data with a previously mapped narrowly tuned glomerulus (VA1v), we identified putative generic features of narrowly tuned glomerular circuits, including higher density of neuronal fibers and synapses, lower degree of OSN lateralization, stronger axo-axonic connections between OSNs, dendro-dendritic connections between many uPNs, and lower degree of presynaptic inhibition on OSN axons. In addition, this work revealed that the dendrites of the single uPN in DL5 contain a substantial amount of autapses interconnecting distant regions of the dendritic tree**.** The comparative analysis of glomeruli allows to formulate synaptic motifs implemented in olfactory circuits with different computational demands.

38 **INTRODUCTION**

39 Olfaction is an anatomically shallow sensory system. In mammals and invertebrates just one
40 synapse separates the sensory periphery from the central brain (Dolan *et al.*, 2018; Liang *et al.*,
41 2010; Owald *et al.*, 2015; Shepherd, 2011; Su *et al.*, 2009). In the olfactory system of *Drosophila*
42 *melanogaster*, the first relay station of synaptic transmission is the antennal lobe (AL), which has a
43 circuit architecture homologous to that of the vertebrate olfactory bulb (Boeckh *et al.*, 1990;
44 Sachse *et al.*, 2021; Shepherd *et al.*, 2021). The fly AL consists of approximately 58 spherical
45 compartments, called glomeruli, which can be distinguished by size, shape and location (Bates *et*
46 *al.*, 2020; Gao *et al.*, 2000; Grabe *et al.*, 2015; Laissue *et al.*, 1999; Vosshall *et al.*, 2000). Each
47 glomerulus receives stereotypic input from axon terminals of olfactory sensory neurons (OSNs),
48 which have their cell bodies and dendrites located in the antennae or maxillary palps (Benton *et*
49 *al.*, 2006; de Bruyne *et al.*, 1999; de Bruyne *et al.*, 2001; Hallem *et al.*, 2004; Shanbhag *et al.*, 1999).
50 All the OSNs innervating a given glomerulus express a typical repertoire of ligand-gated
51 chemoreceptors (Benton *et al.*, 2006; Couto *et al.*, 2005; Fishilevich *et al.*, 2005), which represent
52 a wide range of specifications, binding either a single, few, or many distinct chemicals (Hallem *et*
53 *al.*, 2006; Hallem *et al.*, 2004; Knaden *et al.*, 2012; Münch *et al.*, 2016; Seki *et al.*, 2017; Wicher *et*
54 *al.*, 2021).

55 Most OSNs project bilaterally to the corresponding glomeruli in the left and right AL (Gaudry
56 *et al.*, 2013; Tobin *et al.*, 2017). In the AL, OSNs convey odor signals to excitatory uniglomerular
57 projection neurons (uPNs), which branch only within a single glomerulus, or to inhibitory
58 multiglomerular PNs (mPNs) and inhibitory or excitatory local interneurons (LNs) (Ai *et al.*, 2013;
59 Bates *et al.*, 2020; Cuntz *et al.*, 2007; Kazama *et al.*, 2008; Kazama *et al.*, 2009; Kreher *et al.*, 2008;
60 Masse *et al.*, 2009; Ng *et al.*, 2002; Tanaka *et al.*, 2012; Wilson, 2013). LNs innervate each several
61 glomeruli and are the key modulatory neurons in the AL (Chou *et al.*, 2010; Seki *et al.*, 2010). The
62 highly converging OSNs-to-PN signal transmission (Chen *et al.*, 2005; Jeanne *et al.*, 2015; Masse *et*
63 *al.*, 2009) is lateralized, activating ipsilateral uPNs more strongly than contralateral ones (Agarwal
64 *et al.*, 2011; Gaudry *et al.*, 2013; Tobin *et al.*, 2017). From the AL, uPNs and mPNs relay processed
65 signal information to higher brain centers (Bates *et al.*, 2020; Fiala, 2007; Galizia, 2014; Guven-
66 Ozkan *et al.*, 2014; Jefferis *et al.*, 2007; Keene *et al.*, 2007; Norgate *et al.*, 2006; Strutz *et al.*, 2014).

67 The stereotypic activity pattern elicited by distinct odorants encodes the odor space,
68 represented in a so-called odotopic map of the AL according to the glomerular activation by distinct
69 chemical classes. (Couto *et al.*, 2005; Grabe *et al.*, 2018; Grabe *et al.*, 2015; Knaden *et al.*, 2014;
70 Laissue *et al.*, 2008). Some odorants induce a fixed innate behavior (aversion or attraction),
71 activating characteristically specific glomeruli (Gao *et al.*, 2015; Grabe *et al.*, 2018; Knaden *et al.*,
72 2014; Knaden *et al.*, 2012; Semmelhack *et al.*, 2009). The encoding of hedonic valence already at
73 the level of the AL is important for a fast odor coding. Most odorants are encoded in a
74 combinatorial manner in the fly AL by activating multiple OSNs types expressing broadly tuned

75 receptors and their glomerular circuits, including broadly tuned uPNs (de Bruyne *et al.*, 2001;
76 Galizia, 2014; Masse *et al.*, 2009; Sachse *et al.*, 2016; Seki *et al.*, 2017; Silbering *et al.*, 2007;
77 Silbering *et al.*, 2008; Szyszka *et al.*, 2015). Certain chemoreceptors and their downstream
78 glomerular circuits, however, have evolved a very high specificity and sensitivity to single or very
79 few chemicals (Andersson *et al.*, 2015; Haverkamp *et al.*, 2018; Keesey *et al.*, 2021). These narrowly
80 tuned glomerular circuits often belong to dedicated olfactory pathways, called "labeled lines",
81 which process information regarding single odorants of particular importance for reproduction and
82 survival (Datta *et al.*, 2008; Dweck *et al.*, 2015; Gao *et al.*, 2015; Kurtovic *et al.*, 2007; Stensmyr *et*
83 *al.*, 2012). An extreme example is the DA2 glomerulus, which responds exclusively to geosmin, an
84 ecologically relevant chemical that alerts flies to the presence of harmful microbes, causing the fly
85 to avoid laying eggs at these locations (Stensmyr et al., 2012). This dedicated olfactory pathway
86 and its receptor sequence is conserved throughout evolution (Keesey *et al.*, 2021; Keesey *et al.*,
87 2019). Another example is glomerulus VA1v, which responds to methyl laurate, a pheromone that
88 induces a strongly attractive response in female flies leading to aggregation behavior (Dweck *et al.*,
89 2015). DL5, on the other hand, is an example of a broadly tuned glomerulus, innervated by OSNs
90 activated by several odorants, like E2-hexenal and benzaldehyde (Knaden *et al.*, 2012; Mohamed
91 *et al.*, 2019b; Münch *et al.*, 2016; Seki *et al.*, 2017). This functional diversity suggests differences in
92 neuronal composition and synaptic connectivity between broadly and narrowly tuned glomeruli.
93 A survey of neuronal composition across glomeruli revealed great variation in the numbers
94 of the different types of neurons innervating narrowly and broadly tuned glomeruli (Grabe *et al.*,
95 2016). In general, narrowly tuned glomeruli are innervated by more uPNs and fewer LNs compared
96 to more broadly tuned glomeruli (Chou *et al.*, 2010; Grabe *et al.*, 2016). In addition, narrowly tuned
97 OSNs receive less global LN inhibition than broadly tuned ones (Grabe *et al.*, 2020; Hong *et al.*,
98 2015; Schlegel *et al.*, 2021). For example, in female flies, the narrowly tuned glomerulus DA2
99 contains dendrites of 6-8 uPNs, whereas the broadly tuned glomerulus DL5 houses only 1 or 2 uPNs
100 and has a higher number of innervating LNs. Interestingly, both glomeruli are innervated by the
101 same number of OSNs (Grabe *et al.*, 2016).
102 Little is known, however, about the microarchitecture of the synaptic circuitry in distinct
103 glomeruli and, in particular, about ultrastructural differences between narrowly vs. broadly tuned
104 glomerular circuits. Electron microscopy (EM) allows volume imaging with dense reconstruction of
105 fine neurite branches and synapses in brain tissue at nanometer resolution, necessary to map
106 synapses (Briggman *et al.*, 2006; Cardona *et al.*, 2009; Helmstaedter, 2013; Meinertzhagen, 2018;
107 Rybak, 2013). The first ultrastructural insights into the synaptic connectivity of *Drosophila* olfactory
108 glomeruli were obtained by studies based on serial section transmission EM (ssTEM) (Rybak, 2016;
109 Tobin *et al.*, 2017). Rybak *et al*. (2016) showed that all three basic classes of AL neurons make
110 synapses with each other, while Tobin *et al*. (2017) revealed that the differences in number of
111 innervating uPNs between the left and right DM6 glomeruli are compensated by differences in
112 synaptic strength. With focused ion beam-scanning electron microscopy (FIB-SEM; (Knott *et al.*,

113 2008)) a complete reconstruction of all neurons in the narrowly tuned, pheromone processing
114 glomerulus VA1v was obtained (Horne *et al.*, 2018). Recent technological innovations in ssTEM,
115 FIB-SEM and automated neuron reconstruction have made connectome datasets of the adult
116 *Drosophila* central nervous system available (Li, P. H. *et al.*, 2020; Saalfeld *et al.*, 2009; Scheffer *et*
117 *al.*, 2020; Zheng *et al.*, 2018) and provided complete circuit descriptions of several brain centres
118 (Auer *et al.*, 2020; Bates *et al.*, 2020; Coates *et al.*, 2020; Dolan *et al.*, 2019; Felsenberg *et al.*, 2018;
119 Hulse *et al.*, 2021; Huoviala *et al.*, 2020; Li, F. *et al.*, 2020; Marin *et al.*, 2020; Otto *et al.*, 2020;
120 Schlegel *et al.*, 2021).
121 In an attempt to find answers to how highly specialized olfactory glomerular circuits of
122 dedicated olfactory pathways differ in their signal integration from broadly tuned glomerular
123 circuits, we compared the microarchitecture and synaptic circuitry of a narrowly and a broadly
124 tuned glomerulus (DA2 and DL5). By using a correlative workflow combining transgenic markers
125 with FIB-SEM, in order to identify these glomeruli, we reconstructed OSNs, uPNs and
126 multiglomerular neurons (MGNs), mapped all associated synapses and compared the circuit
127 organization of both glomeruli.
128

129 **RESULTS**

130 ## Volume-based electron microscopy of two different olfactory glomeruli

131 To compare the synaptic circuitries of two olfactory glomeruli known to belong to either
132 narrowly or broadly tuned glomerular types in *Drosophila melanogaster*, we mapped all synapses
133 of glomeruli DA2 (right AL) and DL5 (left AL) in a single female fly (**Figure 1A-B**) with the aid of FIB-
134 SEM. A partial reconstruction of a second DA2 in another fly was used to measure neuronal volume
135 (see Methods). The reconstructions were based on high resolution (4x4x20 nm) datasets (**Figure 1**;
136 **Figure 1 – video 1),** thus allowing reconstruction of the finest neuronal branches (~20 nm diameter;
137 **Figure 1C-D**) as well as mapping chemical synapses (example in **Figure 1E**) in the two volumes of
138 interest (VOI). To restrict the imaging volume to the target VOIs, we employed a correlative
139 approach for the first time for a *Drosophila* EM volume reconstruction. Glomeruli DA2 and DL5
140 were identified by their size, shape and location in brains of transgenic flies (*Orco-GAL4; UAS-*
141 *GCaMP6s*) using the glomerular map of (Grabe *et al.*, 2015). The flies expressed the green
142 fluorescent protein GCaMP6 coupled with calmodulin and M13 (a peptide sequence from myosin
143 light-chain kinase; **Figure 1A-B**). Subsequently, the identified glomeruli were marked by laser
144 branding using a two-photon laser (Bishop *et al.*, 2011). These fiducial marks were apparent under
145 both light (Figure 1A-B) and electron microscopy **(Figure 1C-D)** and facilitated the delimitation of
146 the VOIs during FIB-SEM scanning. We produced two complete FIB-SEM datasets: one for
147 glomerulus DA2 and one for DL5 (pure imaging time for both glomeruli: ~60 h) and a partial dataset
148 for DA2 in a second fly.
149

150 ## Skeleton based neuron reconstruction and synapse identification

151 We reconstructed all neurons within the two VOIs (example neuron: **Figure 1F**) and mapped
152 all their synaptic connections using an iterative skeleton-based reconstruction approach, similar to
153 previously reported procedures (Berck *et al.*, 2016; Schneider-Mizell *et al.*, 2016; Zheng *et al.*,
154 2017) with the aid of the web-based neuron reconstruction software CATMAID
155 (http://www.catmaid.org; RRID:SCR_006278; (Cardona *et al.*, 2009; Schneider-Mizell *et al.*, 2016);
156 **Figure 1 – video 1**). Synapses were identified by their presynaptic transmitter release site, which
157 in *Drosophila* is composed of a presynaptic density called a T-bar, surrounded by synaptic vesicles
158 and apposed postsynaptic elements (**Figure 1E**), as previously described (Fröhlich, 1985; Huang *et*
159 *al.*, 2018; Li, P. H. *et al.*, 2020; Rybak *et al.*, 2016; Trujillo-Cenoz, 1969). All synapses observed in
160 our FIB-SEM data sets were polyadic, i.e. each presynaptic site connected to multiple postsynaptic
161 sites (See example in **Figure 1E**), a feature of insect brain synapses (Hartenstein, 2016; Malun *et*
162 *al.*, 1993; Meinertzhagen *et al.*, 1991; Prokop *et al.*, 2006; Rybak *et al.*, 2016). Some synapses had
163 up to 16 postsynaptic sites (**Figure 2 – figure supplement 1B**), i.e. one T-bar and 16 single synaptic
164 profiles (i.e. sixteen 1:1 single output-input connections). Short neuronal fragments (<10 µm),

165 which could not be connected to any neuronal fiber were designated as "orphans". These
166 fragments represented 4% of the total length of all traced neuronal fibers in DA2 and 6% in DL5
167 and contained about ~12% of all synaptic contacts in both glomeruli.
168
169 **Glomerular neurons: classification, description and inventory**

170 Previous descriptions of the ultrastructural characteristics of the AL in *Drosophila* helped to
171 classify AL neurons into 3 main classes (**Figure 2A**) Olfactory sensory neurons (OSNs),
172 uniglomerular projection neurons (uPNs) and multiglomerular neurons (MGNs; cells that
173 interconnect multiple glomeruli). MGNs are further subdivided into multiglomerular projection
174 neurons (mPNs) and local interneurons (LNs) (Berck *et al.*, 2016; Gruber *et al.*, 2018; Horne *et al.*,
175 2018; Li, P. H. *et al.*, 2020; Rybak *et al.*, 2016; Schlegel *et al.*, 2021; Zheng *et al.*, 2017). Most of the
176 neuronal profiles within the MGN neuron class belong probably to inhibitory local neurons, as this
177 cell type is the most numerous and broadly arborizing of the multiglomerular cell types in the
178 antennal lobe (Chou *et al.*, 2010; Lin *et al.*, 2012). In addition, we observed a few neuronal fibers
179 with an electron-dense and vesicle-rich cytosol, which we interpreted to be either peptidergic
180 neurons (Eckstein *et al.*, 2020; Nässel *et al.*, 2006) or the contralaterally projecting, serotonin-
181 immunoreactive deutocerebral (CSD) neuron, (Coates *et al.*, 2020; Dacks *et al.*, 2006; Eckstein *et*
182 *al.*, 2020; Goyal *et al.*, 2013; Zheng *et al.*, 2017). Except for these neuronal fibers containing
183 abundant electron-dense vesicles, all other neuronal fibers were assigned to either OSNs, uPNs or
184 MGNs based on their morphology (**Figure 2A, B;** see Methods).
185 OSNs formed large, elongated synaptic boutons (**Figure 2A**), had the largest volume/length
186 ratio of all three neuron classes (**Figure 2 – figure supplement 1A**) and displayed the lowest degree
187 of branching intensity of all neurons in both glomeruli (**Figure 2B**). In agreement with what had
188 been observed in other glomeruli (Rybak *et al.*, 2016), the majority of output synapses made by
189 OSN terminals were triads (1:3) and tetrads (1:4). The T-bars of OSN synapses exhibited a large
190 variation in size and some of them were large enough to accommodate 16 postsynaptic contacts
191 (**Figure 2 – figure supplement 1B**)**.** The frequency of large T-bars was much higher in OSNs than in
192 other neuron classes with an average polyadicity (average number of postsynaptic sites at each T-
193 bar) of 6 (1:6; (**Table 1,** row 14). As OSNs had the greatest T-bar and output density along their
194 axons (**Table 1**, row 10-11) they also displayed the largest synaptic ratios (both for the T-bars/input
195 sites and output sites/input sites) of all neuron classes (**Table 1,** row 12-13), which was in line with
196 previous observations (Rybak *et al.*, 2016).
197 The uPNs exhibited the highest degree of branching intensity of the three neuron classes in
198 both glomeruli (**Figure 2A-B**). They showed numerous very fine apical branches that frequently
199 connected multiple times via spines to the same presynaptic site, leading to an entangled 3D shape
200 typical for this neuron class (**Figure 2A**) (Rybak *et al.*, 2016; Schlegel *et al.*, 2021; Tobin *et al.*, 2017).
201 uPNs had the smallest volume/length ratio of all neuron classes (for the DA2: **Figure 2 - supplement**

202 **1A**). In addition to having many fine branches, uPN dendrites also had enlarged regions with almost
203 no cytosol that were packed with large mitochondrial profiles extending over considerable
204 distances. These enlarged profiles showed a larger degree of mitochondria fission (dividing and
205 segregating mitochondrion organelles; personal observation) than the other neuron classes with
206 rather round and compact mitochondria (**Figure 2A**; FIB-SEM image; see data availability). Seven
207 uPNs were found in DA2, confirming light microscopy findings (Grabe *et al.*, 2016). Two of them
208 (PN#1, PN#2; see data availability) branched broadly and innervated the full glomerulus, and
209 received more synaptic input than the other 5 uPNs (PN#3-#7; **see Table S3**), which branched
210 exclusively in sub-regions of the glomerulus, with partial overlap. In addition to abundant clear
211 small vesicles (~20 nm in diameter) (Bates *et al.*, 2020; Strutz *et al.*, 2014; Yasuyama *et al.*, 2003),
212 uPN dendrites also displayed small electron-dense vesicles, as previously reported for PN axon
213 terminals in the mushroom body calyx (Butcher *et al.*, 2012; Yang *et al.*, 2022). These electron-
214 dense vesicles are packed with different types of neuropeptides that act as neuromodulators or
215 co-transmitters (Croset *et al.*, 2018; Eckstein *et al.*, 2020; Gondré-Lewis *et al.*, 2012; Li *et al.*, 2017).
216 In both glomeruli, uPNs had the highest neuronal synaptic input density and the lowest T-bar and
217 output density of the three neuron classes (**Table 1,** row 9-11; DA2 and DL5 differences: see next
218 section). The synaptic ratios (T-bars/input sites and output sites/input sites) were much lower for
219 uPNs than for the other neuron classes (**Table 1,** row 12-13). The majority of uPN dendritic output
220 synapses (feedback synapses) were tetrads in both glomeruli, with an average polyadicity of
221 around 5 (lower than in OSNs; (**Figure 2 – supplement 1; Table 1,** row 14).
222 The majority of the neuronal fibers in both glomeruli belonged to MGNs (**Figure 2A**). MGNs
223 exhibited variable morphology and ultrastructure, as expected, but shared also some
224 ultrastructural features. Their synaptic boutons were formed by thin fibers, thus the volume/length
225 ratio of MGNs was lower than that of OSNs but greater than that of uPNs (**Figure 2 – figure**
226 **supplement 1A**). A similar relationship was found for the number of output sites and the T-bar
227 density along MGN fibers, which were smaller than in OSNs but larger than in uPNs (**Table 1,** row
228 10-11). In contrast, branching intensity in MGNs was larger than in OSNs but smaller than in uPNs
229 (**Figure 2B**). The synaptic ratio of output-to-input sites was around one (**Table 1**, row 12-13). MGNs
230 had the lowest polyadicity (~3) of the three neuron classes (**Table 1**, row 14) and their synapses
231 were mainly triads (**Figure 2 – supplement 1D**). Interestingly, besides the abundant clear small
232 vesicles (~20 nm in diameter), some MGNs had small electron-dense vesicles, most likely housing
233 the neuropeptide sNPF (Nässel *et al.*, 2008).
234
235 **DA2 is more densely innervated and has a higher synapse density than DL5**

236 In our FIB-SEM datasets the volume of glomerulus DA2 was 45% smaller than that of
237 glomerulus DL5 (1500 $\mu m^3$ vs. 2640 $\mu m^3$), which is in agreement with measurements based on light
238 microscopy (DA2 = 1600 $\mu m^3$, DL5 = 2900 $\mu m^3$ (Grabe *et al.*, 2016). We also confirmed that a similar

239 number of OSNs (44-46 OSNs) innervated both glomeruli (**Figure 2C**), and that each glomerulus
240 received OSN innervation from both the ipsilateral and contralateral antennae (Grabe *et al.*, 2016;
241 Vosshall *et al.*, 2000). Also in agreement with (Grabe *et al.*, 2016), the DA2 glomerulus was
242 innervated by 7 uPNs whereas DL5 had a single uPN (**Figure 2C**). MGN cell numbers could not be
243 counted in our study due to their multiglomerular morphology, which also prevented us from
244 tracing MGN fibers to their soma due to our partial volume acquisition (see Methods).
245 To investigate differences between DA2 and DL5 we turned our attention to their glomerular
246 innervation and synaptic composition. We measured the total length (sum in µm) of all neuronal
247 fibers of each neuron class within the DA2 and DL5 (**Figure 2C; Table 1**, row 1). In addition, we
248 counted all T-bars and their output sites (1:1 synaptic contacts) as well as all postsynaptic sites
249 (input sites) for all neuron fibers together and for each neuron class individually (**Table 1, row 2-**
250 **4**). We counted in total ~ 14 000 synaptic contacts and 2648 T-bars in DA2 and ~ 17 000 contacts
251 and 3387 T-bars in DL5 (**Figure 2C, Table 1, row 4**). Most of these synapses were triads and tetrads
252 (**Figure 2 – figure supplement 1B-D**). In order to compare DA2 and DL5 we normalized neuronal
253 length and synaptic numbers to glomerular volume. We then analyzed (**1**) the innervation density,
254 i.e., the length of neuronal fibers per glomerular volume (µm/µm$^3$) and (**2**) the glomerular synaptic
255 density (T-bar #, output site or input site #/µm$^3$). Data are reported in total for all neuronal fibers
256 of each neuron class (**Table 1, row 5-8**) and as an average for neuronal fibers of the respective
257 neuron class **(Figure 3)**. In addition, we compared (**3**) the average polyadicity for each neuron class
258 **(Figure 3)** and **(4)** the average neuronal synaptic density (T-bar, output and input site density along
259 each neuronal fiber) (#/µm) (**Figure 3 – figure supplement 1B**).
260 We observed that the average neuron innervation density of OSNs was 20% higher in DA2
261 than in DL5 (**Figure 3A**), **Table S1**). Also the glomerular synaptic density of input sites, output sites
262 and T-bars along OSNs was significantly higher in DA2 than in DL5 (**Figure 3A**). OSNs in DA2 formed
263 therefore more input sites, and much more T-bars and output sites per glomerular volume than in
264 DL5 (**Table 1**, row 7-8; relative differences: **Table S1**). In contrast, the density of input sites
265 distributed along the length of OSN fibers was similar in DA2 and DL5, whereas T-bar and output
266 site density along the OSN axons was significantly higher in DA2 (**Figure 3 – figure supplement 1A**).
267
268 We then asked if the DA2 glomerulus, due to its higher number of uPNs, also had a higher
269 uPN innervation density and synaptic density of its postsynaptic sites and/or presynaptic sites
270 compared to the DL5 glomerulus, which contains a single uPN. In the DA2, the fibers of the 7 uPNs
271 had almost the same total length as the fibers of the single uPN in the more voluminous DL5 (4652
272 µm in DA2 vs. 5015 µm in DL5; **Table 1**, row 1). The DA2 uPNs had in addition a similar total number
273 of input sites than the single uPN in DL5 (3887 vs. 3955; **Table 1**, row 2). As such, in DA2 the total
274 innervation density of its 7 uPNs was higher as compared to the innervation density of the single
275 uPN in DL5 (**Table 1**, row 5), even though the average innervation density of DA2-uPNs was lower
276 (**Figure 3B**). The total glomerular input density of all uPNs was higher in DA2 as compared to DL5

277 (**Table 1**, row 6). On the other hand, the total glomerular synaptic density of the T-bars and output
278 sites was similar in DA2 and DL5 (**Table 1**, rows 7-8). In line with these results, the neuronal density
279 of T-bars and output sites was less in the DA2 uPNs compared to the DL5 single uPN, whereas the
280 neuronal density of input sites was similar (**Figure 3 – figure supplement 1B; Table 1**, row 9-10).
281 This caused almost twice as high synaptic ratios (T-bars-to-inputs and outputs-to-inputs) in the DL5
282 uPN relative to DA2 uPNs (**Table 1**; row 12-13).
283 We then hypothesized that DA2 will have a lower innervation density of MGNs (mainly LNs)
284 than DL5 as it had been reported that DL5 is innervated by fewer LNs (Chou *et al.*, 2010; Grabe *et*
285 *al.*, 2016). However, we observed the opposite: the innervation density of MGNs was significantly
286 higher in DA2 than in DL5 (**Figure 3C**), with slightly higher total innervation density (**Table 1,** row
287 5**).** Interestingly, only the glomerular input density was significantly higher for DA2 MGNs
288 compared to that found in DL5, not the glomerular synaptic density of output sites or of the T-bars
289 (**Figure 3C**). However, the total glomerular synaptic density of input sites, output sites and T-bars
290 were still higher in DA2 than in DL5 **(Table 1,** rows 6-8**).** Synaptic densities along the MGN fibers
291 were similar in DA2 and DL5 (**Figure 3 – supplement 1**).
292 In summary, the DA2 glomerulus is more densely innervated than DL5 and has a more
293 densely packed neuropil with more synaptic contacts relative to the DL5. The DA2 has a
294 significantly higher innervation density and higher density of T-bars, output and input sites per
295 volume (**Figure 3D**, **Table 1,** row 5-8**).** The degree of synapse polyadicity is also significantly higher
296 in DA2 than in DL5 (**Figure 3D, Table 1,** row 14) due to a shift to higher polyadicity among OSN
297 (**Figure 3A**) and MGN synapses (**Figure 3C**). OSNs show the strongest shift in polyadicity, with
298 tetrads being the most abundant synapse type in DA2 whereas triads are the most abundant in
299 DL5 OSNs (**Figure 2 – figure supplement 1B).**
300
301 **Lateralization of OSN glomerular connectivity**

302 In *Drosophila melanogaster*, the majority of olfactory glomeruli receive bilateral OSN input
303 (Silbering *et al.*, 2011; Stocker *et al.*, 1990; Stocker *et al.*, 1983; Vosshall *et al.*, 2000) see scheme
304 in **Figure 4A**). Recent studies have shown that ipsi- and contralateral OSNs are asymmetric in their
305 synaptic connectivity to other neurons in the majority of the glomeruli (Schlegel *et al.*, 2021; Tobin
306 *et al.*, 2017) and that ipsi- and contralateral OSNs activate uPNs in an asymmetric way (Gaudry *et*
307 *al.*, 2013; Tobin *et al.*, 2017). However, not all glomeruli appear to have the same degree of
308 lateralized OSN connectivity (Schlegel *et al.*, 2021). At least for one narrowly tuned glomerulus
309 (DA1), there is functional evidence that in female flies its uPNs are evenly activated by either ipsi-
310 or contralateral antennal stimulation (Agarwal *et al.*, 2011). We hypothesized that this lack of
311 lateralization could be a feature of other narrowly tuned glomeruli.
312 Ipsi- and contralateral OSNs in DA2 and DL5 were identified based on the location and
313 trajectory of their axons (**Figure 4B**). In both glomeruli, ipsilateral OSN terminals were longer than

314 their contralateral counterparts within the VOI, while polyadicity was stronger in contralateral
315 axons. Synaptic density was not consistently higher or lower in ipsilateral OSNs compared to
316 contralateral ones in DA2 and DL5 (**Figure 4 – figure supplement 1**).
317 We observed that the synaptic output of ipsi- vs. contralateral OSNs was asymmetric, with
318 significant differences in the ipsi- and contralateral OSN output to either uPNs, OSNs or MGNs
319 (**Figure 4C**, DA2 and DL5). In agreement with previous observations in other glomeruli (Schlegel *et*
320 *al.*, 2021), the output fraction to uPNs and OSNs was greater in ipsilateral OSNs than in
321 contralateral ones (**Figure 4C**, DA2 and DL5). Vice versa, the OSN output to MGNs was greater in
322 the contralateral glomerulus than in the ipsilateral side (**Figure 4C**, DA2 and DL5). However, the
323 differences between the medians and means were smaller in DA2 than in DL5 (**Figure 4C**;
324 differences between means: see data availability).
325 Our finding of less lateralized connections in the DA2 (**Figure 4C**, DA2 and DL5) was also
326 observed in another narrowly tuned glomerulus (VA1v; Dweck et al., 2015) for which connectome
327 data is available (Horne *et al.*, 2018). In VA1v, the OSN output to uPNs and MGNs was significantly
328 asymmetric in the same manner as in DA2 and DL5, i.e. with greater ipsilateral OSN output fractions
329 to uPNs and OSNs and greater contralateral OSN output fraction to MGNs (**Figure 4C**). Asymmetry
330 in the VA1v OSN output fractions was even less distinct than in DA2 (regarding both the difference
331 between the median and the mean; **Figure 4C** and data availability). In VA1v, the OSN output
332 fraction to OSNs was similar in ipsi- and contralateral OSNs (**Figure 4C**). In addition, the OSN input,
333 from either sister OSNs or MGNs, was asymmetric in DL5 but not in the narrowly tuned glomeruli
334 (**Figure 4D**). The inputs from uPNs to ipsi- or contralateral OSNs were not compared due to their
335 low numbers.
336 In summary, our data add to the knowledge of lateralized connectivity within olfactory
337 glomeruli and supports the hypothesis that narrowly tuned glomeruli have a lower degree of
338 lateralization of OSN connectivity compared to broadly tuned glomeruli.
339

340 **Glomeruli DA2 and DL5 differ in several features of their circuitry**

341 Next, we asked whether the synaptic circuitries of DA2 and DL5 differ from each other. We
342 counted each synaptic contact (**Table S2 and S3**) and categorized the distinct connection motifs
343 according to the neuron class to which the output and input neuron belonged (**Figure 5A; Table**
344 **S2).** Each connection motif (for example OSN>uPN, i.e., the OSN-to-uPN feedforward connection)
345 was then assessed for its relative synaptic strength, i.e. how many synaptic contacts of this
346 particular connection motif were found compared to the total number of synaptic contacts within
347 the respective circuitry (**Figure 5A-D**; see Methods).
348 We found that neurons from each class made synaptic contacts with each other in DA2 and
349 DL5, as previously reported for other glomeruli (Berck *et al.*, 2016; Horne *et al.*, 2018; Rybak *et al.*,
350 2016; Schlegel *et al.*, 2021; Tobin *et al.*, 2017). In both DA2 and DL5, OSNs provided the strongest

351 relative synaptic output, i.e. 49% of all synaptic connections in DA2 and 43% in DL5 were formed
352 by OSNs (**Figure 5B-C**). Thus, even though DA2 and DL5 had similar numbers of OSNs (44 and 46,
353 respectively), those in DA2 provided a stronger circuit output (14% stronger; **Table S2**) than those
354 in DL5 (**Figure 5B-C)**. In both glomeruli the main OSN output partners were MGNs and uPNs, i.e.
355 27% of all circuitry connections in DA2 and 24% in DL5 were OSN>MGN connections and 20% in
356 DA2 and 18% in DL5 were OSN>uPN connections (**Figure 5B-C**). In DA2, interestingly, each of the 7
357 uPNs received input from almost all OSNs and so could maintain a high degree of convergent signal
358 transmission (**Table S3**). In contrast, OSNs received the lowest relative input of all neuron classes
359 in DA2 and DL5 (7% and 8% respectively; **Figure 5B-C**). In line with previous observations in other
360 glomeruli (Horne *et al.*, 2018; Schlegel *et al.*, 2021), OSNs also made abundant axo-axonic synapses
361 with sister OSNs (2.6% in DA2 and 1.5% in DL5; **Figure 5B-C**). Thus, the relative synaptic strength
362 of the OSN>OSN connection was 70% stronger in DA2 than in DL5 (**Figure 5B-C; Table S2**).
363 The uPNs in both glomeruli had the weakest relative output of all neuron classes within their
364 circuitry, and this was even weaker (38%) in DA2 (**Figure 5B-C; Table S2**). In contrast, the relative
365 synaptic input onto uPNs was greater in DA2 than in DL5 (33% vs. 28%, respectively; **Figure 5B-C;**
366 16% stronger in DA2; **Table S2**), which is in line with our finding that in DA2, the uPNs provide more
367 input sites per unit of glomerular volume than in the DL5 (**Figure 3B-C**). In both glomeruli, the
368 feedback connections from uPNs (depicted in **Figure 5A**), were almost exclusively directed towards
369 MGNs, as previously reported for the broadly tuned DM6 and the narrowly tuned glomerulus VA1v
370 (Horne *et al.*, 2018; Tobin *et al.*, 2017). However, the relative synaptic strength of the uPN>MGN
371 connection was 40% weaker in DA2 than in DL5 (uPN>MGN: 10% in DA2 and 17% in DL5). Only a
372 few cases of uPN>OSN synaptic connections were observed (a total of 16 in DA2 and 26 in DL5)
373 representing a synaptic strength of 0.1% in DA2 and 0.2% in DL5 (**Table S2**). Finally, uPNs in DA2
374 also made 71 reciprocal synaptic connections (representing a synaptic strength of 0.6%; **Table S2**;
375 **Figure 5B**), consistent with electrophysiological evidence for reciprocal synaptic interactions
376 between sister uPNs (Kazama *et al.*, 2009). The single uPN of the DL5 had 54 dendro-dendritic
377 synapses (representing 0.4% of all DL5 synaptic contacts; **Figure 5C**), which were exclusively
378 autapses, i.e. synapses formed by a neuron onto itself. Dendritic uPN autapses exist also in DA2-
379 uPNs, but they were few: we observed only 14 autaptic uPN-uPN connections in DA2, which were
380 mainly located at the two longest uPN dendrites (for further analysis of autapses see next section).
381 MGNs received the strongest input in both glomeruli (60% of the total input in DA2 and 64%
382 in DL5; **Figure 5B-C**). This is in line with the observation that MGNs provided the majority of all
383 traced neuronal fibers in each glomerulus and had the highest innervation density of all neuron
384 classes; **Table 1**). The relative output strength of MGNs was similar in both glomerular circuits
385 (~40% of the total output in each glomerulus; **Figure 5B-C**). MGNs made many reciprocal synapses
386 to each other, accounting for 23% of all synapses in both glomeruli (**Figure 5B-C)**. The relative
387 synaptic strength between MGN>uPN was stronger in DA2 (12%) than DL5 (10%) (**Figure 5B-C**;

388 **Table S2**). The MGN>OSN feedback connection was relatively weak in both glomeruli (5% in DA2
389 vs. 6% in DL5; **Figure 5B-C**) but weaker (25%) in DA2 than in DL5 (**Table S2**).
390 We then looked at the fractional output and input of each neuron class (Figure 5E', E''). In
391 both glomeruli OSNs had a similar proportion of their synaptic output onto uPNs (40%-41%), onto
392 MGNs (55% in both) and onto sister OSNs (4%-5%) (**Figure 5E'**). From the uPNs perspective, over
393 93%-96% of their recurrent synaptic output was directed to MGNs in both DA2 and DL5, and few
394 synapses were directed onto OSNs (~1% of the uPN output; **Figure 5E'**). The uPN>uPN output
395 fraction of the 7 uPNs in DA2 (reciprocal synapses) was twice the uPN output fraction (autaptic) of
396 the single uPN dendrite in DL5 (6% vs. 3%; **Figure 5E'**). MGNs formed synaptic output mainly to
397 other MGNs (58%-59% of the total MGN output in DA2 and DL5). Among MGNs we found also rare
398 cases of autapses. The MGN>uPN output fraction was greater in DA2 (30%) than in DL5 (25%),
399 whereas the MGN>OSN output fraction was smaller in DA2 (12%) than in DL5 (16%; **Figure 5E'**).
400 Turning to the input fractions of each neuron class, we found that in both glomeruli, OSNs
401 received most of their input from MGNs (>50%). In DA2 the input fraction onto OSNs (MGN>OSN)
402 was smaller than in DL5 (63% vs. 78%; **Figure 5E''**). In contrast, the OSN input fraction from sister
403 OSNs was greater in DA2 (35% vs. 20%; **Figure 5E''**). In both glomeruli, the OSNs received only weak
404 uPN input (2%) (**Figure 5E''**). The input fractions onto the 7 uPNs, formed by uPNs, MGNs and OSNs,
405 in the DA2 and the single uPN in DL5 were similar (**Figure 5E''**). Most uPN input was delivered by
406 OSNs (~62% in both glomeruli) and less from MGNs (~36%). The uPN input fraction from other
407 uPNs in DA2 or the autaptic input from the single uPN in DL5 was small (2%; **Figure 5E''**). In DA2
408 the MGNs received a smaller fraction of uPN feedback input than in DL5 (17% vs. 26%; **Figure 5E''**)
409 but a greater OSN input fraction (45% vs. 38%; **Figure 5E''**). The fraction of MGN>MGN input was
410 similar in both glomeruli.
411 To further explore whether the differences in circuitry between DA2 and DL5 reported here
412 might represent features characteristic of narrowly tuned glomeruli, we analyzed connectome
413 data from another narrowly tuned glomerulus (VA1v; (Horne *et al.*, 2018). We calculated the
414 relative synaptic strength between OSNs (n=107), uPNs (n=5) and MGNs (n=74) in the VA1v (**Figure
415 5D; Table S2**). We found that the two narrowly tuned glomeruli shared five circuit features that
416 were different from the broadly tuned glomerulus DL5: (**1**), OSNs in VA1v, as reported above for
417 DA2, displayed a stronger relative feedforward output to uPNs (22%) and to MGNs (32%) (**Figure
418 5D**). The uPNs and MGNs in VA1v, received a larger fraction of OSN input than in DL5 (**Figure 5E''**).
419 (**2**), the OSN>OSN synaptic output was four times stronger (6%) than in DL5 (1.5%; **Figure 5B-D,
420 Table S1**). This was also reflected in the OSN output fraction to sister OSNs (10%), which in VA1v
421 was more than twice that of DL5 (4%; **Figure 5E'**) and in the much greater OSN input fraction (38%)
422 to OSNs in the VA1v than in DL5 (20%; **Figure 5E''**). (**3**), in the VA1v the uPN>uPN relative synaptic
423 output was more than twice that of DL5 (1% vs. 0.4% in DL5; **Figure 5D**), which is in accordance
424 with a much greater uPN output fraction to uPNs (14%) in VA1v than in DL5 (3%) (**Figure 5E'**). (**4**),
425 as observed before in DA2, VA1v uPNs had fewer feedback synapses onto MGNs than in DL5

426 (relative synaptic strength of uPN>MGN connection: 6% vs. 17%; **Figure 5C-D**), also reflected in a
427 smaller output fraction from uPNs to MGNs in VA1v than in DL5 (81% vs. 96%; **Figure 5E'**). In
428 agreement, the MGN input fraction from uPNs in VA1v was much smaller than in DL5 (10% vs. 26%;
429 **Figure 5E''**). (**5**), OSNs in VA1v received a smaller MGN input fraction than DL5 OSNs (60% vs. 78%;
430 **Figure 5E''**).
431 Besides relative differences (stronger or weaker) in DA2 and VA1v connection motifs
432 compared to DL5, two connection motifs were stronger in DA2 and DL5 than in VA1v: **(1)** the
433 MGN>uPN connection showed a synaptic strength of 12% and 10% in DA2 and DL5 vs. 8% in VA1v
434 (**Figure 5B-D, Table S2**). In agreement with this, the MGN output fraction to uPNs (**Figure 5E'**, MGN
435 output) and the MGN input fraction in uPNs was greater in DA2 and DL5 than in VA1v (**Figure 5E''**,
436 uPN input). **(2)**, the relative synaptic strength in MGN>MGN motifs was similar between DA2 and
437 DL5 (23%; **Figure 5B-C**), but weaker in VA1v (17%; **Figure 5D, Table S2**). This was also reflected in
438 a smaller MGN output and input fraction from or to MGNs (**Figure 5E' and E''**).
439 In summary, the two narrowly tuned glomerular circuits studied here shared five circuit
440 features when compared with the broadly tuned glomerular circuit (all glomerular circuit features
441 in DA2, DL5 and VA1v are shown in **Figure 6A**). These features were (1) a stronger OSN>uPN and
442 OSN>MGN connection, (2) a much stronger axo-axonic communication between sister OSNs, (3) a
443 stronger dendro-dendritic connection between uPN dendrites, (4) less feedback from uPNs to
444 MGNs and (5) less feedback from MGNs to OSNs (**Figure 6B**).
445
446 **Autapses in the large DL5 uPN connect distant regions of its dendritic tree**

447 Autapses (synapses made by a neuron upon itself) have seldomly been reported in the
448 *Drosophila* central nervous system (Horne *et al.*, 2018; Takemura *et al.*, 2015). In the DA2
449 glomerulus we found few autapses in uPNs and MGNs (**Figure 5C; Figure 7A**). In the dendritic tree
450 of the single DL5 uPN, on the other hand, three observers registered 54 autaptic connections
451 independently (see Methods). This represents 3% of the output connections of this neuron and
452 0.4% of all synaptic contacts in the whole glomerulus (**Figure 7A**; **Figure 5C; E'**). We found that
453 these autapses were not distributed evenly along the dendritic tree of the DL5 uPN. Some dendritic
454 branches received several autaptic inputs, whereas other had no autaptic input (**Figure 7A**) and we
455 hypothesized that these autapses could connect distant parts of this very large dendritic tree. We
456 thus analyzed the exact location and distribution of their presynaptic and postsynaptic sites (**Figure**
457 **7A**) We discovered a difference in the distribution of the pre- and postsynaptic elements of DL5
458 autapses. Whilst their presynaptic T-bars were evenly distributed at basal (strahler order: 5) and
459 distal regions (strahler order: 1-4), 95% of their postsynaptic sites were located in the most distal
460 region (strahler order 2-1; **Figure 7B-C**). We also calculated the geodesic distance (i.e., along-the-
461 arbor distance) from pre- and post-synaptic sites to the basal root node, which is the node point
462 where the uPN enters the glomerulus and is equivalent to the closest point to the soma in our

463 reconstruction. The geodesic distance to the basal root node from the presynaptic site was
464 significantly shorter than for postsynaptic sites (**Figure 7 – figure supplement 1B**). The pre- and
465 postsynaptic sites of each autapse were either close to each other along the dendritic tree, or
466 distant from each other (see examples in the dendrogram depicted in **Figure 7D**). Thus, the
467 geodesic distance between pre- and postsynaptic sites, (see scheme in **Figure 7E**), as well as the
468 number of branching points between pre- and postsynaptic partners, were bimodally distributed
469 (**Figure 7F-G**). Autapses that connected distant dendritic branches were more frequent than those
470 that connected close dendritic branches (**Figure 7E-G**). In summary, we found abundant autapses
471 within the uPN dendrite of DL5 and they were unevenly distributed, with many output sites located
472 in a few sub-branches connecting distal dendritic regions.

473 **DISCUSSION**

474 We hypothesized that specialized, narrowly tuned olfactory glomeruli differ in their
475 ultrastructure and microcircuitry from broadly tuned glomeruli. By comparing data obtained with
476 dense reconstructions of two narrowly tuned olfactory glomeruli with that of a broadly tuned
477 glomerulus in *Drosophila melanogaster,* we found prominent features of narrowly tuned glomeruli
478 involving synaptic composition, lateralization of sensory input and synaptic circuitry.
479

480 ## Glomerular circuit analysis: a correlative approach

481 The small size of olfactory glomeruli in *Drosophila* gave us the opportunity to reconstruct
482 and analyze the dense connectome of entire glomeruli with volume-based electron microscopy in
483 a reasonable time period. Here we developed a correlative workflow that combines transgenic
484 neuron labeling with near-infra-red-laser-branding for precise volume targeting. We then used FIB-
485 SEM (Bishop *et al.*, 2011) to resolve glomerular networks at the synaptic level. A similar procedure
486 was used recently to investigate single cellular organelles (Ronchi *et al.*, 2021). An advantage of
487 this approach is that it facilitates localization of the volume of interest with high precision and
488 consequently limits to a minimum the volume to be scanned and reconstructed. At the same time,
489 the limitation in volume is a drawback of our workflow, as it was impossible to reconstruct neurons
490 back to their soma. This fact prevented the identification of individual neurons as in other
491 connectome studies (Bates *et al.*, 2020; Berck *et al.*, 2016; Eichler *et al.*, 2017; Horne *et al.*, 2018;
492 Scheffer *et al.*, 2020; Schlegel *et al.*, 2021; Xu *et al.*, 2020; Zheng *et al.*, 2018).
493 We provide data on innervation and synapse density of olfactory sensory neurons (OSNs),
494 uniglomerular projection neurons (uPNs) and multiglomerular neurons (MGNs) in the *Drosophila*
495 antennal lobe (AL). We observed a higher innervation density of all neuron types but mainly by
496 uPNs and MGNs and in parallel higher density of synaptic contacts along OSN terminals in the
497 narrowly tuned DA2 compared with DL5. These results suggest that narrowly tuned glomeruli have
498 a more densely packed neuropil, with more numerous synaptic connections in the feedforward
499 motifs OSN>uPN and OSN>MGN. Overall, our observations on synapse density were comparable
500 with previous reports (Horne *et al.*, 2018; Mosca *et al.*, 2014; Rybak *et al.*, 2016).
501

502 ## Specific features of narrowly tuned glomerular circuits

503 Our analysis revealed circuit features in the narrowly tuned glomerulus DA2 and VA1v
504 that might be adaptations specific of such dedicated glomerular circuits. Nevertheless, future
505 studies, analyzing precise numbers of synaptic connections in more individuals, combined with
506 physiological studies and computational models are required to test this hypothesis.

507 **The OSN>uPN feedforward connection is stronger in narrowly tuned glomeruli**

508 Presynaptic OSN terminals provide the major input to uPNs in insect olfactory glomeruli
509 (Chen *et al.*, 2005; Hansson *et al.*, 2000; Horne *et al.*, 2018; Kazama *et al.*, 2008; Lei *et al.*, 2010;
510 Rybak *et al.*, 2018; Schlegel *et al.*, 2021; Tobin *et al.*, 2017). Here we showed that this connection
511 is stronger in DA2 and VA1v than in DL5 (**Figure 5 and 6**). A strong OSNs>uPN synaptic connection
512 will drive non-linear signal amplification, which improves signal detection at low odor
513 concentrations (Bhandawat *et al.*, 2007; Kazama *et al.*, 2008; Masse *et al.*, 2009; Ng *et al.*, 2002).
514 A larger number of synapses of this type could be an adaptation to improve this amplification
515 effect, as shown by artificial increase of synaptic sites in the AL (Acebes *et al.*, 2001) and in lateral
516 horn dendrites (Liu *et al.*, 2022).
517 Each of the 7 uPNs in DA2 received convergent synaptic input from almost all DA2-OSNs.
518 This is in agreement with reports on the narrowly tuned glomeruli DA1 and VA1v (Agarwal *et al.*,
519 2011; Horne *et al.*, 2018; Jeanne *et al.*, 2015) and for broadly tuned glomeruli (Chen *et al.*, 2005;
520 Kazama *et al.*, 2009; Masse *et al.*, 2009; Tobin *et al.*, 2017; Vosshall *et al.*, 2000). High OSN>uPN
521 convergence is the main driver of highly correlated activity among uPNs in pheromone coding
522 glomeruli in flies as well as moths (Kazama *et al.*, 2009; Rospars *et al.*, 2014). High convergence in
523 the lateral horn improves signal transmission from uPNs to lateral horn neurons without sacrificing
524 speed (Huoviala *et al.*, 2020; Jeanne *et al.*, 2015). In the mushroom body calyces, however, the
525 high degree of convergence is only pursued for DA2 uPNs, which converge onto few Kenyon cells,
526 whereas VA1v uPNs synapse randomly onto many dispersed Kenyon cells (Caron 2013; Zheng
527 2020; Li 2020), indicating diverse signal integration in the mushroom body.
528 From our study, we hypothesize that in narrowly tuned glomerular circuits, which have
529 more uPNs, the maintained strong OSN>uPN convergence, improve signal transmission accuracy.
530 Secondly, a stronger OSN>uPN connection might compensate for the lack of OSN>uPN signal
531 transmission sites in the case of odorants activating OSNs in a single glomerulus.

532 **Reciprocal connections between sister OSNs and sister uPNs are stronger in narrowly tuned**
533 **glomeruli**

534 The reciprocal OSN-OSN synapse is generally stronger in narrowly tuned glomeruli DA1, DL3
535 and DL4, compared to broadly tuned glomeruli DL5, DM6, DM3 and DM4 (Dweck *et al.*, 2015;
536 Ebrahim *et al.*, 2015; Grabe *et al.*, 2016; Knaden *et al.*, 2012; Schlegel *et al.*, 2021; Seki *et al.*, 2017;
537 Suh *et al.*, 2004; Tobin *et al.*, 2017). A high degree of axo-axonic synapses between sister OSNs was
538 also found in VA1v (Horne *et al.*, 2018; Schlegel *et al.*, 2021)and DA2 but not in the DL5 (this study).
539 Hence, we suggest that a strong OSN-OSN connection is a characteristic feature of the synaptic
540 circuitry of narrowly tuned olfactory glomeruli. Axo-axonic connections have also been reported
541 between gustatory and mechanosensory neurons in *Drosophila* larvae (Miroschnikow *et al.*, 2018)
542 and in the olfactory epithelium and the olfactory bulb of vertebrates (Hirata, 1964; Shepherd *et*

543 *al.*, 2021). In vertebrates, axo-axonic synapses between excitatory sensory neurons are involved in
544 correlated transmitter release (Cover *et al.*, 2021), reminiscent of correlated uPN activity due to
545 reciprocal synaptic and electric coupling in the *Drosophila* AL and LH (Huoviala *et al.*, 2020; Kazama
546 *et al.*, 2009). A strong OSN-OSN connection also has the potential to increase the correlation of
547 OSN spiking events and therefore facilitate a robust OSN signal (de la Rocha *et al.*, 2007).
548 Reciprocal dendro-dendritic synapses between sister uPNs are reported here for the DA2
549 have been reported previously also for glomeruli DM6, DM4, VA7 and VA1v (Horne *et al.*, 2018;
550 Kazama *et al.*, 2009; Rybak *et al.*, 2016; Tobin *et al.*, 2017). These types of synapses enhance uPN
551 signal correlation (Kazama *et al.*, 2009), as reported for mitral and tufted cells of the vertebrate
552 olfactory bulb, the circuit equivalent to PNs of insect ALs (Christie *et al.*, 2005; McTavish *et al.*,
553 2012; Shepherd *et al.*, 2021). In *Drosophila* multiple uPNs could induce correlated PN
554 depolarization events, which improve the signal-to-noise-ratio of PN signal transmission (Chen *et*
555 *al.*, 2005; Jeanne *et al.*, 2015; Kazama *et al.*, 2009).
556 In summary, our data give evidence that reciprocal OSN-OSN and uPN-uPN connections are
557 a prominent feature of the synaptic circuit of narrowly tuned glomeruli. We suggest that those
558 reciprocal OSN-OSN and uPN-uPN connections support correlation of neuronal activity and
559 therefore boosts signal induced depolarization events. This will in turn enhance the signal-to-noise
560 ratio (accuracy) and transmission probability of weak and/or irregular odorant input, increasing
561 processing speed.

562 **Less lateralization in the OSN bilateral connectivity in narrowly tuned glomeruli**

563 In *Drosophila*, most OSN axons project bilaterally and form synapses in their corresponding
564 glomerulus on both the left and right brain hemispheres (Couto *et al.*, 2005; Kazama *et al.*, 2009;
565 Schlegel *et al.*, 2021; Silbering *et al.*, 2011; Stocker *et al.*, 1990; Tobin *et al.*, 2017; Vosshall *et al.*,
566 2000). This is rarely observed in other insects and absent in vertebrates (Anton *et al.*, 2003; Dalal
567 *et al.*, 2020; Galizia *et al.*, 1998; Hansson *et al.*, 2000; Masson *et al.*, 1990; Parthasarathy *et al.*,
568 2013; Stocker *et al.*, 1983). In the mammalian olfactory system, bilateral comparison of olfactory
569 input only occurs in higher brain centers (Dalal *et al.*, 2020). In flies, bilateral sensory input enables
570 them to discriminate odor sources of different spatial origin through bilateral comparison of
571 olfactory stimulation (Borst *et al.*, 1982; Duistermars *et al.*, 2009; Gaudry *et al.*, 2013; Mohamed
572 *et al.*, 2019a; Taisz *et al.*, 2022). Asymmetric OSN connectivity, shown for many olfactory OSNs
573 (Schlegel *et al.*, 2021; Tobin *et al.*, 2017) seems to be the origin of a bilateral contrast in the uPN
574 response (Agarwal *et al.*, 2011; Gaudry *et al.*, 2013; Taisz *et al.*, 2022; Tobin *et al.*, 2017), and is
575 most likely the key to precise odor source localization (Taisz *et al.*, 2022). Bilateral comparison is
576 also used in the lateral horn (a higher olfactory brain center in *Drosophila*) for odorant position
577 coding (Mohamed *et al.*, 2019a). However, not all glomeruli are similar in the magnitude of
578 bilateral asymmetry with respect to their OSN connectivity (Schlegel *et al.*, 2021) or their uPN
579 responses (Agarwal *et al.*, 2011).

580 In agreement with observations in other olfactory glomeruli (Schlegel *et al.*, 2021; Tobin *et*
581 *al.*, 2017), we found that glomeruli DL5, DA2 and VA1v (data from: (Horne *et al.*, 2018) have
582 ipsilaterally asymmetric OSN synaptic output to excitatory uPNs and sister OSNs and contralaterally
583 an enhanced OSN>MGN output (**Figure 4**). We believe that, in agreement with a recent study,
584 these asymmetric connections determine a strong left-right-contrast in the uPN response, akin to
585 a "winner-takes-all" principle (Taisz *et al.*, 2022).
586 We also observed that the degree of bilateral OSN asymmetry in DA2 and VA1v was much
587 weaker than in DL5 (**Figure 4**). Weakly lateralized OSN connectivity is perhaps insufficient to induce
588 an adequate bilateral contrast necessary for odor source localization. Recent work supports this
589 idea by showing the importance of the interplay of asymmetric OSN signaling and LN inhibition to
590 enhance the bilateral contrast of uPN activity and to facilitate navigation (Taisz *et al.*, 2022).
591 Why do these narrowly tuned glomeruli have weaker bilateral contrast than broadly tuned
592 glomeruli? The answer could lie in the ecological significance of the individual odorants. Geosmin,
593 encoded by glomerulus DA2 (Stensmyr *et al.*, 2012), and the pheromone methyl laurate, encoded
594 by glomerulus VA1v (Dweck *et al.*, 2015), act at short distances, mainly when the fly is walking and
595 not flying. Perhaps, the behavioral response to geosmin or methyl laurate does not need a precise
596 odor source location. On the other hand, food odor detection at a distance, which happens mainly
597 at flying conditions, needs continuous processing of odor position and body alignment to navigate
598 towards the odor source (Demir *et al.*, 2020; Thoma *et al.*, 2015). The bilateral OSN projection onto
599 uPNs in DA2 and VA1v potentially has a distinct function other than odor position coding and could,
600 via the enhancement of the effect of convergence of OSN>uPN signal transmission, enhance odor
601 signal amplification (Bhandawat *et al.*, 2007; Jeanne *et al.*, 2015; Kazama *et al.*, 2009; Masse *et al.*,
602 2009)

603 **Distinct synaptic integration of local modulatory neurons in narrowly tuned glomeruli**

604 MGNs are composed of multiglomerular projection neurons (mPNs) that project directly to
605 the LH (Bates *et al.*, 2020; Jefferis *et al.*, 2007; Strutz *et al.*, 2014) and inhibitory and excitatory local
606 interneurons (LNs) that interconnect the AL glomeruli (Chou *et al.*, 2010; Liu *et al.*, 2013; Masse *et*
607 *al.*, 2009; Okada *et al.*, 2009; Seki *et al.*, 2010). Since LNs are the most numerous and broadly
608 arborizing of the multiglomerular cell types in the AL (Chou *et al.*, 2010; Lin *et al.*, 2012), we focus
609 our discussion on these. Multiglomerular LNs are crucial for the modulation of the OSN>uPN signal
610 transmission (Chou *et al.*, 2010; Galizia, 2014; Masse *et al.*, 2009; Seki *et al.*, 2010; Szyszka *et al.*,
611 2015).
612 Previous observations have shown that glomeruli DA2 and VA1v have a lower number of
613 innervating LNs (Chou *et al.*, 2010; Grabe *et al.*, 2016) and receive less global interglomerular LN
614 inhibition than broadly tuned glomeruli (Hong *et al.*, 2015). We therefore assumed that DA2 or
615 VA1v would have a lower LN innervation density and less LN synaptic integration in their circuitry.
616 However, we did not observe a general lower synaptic integration in DA2 (**Figure 5**) and found a

617 greater MGN innervation density, and a higher density of input sites than in DL5. VA1v MGNs on
618 the other hand received less synaptic input and provided less output in its glomerular circuit than
619 MGNs in DL5.

620 Taking a closer look at particular synaptic connection motifs of MGNs we saw that narrowly
621 tuned glomeruli had a relatively weak uPN>MGN feedback (**Figure 6**). uPN feedback onto LNs and
622 their reciprocal connection (LN>uPN) were reported in *Drosophila* and other insects, such as honey
623 bees, cockroaches and moths, but their function is still poorly understood (Boeckh *et al.*, 1993;
624 Sachse *et al.*, 2002; Sun *et al.*, 1997). In the honey bee reciprocal dendro-dendritic synapses
625 between excitatory and inhibitory neurons enhance signal contrast and the reliability of true signal
626 representations throughout the AL (Sachse *et al.*, 2002; Yokoi *et al.*, 1995). Here we could not
627 differentiate the LN types involved in the uPN>MGN synaptic motif. However, the prevailing
628 uPN>LN synapses involve mainly widespread pan-glomerular LNs in the adult (Horne et al., 2018)
629 and larval AL (Berck et al., 2016), which are important for combinatorial coding (Galizia, 2014;
630 Sachse *et al.*, 2016). Thus, weaker uPN>MGN feedback in the narrowly tuned DA2 and VA1v circuits
631 might be a compensatory mechanism to lower the computational demand of interglomerular
632 communication for odor identity coding.

633 We also observed that OSNs received less MGN in the narrowly tuned DA2 and VA1v than
634 in the DL5, suggesting that the OSNs in DA2 and VA1v receive relatively weak presynaptic
635 inhibition. Pan-glomerular GABAergic LNs induce presynaptic inhibition at OSN presynaptic site
636 (Berck *et al.*, 2016; Schlegel *et al.*, 2021). These inhibitory LNs are drivers of balanced glomerular
637 gain control and are a key player for odor identity coding, balancing incoming and alternating odor
638 intensities (Asahina *et al.*, 2009; Galizia, 2014; Hong *et al.*, 2015; Olsen *et al.*, 2008; Root *et al.*,
639 2008; Sachse *et al.*, 2016; Silbering *et al.*, 2008; Szyszka *et al.*, 2015; Wang, 2012). Our data support
640 these observations and provide an argument for why narrowly tuned OSNs receive much lower
641 inhibition during AL stimulation with odorants activating other OSN populations (Hong *et al.*, 2015).
642 Even though DA2 and VA1v might receive less interglomerular inhibition, their OSN>MGN output
643 is still strong, in agreement with studies showing that throughout the AL, global lateral inhibition
644 mediated by LNs scales with general OSN activation (Hong *et al.*, 2015; Olsen *et al.*, 2008).

645 In summary, narrowly tuned circuits are probably influenced more strongly by
646 intraglomerular than by interglomerular modulation. Narrowly tuned circuits perhaps have greater
647 computational capacities in intraglomerular modulation of signal transmission, which could be
648 important for example for PN fine-tuning and response adjustment (Assisi *et al.*, 2012; Ng *et al.*,
649 2002).

650 Above we discussed putative generic features of narrowly tuned glomerular circuits.
651 Besides these circuit features, we found a strong MGN>MGN connection in the aversive glomerular
652 circuits DA2 and DL5 in contrast to a much weaker MGN>MGN connection in the attractive
653 glomerulus VA1v (Dweck *et al.*, 2015; Knaden *et al.*, 2014; Knaden *et al.*, 2012; Mohamed *et al.*,
654 2019b; Stensmyr *et al.*, 2012). Why do aversive olfactory circuits have a stronger MGN>MGN

655 connection than attractive circuits? In the larval *Drosophila* AL, reciprocal LN>LN synapses induce
656 disinhibition induced by a strong connection between the pan-glomerular LNs and a bilateral
657 projecting LN, the Keystone LN, which synapses strongly onto pan-glomerular LNs and selectively
658 onto OSNs, which are activated by attractive food odors. This is thought to be a key feature to
659 switch from homogenous to heterogeneous presynaptic inhibition and therefore to a selective gain
660 control enhancing contrast between attractive and aversive odor activation (Berck *et al.*, 2016).
661 Such balanced inhibitory systems could also be present in the adult *Drosophila* AL, reflected in the
662 strong LN>LN connection in DA2 and DL5. Disinhibition of interglomerular presynaptic inhibition in
663 aversive glomeruli circuits might be important for the fly to stay vigilant to aversive odors, while
664 perceiving attractive cues, for example during feeding conditions so that a fast switch in behavior
665 can be initiated if necessary.
666

667 **Autaptic connection within the dendritic tree of a single uPN**

668 We observed autapses along the large dendritic tree of the single DL5-uPN. To our
669 knowledge, this is the first report of bulk dendro-dendritic autapses in the *Drosophila* olfactory
670 system, indicating a cell-type specific occurrence of autapses in the DL5-uPN as reported for other
671 cell types in the optic lobe (Takemura et al., 2015). Autapses are also reported to be present at
672 different frequencies in different types of neurons in the mammalian brain (Bacci *et al.*, 2006;
673 Bekkers, 1998; Bekkers, 2009; Ikeda *et al.*, 2006; Saada *et al.*, 2009; Tamás *et al.*, 1997; Van der
674 Loos *et al.*, 1972). In *Drosophila,* most uPNs are cholinergic (Croset *et al.*, 2018; Kazama *et al.*, 2008;
675 Tanaka *et al.*, 2012; Yasuyama *et al.*, 2003; Yasuyama *et al.*, 1999) and the DL5-uPN autapses
676 reported here might activate either nicotinic or muscarinic acetylcholine postsynaptic receptors**.**
677 Muscarinic acetylcholine receptors have an inhibitory effect in the Kenyon cells of the mushroom
678 body (Bielopolski *et al.*, 2019), but mediate excitation in the AL (Rozenfeld *et al.*, 2019)**.**
679 What could be the function of these autaptic feedback loops within the DL5-uPN dendritic
680 tree? Recent studies in vertebrates show that excitatory autapses enhance neuron bursting and
681 excitability (Guo *et al.*, 2016; Wiles *et al.*, 2017; Yin *et al.*, 2018). Autaptic inhibitory connections
682 have been implicated in circuit synchronization, spike-timing precision, self-stabilization of
683 neuronal circuits and feedback inhibition (Bacci *et al.*, 2006; Bekkers, 1998; Ikeda *et al.*; Saada *et*
684 *al.*, 2009; Tamás *et al.*, 1997; Van der Loos *et al.*, 1972).
685 Autapses in the DL5 uPN form mainly long-distance feedback loops, connecting distinct
686 dendritic subtrees and the basal dendrite region (closer to the soma) with distal branches. This
687 spatial segregation is similar to the distribution of non-autaptic pre- and postsynaptic sites in
688 *Drosophila* uPNs, where presynapses are located more frequently at basal dendrites than
689 postsynapses (Rybak *et al.*, 2016) and other insects, such as *Periplaneta americana* and moths (Lei
690 *et al.*, 2010; Malun, 1991; Sun *et al.*, 1997). Dendro-dendritic autaptic feedback loops connecting
691 basal to distal branches and distinct dendritic subtrees of a large dendritic tree might facilitate

692 activity correlation between distant dendritic subunits, as described for non-autaptic, reciprocal
693 uPN>uPN connections (Kazama *et al.*, 2009). This could be important in a large compartmentalized
694 dendrite that receives inhomogeneous excitation by several OSNs at distinct dendritic sites, in
695 order to enhance synchronized depolarization events along the dendrite, supporting signal
696 integration (Graubard *et al.*, 1980; Tran-Van-Minh *et al.*, 2015). Clustered autapses could mediate
697 local signal input amplification for distinct dendritic subunits (Kumar *et al.*, 2018; Liu *et al.*, 2022).
698 Autaptic contacts, finally, could be able to shift the uPN membrane depolarization towards the
699 spiking threshold, and enhance the firing probability during activation.
700 In conclusion, we provide a comprehensive comparative analysis of the ultrastructure and
701 synaptic circuitry of two functionally diverse olfactory glomeruli with distinct computational
702 demands, processing either single odorant information in a dedicated olfactory pathway (DA2) or
703 input regarding several odorants and taking part in combinatory coding across distributed
704 glomeruli (DL5). Our work provides an opportunity to gain insight into variations in network
705 architecture and provides fundamental knowledge for future understanding of glomerular
706 processing. By comparing our data with those from another narrowly tuned glomerulus (VA1v), we
707 distilled prominent circuit features that suggest that narrowly tuned glomerular circuits encode
708 odor signals with a weaker left-right-contrast, improved accuracy, stronger signal amplification and
709 stronger intraglomerular signal modulation relative to broadly tuned glomeruli. Our findings reveal
710 the existence of autapses in olfactory glomeruli and indicate that dendro-dendritic autapses play
711 an important role in dendritic signal integration.

712 **MATERIAL AND METHODS:**

713 **Fly line and fly rearing**

714 Flies of the genotype *Orco-GAL4; UAS-GCaMP6s* (Vosshall *et al.*, 2000) were obtained from the
715 Bloomington *Drosophila* Stock Center (https://bdsc.indiana.edu) and reared on standard
716 *Drosophila* food at 25°C and 70% humidity on a 12 h:12 h day:night cycle. Seven-days old female
717 flies were used. In these flies, Orco-positive olfactory sensory cells emit green fluorescence, making
718 possible to identify individual glomeruli.

719

720 **Brain dissection and fixation for Focus Ion Beam Microscopy-Scanning electron microscopy (FIB-**
721 **SEM)**

722 Two 7-day old female flies were anesthetized with nitric oxide (with Sleeper TAS; INJECT+MATIC,
723 Switzerland) and decapitated with forceps. Heads were dipped for one minute in 0.05% Triton X-
724 100 in 0.1M Sorensen's phosphate buffer, pH 7.3 and transferred to a droplet of freshly prepared
725 ice-cooled fixative (2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1M Sørensen's
726 phosphate buffer, pH 7.3; as in (Karnovsky, 1965). The proboscis was removed and the back of the
727 head was opened to improve fixative penetration. After 5-10 minutes, the brain was dissected out
728 of the head capsule and post-fixed for two hours on ice. Fixation was stopped by rinsing the brain
729 several times in ice-cooled 0.1M Sørensen's phosphate buffer, pH 7.3 (after (Rybak *et al.*, 2016)).

730

731 **Laser branding of glomeruli for identification during FIB-SEM microscopy**

732 To identify the glomeruli of interest at the ultrastructural level and to limit to a minimum the
733 volume of tissue to be scanned with FIB-SEM, near-infrared laser branding (NIRB,(Bishop *et al.*,
734 2011). Glomeruli of interest were first located with light microscopy in brains of *Orco-GAL4; UAS-*
735 *GCaMP6s* flies using a confocal microscope (ZEISS LSM 710 NLO, Carl Zeiss, Jena, Germany), a 40x
736 water immersion objective (W Plan-Apochromat 40x/1.0 DIC VIS-IR, Carl Zeiss, Jena, Germany), a
737 laser wavelength of 925 nm at 30% laser power and ZEN software (Carl Zeiss, Jena, Germany). Once
738 glomeruli DA2 or DL5 were identified by means of location, shape and size the volume of interest
739 (VOI) was tagged with fiducial marks ("laser-branded") close to the borders of the glomerulus
740 (**Figure 1A-B**), using an infrared Chameleon Ultra diode-pumped laser (Coherent, Santa Clara, USA)

741 at wavelength 800 nm and at 75-90% of laser power). Two laser scan rounds were performed for
742 each induced fiducial brand. DA2 (right AL) and DL5 (left AL) were laser-branded in the same fly. A
743 second glomerulus DA2 was marked in the right AL of another fly.
744

745 **Transmission Electron Microscopy**

746 Brains were rinsed with 2.5% sodium-cacodylate buffer and post-fixed in 1% osmium tetroxide and
747 1% potassium ferrocyanide in cacodylate buffer for 2 hours. After rinsing with cacodylate buffer
748 the brains were dehydrated with a graded acetone series (30%-100% acetone), including an
749 additional *en bloc* staining step in-between, in which the brains were incubated in 1% uranyl
750 acetate in 50% acetone for 30 minutes in the dark, and gradually infiltrated with Araldite (glycerol-
751 based aromatic epoxy resins; Serva, Germany). In the final step, the tissue was embedded in pure
752 resin and left in a 60°C incubator to polymerize for 48h. Resin blocks were trimmed with a Reichert
753 UltraTrim microtome (Leica, Deer Park, USA) and the fiducial laser marks were then located in
754 semi-thin sections. To check tissue quality before performing high-resolution volume-based
755 electron microscopy, serial sections 50 nm in thickness were cut with a diamond knife (Ultra 45°,
756 Diatome, Switzerland) on a Reichert Ultracut S ultramicrotome (Leica, Deer Park, Germany),
757 collected on single slot grids (2 x 1 mm), and imaged with a JEM 1400 electron microscope (Jeol,
758 Freising, Germany) operated at 80 kV. Digital micrographs were obtained with a Gatan Orius SC
759 1000 CCD camera (Gatan Orius SC 1000; Gatan, Pleasanton, USA) controlled with the Gatan
760 Microscopy Suite software Vers. 2.31.734.0.
761 .

762 **Focused Ion Beam-Scanning Electron Microscopy (FIB-SEM)**

763 Before serial Focused Ion Beam milling and Scanning Electron Microscopy imaging (FIB-SEM; (Knott
764 *et al.*, 2008; Xu *et al.*, 2017), the surface of the trimmed block was coated with a conductive carbon
765 layer to prevent charging artifacts. A FEI Helios NanoLab G3 UC (FEI, Hilsboro, USA) was used for
766 FIB-SEM process. The laser marks used to landmark the VOI were visible across the surface of the
767 block. The VOI surface was protected via a local deposition of platinum using a gas injection system
768 for subsequent ion and electron beam deposition. The material surrounding the VOI at the front
769 and the side was removed to reduce re-deposition of material during FIB-SEM. Serial images across

770 the entire VOI were generated by repeated cycles of milling slices orthogonal to the block surface
771 via FIB and imaging via SEM the newly exposed surface. The tissue was milled with a focused beam
772 of gallium ions using FEI's Tomahawk ion column (accelerating voltage: 30 kV, beam current: 790
773 pA, milling steps: 20 nm). FEI's Elstar electron column was used to create the backscattered
774 electron contrast images using an In-Column Detector (accelerating voltage. 3kV; 1.6 nA; dwell
775 time: 10 µs). The DA2 and DL5 volumes in the first fly were imaged with a resolution of 4.9 x 4.9 x
776 20 $nm^3$/vox (DA2: 769 images with 4096 x 3536 pix; DL5: 976 images with 5218 x 3303 pix). The
777 volume of a second DA2 in a second fly was imaged with a resolution of 4.4 x 4.4 x 20 $nm^3$/vox (571
778 images with 4096 x 3536 pix). The milling/imaging cycles were controlled with the Auto Slice and
779 View 4.0 software (FEI, Hilsboro, USA).
780
781 **Image alignment, 3D reconstruction and segmentation**
782 FIB-SEM image stacks were aligned by maximizing the Pearson correlation coefficient of the central
783 part of two consecutive images using template matching from the openCV library
784 (https://opencv.org). Dense reconstructions of the glomeruli were produced by manually tracing
785 all neuronal fibers and by annotating all synapses within the two glomeruli, using a skeleton-based
786 reconstruction procedure similar to previous approaches (Berck *et al.*, 2016; Schneider-Mizell *et*
787 *al.*, 2016; Zheng *et al.*, 2017). Up to five independent tracers and two reviewers participated in an
788 iterative reconstruction process using the web-based reconstruction software CATMAID
789 (http://www.catmaid.org; RRID:SCR_006278; (Saalfeld *et al.*, 2009; Schneider-Mizell *et al.*, 2016);
790 **Figure 1D, Figure 1 – video 1**), performing a dense reconstruction of synaptic neuropil. In a second
791 fly, neurons of a DA2 glomerulus were manually reconstructed with the volume-based
792 reconstruction method TrakEM2 (Cardona *et al.*, 2012), an ImageJ (Fiji) plugin
793 (https://imagej.net/TrakEM2).
794
795 **Neuron visualization**
796 Reconstructed neurons were visualized using CATMAID 3D visualization (http://www.catmaid.org
797 and using Blender 3D, an open-source 3D software (https://www.blender.org/; **Figure 7 – figure**
798 **supplement 1**). Neuron data from CATMAID were imported and shaded by Strahler order using an

799 existing CATMAID plugin for Blender (https://github.com/schlegelp/CATMAID-to-Blender; Schlegel
800 et al., 2016). Volume-based reconstructions were visualized as surface shapes in CATMAID
801 imported from TrakEM2 (https://imagej.net/TrakEM2).
802
803 **Glomerular border definition**
804 The definition of the boundary between olfactory glomeruli was based on the combination of
805 several structural features: the spatial position of pre- and postsynaptic elements along OSN axons,
806 the position of the majority of uPN postsynaptic sites, the faint glial leaflets scattered at the
807 periphery of the glomerulus, and the fiducial laser marks (**Figure 1B, D**).
808
809 **Neuron identification**
810 Neuronal fibers were assigned to one of three pre-defined neuron classes: OSNs, uPNs, and MGNs.
811 The classification was based on their 3D shape (**Figure 2A**), their branching intensity (**Figure 2B**),
812 the average diameter of their fibers (neuronal profiles: **Figure 2A -** FIB-SEM image; exemplary
813 volume-based reconstruction), the ratio of T-bars-to-input sites and the size of their T-bars, which
814 were either "small" (few postsynaptic connections) or "large" (many postsynaptic connections
815 **Figure 2 – supplement 1B-D)**. In addition, several intracellular features helped to classify neuron
816 classes: the shape and appearance of mitochondria, the size and electron density of vesicles and
817 the amount of synaptic spinules (small filopodia-like invaginations of neighboring cells (**Figure 2A**
818 **-** FIB-SEM image; (Gruber *et al.*, 2018). OSNs and uPNs could be counted, due to their uniglomerular
819 character, by means of the identification of the axons (OSNs) or main dendrites (uPNs) entering
820 the glomerulus. The number of MGNs could not be counted because of their pan-glomerular
821 projection patterns in the AL. Ipsi- and contralateral OSNs in DA2 and DL5 were identified based
822 on the trajectory of axonal fibers and their entry location in each glomerulus, (example neurons:
823 **Figure 4B).** Ipsilateral OSNs reach the glomerulus from the ipsilateral antennal nerve and leave the
824 glomerulus towards the antennal lobe commissure (ALC: (Tanaka *et al.*, 2012). Contralateral OSNs
825 reach the glomerulus projecting from the ALC.
826
827 **Data analysis**

With the aid of the web-based software CATMAID (http://www.catmaid.org) the following properties were quantified: glomerular volume, neuronal fiber length (in µm), number of fiber branching points, number of synaptic input and output sites and T-bars (see data availability). In a second fly, the volume of neurons in DA2 was measured (**Figure 2 – supplement 1A**) with the aid of TrakEM2 (Cardona *et al.*, 2012), an ImageJ (Fiji) plugin (https://imagej.net/TrakEM2). The following calculations were performed:

1. $Innervation\ density = \frac{total\ neuron\ length\ (\mu m)}{glomerular\ volume\ (\mu m^3)}$,
    a. calculated as a ratio: (1) the sum of all neuronal fibers of each neuron class or (2) all together (**Table 1**) or (3) for each neuron individually (**Figure 3**)
2. $Glomerular\ synaptic\ density = \frac{\#\ of\ synaptic\ inputs, -\ outputs\ or\ T-bars}{glomerular\ volume\ (\mu m^3)}$,
    a. calculated as a ratio: (1) the sum of all neuronal fibers of each neuron class or (2) all together (**Table 1**) or (3) for each neuron individually (**Figure 3**)
3. $Neuronal\ synaptic\ density = \frac{\#\ of\ synaptic\ inputs -, outputs\ or\ T-bars}{neuronal\ fiber\ length\ (\mu m)}$ (**Table 1; Figure 3 – figure supplement 1**)
4. $Synaptic\ ratios = \frac{\#\ of\ T-bars\ or\ outputs}{inputs}$ (represents the average for each neuron class; **Table 1**)
5. $Polyadicity = \frac{\#\ of\ outputs}{T-bars}$ (represents the average number of postsynaptic sites at a T-bar of each neuron class; **Table 1 and Figure 1E**)
6. $\text{Relative differences} = \frac{respective\ value\ target\ glomerulus - value\ \ source\ \ glomerulus}{source\ glomerulus} \times 100$ (**Table S1; Table S2**)
7. $\text{Relative synaptic strength} = \frac{\#\ of\ synaptic\ contacts\ from\ neuron\ class\ A\ to\ B}{\#\ all\ synaptic\ contacts\ in\ corresponding\ glomerulus}$ (**Table S1; Table S2**)
8. $\text{Fraction of output} = \frac{\#\ of\ outputs\ of\ neuron\ class\ A\ directed\ to\ neuron\ class\ B}{total\ \#\ of outputs\ of\ neuron\ class\ A} \times 100$
9. $\text{Fraction of input} = \frac{\#\ of\ \ inputs\ from\ neuron\ class\ A\ from\ class\ B}{total\ \#\ of\ inputs\ of\ neuron\ class\ A} \times 100$

Graphs were made with the programming language R and RStudio (R Core Team, 2018) using the packages 'ggplot2' and 'reshape' (see data availability) or with Python (see data availability). EM

854 and fluorescence images were visualized with ImageJ (Fiji) (http://fiji.sc/; (Schindelin *et al.*, 2012)
855 (and All figures were compiled with Adobe Illustrator CS5 software (Adobe Inc.).
856 Statistical analysis was performed with R Studio (R Studio Team, 2016) using the packages
857 'ggsignif'. Differences between samples DA2 and DL5 or between ipsilateral and contralateral OSNs
858 were tested for significance with a two-sided student's t-test if sample size was normally
859 distributed, or with Wilcoxon two sample test if the data was not normally distributed (noted in
860 figure legend). Data is in all cases represented as mean + standard deviation.
861

862 **Analysis of autapses**

863 The location of autapses, the measurement of their geodesic distance (distance along the neuronal
864 dendrite) and the number of branching points from point A (presynaptic site) to B (postsynaptic
865 profile) was analyzed with Python using the package 'neuroboom'
866 https://github.com/markuspleijzier/neuroboom (see also data availability).
867

868 **Data availability**

869 Datasets will be available through the public CATMAID instance:
870 https://catmaid.ice.mpg.de/catmaid_2020.02.15/. Neurons are named according to their neuron
871 classification. The neuroboom Python package was used for dendrogram analysis, available at
872 https://github.com/markuspleijzier/neuroboom and https://pypi.org/project/neuroboom/.
873 Further newly generated source codes can be made available upon request. Source data files are
874 provided (see source data files).
875

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1405 **FIGURES**

1406 **Figure 1: A correlative approach to analyze the ultrastructure of identified olfactory glomeruli**
1407 **A-B:** Two-photon laser scans of the antennal lobes in *Orco-Gal4; UAS-GCaMP6s* flies where Orco-
1408 positive olfactory sensory neurons (OSNs) in the glomerular neuropils were labeled by GCaMP
1409 (green fluorescence). Glomeruli DA2 (A) and DL5 (B) are encircled. Schematics show their relative
1410 position in the antennal lobe. Once the glomeruli of interest were identified, glomerular borders
1411 were marked with fiducial marks (white triangles) via laser branding, which enabled their
1412 identification at the ultrastructural level. **C-D**: Representative images of the same glomeruli (DA2
1413 in **C** and DL5 in **D**) obtained with focused-ion-beam electron microscopy (FIB-SEM), showing their
1414 ultrastructure. Asterisks indicate the main neurite of uniglomerular projection neurons entering
1415 the glomerulus. White triangle shows a 2-photon laser mark (see also **A** and **B**). **E**: FIB-SEM image
1416 of a polyadic synapse: the presynaptic site (red arrowhead) is composed of a T-bar shaped
1417 presynaptic density surrounded by small vesicles and is opposed by several postsynaptic profiles
1418 (cyan dots). Scheme of a tetrad synapse: a presynaptic site with its T-bar (red arrowhead) forms
1419 four output connections (arrows) with four postsynaptic input sites (cyan dots). **F:** A skeleton-based
1420 reconstruction of an OSN axon terminal (green line) with presynaptic (red dots) and postsynaptic
1421 sites (cyan dots). The dark grey shading surrounding the OSN trace represents the volume-based
1422 reconstruction of the same neuron. Tracing and reconstruction were performed within the FIB-
1423 SEM dataset (light grey area).
1424

1425 **Figure 2: Neuron classification and neuronal composition of the DA2 and DL5 glomeruli**
1426 **A**: Example FIB-SEM images (left column), volumetric neuronal reconstructions (middle column),
1427 and skeleton-based neuron traces (right column) of a representative example of each neuron class:
1428 OSNs (green), uniglomerular projection neurons (uPNs, red) and multiglomerular neurons (MGNs,
1429 blue). The ultrastructure of neurons, including T-bars (black triangle), mitochondria (asterisks) and
1430 spinules (white triangle) are indicated. Exemplar volumetric reconstructions (middle column) show
1431 the general morphology of each neuron class. Presynapses and postsynapses are indicated with
1432 red and cyan dots on the skeleton traces (right column). **B:** Average branching intensity (branching
1433 points per µm of neuronal-fiber length) of each neuron class OSNs, uPNs and MGNs in DA2 and
1434 DL5. Data represent mean+ standard deviation (error bars). Data points represent single values.
1435 Means were compared using Wilcoxon two-sample test. No significant differences of branching
1436 points/µm in OSNs or MGNs between glomeruli were found (significance was not tested for uPNs
1437 due to the presence of a single uPN in DL5). **C**: Schematic summary indicating, for each glomerulus,
1438 its volume (in $\mu m^3$), the number of neurons of each class (MGNs were not counted), the total fiber
1439 length of all neurons for each neuron class and the total number of single synaptic contacts for
1440 each glomerulus.
1441

1442 **Table 1. Glomerular innervation and synaptic composition**
1443 Quantitative neuronal data comparing glomeruli DA2 and DL5, detailing glomerular innervation
1444 and synaptic properties for each neuronal class: OSNs (green), uPNs (red) and MGNs (blue) and the
1445 sum of all of them. **Row 1**: Total length of all neurons of each neuron class and total length for all
1446 neurons in each glomerulus. **Row 2-4**: Synaptic counts: input sites (inputs), output sites (outputs)

1447 and T-bars (T-bars). **Row 5**: Innervation density: total neuron length (µm; row 1)/glomerular
1448 volume (µm$^3$); glomerular volume: DA2=1500 µm$^3$ and DL5=2600 µm$^3$ (see Figure 1C). **Row 6-8**:
1449 Total synaptic density per unit of glomerular volume (µm$^3$): sum of all input sites (inputs), output
1450 sites (outputs) and T-bars of each neuron class or of all neurons/glomerular volume. **Row 9-11**:
1451 Average synaptic density along neuronal fibers (illustrated also in Figure 3 – supplement 1):
1452 number of inputs, outputs or T-bars/neuron length (µm). **Row 12-13:** Average synaptic ratios: the
1453 ratio of T-bars-to-inputs or outputs-to-inputs. **Row 14:** Polyadicity: the average number of
1454 postsynaptic sites at each T-bar in DA2 and DL5. The ratios in rows 12-14 were calculated based on
1455 synaptic counts normalized to neuron length (rows 9-11). The color shading highlights values that
1456 have a relative difference greater than 20% (see relative differences Table S1) between DA2 and
1457 DL5. Dark shades highlights values that are greater in DA2 than in DL5 (green (OSNs), red (uPNs),
1458 blue (MGNs)) and light colors highlight values that are less in DA2 than in DL5.
1459

1460 **Figure 3: Innervation density and synaptic density in DA2 and DL5**
1461 **A-E**: The average glomerular innervation density of OSNs (**A**), uPNs (**B**), MGNs (**C**) and collectively
1462 of all glomerular neurons (**D**); the average synaptic density of input sites (inputs), output sites
1463 (outputs) and T-bars and the average polyadicity. Innervation density: length (µm) of each
1464 neuronal fiber normalized to one µm$^3$ of glomerular (glom.) volume. Synaptic density: number of
1465 input sites, output sites or T-bars of each neuronal fiber normalized to one µm$^3$ of glomerular
1466 volume. Polyadicity: average number of single output sites per T-bar in each neuronal fiber. Data
1467 for DA2 shown in dark colors and for DL5 in light colors. Number of neurons in DA2: OSNs (green)
1468 n= 44; uPNs (red) n= 7; MGNs (blue) n=180; all neurons n=231, in DL5: OSNs n=46; uPN n=1; MGNs
1469 n=221; all neurons n=268. Data represent mean + standard deviation (error bars). Data points
1470 represent single values. Means were compared using either Student's t-test (OSNs) or Wilcoxon
1471 two-sample test (MGNs and all neurons). uPNs were not compared, since the DL5 has only one.
1472 Significance value: $p>0.05$ (not significant, no star), $p\leq0.05$ (*), $p\leq0.01$ (**), $p\leq0.001$ (***). Values
1473 are provided at data availability; polyadicity values are listed in Table 1, row 14.
1474

1475 **Figure 4: Lateralization of OSN terminals in the antennal lobes**
1476 **A**: Illustration of an ipsilateral (dark green) and a contralateral (light green) OSN with dendrites in
1477 the corresponding antennae and their axonal projections to the ipsilateral olfactory glomerulus in
1478 the antennal lobe (AL) (dashed rectangle). **B**: Exemplary skeleton traces of an ipsilateral (dark
1479 green) and a contralateral (light green) OSN terminal inside glomerulus DA2. The ipsilateral OSN
1480 axons reach the glomerulus via the ipsilateral antennal nerve (arrow down) and leave the
1481 glomerulus towards the AL commissure (arrow up) while OSN axons originating at the contralateral
1482 antenna reach the glomerulus via the AL commissure. Red dots: presynapses; blue dots:
1483 postsynapses. **C**: Boxplots showing the fraction of synaptic output to uPNs (in red), - to OSNs (in
1484 green) or - toMGNs (in blue), , for the ipsilateral OSNs (dark green boxplot) and contralateral OSNs
1485 (light green), respectively, in the DA2, DL5 and VA1v glomeruli (Horne *et al.*, 2018). **D**: Boxplots
1486 showing the fraction of synaptic input of the same ipsilateral and contralateral OSNs that they
1487 receive from OSNs and MGNs. Connection polarity is indicated by arrows in the schematic neuronal
1488 drawings on the left of each plot. Dots represent single values. Means were compared using

Student's T-test. Significance value: $p>0.05$ (not significant, no star)), $p\leq0.05$ (*), $p\leq0.01$ (**), $p\leq0.001$ (***). Mean and Median values are provided at data availability.

**Figure 5: Strength of synaptic connections between neuron classes in the circuitry of DA2, DL5 and VA1v.**

**A:** Schematic representation of principal connection motifs between the neuron classes OSNs (green), uPNs (red) and MGNs (blue). The synaptic flow directed towards uPNs is a feedforward and that directed towards OSNs or from uPNs to MGNs defined as a feedback connection (arrows). **B-D**: Alluvial diagrams of the glomerular circuitry in DA2 (**B**), DL5 (**C**) and VA1v (**D**). Each diagram shows the relative synaptic strength calculated as the proportion of 1:1 single synaptic contacts between each neuron class in relation to the total number of synaptic contacts in their respective glomerulus. The synaptic strength between each neuron class, given as percentage, is indicated by the thickness of the lines. The proportions (as percentage) of output (left side) or input (right side) are illustrated by colored rectangles to the left or right of each alluvial diagram. The total number of synaptic contacts is indicated below the diagrams. Percentages of the relative synaptic strength and synaptic counts are listed in the supplementary Table S1. **E:** Stacked bar charts depict output (E') and input (E'') fractions (given as percentages) of each neuron class: OSNs (green), uPNs (red), MGNs (blue), schematically illustrated next to the bar charts respectively, to each of the other neuron classes for glomeruli DA2, DL5 and VA1v. Fractions are color-coded according to the neuron class of the respective connecting partner.

**Figure 6: Differences in connectivity strength in glomeruli DA2, DL5 and VA1v**

**A**: Schematic representation of synaptic connection motifs (arrows) between OSNs (green), uPNs (red), and MGNs (blue) in glomeruli DA2, DL5 and VA1v. The number of neurons of each class or truncated neuronal fibers (in brackets) is noted in the corresponding circle. **B**: Schematics of connection motifs (left) that are jointly stronger or weaker in DA2 and VA1v than in DL5. The relative differences (as percentage) between DA2 and DL5 as well as VA1v and DL5 are illustrated as arrows up (stronger) or arrows down (weaker) according to their intensity (see legend at the bottom) from the perspective of the target glomerulus (defined in the table header). The values of relative differences are listed in the Table S2.

**Figure 7: Distribution of pre- and postsynaptic partners of autapses in the uPN dendrite of the DL5**

**A**: Distribution of autaptic presynaptic (red dots) and postsynaptic sites (cyan dots) mapped in a dendrogram of the dendrite of the single uPN in glomerulus DL5. The basal root node (black dot) represents the entry site of the uPN dendrite into the glomerulus (closest point to its soma). Clustering of autaptic input sites along some branches are encircled. **B**: Simplified representation of the uPN's dendrogram illustrating the distinct strahler orders, at distal branches (1-4) and at basal branches (5-8); see legend on the right). **C**: Distribution of autaptic presynaptic (left) and postsynaptic input sites (right) along the dendrite, as proportions at each corresponding strahler order (color coded). Note that autaptic postsynaptic sites are located almost exclusively at the most distal dendritic branches. **D**: Dendrogram of the DL5-uPN showing the distribution of presynaptic sites (triangles) and postsynaptic sites (circles) of selected autapses (indicated by same color). Distant pairs of pre- and postsynapses (long geodesic distance) are indicated by numbers

whereas closely attached synaptic sites (short geodesic distance) are encircled and labelled with letters. **E**: Schematic of the dendrogram illustrating the location of the presynaptic (red dot) and postsynaptic (cyan dot) sites of a single autapse, the geodesic distance between them, i.e. the distance along the dendrite (µm), and the number of branching points (orange dots) between the pre- and postsynaptic components of the same autapse. **F**: Number of autapses with distinct geodesic distances between their pre- and postsynapses (illustrated in E). **G**: Number of autapses with the number of branch points between their pre- and postsynapses counted along the uPN dendrite (illustrated in E).


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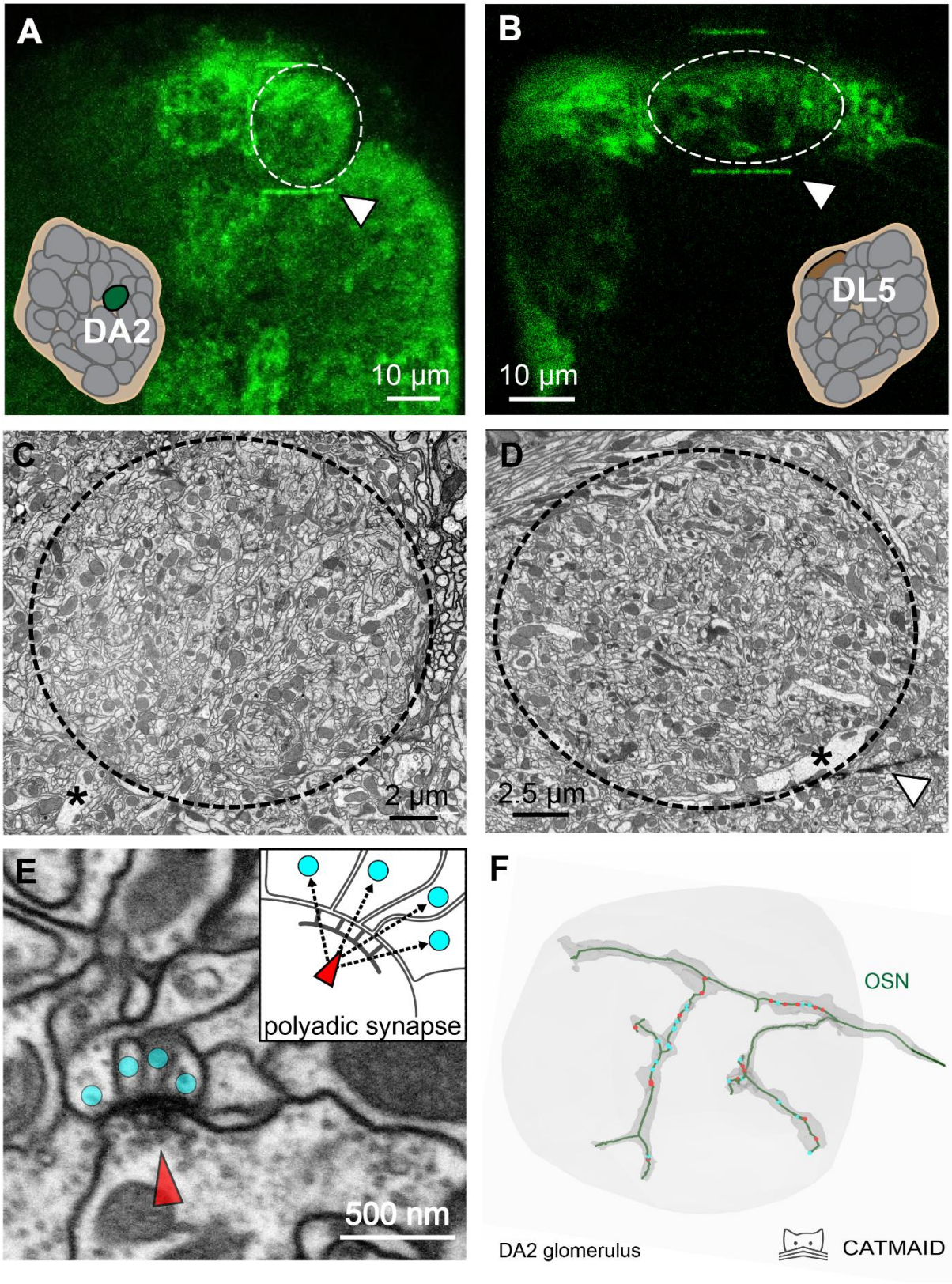

A
DA2
10 µm
B
DL5
10 µm
C
2 µm
D
2.5 µm
E
polyadic synapse
500 nm
F
OSN
DA2 glomerulus
CATMAID

**Figure 1**

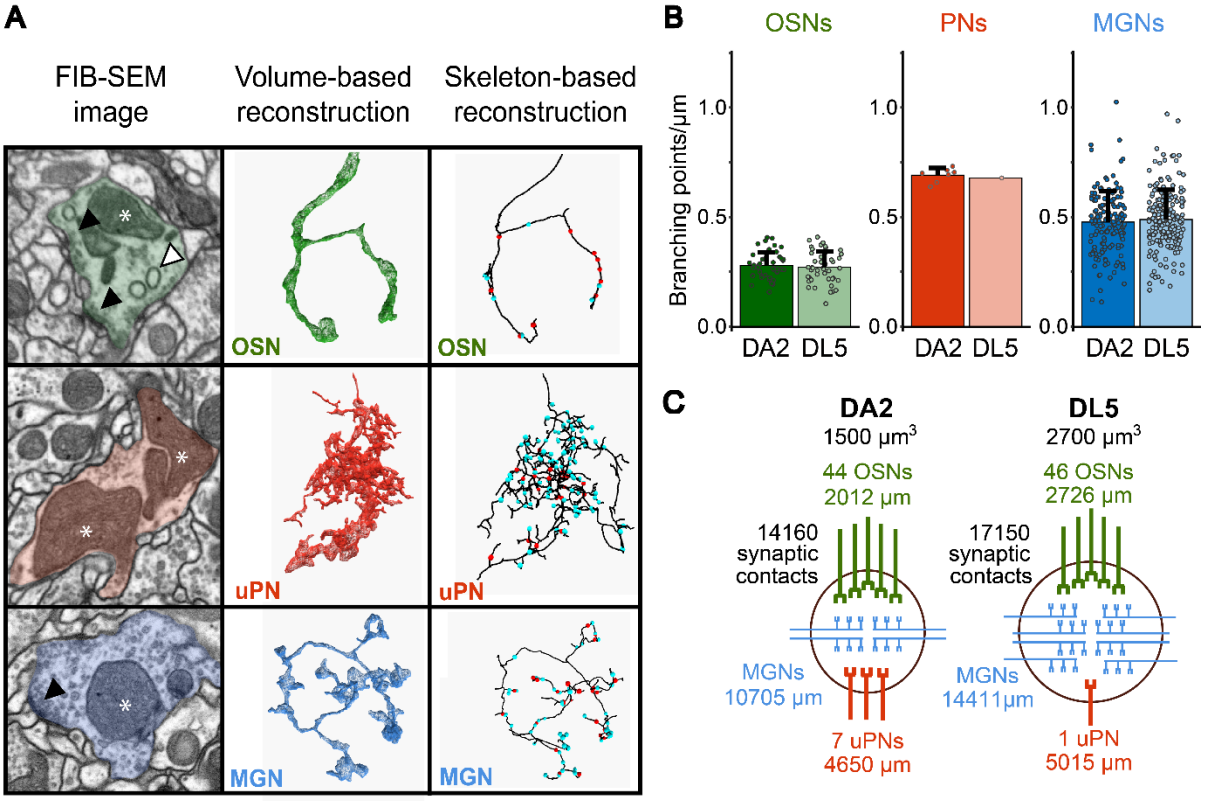

A
FIB-SEM image
Volume-based reconstruction
Skeleton-based reconstruction
OSN
OSN
uPN
uPN
MGN
MGN
B
OSNs
PNs
MGNs
Branching points/µm
1.0
0.5
0.0
DA2
DL5
C
DA2
1500 µm3
44 OSNs
2012 µm
14160 synaptic contacts
MGNs
10705 µm
7 uPNs
4650 µm
DL5
2700 µm3
46 OSNs
2726 µm
17150 synaptic contacts
MGNs
14411µm
1 uPN
5015 µm

**Figure 2**

| Row | Values | Unit | OSNs | | uPNs | | MGNs | | all neurons | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | DA2 | DL5 | DA2 | DL5 | DA2 | DL5 | DA2 | DL5 |
| 1 | Total neuronal length | µm | 2012 | 2727 | 4652 | 5015 | 10705 | 14411 | 17370 | 22153 |
| 2 | Total synaptic counts | input | 868 | 1083 | 3887 | 3955 | 7229 | 9018 | 11984 | 14056 |
| 3 | | output | 6671 | 6828 | 1624 | 3108 | 5659 | 6749 | 13954 | 16685 |
| 4 | | T-bars | 1063 | 1213 | 322 | 602 | 1263 | 1572 | 2648 | 3387 |
| 5 | Total innervation density (sum of length of all neuronal fibers/glomerular volume) | µm/µm$^3$ | 1.26 | 1.05 | 2.91 | 1.93 | 6.69 | 5.54 | 10.86 | 8.52 |
| 6 | Total glomerular synaptic density (total synaptic counts/glomerular volume) | inputs/µm$^3$ | 0.54 | 0.42 | 2.43 | 1.52 | 4.52 | 3.47 | 7.49 | 5.41 |
| 7 | | outputs/µm$^3$ | 4.17 | 2.63 | 1.02 | 1.20 | 3.54 | 2.60 | 8.72 | 6.42 |
| 8 | | T-bars/µm$^3$ | 0.66 | 0.47 | 0.20 | 0.23 | 0.79 | 0.60 | 1.66 | 1.30 |
| 9 | Neuronal synaptic density (synaptic counts/neuronal length) | inputs/µm | 0.42 | 0.39 | 0.83 | 0.79 | 0.62 | 0.59 | 0.59 | 0.56 |
| 10 | | outputs/µm | 3.37 | 2.62 | 0.33 | 0.62 | 0.52 | 0.51 | 1.06 | 0.87 |
| 11 | | T-bars/µm | 0.53 | 0.46 | 0.07 | 0.12 | 0.12 | 0.12 | 0.19 | 0.18 |
| 12 | Synaptic ratio | T-bars/inputs | 1.31 | 1.27 | 0.08 | 0.15 | 0.23 | 0.24 | 0.43 | 0.42 |
| 13 | | outputs/inputs | 8.29 | 7.29 | 0.40 | 0.79 | 1.04 | 1.11 | 2.40 | 2.17 |
| 14 | Polyadicity | outputs/T-bars | 6.35 | 5.70 | 4.95 | 5.16 | 3.22 | 2.64 | 3.88 | 3.17 |

**Table 1**

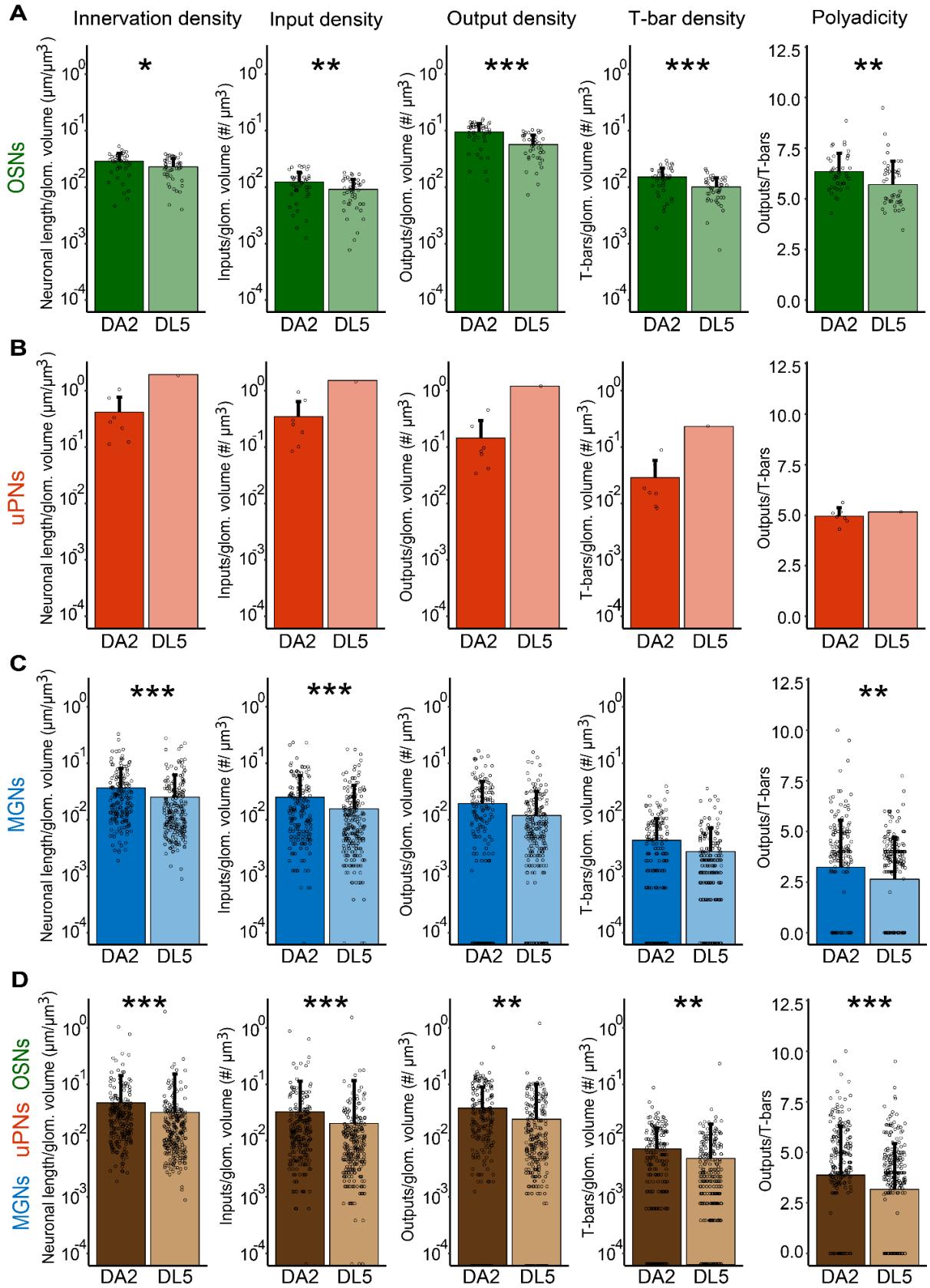

A
Innervation density
Input density
Output density
T-bar density
Polyadicity
OSNs
Neuronal length/glom. volume (µm/µm3)
Inputs/glom. volume (#/ µm3)
Outputs/glom. volume (#/ µm3)
T-bars/glom. volume (#/ µm3)
Outputs/T-bars
DA2
DL5
B
uPNs
C
MGNs
D
MGNs uPNs OSNs

**Figure 3**

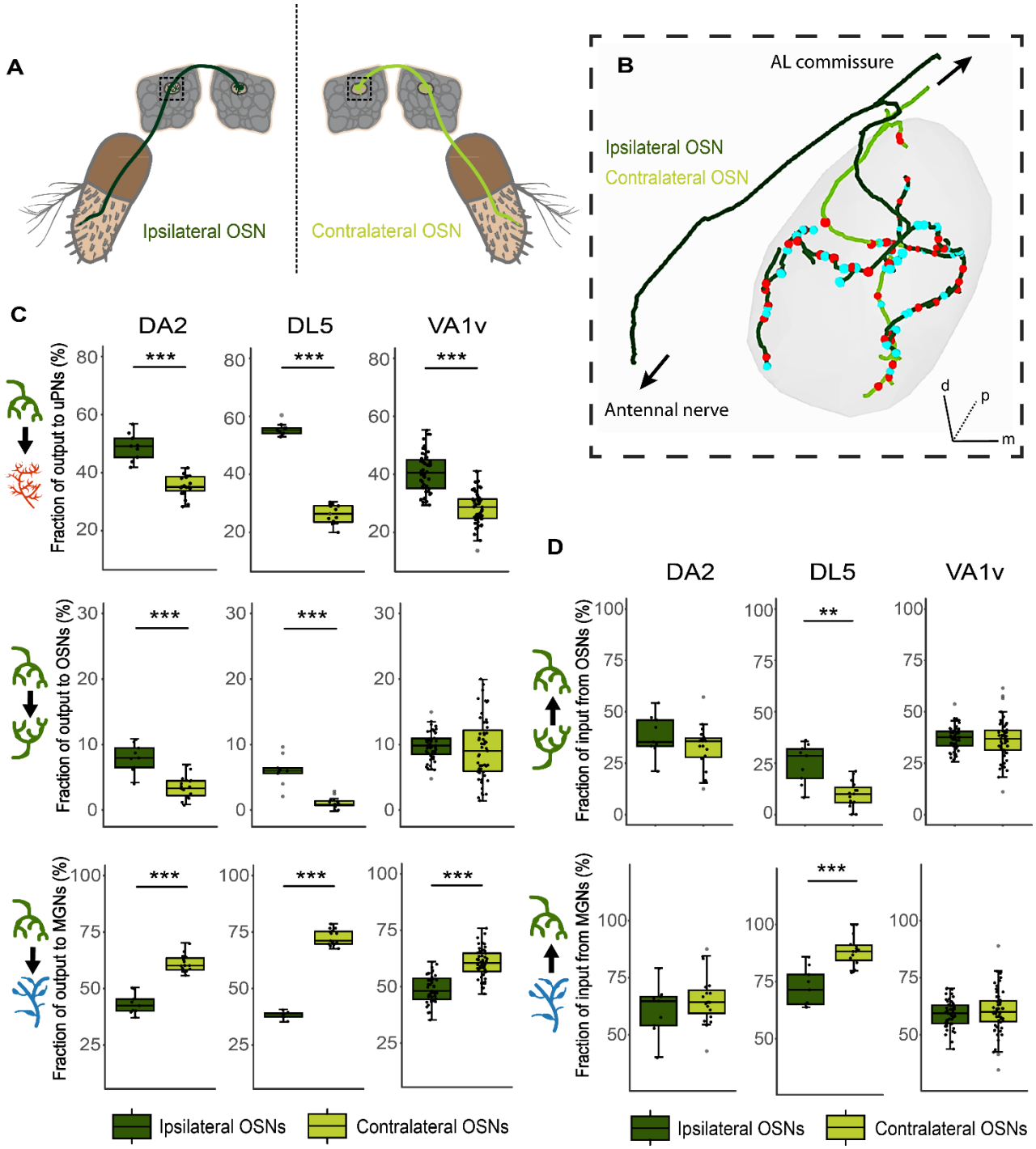

A
Ipsilateral OSN
Contralateral OSN
B
AL commissure
Ipsilateral OSN
Contralateral OSN
Antennal nerve
d
p
m
C
DA2
DL5
VA1v
Fraction of output to uPNs (%)
Fraction of output to OSNs (%)
Fraction of output to MGNs (%)
***
D
Fraction of input from OSNs (%)
Fraction of input from MGNs (%)
**
Ipsilateral OSNs
Contralateral OSNs

**Figure 4**

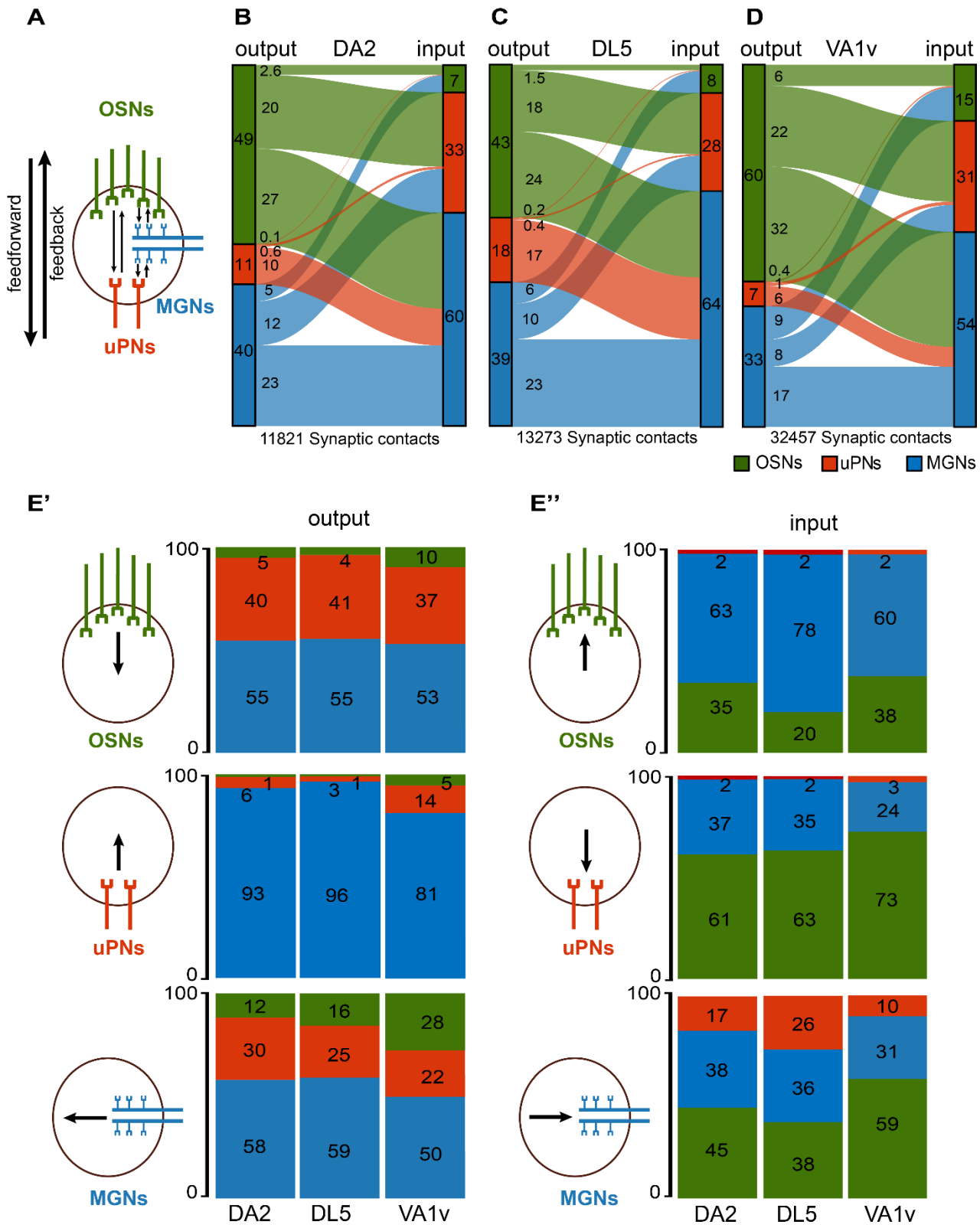
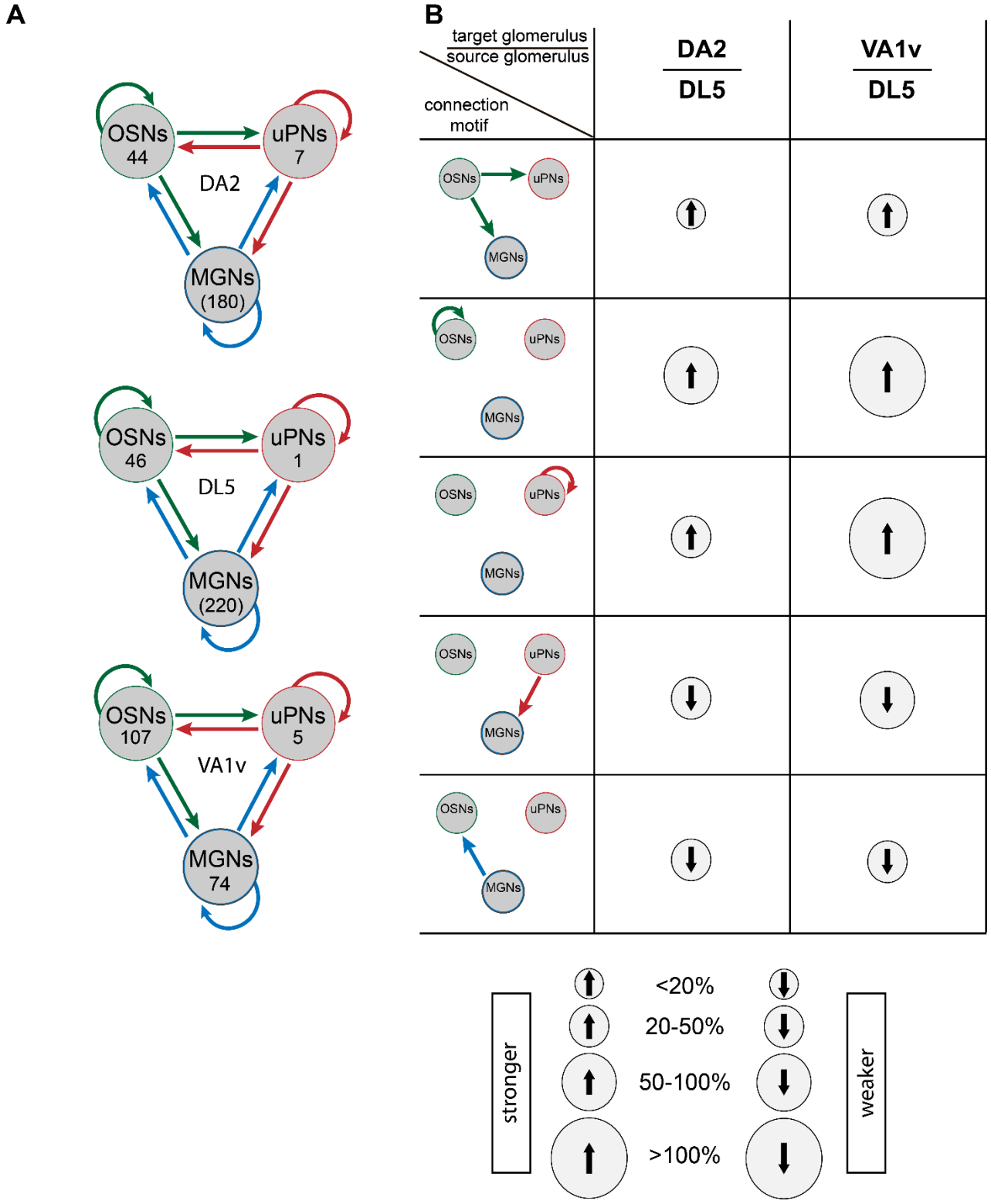

A
OSNs
feedforward
feedback
MGNs
uPNs
B
output
DA2
input
11821 Synaptic contacts
C
output
DL5
input
13273 Synaptic contacts
D
output
VA1v
input
32457 Synaptic contacts
OSNs
uPNs
MGNs
E'
output
E''
input
OSNs
uPNs
MGNs
DA2
DL5
VA1v

**Figure 5**

2077




2078
2079 **Figure 6**

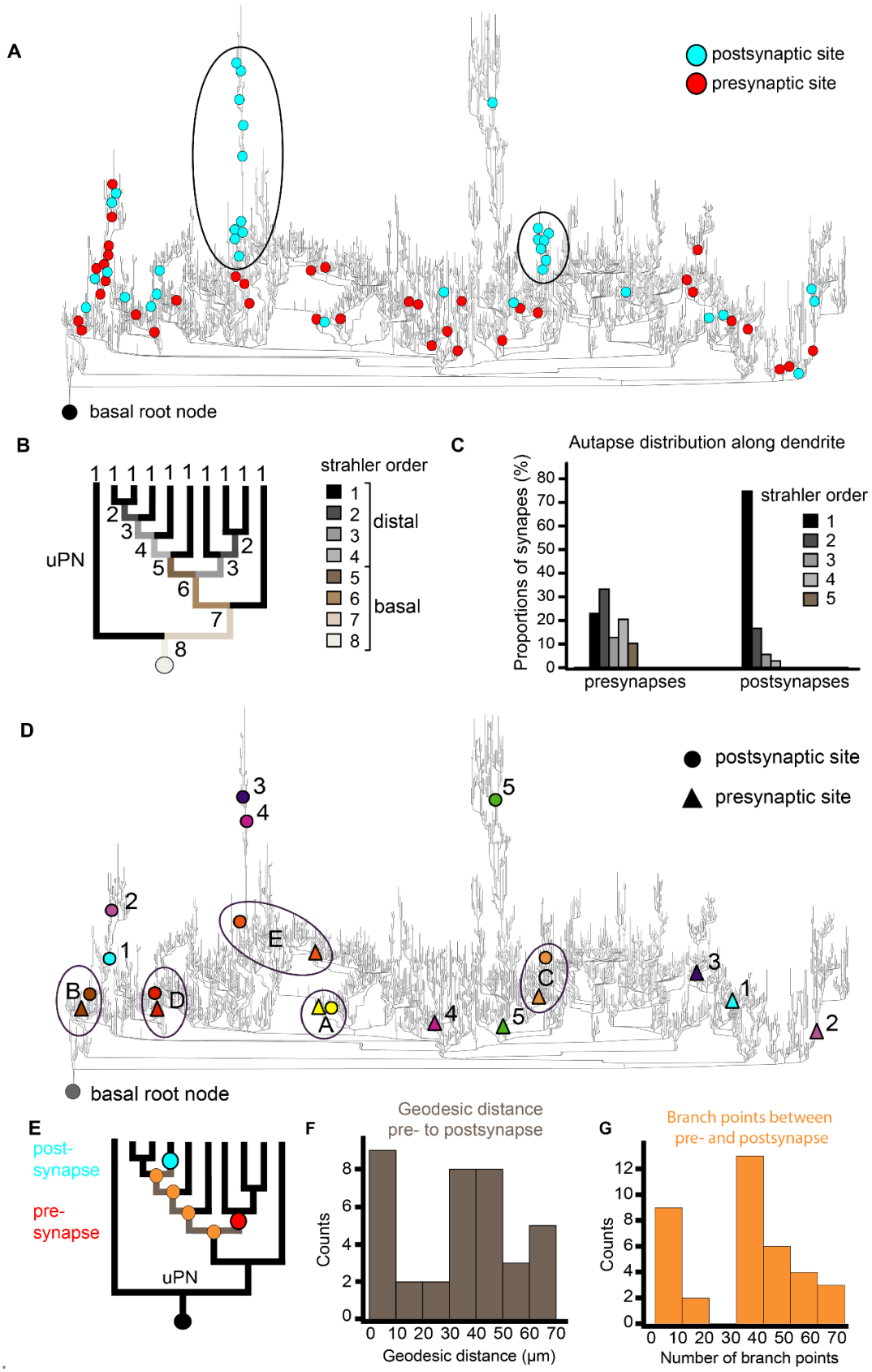

A
postsynaptic site
presynaptic site
basal root node
B
uPN
strahler order
distal
basal
C
Autapse distribution along dendrite
Proportions of synapses (%)
strahler order
presynapses
postsynapses
D
postsynaptic site
presynaptic site
basal root node
E
post-synapse
pre-synapse
uPN
F
Geodesic distance pre- to postsynapse
Counts
Geodesic distance (µm)
G
Branch points between pre- and postsynapse
Counts
Number of branch points

**Figure 7**

2082 **SUPPLEMENTARY FIGURES**

2083 **Figure 1 -- Video 1: FIB-SEM dataset of a DA2 glomerulus with highlighted uPN reconstruction**
2084 (see extra file).
2085 The video shows a full FIB-SEM scan of a DA2 glomerulus at pixel resolution 4x4x20 nm, with the
2086 neuron trace of a single uniglomerular projection neuron (uPN#2) highlighted in yellow.

2087 **Figure 2 – figure supplement 1: Neuronal volume and polyadicity**

2088 **A**: Ratio between neuronal fiber volume and length in glomerulus DA2. Data represent mean +
2089 standard deviation (error bars) (OSNs n=30; uPNs n =5; MGNs n = 15). **B-D**: Frequency of T-bars
2090 associated with a number of postsynaptic contacts (Polyadicity) in OSNs (**B**), uPNs (**C**) and MGNs
2091 (**D**) in DA2 (dark shade) and DL5 (light shade)

2092 **Supplementary Table S1: Relative differences of innervation and synaptic composition**
2093 **between glomeruli DA2 and DL5**

2094 The Table lists the relative differences between DA2 and DL5 (see Methods for calculations).
2095 Values that are at least 20% greater in DA2 than in DL5 are highlighted in dark shades and values
2096 that are at least 20% less in DA2 than in DL5 are highlighted in light shades.

2097 **Figure 3 – figure supplement 1: Synaptic density along neuronal fibers in DA2 and DL5**

2098 Counts of synaptic inputs, synaptic outputs and T-bars normalized to 1 µm of neuronal length
2099 along OSN, uPN or MGN fibers and collectively for all neurons within glomeruli DA2 (dark colors)
2100 and DL5 (light colors). DA2: OSNs (green) n= 44; uPNs (red) n= 7; MGNs (blue) n=180; all neurons
2101 n=231. DL5: OSNs n=46; uPN n=1; MGNs n=221; all neurons n=268. Data represent mean +
2102 standard deviation (error bars). Data points represent single values. Means are compared using
2103 either Student's T-test (in OSNs) or Wilcoxon two-sample test (in MGNs and all neurons). The
2104 uPNs of the DA2 are not compared to the single uPN of the DL5. Significance value: $p>0.05$ (not
2105 significant, no star), $p\leq0.05$ (*), $p\leq0.01$ (**), $p\leq0.001$ (***). Values are listed in Table 1, row 9-11.

2106 **Figure 4 – figure supplement 1: Properties of ipsi- and contralateral OSNs.**

2107 **A:** Boxplots for total neuronal-fiber length and synaptic density (inputs, outputs, T-bars per unit
2108 of neuronal fiber length) of ipsilateral (dark green) and contralateral OSN terminals (light green).
2109 Dots represent single values. Means were compared using Stu
2110

2111 dent's T-test. Significance value: p≤0.05 (*), p≤0.01 (**), p≤0.001 (***).

2112 **Supplementary Table S2**: **Synaptic connectivity and relative differences between DA2, DL5 and**
2113 **VA1v**

2114 Synapse counts and synaptic strength of each connection type in DA2, DL5 and VA1v. Three
2115 comparisons are shown: DA2 compared with DL5 (top table), VA1v with DL5 (middle) and VA1v
2116 with DA2 (bottom). The relative synaptic strength (rel syn strength) of each connection type is
2117 listed on the left side and the relative differences (rel differences) are listed on the right side.

2118 **Supplementary Table S3: Connectivity of single neurons in DA2** (see extra file)

2119 **Supplementary Table S4: Connectivity of single neurons in DL5** (see extra file)

2120 **Figure 7 – figure supplement 1: Distribution of synapses and autapses along the DL5 uPN**
2121 **dendrite in DL5**
2122 **A**: 3D-reconstruction of the dendritic tree of the single uPN in glomerulus DL5 shown as skeleton
2123 trace with artificial thickness according to Strahler order. Autapses are shown as cyan dots. The
2124 entry site of the uPN main dendrite into the glomerulus (point closest to the soma) is the basal
2125 root node. **B**: Number of autaptic presynaptic (red) and postsynaptic sites (cyan) according to
2126 their geodesic distance to the basal root node point (indicated with a black circle in A). **C**:
2127 Proportional distribution of all presynapses and postsynapses (excluding autaptic connections) in
2128 the DL5 uPN at each strahler order (see legend inset). Note the high proportion of postsynaptic
2129 sites on most distal dendritic branches.

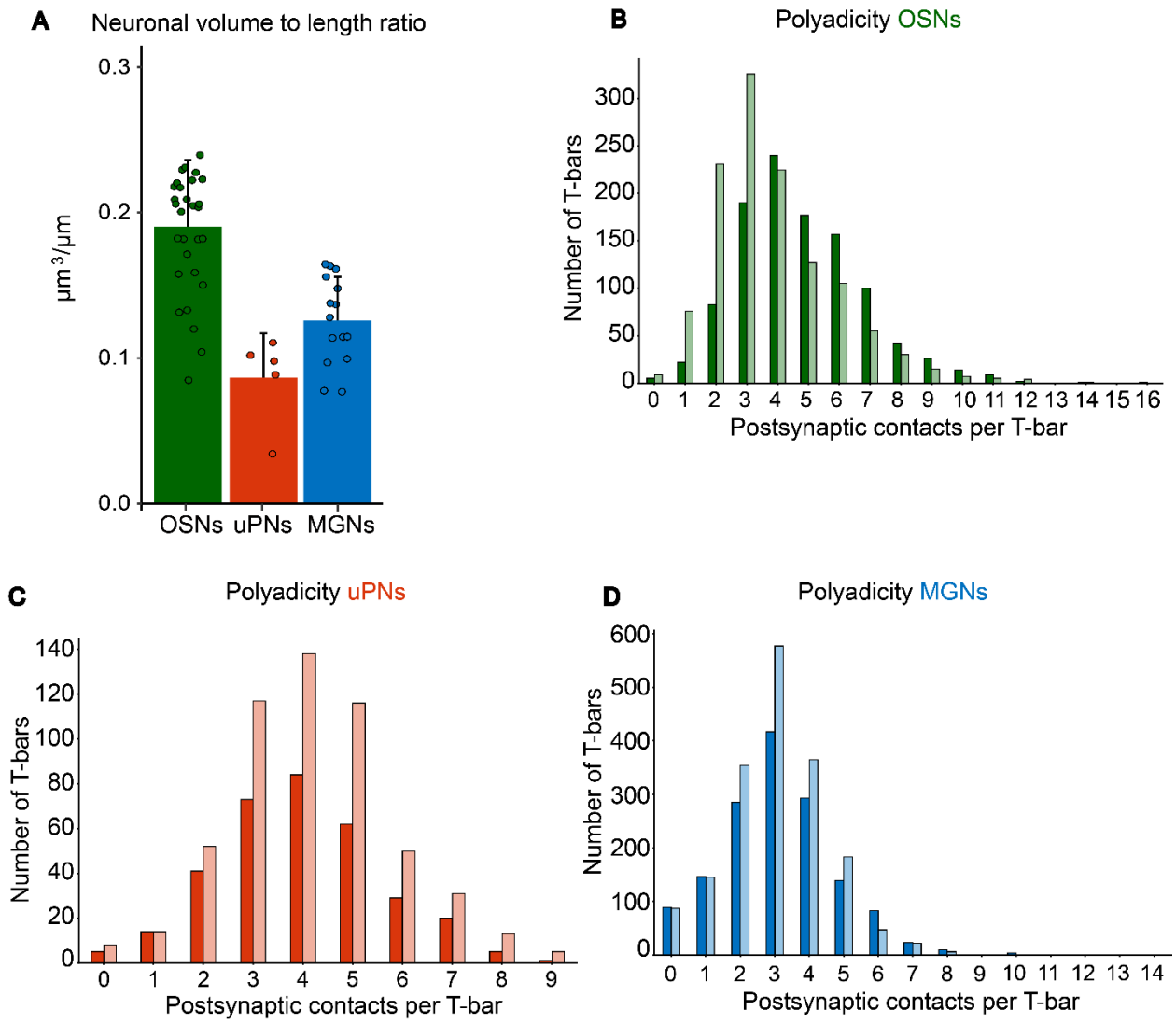



**Figure 2 – figure supplement 1**

| Row | Values | Unit | OSNs | uPNs | MGNs | all neurons |
|---|---|---|---|---|---|---|
| | | | Relative differences between DA2 and DL5 | | | |
| 1 | Total neuronal length | µm | -26 | -7 | -26 | -22 |
| 2 | Total synaptic counts | input | -20 | -2 | -20 | -15 |
| 3 | | output | -2 | -48 | -16 | -16 |
| 4 | | T-bars | -12 | -47 | -20 | -22 |
| 5 | Total innervation density (sum of length of all neuronal fibers/glomerular volume) | µm/µm$^3$ | 20 | 51 | 21 | 27 |
| 6 | Total glomerular synaptic density (total synaptic counts/glomerular volume) | inputs/µm$^3$ | 30 | 60 | 30 | 39 |
| 7 | | outputs/µm$^3$ | 59 | -15 | 36 | 36 |
| 8 | | T-bars/µm$^3$ | 42 | -13 | 31 | 27 |
| 9 | Neuronal synaptic density (synaptic counts/neuronal length) | inputs/µm | 8 | 5 | 5 | 6 |
| 10 | | outputs/µm | 29 | -47 | 2 | 21 |
| 11 | | T-bars/µm | 16 | -45 | -5 | 7 |
| 12 | Synaptic ratio | T-bars/inputs | 3 | -47 | -6 | 2 |
| 13 | | outputs/inputs | 14 | -50 | -6 | 11 |
| 14 | Polyadicity | outputs/T-bars | 11 | -4 | 22 | 22 |

**Supplementary Table S1**

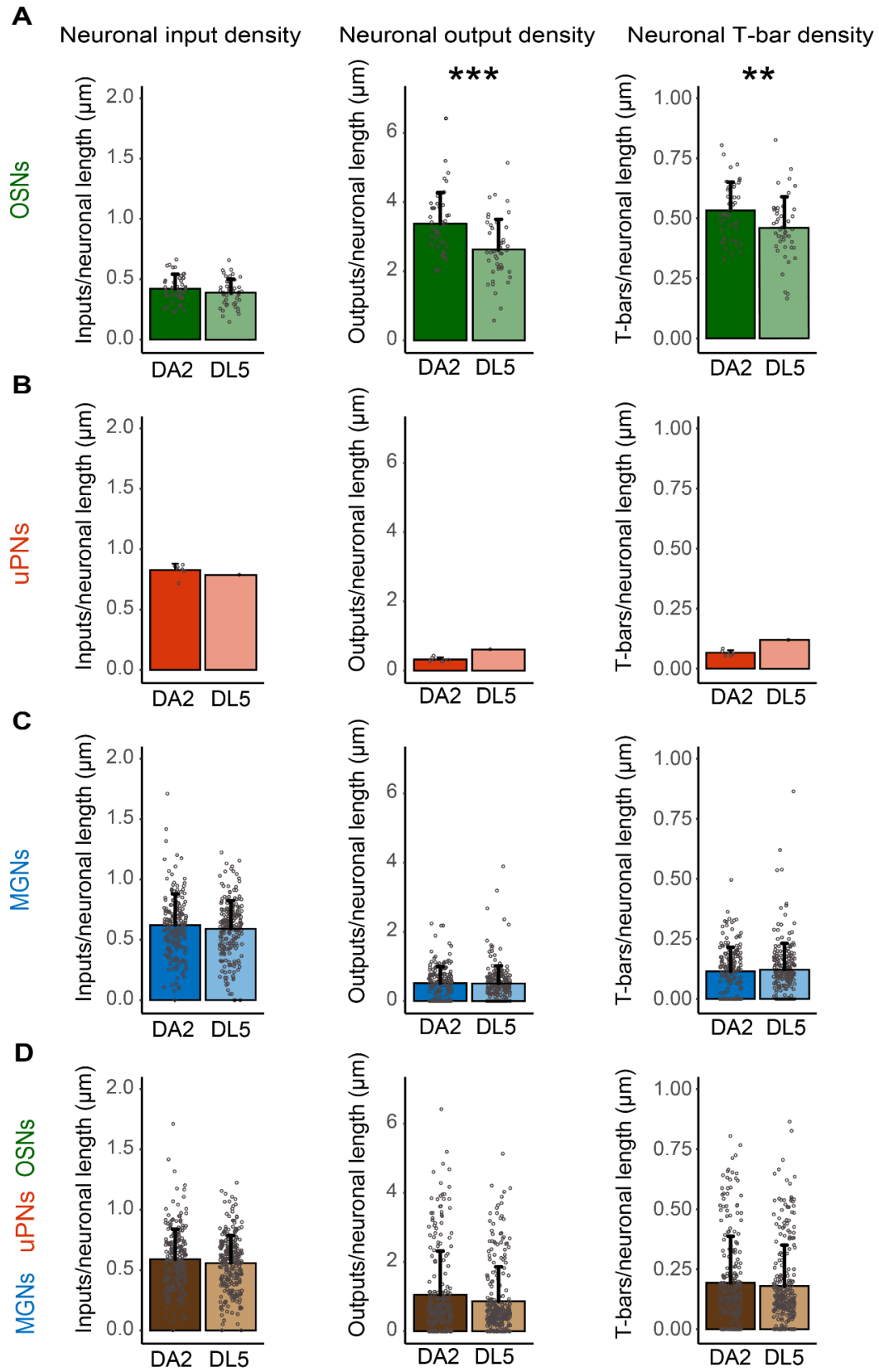



**Figure 3 – figure supplement 1**

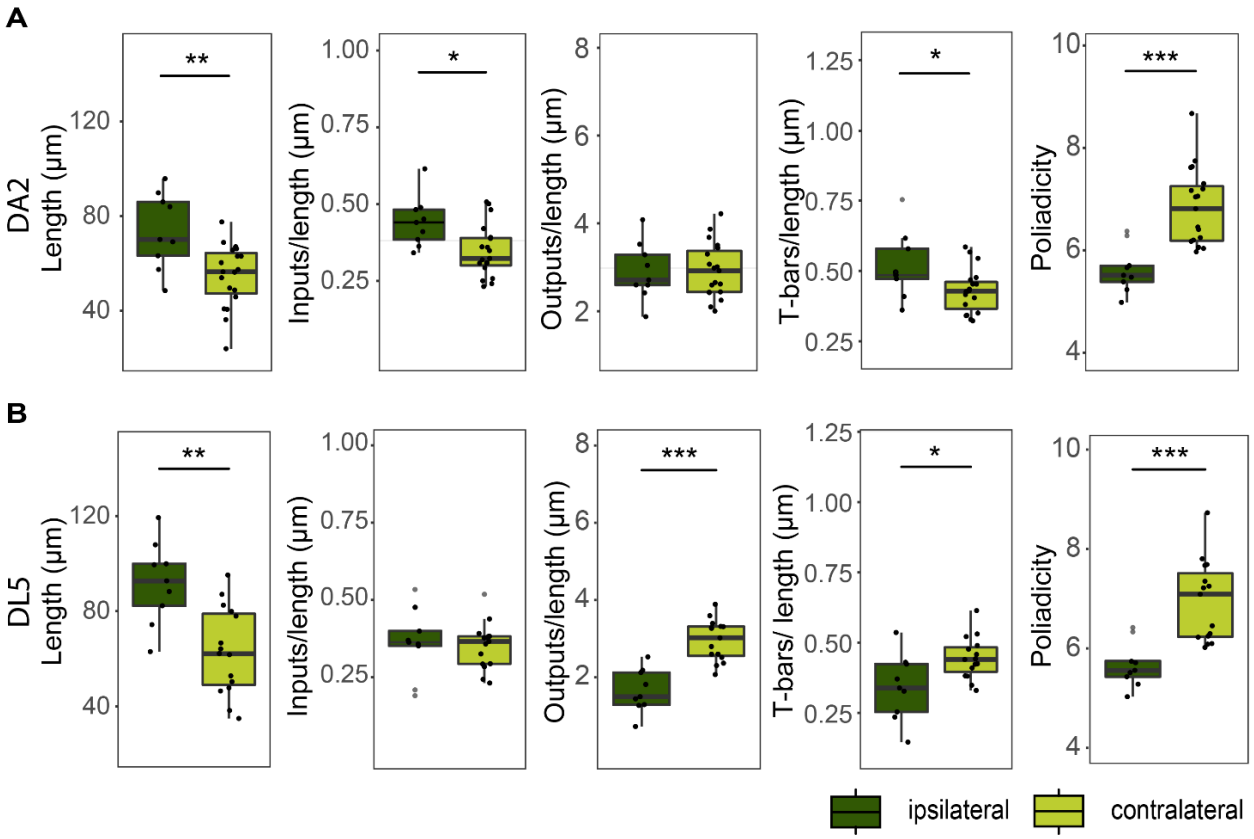



**Figure 4 – figure supplement 1**

| neuron class | connection motif | DA2 | rel syn strength (%) | rel out classes (%) | rel in classes (%) | DL5 | rel syn strength (%) | rel out classes (%) | rel in classes (%) | rel difference (%) | rel out difference (%) | rel in difference (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OSNs | OSNs>uPNs | 2365 | 19.99 | 49.48 | 7.37 | 2354 | 17.76 | 43.44 | 7.89 | 12.52 | 13.93 | -6.63 |
| | OSNs>MGNs | 3186 | 26.92 | | | 3201 | 24.15 | | | 11.47 | | |
| | OSNs>OSNs | 305 | 2.58 | | | 202 | 1.52 | | | 69.11 | | |
| uPNs | uPNs>OSNs | 16 | 0.14 | 10.92 | 32.62 | 26 | 0.20 | 17.50 | 28.07 | -31.08 | -37.63 | 16.18 |
| | uPNs>MGNs | 1205 | 10.18 | | | 2240 | 16.90 | | | -39.75 | | |
| | uPNs>uPNs | 71 | 0.60 | | | 54 | 0.41 | | | 47.26 | | |
| MGNs | MGNs>OSNs | 551 | 4.66 | 39.60 | 60.01 | 818 | 6.17 | 39.06 | 64.03 | -24.56 | 1.38 | -6.28 |
| | MGNs>uPNs | 1424 | 12.03 | | | 1313 | 9.91 | | | 21.47 | | |
| | MGNs>MGNs | 2711 | 22.91 | | | 3046 | 22.98 | | | -0.32 | | |
| | SUM | 11833 | 100 | 100 | 100 | 13254 | 100 | 100 | 100 | | | |

**Supplementary Table S2**

2138

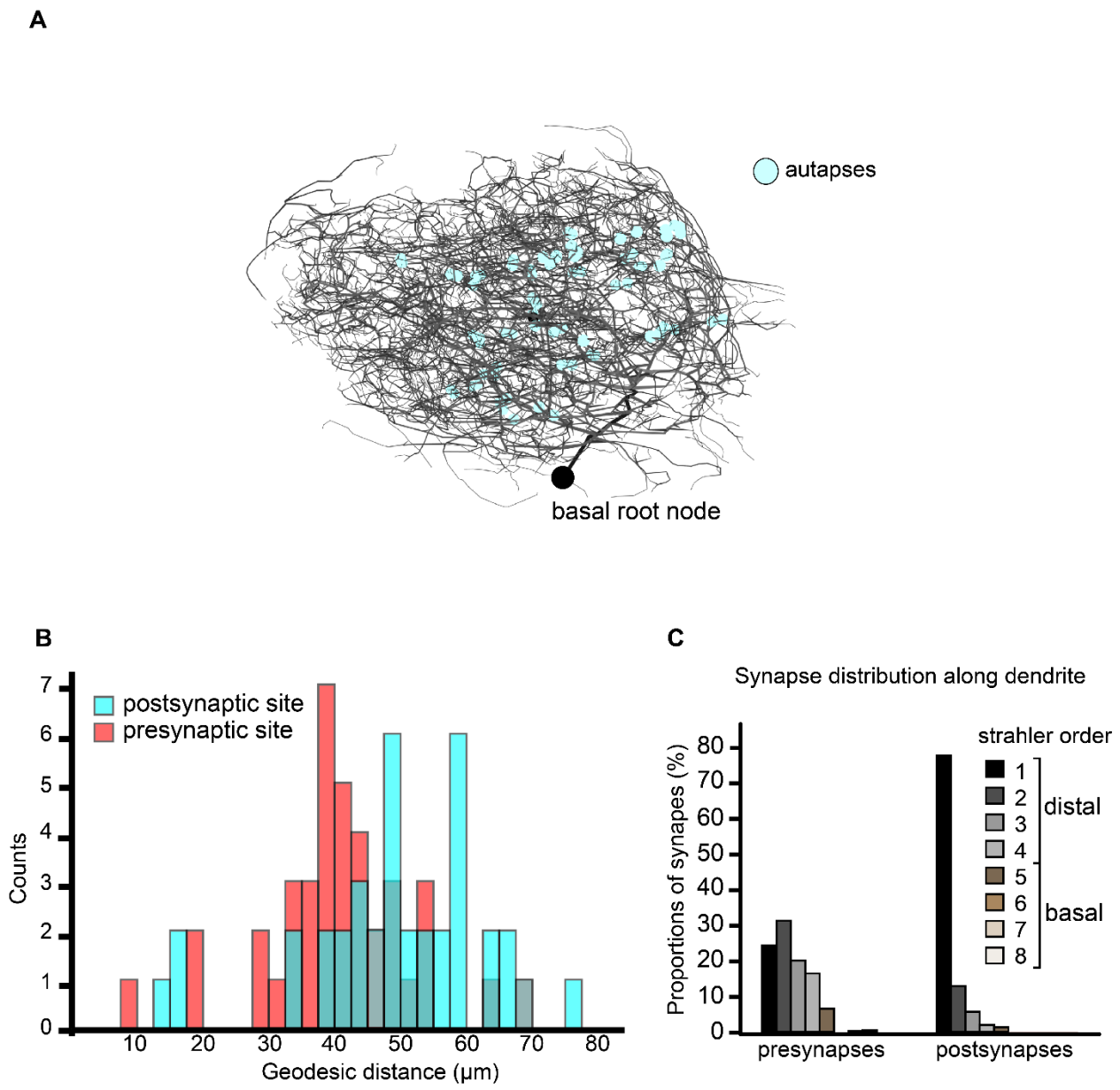
| neuron class | connection motif | VA1v | rel syn strength (%) | rel out classes (%) | rel in classes (%) | DL5 | rel syn strength (%) | rel out classes (%) | rel in classes (%) | rel difference (%) | rel out difference (%) | rel in difference (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OSNs | OSNs>uPNs | 7226 | 22.26 | 59.84 | 15.51 | 2354 | 17.76 | 43.44 | 7.89 | 25.35 | 37.76 | 96.53 |
| | OSNs>MGNs | 10295 | 31.72 | | | 3201 | 24.15 | | | 31.33 | | |
| | OSNs>OSNs | 1901 | 5.86 | | | 202 | 1.52 | | | 284.30 | | |
| uPNs | uPNs>OSNs | 117 | 0.36 | 6.83 | 30.67 | 26 | 0.20 | 17.50 | 28.07 | 83.76 | -60.96 | 9.26 |
| | uPNs>MGNs | 1801 | 5.55 | | | 2240 | 16.90 | | | -67.17 | | |
| | uPNs>uPNs | 300 | 0.92 | | | 54 | 0.41 | | | 126.86 | | |
| MGNs | MGNs>OSNs | 3016 | 9.29 | 33.33 | 53.82 | 818 | 6.17 | 39.06 | 64.03 | 50.56 | -14.68 | -15.96 |
| | MGNs>uPNs | 2430 | 7.49 | | | 1313 | 9.91 | | | -24.42 | | |
| | MGNs>MGNs | 5371 | 16.55 | | | 3046 | 22.98 | | | -27.99 | | |
| | SUM | 32457 | 100 | 100 | 100 | 13254 | 100 | 100 | 100 | | | |

2139 **Supplementary Table S3**

| neuron class | connection motif | VA1v | rel syn strength (%) | rel out classes (%) | rel in classes (%) | DA2 | rel syn strength (%) | rel out classes (%) | rel in classes (%) | rel difference (%) | rel out difference (%) | rel in difference (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OSNs | OSNs>uPNs | 7226 | 22.26 | 59.84 | 15.51 | 2365 | 19.99 | 49.48 | 7.37 | 11.40 | 20.92 | 110.48 |
| | OSNs>MGNs | 10295 | 31,.2 | | | 3186 | 26.92 | | | 17.82 | | |
| | OSNs>OSNs | 1901 | 5.86 | | | 305 | 2.58 | | | 127.25 | | |
| uPNs | uPNs>OSNs | 117 | 0.36 | 6.83 | 30.67 | 16 | 0.14 | 10.92 | 32.62 | 166.60 | -37.41 | -5.96 |
| | uPNs>MGNs | 1801 | 5.55 | | | 1205 | 10.18 | | | -45.51 | | |
| | uPNs>uPNs | 300 | 0.92 | | | 71 | 0.60 | | | 54.06 | | |
| MGNs | MGNs>OSNs | 3016 | 9.29 | 33.33 | 53.82 | 551 | 4.66 | 39.60 | 60.01 | 99.57 | -15.84 | -10.33 |
| | MGNs>uPNs | 2430 | 7.49 | | | 1424 | 12.03 | | | -37.78 | | |
| | MGNs>MGNs | 5371 | 16.55 | | | 2711 | 22.91 | | | -27.76 | | |
| | SUM | 32457 | 100 | 100 | 100 | 11833 | 100 | 100 | 100 | | | |

| color code | rel syn strength (%) | <5 | 5-10 | 10-15 | 15-20 | >20 |
|---|---|---|---|---|---|---|
| | rel difference (%) | <10 | 10-20 | 20-50 | 50-100 | >100 |

**Supplementary Table S4**




2145
2146 **Figure 7 – figure supplement 1**