# Integration of the olfactory code across dendritic claws of single mushroom body neurons

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In the olfactory system, sensory inputs are arranged in different glomerular channels, which respond in combinatorial ensembles to the various chemical features of an odor. We investigated where and how this combinatorial code is read out deeper in the brain. We exploited the unique morphology of neurons in the *Drosophila* mushroom body, which receive input on large dendritic claws. Imaging odor responses of these dendritic claws revealed that input channels with distinct odor tuning converge on individual mushroom body neurons. We determined how these inputs interact to drive the cell to spike threshold using intracellular recordings to examine mushroom body neurons: these cells receive different types of input and require those inputs to be coactive to spike. These results establish the mushroom body as an important site of integration in the fly olfactory system.

The olfactory system of Drosophila is an excellent platform for studying how sensory information is transformed as it passes through the successive layers of a neural circuit. Although the fly olfactory circuit is organized similarly to more complex organisms, it is numerically simpler and has been mapped with cellular resolution. Sensory input comes in from a large set of olfactory receptor neurons (ORNs), each of which has a particular chemical sensitivity<sup>1,2</sup>. These inputs are organized into distinct channels in the antennal lobe, as ORNs that express the same type of olfactory receptor converge to form synapses with a cognate set of projection neurons in structures termed glomeruli<sup>3</sup>. There are 54 different glomerular channels that have been identified in Drosophila<sup>4</sup>, and the constellation of different physiochemical features in an odor evokes widely distributed patterns of activity across these channels. In Drosophila, the main transformations at the first synapse in the circuit are noise reduction and gain control<sup>5-8</sup>. These transformations distribute activity more uniformly among the glomerular channels in the projection neuron layer than the ORNs<sup>5</sup>. This ensures that the combinatorial capacity of the system is used effectively and theoretically enables a simple linear neural network to more easily discriminate odors<sup>5</sup>. The set of, on average, three projection neurons present in each glomerular channel<sup>9–11</sup> then transmits information in nearly identical spike trains<sup>12</sup> to downstream brain areas, the lateral horn and mushroom body.

The transformation from the projection neurons to the mushroom body is of particular interest, as the mushroom body is essential for olfactory learning and memory<sup>13–16</sup>. The mushroom body is composed of 2,000 neurons known as Kenyon cells<sup>17</sup>. In comparison with projection neurons, which typically respond to a wide range of odors, Kenyon cells have much more odor-selective responses<sup>18–21</sup>. Consequently, the densely combinatorial odor representations in the projection neuron layer are transformed into sparse response patterns across the Kenyon cell population. The high-specificity of Kenyon cell responses is thought to underlie the accuracy of memory formation<sup>22</sup>, and sparse representations are in fact a general feature of deeper layers of the brain, including the olfactory cortex in mammals<sup>23–25</sup>. However, we understand very little of how Kenyon cells achieve such selective responses.

Projection neuron–Kenyon cell synapses are remarkable structures. They are among the largest synapses in the *Drosophila* brain and have a striking morphology<sup>26,27</sup>. The synaptic terminals of projection neurons form large ~5- $\mu$ m boutons that are enwrapped by correspondingly large claw-like dendritic structures of Kenyon cells. Although fine distinctions between dendritic specializations can be resolved at high resolution<sup>28</sup>, we will refer to these contacts as Kenyon cell claws (as in ref. 26). Ultrastructural studies have shown that each Kenyon cell claw contacts a single projection neuron bouton, establishing the claw as the anatomical unit of projection neuron input to these cells<sup>26</sup>. Kenyon cells typically have five to seven such claws<sup>26</sup>, and a recent study examining the anatomy of projection neuron convergence onto individual Kenyon cells found that connectivity at this layer was random and that projection neurons from different glomeruli were no more likely to wire together than chance<sup>29</sup>.

The unusual morphology of projection neuron–Kenyon cell connections presents us with the opportunity to investigate the response properties of individual synaptic sites from a functional perspective. First, using *in vivo* dendritic imaging, we directly examined the odor response properties of individual synaptic sites. This approach enabled us to determine whether functionally distinct inputs converge onto individual Kenyon cells. Second, to understand how Kenyon cells integrate synaptic input, we used optogenetic methods to provide precisely controlled input to the claws and intracellular recordings to examine the postsynaptic response. The large size of projection

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Figure 1 Imaging dendritic claws of Kenyon cells reveals distinct odor responses. (a) Schematic of the Drosophila olfactory circuit. Projection neurons (PNs) in the antennal lobe (AL) send axons to mushroom body calyx (CX), where they make synaptic contact with Kenyon cells (KCs) via large synaptic boutons that are enwrapped by claw-shaped dendrites. Projection neuron axons ultimately terminate in the lateral horn (LH). (b) An example Kenyon cell with five claws labeled with GCaMP3 and the anatomical marker myr-tdTomato. Image is a projection of a z series that bracketed the cell body and dendritic region of the mushroom body. Boxes demarcate claws whose odor responses were characterized. Note that, in this particular example, we imaged four of the total five claws (fifth claw located on bottom left, not demarcated). Scale bar represents 5  $\mu$ m. (c) Odor response time courses ( $\Delta G/R$ ) from dendritic claws shown in **b**. Responses from individual trials are shown in black (n = 3) and mean responses in red. Odor valve opening is at t = 0. Note the distinct odor responses in claws 3 and 4, despite their close physical proximity. (d)  $\Delta G/R$  responses of each claw from **b** to a panel of ten odorants (three trials per odor).

neuron–Kenyon cell synapses enabled us to relate the connectivity that we observed anatomically to the functional responses that we measured electrophysiologically. We found that Kenyon cells received convergent input from different channels and required several inputs to be active to spike. Notably, these cells achieved their response specificity despite essentially linear additivity of synaptic inputs. Overall, our results indicate that a fundamental aspect of the transformation at this layer is the integration of combinatorial patterns of glomerular activity by individual Kenyon cells.

#### RESULTS

#### Functional imaging of dendritic odor responses

We examined the odor response profiles of individual dendritic claws in vivo using two-photon imaging. We used a stochastic genetic approach<sup>30</sup> to label single Kenyon cells with both the calcium indicator GCaMP3 (ref. 31) and the anatomical marker myr-tdTomato. tdTomato enabled easy identification of Kenyon cell claws in vivo, whereas GCaMP3 reported the postsynaptic response to projection neuron input, which leads to Ca2+ influx via calcium-permeable acetylcholine receptors<sup>32</sup> as well as a potential contribution from voltagegated calcium channels. We examined odor responses of the claws of individually labeled Kenyon cells using a panel of ten different odorants (Fig. 1). Individual claws exhibited responses that were consistent across multiple presentations and differences between odor response profiles were readily apparent (Fig. 1c,d). For example, claws 1 and 2 showed similar responses to several odors, but could be distinguished by their differential responses to pine and trans-2-hexanal (Fig. 1d). Even claws in close spatial proximity were able to exhibit markedly different odor response profiles (Fig. 1c,d and Supplementary Movie 1). Our sampling of Kenyon cell claws was not comprehensive for every Kenyon cell; however, our goal was to determine whether or not the claw response profiles that we did measure were distinct. Overall, these findings indicate that we could reliably measure odor response profiles at individual dendritic sites in vivo. In addition, for the example Kenyon cell, we observed that different claws exhibited clearly distinct odor response profiles.

To test whether the convergence of inputs with different odor tuning is a prevailing feature of connectivity in the mushroom body, we examined claw response profiles from a population of Kenyon cells (n = 34). We commonly observed that claws of the same Kenyon cell had distinct response profiles (**Fig. 2a**). In some cases, profiles were completely different, whereas, in other cases, there was a prominent difference in the response to just one odor (**Fig. 2a**). We quantified the similarity of claw odor tuning profiles using Pearson's correlation.



For each Kenyon cell, we calculated the correlation between all pairwise combinations of its claws (Fig. 2b). The majority of Kenyon cells received distinct inputs on their claws. We found that 13 of 34 Kenyon cell had pairs of claws with correlation *r* < 0, 25 of 34 Kenyon cells had claws with correlation r < 0.5 and nearly all of the Kenyon cells (30 of 34) had claws with correlations r < 0.9. It is important to note that this measure of tuning curve distinctiveness likely represents a lower bound, as we were only able to test ten odors in these experiments as a result of photobleaching of the claws. Nevertheless, even within these constraints, it was clear that the dominant feature of the results was the differences in claw tuning. These experiments directly demonstrate that Kenyon cell dendrites receive convergent inputs with functionally distinct odor tuning properties, corroborating anatomical measures of convergence<sup>29</sup> and indicating that Kenyon cells meet the first criterion to serve as integrators of the olfactory combinatorial code.

An important question regarding connectivity at this layer is whether there is any relationship between the tuning properties of the different synaptic inputs to a Kenyon cell. A recent anatomical study indicated that convergence of different projection neuron types is random in the mushroom body<sup>29</sup>. We used our functional characterization to assess the relationship between the odor response properties of different claws on a Kenyon cell. We found that claws connected to the same Kenyon cell had significantly more similar odor response profiles than claws from different cells (P < 0.001; **Fig. 2c**). This suggests that projection neurons with similar tuning profiles have a tendency to connect to the same Kenyon cell. To account for the possibility that cell-wide changes in membrane potential contribute to a correlation in calcium signals from the claws<sup>33</sup>, we constructed tuning profiles using only odors that did not evoke a response at the cell's soma, and

Figure 2 Kenyon cell dendrites collect inputs with diverse odor response profiles. (a) Odor response profiles of dendritic claws and cell bodies from five different Kenyon cells (1-5). All of the claws imaged from a single Kenyon cell are presented in a single row with each subpanel depicting the results from a single claw (or soma). Points are responses to a single odor presentation, whereas bars represent the median. Claws are arranged by the order in which they were imaged; odors are ordered for each cell based on the sorted response of the first imaged claw. (b) Pairwise correlations of claw response profiles (n = 34 Kenyon cells). Correlations of claws from the same Kenyon cell are demarcated in a box. Kenyon cells are ordered according to the minimal correlation score. Of the 34 total Kenyon cells, 13 cells had pairs of claws with correlations below 0, whereas 25 cells had pairs with correlations below 0.5. Numbers refer to the Kenyon cells shown in a. (c) Correlation between claws was higher than expected by chance. Shuffled data set was generated by calculating correlations on random sets of claws (gray bands indicate 99% confidence intervals, see Online Methods). Excluded data set was generated by excluding odors that evoked somatic responses for each Kenyon cell separately and calculating pairwise correlation on the reduced response profiles. Note that the negative correlation peak is a result of sparse responses to different odors (for example, claws that respond to different odors will appear negatively correlated). (d) Somatic responses as a function of responding claws. The height of each bar indicates the number of instances where n claws of a Kenyon cell responded to an odor. The black fill denotes instances in which there was also a somatic response in at least two of the three odor presentations. Blue curve shows the probability of somatic response as a function of number of responding claws.



then examined the correlation between claw tuning for this reduced set of odors. Of course, by doing this we removed not only artifactual correlations that arise in the claws from cell-wide voltage effects, but also true correlations that actually drive somatic responses. Even with this constraint, we observed that tuning curves of claws from the same Kenyon cell were more correlated than would be expected by chance. Nevertheless, it remains possible that some feature of cellular physiology other than projection neuron input contributes to the similarity of claw odor tuning. Consequently, we used a more direct approach to test projection neuron–Kenyon cell connectivity optogenetically (see below).

We next examined the relationship of claw activation patterns to the somatic calcium signals that we measured. Calcium signals at the cell body reflect spiking output in cell types from many different systems<sup>31,34,35</sup>, including projection neurons in the antennal lobe<sup>36</sup>. Our goal was to determine whether somatic responses required activation of multiple claws, which would indicate that Kenyon cell responses truly reflect the integration of different input channels. We first determined the number of claws that responded significantly to each odor. Most odors evoked a response in only one or two claws, although there was a wide distribution that ranged up to seven activated claws (**Fig. 2d**). We then assessed whether the odor also evoked a response from the cell's soma (**Fig. 2d**). This revealed that reliable somatic signals typically required the activation of multiple claws. Many odors activated one or two claws, but only in a small proportion of those cases was there an accompanying somatic response. In contrast, when an odor activated several claws, somatic responses were observed much more frequently. In fact, odors that activated six or seven claws invariably elicited a somatic response. It should be noted, however, that we were unable to establish the relationship between these somatic signals and Kenyon cell spiking activity. This would require simultaneous imaging and electrophysiological recordings. Unfortunately, intracellular recordings perturbed the concentration of GCaMP inside the cell, and extracellular recordings were unable to detect Kenyon cell spikes. Thus, to directly address the relationship between claw activation and Kenyon cells spiking, we combined intracellular recordings with optogenetic stimulation of the inputs.

#### Optogenetic characterization of connectivity

By optogenetically controlling a defined set of projection neurons, we sought to determine the number and strength of synaptic contacts that are required to evoke a spiking response from a Kenyon cell (**Fig. 3**). We expressed ChR2-YFP in a subset of ~13 projection neurons innervating 3 of the 54 total glomeruli using the *Mz19-Gal4* driver<sup>37</sup>. Cell-attached recordings in projection neurons confirmed that light-evoked spiking rates were similar to those observed during strong

Figure 3 Detecting functional and anatomical connectivity between projection neurons and Kenyon cells using ChR2-based stimulation. (a) Experimental schematic. ChR2-YFP was expressed in projection neurons innervating 3 of the 54 total glomeruli. Whole-cell Kenyon cell recordings enabled detection of functional connectivity and intracellular dye-filling enabled characterization of anatomical connectivity. (b) Examples of projection neuron-Kenyon cell connections. Magenta represents individual dye-filled Kenyon cell claws. Green represents YFP-expressing projection neuron boutons. Right, merge showing contact. Scale bar represents 2 µm. (c) Projection neuron spiking responses to photostimulation. (d) Postsynaptic responses of an example Kenyon cell. Black traces represent membrane potential recorded on a sample of individual trials, and magenta traces represent the mean (n = 50 trials). Blue shading indicates timing of photostimulation. Note that, for this Kenyon cell, the strongest stimulation evoked a response containing several spikes (denoted by an asterisk). (e) Comparison of the number of connected claws expected from random connectivity (blue bars) with the number observed experimentally (red dots). Blue bars denote the 99% confidence interval of the expected probability of each connection level, calculated using a binomial distribution with probability of connection P = 0.05 (Online Methods). Note that we observed two Kenyon cells connected via five claws in our sample of 39 connected cells, whereas the expected probability of observing five connected claws if connectivity were random is P < 0.00001. (f) Mean projection neuron spiking rates (n = 8) obtained with different photostimulation durations. (g) Overlay of single trial responses of a Kenyon cell to increasing projection neuron photostimulation showing the increase in the evoked response, which culminates with a single spike (denoted by an asterisk). The same Kenyon cell is shown as in d.



odor responses (**Fig. 3f**), although the dynamics of projection neuron spiking were different, as there was a transient peak in firing that was more prominent with ChR2-based stimulation than in a typical odor response<sup>6</sup> (**Fig. 3b**). We performed whole-cell recordings from randomly selected Kenyon cells while stimulating the projection neurons with light pulses of different durations. When we found a connected Kenyon cell, excitatory postsynaptic responses were clear (**Fig. 3d**) and, in rare cases, crossed spike threshold (**Fig. 3d,g**). By dye-filling the neurons, we were able to determine *post hoc* the number of projection neuron–Kenyon cell connection sites. We recorded from 80 Kenyon cells, of which 39 exhibited a clear synaptic response following projection neuron stimulation, and were adequately filled to visualize the entire dendritic tree.

We then examined the connectivity of these cells with confocal microscopy, identifying projection neuron boutons using the ChR2-YFP label and Kenyon cell claws via the intracellular dye-fill. Projection neuron–Kenyon cell synapses were clearly visible, as fine Kenyon cell processes typically formed a grasping or ring-type morphology wrapped around a large globular projection neuron bouton (**Fig. 3c**). For each Kenyon cell that exhibited a clear postsynaptic response, we were able to identify at least one site of anatomical contact, and often more. Pharmacological blockade revealed that Kenyon cell responses were mediated by spike-dependent synaptic transmission (**Supplementary Fig. 1**). We characterized anatomical connectivity in terms of the number of connected claws, that is, a claw contacted by at least one ChR2-expressing projection neuron bouton. We used this definition because, in a small number of cases, a single Kenyon cell claw exhibited a complex morphology, showing contacts with more than one labeled projection neuron bouton (**Supplementary Fig. 2**). Across our data set, we found Kenyon cells connected via one, two, three and even five claws (n = 17, 14, 6 and 2 Kenyon cells, respectively; **Supplementary Movie 2**).

We compared these results to the connectivity levels expected if projection neurons converged randomly onto Kenyon cells. If connectivity is random, the probability of observing a connection between this set of projection neurons and a Kenyon cell is dictated simply by the proportion of projection neuron boutons that are labeled with the Mz19 driver. We estimated this proportion by directly counting the number of ChR2-expressing boutons in Mz19 flies and comparing this to the total number of projection neuron boutons from estimates

Figure 4 Additivity of synaptic input in Kenyon cells. (a) Average time courses of Kenyon cell membrane potential in response to different durations of photostimulation (color bar). Kenyon cell responses are grouped according to the number of claws receiving direct projection neuron input. (b) Response magnitudes increased with number of connected claws (CC). Bottom, each line depicts the mean peak responses of Kenyon cells with n connected claws. Top, mean peak responses for each individual Kenyon cell grouped by the number of connected claws. (c) Multiple synaptic inputs interacted sublinearly in Kenyon cells. Expected response amplitudes were calculated for each duration of photostimulation (b), assuming linear additivity of inputs across multiple claws. Experimentally observed responses for cells with three and five active claws fell below this linear prediction, indicating that multiple inputs interact sublinearly. Bars represent ±s.e.m. (d) Overlay of Kenyon cell membrane potential responses to combined photostimulation and somatic current injection, with current delivered at three different times relative to photostimulation. Observed responses (dark blue) closely matched those expected from linear summation (light blue) of isolated light and current (L + C) responses. (e) Observed/expected ratio for current and light stimulations across a range of different relative timings (n = 7 Kenyon cells).

based on projection neuron-labeling experiments<sup>18</sup>. This yielded a proportion of 0.05. An independent estimate of the fraction of Mz19 boutons yielded a very similar value of 0.04 (ref. 38). We determined the number of connected claws expected for random convergence in a sample of 80 Kenyon cells by drawing from a binomial distribution with a probability of connection of 0.05 and the total number of claws for each Kenyon cell derived from the distribution that we observed experimentally (Supplementary Fig. 3). We repeated this process 1,000 times and used the results to derive the 99% confidence interval for the number of Kenyon cells expected to be found at each connectivity level. Our data set contained significantly higher numbers of Kenyon cells connected via two, three and five claws and lower numbers of cells connected via one claw than would be expected from chance (*P* < 0.001; Fig. 3e). Even if we used the connection probability of 0.14 observed in ref. 29, the number of Kenyon cells that we found connected via three and five claws was still higher than expected from random convergence. Overall, our results suggest that convergence of this set of projection neurons is not random; instead they tend to wire together when they connect to downstream Kenyon cells.

## Linear additivity of dendritic inputs

Having established anatomical levels of connectivity for each recorded Kenyon cell, we next examined the postsynaptic response under these stimulation conditions. Kenyon cells responded with an initial depolarization, which subsequently plateaued at a steady membrane potential (**Fig. 4a**). The plateau occurred roughly 30 ms following stimulus onset, irrespective of the number of activated claws. After this point, even though projection neuron spiking persisted at >100 spikes per s, the membrane potential got no closer to spike threshold. However, the rate of depolarization in the initial time window was greater when more claws were activated (**Supplementary Fig. 4**). Consequently, the membrane potential climbed to a higher level for Kenyon cells with more activated claws (**Fig. 4b**). These results suggest that the additivity of inputs from multiple claws is an important determinant of whether cells cross spike threshold. Consequently, we turned our attention to how inputs from different claws interact.

To examine the additivity of inputs from different claws, we compared responses of Kenyon cells with different numbers of connected claws. Do claws interact synergistically, increasing the drive toward spike threshold when several inputs are activated together, or do they simply add linearly? Synergistic interaction would occur if Kenyon



cells possess voltage-gated channels that boost response amplitudes, which has been observed in other insects<sup>39-41</sup>. Linear or sublinear additivity would arise if dendrites had purely passive cable properties, as assumed in a previous model of *Drosophila* Kenyon cells<sup>18</sup>. We compared the responses observed with multiple active inputs to the response expected from linear summation of a single input (Fig. 4c). Average response amplitudes for Kenyon cells connected via one claw were measured for each of the eight photostimulus durations. The response amplitudes expected for Kenyon cells connected via two, three or five claws were calculated by directly factoring up the amplitudes observed with Kenyon cells connected via one claw. These expected values were compared against the experimentally observed results (Fig. 4c). We found that response amplitudes of Kenyon cells with two connected claws were not significantly different from doubling the responses observed upon activation of a single claw. For Kenyon cells with three connected claws, there was a trend toward a sublinear interaction between claws; this was more prominent with the Kenyon cells we found connected via five claws (n = 2, an inevitably rare occurrence given that we had to label smallnumbers of projection neurons in order to clearly visualize projection neuron-Kenyon cell synaptic connections). We found no evidence for supralinearity when examining Kenyon cell responses to any of the different photostimulation regimes. This was true for both prolonged photostimulation durations that lead to a plateaued response, as well as brief stimulations where responses remained in the initial rapid depolarization phase. It is important to note that under these conditions, Kenyon cells are receiving high rates of projection neuron input in a short time window. The 25-ms stimulation evokes seven

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Figure 5 Kenyon cells require activation of multiple dendritic claws to spike. (a) Histogram depicting both subthreshold (grayscale heatmap) and suprathreshold (z axis) responses to projection neuron photostimulation in synaptically connected Kenvon cells (n = 39 Kenyon cells). Colored frames demarcate cells according to the number of connected claws; colors are as depicted in Figure 4b. Bar height indicates the number of spikes above spontaneous firing. Note the sparsity of spiking responses in this data set, selected solely for synaptic connectivity to ChR2-expressing projection neurons, where only two cells showed reliable spiking responses (>0.5 spikes per trial): one connected via three claws and another via five. (b) Example Kenvon cell spiking responses to photostimulation. In this data set (distinct from **a**), n = 191Kenyon cells were recorded and those exhibiting a reliable spiking response were selected for anatomical analysis to determine the number of connected claws for each cell. Each column of rasters shows an example of spiking activity observed for a particular level of connectivity. The duration of photostimulation is shown at left and timing is indicated by the blue bar. (c) Number of spikes evoked by photostimulation in Kenyon cells with different numbers of claws connected to ChR2-expressing projection neurons (n = 13 spiking Kenyon cells). (d) Proportion of Kenyon cells exhibiting a spiking response across different levels of connectivity. Kenyon cells with four to six connected claws combined for analysis. Inset, blue



points indicate expected distribution of connectivity levels (99th percentile confidence interval) and red points indicate observed frequency of spiking cells with different numbers of connected claws (Online Methods).

projection neuron spikes on average during the light pulse, so Kenyon cells with multiple projection neuron connections receive high frequency input from several projection neurons in this brief period. Thus, these conditions were well-suited for observing synergistic interactions that might arise when multiple inputs arrive within a narrow integration window. Nevertheless, we consistently observed that claws interact linearly or sublinearly.

To further address how inputs are integrated in time, we examined Kenyon cell responses to two precisely timed inputs: current injection at the soma and synaptic stimulation at the dendrites. We delivered a brief (3 ms) pulse of current together with 10-ms photostimulation, systematically varying the relative timing of these two inputs. This enabled us to test whether there is a particular time window in which these two inputs add synergistically. We compared the experimentally observed responses to that expected from linear summation of responses to isolated synaptic and current stimulation. We found a close match between our observations and the predicted linear response, indicating that Kenyon cells are not particularly sensitive to the relative timing of these two inputs (Fig. 4d,e). We also tested whether synaptic responses showed any voltage dependence. We optogenetically stimulated inputs while holding the Kenyon cell at different membrane potentials, but found no indication of voltage-dependent amplification of the synaptic response (Supplementary Fig. 5a,b). In addition, current injection experiments showed no evidence of a nonlinear

component in the Kenyon cell membrane potential response other than spike threshold (**Supplementary Fig. 5c,d**). Altogether, these findings support a model in which synaptic inputs are integrated linearly or sublinearly, with no apparent voltage-dependent boosting of the synaptic response. Although we found no indication that Kenyon cells are especially sensitive to coincident input, interactions across claws are clearly important determinants of response amplitude, and likely contribute to the sparsity of spiking responses in the Kenyon cells.

#### Kenyon cells require activation of multiple claws to spike

The most notable feature of this data set was the scarcity of spiking responses. Although photostimulation evoked large-amplitude depolarizations in numerous cases, spiking responses were only rarely observed (**Fig. 5a**). Only 2 of 39 Kenyon cells climbed above spike threshold with sufficient reliability to resemble an odor response (that is, >0.5 spikes per trial). These responses were found only in cells with three or five connected claws (**Fig. 5a**). We did observe weak light-evoked spiking in two other Kenyon cells with lower levels of connectivity; however, these cells showed atypical spontaneous firing and their responses were not as reliable. These results suggest that Kenyon cells require strong projection neuron input to multiple claws to evoke a significant spiking response.

To directly address the relationship between numbers of connected claws and spiking, we used the same approach, but instead carried out a targeted search for spiking cells. In an entirely separate data set, we recorded from randomly selected Kenyon cells (n = 191), searching for cells that spiked reliably in response to photostimulation and then selecting these for anatomical analysis of connectivity. We found 13 reliably responding cells that were adequately dye-filled (Fig. 5b). We found a range of connectivity amongst these spiking cells, with cells connected to ChR2-expressing projection neurons via one, two, three, four and six claws (n = 2, 3, 1, 6 and 1 Kenyon cells, respectively). To our surprise, the number of spikes evoked by photostimulation was not strongly dependent on the number of connected claws (linear regression  $R^2 = 0.02$ ; Fig. 5c). However, although it was rare for a Kenyon cell to be connected via several claws to the few projection neurons labeled in this experimental situation, most of the cells that spiked did have ChR2-positive connections on multiple claws. We estimated the proportion of Kenyon cells that we expected to be connected via *n* claws, based on our observations of connectivity from the previous experiment where we characterized connectivity of all synaptically responding Kenyon cells. We then used this expected distribution to calculate the proportion of spiking responses for cells with different numbers of connected claws (Fig. 5d). We found a clear relationship between the number of claws receiving strong input and the proportion of Kenyon cells exhibiting a spiking response. In particular, there was a prominent increase in the proportion of spiking responses between three and four contacted claws. Kenyon cells have seven claws on average (Supplementary Fig. 3), so these results suggest that strongly activating more than half of the dendritic inputs is required to drive a Kenyon cell to spike. The fact that different Kenyon cells require different numbers of active inputs may arise in part because there is a wide range of synaptic strengths for individual projection neuron-Kenyon cell connections (Supplementary Fig. 6).

The outcome of these optogenetic experiments was generally consistent with our dendritic imaging results (**Fig. 2d**). In both cases, activation of several claws was required to drive either a spiking response or a somatic calcium signal. However, responses were very rarely observed when a single claw was active, both when imaging odor responses (**Fig. 2d**) and with optogenetic stimulation (**Fig. 5**). These results indicate that Kenyon cells have a strong, but not absolute, requirement for activation of multiple claws to exhibit a reliable spiking response.

## DISCUSSION

There are two basic requirements that must be fulfilled to show that neurons read the combinatorial code of the early olfactory layers: they receive convergent input from functionally distinct glomerular channels and require activation of multiple inputs to respond. Using in vivo imaging of the individual synaptic inputs, we found that Kenyon cells receive inputs with functionally distinct odor response properties. By optogenetically stimulating a defined subset of projection neurons and intracellularly recording from postsynaptically connected Kenyon cells, we found that several of those inputs must be activated to evoke a spiking response in a downstream Kenyon cell. These results indicate that Kenyon cells respond to specific combinations of coactive glomerular channels. This is likely the basis for their highly stimulus-specific response properties. Notably, we found that synaptic summation is essentially linear in Kenyon cells, and multiple lines of evidence suggest that Kenyon cell dendrites have purely passive cable properties. Using patterned photostimulation, others have shown that neurons in olfactory cortex respond selectively to particular patterns of glomerular activation<sup>42</sup>. Thus, the integration of different channels is likely to be a fundamental aspect of the transformation at the third layer of the olfactory system.

Convergence of glomerular channels on Kenyon cell dendrites Anatomical studies originally suggested that the mushroom body might be an important site for the convergence of different glomerular channels. Projection neurons send wide-ranging projections in the calyx of the mushroom body, and the synaptic terminals of different projection neuron types intermingle in this area<sup>37,43</sup>. Moreover, single-cell labeling suggests that the dendrites of individual Kenyon cells extend rather widely in the mushroom body calyx<sup>28,30,44</sup>, suggesting they could collect input from different projection neuron types. Retrogradely tracing the inputs to individual Kenyon cells showed that projection neurons of different glomerular origin converge onto individual Kenyon cells<sup>29</sup>. This study found no statistical structure in the probability of different projection neurons converging onto the same Kenyon cell, suggesting that connectivity at this layer is random. This result contrasts somewhat with earlier anatomical studies that found that different projection neuron types show coarsely regionalized projections, although there was extensive overlap between different projection zones. It has also been shown that different subtypes of Kenyon cells tend to innervate particular regions of the mushroom body calyx<sup>37,43</sup>. Consistent with this loose regionalization, one study reported a correlation in the projection patterns of particular types of projection neurons and Kenyon cells<sup>37</sup>. Altogether, the evidence from these global mapping studies supports a model in which projection neuron-Kenyon cell connectivity is probabilistic and regionally biased rather than completely random.

We took a functional approach to the question of convergence by directly imaging odor responses of individual synaptic sites. Our results indicate that functionally distinct inputs converge onto the dendritic trees of individual Kenyon cells. When we examined the similarity of odor response profiles for the claws of a given Kenyon cell, we found that they were more similar to one another than they were to claws from different cells. This suggests that projection neurons with similar tuning properties have a tendency to converge onto the same Kenyon cells (Fig. 2c). In addition, when we used ChR2 stimulation to examine the relationship between anatomical and functional connectivity, the levels of connectivity that we observed were significantly different from that predicted by random convergence (*P* < 0.001; Fig. 3e). Overall, our results were more consistent with the model of regionalized, probabilistic connectivity derived from global mapping of projection neuron and Kenyon cell projections than that of entirely random convergence supported by retrograde labeling. Our functional approach may have proved more sensitive to detecting correlations in connectivity than a purely anatomical one; correlated response properties are important determinants of wiring in many neural circuits. Nevertheless, the dominant theme of our results was that functionally distinct inputs converge onto the dendrites of individual Kenyon cells. Convergence is likely a core feature of this layer of the olfactory circuit, as transsynaptic tracing has shown that neurons in the piriform cortex receive mitral cell input originating from several different glomeruli<sup>45</sup>.

## Linear integration of dendritic inputs

A recent study investigated the input-output transformation of Kenyon cells by imaging activity in projection neuron boutons and Kenyon cell axons<sup>33</sup>. Examining odor-evoked activity in these different cells, the authors found that the summed activity of the projection neuron inputs correlates well with the likelihood of an axonal response in a postsynaptic Kenyon cell. In contrast, we used an electrophysiological approach to examine synaptic responses to optogenetically controlled projection neuron inputs, enabling us to investigate the integration of inputs from the dendritic claws. By recording from randomly

selected Kenyon cells and reconstructing their morphology *post hoc*, we established both functional and anatomical measures of connectivity for each cell recorded. Comparing responses of Kenyon cells with different levels of connectivity allowed us to determine how different synaptic inputs interact and to establish the relationship between synaptic input and spiking in these cells.

Dendritic inputs from different sites could interact synergistically; for example, by summating to activate voltage-gated channels that boost the synaptic response. Such synergistic interactions have been observed in mammalian barrel cortex, where coordinated input to different dendritic sites from different sensory modalities elicits a distinct bursting response mode in these cells<sup>46</sup>. Synergistic interactions could make it easier for Kenyon cells to respond selectively to particular input patterns; they could enable a clear distinction between activation of a small set of inputs that is not sufficient to bring the cell to spike and activation of a larger, supra-threshold number of inputs. Electrical stimulation experiments in locust Kenyon cells have shown that synaptic responses are amplified and temporally sharpened by a voltage-dependent mechanism<sup>39,40</sup>. In contrast, our results with Drosophila Kenyon cells revealed that claws interact linearly when small numbers are coactive, and sublinearly when larger numbers are stimulated, likely as a result of shunting effects on the dendritic tree. Note that our stimulation conditions were designed to deliver high-frequency projection neuron input in a narrow time window, conditions that are well-suited to reveal synergistic interactions between coincident input. In separate experiments, we examined the interaction between two precisely timed inputs: synaptic stimulation at the dendrites and current injection at the soma (Fig. 4d,e). We examined the additivity of these inputs at a variety of different relative timings, and again found no evidence for synergistic effects that would indicate heightened sensitivity to coincident input. Rather, our results are consistent with a model in which Kenyon cell dendrites serve as passive cables that convey and integrate synaptic input. Notably, this may enable Kenyon cells to take advantage of the format of projection neuron population activity. The antennal lobe transforms olfactory signals so that population representations are more linearly separable in the projection neurons than in the ORNs<sup>6</sup>. By acting as linear integrators, the Kenyon cell dendrites would be well-suited to separate distinct projection neuron activity patterns.

Our results are reminiscent of findings from third-order neurons in vertebrates. A recent study of cells in the dorsal telencephalon of zebrafish showed that these cells exhibit no special sensitivity to synchronous mitral cell inputs<sup>47</sup>. Rather, input synchronization was important to control the precise timing of dorsal telencephalon spikes. Similarly, in mammalian olfactory cortex, synaptic inputs arrive in alternating waves of excitation and inhibition, which enforce temporally precise spiking, phase-locked to this oscillation cycle<sup>25</sup>. In the case of the dorsal telencephalon neurons, the main factor driving cells to spike threshold is a large, slow depolarization, whereas the oscillatory synaptic drive governs the timing of spikes. This is very similar to intracellular recordings of Kenyon cell odor responses: large depolarizations are observed, with high-frequency (although non-periodic) fluctuations riding on top<sup>18,48</sup>. As such, it seems likely that the main factor driving Kenyon cells to spike threshold in Drosophila, similar to dorsal telencephalon neurons, is the slow wave of depolarization that arises from the summation of projection neuron input from several dendritic claws.

#### Kenyon cells detect coactive input from multiple channels

The linear and sublinear additivity of claws is likely an important factor contributing to the sparsity of Kenyon cell spikes. When examining synaptic responses, we found only a few cases in which projection neuron stimulation evoked a response that was consistently above spike threshold in the postsynaptic Kenyon cell. These Kenyon cells were connected via several claws: three claws for one cell, five for the other. To characterize the relationship between connectivity and spiking, we carried out an extensive series of recordings in which we searched specifically for Kenyon cells that spike in response to photostimulation. We found a range of connectivity in the set of responding cells. We compared across these different cells to examine the effects of increasing connectivity on spiking characteristics of Kenyon cells. To our surprise, we found no correlation between the number of contacted claws and the magnitude of the spiking response. Kenyon cells tended to either respond or not, similar to the odor-evoked responses of these cells, which also do not span a wide range of spike rates<sup>18</sup>. However, when we examined the likelihood of Kenyon cell responses as a function of the number connected claws, we observed a strong relationship. This analysis revealed that there was a marked increase in the proportion of responding cells receiving input on four or more claws. Kenyon cells typically have five to seven claws, so this suggests that the majority of a Kenyon cell's claws need to be coactive to evoke an odor-like response.

This requirement was not absolute, however, as we found cells with reliable spiking responses with lower levels of connectivity, and even a very small number of Kenyon cells that spiked when connected via only a single claw (2 of 191 total recorded Kenyon cells). Although it is possible that some Kenyon cells require activation of all their claws, it seems likely that most Kenyon cells require only a subset of their inputs to be active to spike. As there are many different projection neuron input patterns that could activate a subset of the Kenyon cells claws, these results indicate that Kenyon cells encode input patterns in a degenerate manner and that several different input patterns could effectively drive the cell. This is consistent with the odor response properties of Kenyon cells; although these cells are much more odorselective than projection neurons, they do occasionally respond to multiple odors<sup>18,19,48</sup>. Thus, although Kenyon cells' requirement for multiple coactive inputs certainly contributes to their stimulus selectivity, that selectivity is not absolute. Moreover, inhibitory circuit elements could potentially be important for controlling this selectivity, a possibility we have not addressed here<sup>49</sup>. Together, these considerations indicate that Kenyon cells are likely to be degenerate decoders that respond to several related projection neuron response patterns.

We found that Kenyon cells derive their highly specific response properties by integrating over diverse input channels, effectively reading the combinatorial odor code. Why would such selective responses be constructed from synaptic inputs with widely divergent tuning? One possibility is that it gives the mushroom body the flexibility to support olfactory learning. Given that each Kenyon cell is connected to inputs with very different odor tuning, adjusting the synaptic strength of one of the inputs could markedly alter the cell's response properties. In addition, this convergent connectivity is likely important for diversifying the odor response properties of the Kenyon cells relative to the projection neurons. This diversification could enable the network to transition from broadly tuned projection neurons to narrowly tuned Kenyon cells<sup>18–20,50</sup> while maintaining the capacity to represent many different odors.

## METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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#### AUTHOR CONTRIBUTIONS

E.G. and G.C.T. designed the experiments. E.G. performed the experiments and analyzed the data. E.G. and G.C.T. wrote the manuscript.

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The authors declare no competing financial interests.

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#### **ONLINE METHODS**

*In vivo* dendritic imaging. To label small numbers of Kenyon cells with GCaMP3 and myr-tdTomato, we used a recombination-based approach known as mosaic analysis with a repressible cell marker (MARCM)<sup>30</sup>. We constructed heterozygous flies by crossing a reporter strain of genotype: hs-FLP, tubP-Gal80, neoFRT(19A); UAS-GCaMP3; myr-tdTomato/TM3, ser to a driver strain of genotype: neoFRT; OK107-Gal4. (UAS-myr-tdTomato was provided courtesy of T. Clandinin, Stanford University) Mitotic recombination between the FRT sites in these animals causes loss of the Gal80 repressor, releasing expression of GCaMP3 and myr-tdTomato by the OK107-Gal4 driver. Recombination is initiated by flippase, which is under control of a heat-shock inducible promoter. Although the recombination and subsequent expression occurs stochastically in a subset of Kenyon cells, the size of the set of labeled Kenyon cells is influenced by the duration of the heat-shock induction. To generate sparsely labeled animals with an experimentally practicable frequency, we empirically derived the following labeling procedure.

We collected eggs laid in a 2-h time window, transferred them to a vial of fresh food and left them to develop for 4 d at 25 °C. Heat shock was carried out by transferring larvae in food vials to a 37 °C water bath for 20–30 min. The brief duration of this heat shock increased the probability of labeling only one or two Kenyon cells per mushroom body. We conducted the heat shock 4 d post-hatching to increase the probability of labeling  $\sigma'/\beta'$  Kenyon cells, which are born at this time during development<sup>51</sup> and are known to be more responsive to odors than other Kenyon cell types<sup>18</sup>. This protocol enabled us to label one or two Kenyon cells in, on average, one of every four flies (that is, eight brain hemispheres). We imaged dendritic responses in 2–5-d-old female flies produced by this protocol.

Animal preparation. 2–5-d-old female flies were prepared for imaging as described<sup>19</sup>. To minimize brain movement in the head capsule, it was sometimes necessary to immobilize the proboscis by epoxying it in its socket. The peri-neural sheath was left intact for all imaging experiments. Extracellular saline contained 103 mM NaCl, 3 mM KCl, 4 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, 5 mM N-tris(hydroxymethyl) methyl-2-aminoethane-sulfonic acid, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM trehalose and 10 mM glucose. Saline osmolarity was adjusted to 275 mOsm with sucrose if necessary and equilibrated to pH 7.3 by constantly bubbling with a mixture of 95% O<sub>2</sub>/5% CO<sub>2</sub>.

**Odor delivery.** Odor stimuli consisted of 1-hexanol (Sigma), 2-phenylethanol, pentyl acetate, methyl benzoate, trans-2-hexanal, geraniol, apple cider vinegar (Richfood) and three essential oils (Aura Cacia): peppermint, pine and orange. Odors were presented in pseudo-random order in blocks; no odor was presented twice in succession. At least three blocks of trials were presented in every experiment. Odors were delivered at a concentration of 5% saturated vapor using a custom-built odor delivery system<sup>19</sup>. We chose this relatively high odor concentration to increase the likelihood of evoking odor responses in the Kenyon cells. Overall air flow was 1 l min<sup>-1</sup>. Odor pulses were 1 s in duration, and the interstimulus interval was 18 s. Odor delivery was monitored using a photoionization detector (Aurora Scientific) and was highly reliable across trials.

**Dendritic calcium imaging.** Two-photon imaging was carried out using a Prairie Ultima system (Prairie Technologies) and a Chameleon Ti-Sapphire laser (Chameleon XR, Coherent) tuned to 920 nm. Laser power was adjusted to deliver the minimal amount of power that still allowed detection of the dendritic processes based on myr-tdTomato fluorescence (no more than 8 mW at the sample). Emission fluorescence was collected through a HQ607/45-2p filter (Chroma Technologies). All images were acquired using the Olympus LUMPlanFl/IR 60×, 0.90 NA water-immersion objective. Imaging planes were selected to maximize the cross-sectional area of each claw. Typical frames were 120 × 150 pixels, acquired with a pixel dwell time of 4  $\mu$ s, yielding frame rates near 12 Hz. Frame scans were acquired as 11-s trials, from 4 s before odor valve opening, though the 1-s odor pulse, until 6 s after odor valve closing.

**Imaging analysis.** Data were analyzed using custom routines written in Matlab (Mathworks). To correct for motion artifacts, we aligned frames from the same trial by calculating a translational-based discrete Fourier analysis<sup>52</sup> on the anatomical signal from the red channel and applying the translation to both red and green channels. Given that each individual frame was

too noisy to allow for movement correction, three sequential frames were averaged, then median filtered, and the resulting translation was applied to the middle frame.

A region of interest (ROI) was selected manually around each claw using the red myr-tdTomato signal. The boundaries of the ROI were tailored to the image of the claw by examining pixel intensity values throughout the duration of the experiment. Pixels that never exhibited intensity value greater than those observed in a 'background' area of the frame were excluded from the claw ROI.

We quantified claw activity in a series of steps. First, the mean fluorescence signal (F) from all pixels in the ROI was calculated for the green (GCaMP) and red (myr-tdTomato) channels individually. Second, fluorescence values were adjusted for photo-bleaching by fitting an exponential function to the fluorescence values recorded on each trial. The correction was only applied to the red channel, as the green channel did not display photo-bleaching (in addition, the green channel values were subtracted from green baseline; thus, it is of less consequence). Third, to exclude movement artifacts, frames in which the red signal was 2 s.d. below the average were discarded from the time series. If a trial had more than 65%of frames discarded from the baseline or response periods, the entire trial was discarded. Fourth, a  $\Delta F_{\text{Green}}/F_{\text{red}}$  value was calculated for each frame using the formula:  $(F(t)_{\text{Green}} - Fb_{\text{Green}})/F(t)_{\text{Red}}$ , where F(t) is the fluorescence signal for a frame at time (t) and Fb is the baseline fluorescence (averaged over the 3 s before odor valve opening). We quantified the overall magnitude of odor responses for each ROI by summing  $\Delta G/R$  values that were greater than 2 s.d. above baseline. If no such frames were found, the response was defined as zero; we made no attempt to calculate negative responses.

**Calculating tuning correlations with random connectivity.** Correlations between claw tuning were calculated using Pearson's *r*. To generate the distribution of claw tuning curve correlations assuming random connectivity shown in **Figure 2c**, we first pooled together all the claw tuning curves. We then simulated a population of 34 cells by drawing response tuning curves from the pool based on the experimentally determined distribution of Kenyon cell claws (**Supplementary Fig. 3**). All pairwise correlations in this set of simulated Kenyon cells was calculated, and this process was repeated 1,000 times to determine the variance of the expected results.

**ChR2-based projection neuron stimulation.** For projection neuron photostimulation experiments, we used 4–7-d-old heterozygous females generated by crossing Mz19-Gal4 (refs. 37,53) to UAS-ChR2-YFP (line C in ref. 54). This driver labels 13 projection neurons from 3 different glomeruli (DA1, DC3 and VA1d). Progeny were collected on the day of eclosion and transferred onto agar plates, where they were supplied with yeast paste supplemented with 5 mM all-trans-retinal (Sigma). Flies fed on these plates for 3 d at 25 °C in the dark to maintain the retinal in the all-trans state. The plates were supplemented with fresh retinal-containing yeast paste every 2–3 d, and flies were used for recordings during the next 2–3 d.

Flies were prepared as for imaging experiments, except the antennae were surgically removed to reduce baseline synaptic activity<sup>55</sup> and facilitate the detection of light-evoked responses. In addition, the peri-neural sheath was carefully removed with fine forceps. For cell-attached recordings of projection neurons, the head was tilted backwards and the antennae positioned on the upper side of the recording platform. The cuticle between the eyes and the antennae was then removed completely to expose the antennal lobes. Extracellular saline was identical to the saline used in the dendritic imaging experiment, except for the experiments shown **Supplementary Figure 1**, when it was supplemented with either 1  $\mu$ M TTX or 100 $\mu$ M mecamylamine.

Light stimulation was delivered using a blue LED (470 nm) mounted on a collimated holder (Thorlabs LEDC1) in the fluorescence light path of an Olympus BX51 microscope. We controlled the light using a driver (Thorlabs LEDD1B) to provide 1-A current to the LED and custom written Matlab routines to control stimulus timing. Light stimulation was delivered through a LUMPlanFl/IR 60×, 0.90 NA water-immersion objective, providing extensive wide-field illumination of the preparation. Photostimulus durations of 1, 2, 5, 10, 25, 50, 100 and 250 ms were presented in a randomly interleaved fashion.

**Electrophysiology.** Whole-cell recordings from Kenyon cells were performed as described previously<sup>18</sup> except that the saline in the recording pipette contained

 $250 \ \mu$ M Alexa 568 hydrazide (Molecular Probes), which was added fresh to the internal solution from a 1 mM stock immediately before each experiment (internal solution adjusted so final osmolarity was 265 mOsm).

We assessed the quality of Kenyon cell recordings by injecting each cell with a current ramp at three separate times during the experiment and determined spiking threshold. Cells in which spikes could not be detected were excluded from the analysis. Although some cells that showed no response to photostimulation were analyzed fully in the data set, we often aborted the recordings of cells that did not show any sign of functional connection during the recording session. If, after three repeats of the entire photostimulation protocol, there was no obvious response in the Kenyon cell, the recording was typically aborted. For the photostimulation experiment targeting spiking cells (**Fig. 5c,d**), 10 repeats of 25 ms and 250 ms were given at the beginning of the recording and if no spikes were observed the recording was aborted.

Cell-attached projection neuron recordings were done under similar conditions only with the fly's head tilted backwards and marginally wider pipettes to minimize spontaneous break-in. Typical seal resistances were 60–100 MOhm. A strong current pulse (500 pA for 500 ms) was delivered at the end of each recording to verify that spikes could not be evoked and therefore no spontaneous break-in had occurred.

We calculated the magnitude of Kenyon cell responses to photostimulation by averaging membrane potential traces for each stimulus duration and then measuring the difference between the peak of the response and baseline. Spiking responses were quantified by determining the number of spikes in a time window from stimulus onset to 200 ms after stimulus offset and then subtracting mean baseline spiking, calculated in an identical time interval before stimulus onset (although most cells had a baseline rate of zero).

To quantify the range of projection neuron–Kenyon cell synaptic strengths, we measured the amplitudes of individual excitatory postsynaptic potentials (EPSPs) from Kenyon cells connected via a single claw. Using the 25-ms photostimulation trials, we selected individual trials in which EPSPs were identifiable, manually marked the base and peak of the first EPSP in each train, and took the difference as the EPSP amplitude.

Imaging projection neuron-Kenyon cell connections. To effectively visualize anatomical connectivity between projection neurons and Kenyon cells, we dyefilled Kenyon cells intracellularly with Alexa Fluor 568 hydrazide (Invitrogen), which was necessary to discern their fine dendritic processes. However, combining these intracellular fills with standard immunocytochemistry protocols caused a marked reduction in the signal from the dye. Consequently, we directly imaged brains following a brief fixation. Immediately after the recording, the brain was dissected out of the head capsule and transferred to 0.1 M phosphate buffer, pH 7.3. Paraformaldehyde (Electron Microscopy Sciences) was then added to a final concentration of 4% and the brain was rocked for 10 min at 20–25 °C. After this fixation, the brain was washed three times with phosphate buffer, mounted directly on a slide with Vectashield (Vector Laboratories) and imaged. Confocal stacks (0.4- $\mu$ m z steps) were acquired with a Zeiss LSM-510 using a 63× 1.4 NA oil-immersion objective. Connections between projection neuron boutons and Kenyon cell claws were scored blind to the results of functional connectivity, using ImageJ software<sup>56</sup>.

Analyzing connectivity. To estimate the connectivity levels expected from random convergence of projection neurons (Fig. 3e), we calculated connection probability using two independent measures of the proportion of projection neuron boutons labeled with the Mz19 driver. First, we manually counted the number of ChR2-expressing projection neuron boutons in Mz19-Gal4; UAS-ChR2-YFP flies. This was done using the ImageJ Cell Counter plugin (http://rsbweb.nih. gov/ij/plugins/cell-counter.html). The total number of projection neuron boutons in the mushroom body was taken from published counts<sup>18</sup>. Calculated this way, we estimated the proportion of Mz19 boutons at 0.05. A previously published estimate of the proportion of Mz19 boutons across several brains yielded a median of 0.04 (ref. 38). For our calculations, we used a probability of connection of 0.05 (upper quartile in ref. 38).

We used this connection probability to estimate the number of Kenyon cell claws connected to Mz19 projection neurons. We simulated a population of 80 Kenyon cells, drawing the total numbers of claws on each cell from the probability distribution that we measured experimentally (**Supplementary Fig. 3**). We then calculated the number of contacted claws for each of these cells using the binomial distribution with P = 0.05 and n the number of claws on each of the cells. This simulated the number of connected claws expected in a single experiment with 80 Kenyon cells. We repeated this simulated experiment 1,000 times to determine the variance of the expected results. In this way, we estimated the distribution of the number of connected claws expected if connectivity is random.

To generate the distribution of connectivity levels expected for the photostimulation experiment in which we searched for spiking cells (**Fig. 5d**), we derived a parametric description of the connectivity levels we observed in our first photostimulation data set. We fit the observed distribution of connected claw numbers using the EzyFit package from Frederic Moisy on the Matlab file exchange (http:// www.mathworks.com/matlabcentral/fileexchange/10176-ezyfit-2-41). We tested all the discrete distributions and found the geometric distribution was the closest fit. These parameters were then used to simulate a population of 80 Kenyon cells with numbers of connected claws drawn from a geometric distribution. We generated 1,000 such samples of 80 Kenyon cells to establish the mean and variance of the number of those 80 cells connected with different numbers of claws. Note that we used the binomial distribution for **Figure 3e**, as our objective was to test the observed distribution against the distribution expected from random connectivity, whereas, in **Figure 5d**, we used the geometric distribution, as our objective was to fit the data as accurately as possible.

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