Odor mixtures of opposing valence unveil inter-glomerular crosstalk in the *Drosophila* antennal lobe

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Evaluating odor blends in sensory processing is a crucial step for signal recognition and execution of behavioral decisions. Using behavioral assays and 2-photon imaging, we have characterized the neural and behavioral correlates of mixture perception in the olfactory system of *Drosophila*. Mixtures of odors with opposing valences elicit strong inhibition in certain attractant-responsive input channels. This inhibition correlates with reduced behavioral attraction. We demonstrate that defined subsets of GABAergic interneurons provide the neuronal substrate of this computation at pre- and postsynaptic loci via GABA\(_B\)- and GABA\(_A\)- receptors, respectively. Intriguingly, manipulation of single input channels by silencing and optogenetic activation unveils a glomerulus-specific crosstalk between the attractant- and repellent-responsive circuits. This inhibitory interaction biases the behavioral output. Such a form of selective lateral inhibition represents a crucial neuronal mechanism in the processing of conflicting sensory information.
An important role of an animal’s brain is to encode, integrate, and interpret olfactory information from the surrounding environment in order to translate this sensory input into a relevant behavioral output. However, most, if not all, odors encountered are not single molecular compounds, but rather complex blends that vary in both valence and ratio of their individual components. Mixture processing has been well studied in vertebrates\(^2\)\(^,\)\(^3\) and invertebrates\(^4\)\(^,\)\(^5\). However, the origin and the underlying neuronal mechanisms and its correlate to the behavioral output are still unresolved.

The simplicity of the olfactory system of *Drosophila melanogaster* makes it a favorable model to study mixture processing. The fly’s ability to detect odors with olfactory receptor neurons (ORNs) housed in olfactory sensilla on the antennae and the maxillary palps\(^6\). Most of the ~50 ORN types express one (or two) odorant receptors (ORs) together with the co-receptor (Orco)\(^7\). All ORNs expressing the same OR innervate the same glomerulus in the antennal lobes (ALs)\(^8\)\(^,\)\(^9\), where they synapse onto projection neurons (PNs)\(^10\). Glomeruli are interconnected by local interneurons (LNs) which are mainly GABAergic and synapse onto both ORNs and PNs\(^11\)\(^,\)\(^12\). The multimodal innervation of most LNs supports the idea of their role in global inhibition, thereby ensuring gain control\(^13\)\(^,\)\(^14\)\(^,\)\(^15\). Nonetheless, some of the inhibitory LNs were shown to connect defined subsets of glomeruli\(^13\)\(^,\)\(^16\) and might contribute to mixture processing.

Mixture interactions are influenced by the composition and concentration of each component within an odor blend\(^17\). Recent behavioral studies in flies showed that the attraction of a mixture can be predicted by the behavioral responses towards the individual mixture constituents\(^18\)\(^,\)\(^19\). Another study in mice showed that aversive odors can neutralize attractive odors in a blend, or even turn the mixture into a repellent\(^20\). However, it still remains elusive how the olfactory circuitry accomplishes mixture processing and where the neuronal correlate to the fly’s decision is located along the olfactory pathway.

In order to dissect mixture processing in the fly, we used binary blends of odors having opposing valences and established a mixture ratio at which the repellent odor starts to significantly reduce the attraction towards the mixture. We demonstrate that certain glomeruli contribute differentially to the mixture processing through interglomerular crosstalk mediated by GABAergic inhibition, which bias the behavioral output.

**Results**

**Establishing mixture ratios of odors with opposing valence.** To investigate how odor blends are perceived, processed, and evaluated by the fly’s olfactory system, we chose binary mixtures of odors with opposing valences and first determined the ratio of the mixture components at which the repellent odor reduces the behavioral attraction to the mixture. We used the FlyWalk\(^{21}\), a behavioral bioassay monitoring odor-guided walking behavior of individual flies (Fig. 1a). Presenting attractive odors usually results in upwind movement, while repellent odors reduce the flies’ movement\(^{21}\). As a starting point, we picked ethyl acetate (10\(^{-2}\)) as an attractive odor\(^{18}\), benzaldehyde (10\(^{-1}\)) as an aversive odor\(^{21,22}\) and their binary mixture. In this experiment, flies showed the same attraction to the mixture as to ethyl acetate alone (Fig. 1b, c). We next kept the concentration of the aversive odor constant and blended it with a lower concentration (10\(^{-3}\)) of ethyl acetate. Although ethyl acetate on its own was still highly attractive, the attraction towards the binary mixture was significantly reduced (Fig. 1d, e). Hence, we had identified the concentration at which benzaldehyde starts to reduce the attraction to the mixture. We define, hereinafter, the attractive mixture as MIX(+) and the mixture with reduced attraction as MIX(−). To verify that the determined mixture ratio was consistent regardless the behavioral paradigm, we employed an additional two-choice bioassays, the T-maze (Fig. 1f). The flies had to choose between the solvent control (mineral oil) and either the single odors or their binary mixtures MIX(+) or MIX(−).

In line with our FlyWalk data, MIX(+) was equally attractive as the attractive odor, while the flies showed a significantly reduced attraction to MIX(−) (Fig. 1g).

**Glomeruli activated by the attractant are inhibited by MIX(−).** Having established the behavioral output, we next asked how the ratio-dependent switch is encoded in the fly’s olfactory system. As it has been shown that the internal state can influence odor-guided behavior as well as odor-evoked responses in the AL\(^{23}\), we kept the internal state of the flies constant among different experiments (see Methods). Ethyl acetate and benzaldehyde evoke activity in, mostly, non-overlapping glomeruli\(^24\)\(^,\)\(^25\). We first focused on the AL output to analyze whether any mixture processing in form of lateral excitation\(^26\)\(^,\)\(^27\)\(^,\)\(^28\) and/or lateral inhibition\(^12\)\(^,\)\(^27\)\(^,\)\(^28\) was taking place. We expressed GCaMP6s\(^{29}\) in PNs under control of *GHI14-Gal4*, which labels most of the uniglomerular PNs\(^30,31\). Using two-photon imaging we monitored odor-evoked signals in PNs applying the same odor delivery system and odor concentrations as used in our behavioral experiments (Fig. 2a). We verified the stimuli using a photoionization detector and SPME GC-MS (Supplementary Fig. 1). We annotated and analyzed all glomeruli that we could confidently identify based on their anatomical position using the in vivo 3D AL atlas (i.e., 34 glomeruli in total)\(^{30}\) (Supplementary Fig. 2). Using ethyl acetate (10\(^{-2}\)), benzaldehyde (10\(^{-1}\)) and their mixture (MIX(+) ), we observed that the mixture was linearly represented (Fig. 2b, d and Supplementary Fig. 2a). Ethyl acetate evoked the strongest response in glomeruli DM1, DM2, DM3, and DM4, while benzaldehyde induced strong responses in DL1 and DL5, which is in line with previous data\(^{25}\). In general, glomeruli DL1 and DL5 are mostly activated by aversive odors, while glomeruli DM1-DM4 mainly respond to attractive odors and belong to a small and special subset of valence-specific glomeruli\(^22\). Hence, we name this subset of glomeruli henceforth attractant-responsive or repellent-responsive glomeruli. Interestingly when we measured PN responses to the mixture with reduced attraction, i.e., MIX(−), we noticed a strong inhibition in four out of the 34 glomeruli compared to their activity to the single odor component (Fig. 2c, e and Supplementary Fig. 2b). Notably, these inhibited glomeruli are the four most responsive glomeruli to ethyl acetate. To visualize the odor representations we employed a principal component analysis. MIX(+) was located between its individual components, while MIX(−) was clustered with the repellent odor benzaldehyde (Fig. 2f).

We next examined whether the mixture inhibition is concentration- or ratio-dependent. We established a second pair of MIX(+) and MIX(−) in the FlyWalk by reducing the concentrations 10-fold and measured responses in PNs (Fig. 2g\(^\pm\)j and Supplementary Fig. 2c, d). In line with our previous results, only the four attractant-responsive glomeruli were inhibited when stimulated with MIX(−), while the other 30 glomeruli showed a linear mixture representation. We conclude that inhibition of these four attractant-responsive glomeruli is dependent on the ratio between the attractive and aversive odor and is correlated with a reduced behavioral attractiveness of the odor mixture.

**Identity of inhibited glomeruli depends on the repellent.** To address whether other binary mixtures of attractive and aversive compounds induce the same kind of mixture interactions, we tested other odor combinations. We wondered whether activation
solely one of the two repellent-responsive glomeruli is sufficient to induce mixture interactions. To investigate this we selected the odor methyl salicylate, which is a Drosophila repellent and activates only glomerulus DL1 at the used concentration of $10^{-3}$ (Supplementary Fig. 3a, b). We blended methyl salicylate with different concentrations of ethyl acetate and determined another set of MIX($+$) and MIX($-$). In this odor combination, behavioral attraction towards high concentration of ethyl acetate ($10^{-2}$) was not affected, while the attraction of the lower concentrated attractant ($10^{-3}$) was significantly reduced by the repellent in the mixture (Fig. 3a, b). When testing the different mixtures in calcium imaging experiments, we found inhibition in only two out of the four attractant-responsive glomeruli (DM1 and DM4) during stimulation with MIX($-$), while MIX($+$) was linearly represented (Fig. 3a, b and Supplementary Fig. 3a, b). This implies that activation of glomerulus DL1 might...
induce inhibition of DM1 and DM4, while activation of DL5 might be required for inhibition of DM2 and DM3 — an assumption that we pursue in more detail in the next section.

We then asked whether the mixture-induced inhibition occurs also in other glomeruli that are activated by attractive odor compounds. We therefore chose balsamic vinegar as it is one of the most attractive odors for vinegar flies. Moreover, as it contains ethyl acetate, it activates overlapping but also additional glomeruli (Supplementary Fig. 3c). Again, we determined the concentrations at which the attraction to balsamic vinegar was
Fig. 2 Glomeruli responding to the attractive odor reveal mixture inhibition. a Schematic of odor delivery system connected to 2-photon microscope. FM flowmeter, cont. continuous, O1/O2 odor 1/odor 2. b, c Representative odor-evoked calcium responses in PNs from three focal planes. Gray-scale images represent AL structure with identified glomeruli. Calcium responses are shown to ethyl acetate (10^−2/10^−3), benzaldehyde (10^−3), MIX (+) and MIX (−). Scale bar = 20 μM. d Mean PN activity of strongest activated repellent-responsive (DL1, DL5, red) and attractant-responsive glomeruli (DM1, DM2, DM3, DM4, blue-green) during stimulation with ethyl acetate (10^−2, blue-green), benzaldehyde (10^−1, red) and their binary mixture (MIX(+), yellow). Odor responses of all annotated glomeruli (in total 34) are shown in Supplementary Fig. 2. Upper panel, averaged time traces of calcium signals with SEM seems not to take place in these cases. We noted that stimulation of the T-maze that allowed us to monitor strong repulsion in odor-stimulated flies. We therefore hypothesize that not all activated glomeruli might be crucial for odor valence coding and that only very few, special glomeruli show valence-specificity – an assumption that needs to be tested further. Notably, Or10a−/− flies showed still a strong repulsion to benzaldehyde suggesting that this avoidance is mediated through several channels in a combinatorial way.

Specific glomerular crosstalk. Our results thus far show that glomeruli DL1 and DL5 might distinctively inhibit the four attractant-responsive input channels. We, therefore, postulate a glomerulus-specific crosstalk between the attractant- and repellent-responsive glomeruli. To test this, we first investigated the effect of selectively silencing the input to the repellent-responsive glomeruli at a functional and behavioral level. To do so, we monitored calcium signals from PNs after stimulation with MIX (+), MIX (−), and the individual odors benzaldehyde and ethyl acetate in flies where DL1 or DL5 were individually silenced using a mutant background of Or10a or Or7a, respectively (Fig. 4a–f). Both mutants revealed no odor-evoked PN activity in the corresponding glomerulus, indicating that lateral excitation seems not to take place in these cases. We noted that stimulation with MIX (+) did not result in any inhibition in flies bearing one of the two mutant backgrounds (Supplementary Fig. 4a, b). However, the MIX (−)-induced inhibition in DM1 and DM4 was abolished in flies with a non-functional DL1 glomerulus, while the inhibition of glomeruli DM2 and DM3 was still visible (Fig. 4b, c). Interestingly, when we silenced DL5, the MIX (−)-induced inhibition was abolished in DM3 and reduced in DM2, while DM1 and DM4 were unaffected (Fig. 4e, f).

We then wondered whether silencing the repellent-responsive receptors would also affect the behavioral output. We turned to the T-maze that allowed us to monitor strong repulsion in odor-guided behavior (Fig. 4g). Using w1118 as a control line, we observed a robust repulsion to benzaldehyde (10^−1), while both concentrations of ethyl acetate (10^−2 and 10^−3) were highly attractive (Fig. 4h). Notably, when the input to DL1 was silenced, mutant flies were attracted to MIX (−), while they were still repelled by benzaldehyde alone. As the inhibition of the attractant-responsive glomeruli DM1 and DM4 was subsequently abolished, the modified behavioral output indicates the importance of these glomeruli for behavioral attraction. Indeed, silencing the input to these two glomeruli by expressing UAS-Kir2.135, odor attraction towards ethyl acetate as well to both mixtures was abolished and even shifted to aversion for MIX (+) and MIX (−) (Supplementary Fig. 4c). This finding is consistent with previous studies showing the significant role of Or42b and Or59b (i.e. DM1 and DM4) for flies’ odor attraction. Surprisingly, silencing glomerulus DL5 did not change the flies’ preference towards any of the tested odors (Fig. 4h). We therefore hypothesize that not all activated glomeruli might be crucial for odor valence coding and that only very few, special glomeruli show valence-specificity – an assumption that needs to be tested further. Notably, Or10a−/− flies showed still a strong repulsion to benzaldehyde suggesting that this avoidance is mediated through several channels in a combinatorial way.

We next asked whether activation of a specific ORN population that responds to aversive odors is sufficient to induce the observed inhibition. To do so, we replaced the aversive odor by optogenetic activation of the repellent-responsive glomeruli, while we simultaneously stimulated the antennae with ethyl acetate. The red-shifted channelrhodopsin CaChrimson37 was expressed in Or10a- or Or7a-expressing ORNs, respectively, while we monitored calcium signals in PNs (Fig. 5a, d). We first calibrated the light intensity required to evoke activity that simulated the physiological response to odor stimulation (Fig. 5b, e and Supplementary Fig. 5a, b, f, g). Artificial photoactivation of Or10a-expressing ORNs (i.e. DL1) inhibited the calcium responses to ethyl acetate in glomeruli DM1 and DM4 (Fig. 5c, and Supplementary Fig. 5d, e). On the other hand, when we photoactivated DL5 during stimulation with ethyl acetate, the activation of DM3 was significantly inhibited (Fig. 5f and Supplementary Fig. 5i, j). However, excitation of glomerulus DM2 was only slightly reduced by artificial activation of either Or7a- or Or10a-expressing ORNs. Possibly, inhibition of DM2 might require co-activation of both repellent-responsive glomeruli DL1 and DL5 and weakly activated glomeruli. As expected, control flies (i.e. flies fed on artificial food with no all-retinal) showed no activation in DL1 or DL5 with light stimulation, and displayed an unmodified activation of the
attractant-responsive glomeruli during light and ethyl acetate stimulation (Supplementary Fig. 5c, e, h, j). These results are consistent with the data obtained by silencing single ORN types (Fig. 4).

Next we wondered whether the behavioral response to MIX(−) could be mimicked by replacing the aversive odor with optogenetic activation of the two repellent-responsive glomeruli. CsChrimson was expressed in Or10a- or Or7a-expressing ORNs and the behavioral response to ethyl acetate was monitored in the T-maze (Fig. 5g). Artificial activation of DL1 and DL5 combined with stimulation of ethyl acetate at 10−2 was as attractive as ethyl acetate alone which resembles the response to MIX(+). As predicted, a lower concentration of ethyl acetate (10−3) combined with artificial activation led to significantly reduced attraction and mimicked the behavioral response to MIX(−). Notably, phot activation of DL1, DL5, or both resulted in aversive behavior.
Fig. 3 Different binary mixtures evoke glomerulus-specific inhibitions. a, b Upper panel, box plots represent net upwind displacement in the FlyWalk within 4 s following stimulation with ethyl acetate (10^{-2} and 10^{-3}, blue-green/bright blue-green), methyl salicylate (10^{-3}, magenta) and their binary mixtures (MIX(+) and MIX(−), yellow/orange). Colored dots and gray lines represent individual flies (n = 30, Wilcoxon signed rank test). Lower panel, mean PN activity of strongest activated attractant- and repellent-responsive glomeruli during stimulation with the odors used in the FlyWalk (n = 6, paired t-test). c Left, box plots represent net upwind displacement in the FlyWalk within 4 s following stimulation with balsamic vinegar (10^{-2}, blue), benzaldehyde (10^{-1}, red), and their binary mixture (MIX(−), orange) (n = 30, Wilcoxon signed rank test). Right, mean PN activity of strongest activated attractant- and repellent-responsive glomeruli during stimulation with the odors used in the FlyWalk. (n = 6, paired t-test). d Left, box plots represent net upwind displacement in the FlyWalk within 4 s following stimulation with balsamic vinegar (10^{-2}, blue), geosmin (10^{-1}, pink), and their binary mixture (MIX(−), orange, n = 30, Wilcoxon signed rank test). Right, mean PN activity of strongest activated attractant- and repellent-responsive glomeruli during stimulation with the odors used in the FlyWalk (n = 6, paired t-test). Odom responses to the different mixture combinations of all annotated glomeruli (in total 34) are shown in Supplementary Fig. 3.

![Diagram](image1.png)

**Fig. 4** Selective silencing of input channels reveals glomerulus-specific inhibition. a Schematic of the experimental design: Or10a-expressing ORNs (targeting DL1) are not functional in a Or10a−/− mutant background. Color code indicates glomerulus-specific activation by the attractive (bright blue-green) or repellent (red) odor. b Representative odor-evoked calcium responses in PNs from three focal planes of Or10a−/− mutant fly expressing UAS-GCaMP6s in PNs. Gray-scale images represent the AL structure highlighting the attractant- (DM1, DM2, DM3, and DM4) and repellent-responsive glomeruli (DL1 and DL5) with colored circles. Calcium responses are shown to stimulation with ethyl acetate (10^{-1}, blue-green) or repellent (red) odor. c Schematic of the experimental design: Or7a-expressing ORNs (targeting DL5) are not functional in a Or7a−/− mutant background. Color code indicates glomerulus-specific activation by the attractive (bright blue-green) or repellent (red) odor. d Representative gray-scale and pseudocolored images of odor-evoked calcium responses in PNs from three focal planes of a Or7a−/− mutant fly expressing UAS-GCaMP6s in PNs. Calcium responses are shown to stimulation with ethyl acetate (10^{-3}, benzaldehyde (10^{-1}), and their binary mixture (MIX(−)). Scale bar = 20 μM. e Mean PN activity of repellent- and attractant-responsive glomeruli during stimulation with ethyl acetate (10^{-3}, bright blue-green), benzaldehyde (10^{-1}, red), and MIX(−) (orange) in Or10a−/− mutant flies. Individual flies are given by individual dots and lines; mean is indicated by black thick line (n = 10, paired t-test). Pairwise comparisons of mixture responses to the response with the strongest single component (i.e. ethyl acetate or benzaldehyde) are shown for each animal. d Schematic of the experimental design: Or7a−/− expressing ORNs (targeting DL5) are not functional in a Or7a−/− mutant background. Color code indicates glomerulus-specific activation by the attractive (bright blue-green) or repellent (red) odor. e Representative gray-scale and pseudocolored images of odor-evoked calcium responses in PNs from three focal planes of a Or7a−/− mutant fly expressing UAS-GCaMP6s in PNs. Calcium responses are shown to stimulation with ethyl acetate (10^{-3}, benzaldehyde (10^{-1}), and their binary mixture (MIX(−)). Scale bar = 20 μM. f Mean PN activity of repellent- and attractant-responsive glomeruli during stimulation with ethyl acetate (10^{-3}, bright blue-green), benzaldehyde (10^{-1}, red), and MIX(−) (orange) in Or7a−/− mutant flies (n = 12, paired t-test). g Schematic of the T-maze assay. h Box plots showing behavioral preference indices in the T-maze of Or10a−/− mutant (pink), Or7a−/− mutant (purple), and w1118 flies (gray) to the odors benzaldehyde (10^{-3}, ethyl acetate (10^{-2}/10^{-3}), MIX(+), and MIX(−) against the solvent control (MOL). (n = 15−19, one-way ANOVA with posthoc Tukey test, **p < 0.01). Filled boxes are significantly different from zero, empty boxes not (Student’s t-test).
Fig. 5 Optogenetic activation of repellent-responsive glomeruli unveils glomerular crosstalk. a Schematic of the experimental design: artificial activation of CsChrimson by red light in Or10a-expressing ORNs (targeting DL1) during stimulation with ethyl acetate. Color code reflects activation by light (red) or attractive odor (bright blue-green). b Calcium signals (time traces and pseudocolored images) of glomerulus DL1 to photoactivation with increasing intensities of 619 nm light for 2 s. Gray boxes indicate light stimulation. Lines represent averaged response, shadows give SEM (n = 3). Dashed box marks the light intensity used for further experiments. c Mean PN activity of repellent- and attractant-responsive glomeruli during stimulation with either light (red dots), ethyl acetate (10−3, bright blue-green dots), or both combined (additional red rectangles) in flies expressing CsChrimson in ORNs of DL1 and GCaMP3 in PNs (n = 19, paired t-test). d Schematic of the experimental design: artificial activation of CsChrimson by red light in Or7a-expressing ORNs (targeting DLS) during stimulation with ethyl acetate. e Same experiment as in b but for CsChrimson expression in ORNs of DLS (n = 4). f Same experiment as in c but for CsChrimson expression in ORNs of DLS (n = 21, paired t-test). g Box plots showing preference indices in the T-maze assay of flies with artificial activation of ORNs expressing Or10a (DL1), Or7a (DLS) or both via CsChrimson by red light alone (bulb) or red light combined with an odor (bulb + odor) against the dark arm of the T-maze without (Blank) or with solvent (MOL) (n = 22-24, one-way ANOVA with posthoc Sidak’s multiple comparisons test, *p < 0.05, ***p < 0.001). Treatment and genotypes are indicated by the table below. In our assay, control flies (no all-trans retinal) showed reproducible slight attraction to light. Based on this, we used the control flies as the comparison point for calculating optogenetically driven avoidance.

Mixture inhibition is mediated by GABA. We next turned our attention to the neuronal mechanism. Most of the odor-induced inhibitions in the Drosophila AL are mediated by the inhibitory neurotransmitters GABA, which binds to GABAA and GABAB receptors12,27,28, and glutamate, which opens glutamate-gated chloride channels (GluCl)39.

In order to block GABAergic and/or glutamatergic receptors in the AL we applied the antagonist CGP54626 (50 µM) to silence GABAergic receptors and picrotoxin (100 µM) to block the Rdl subunit of the GABAergic receptor and the GluCl. We simultaneously monitored the odor-induced calcium signals in PNs. By blocking GABAergic receptors, we noticed a reduction in the MIX(−)–induced inhibition in the four attractant-responsive glomeruli compared to the saline or wash-out situation (Fig. 6a, b). To quantify this reduction, we calculated the differences between the normalized peak responses upon stimulation with MIX(−) and the attractant alone (Fig. 6c). As expected, the peak response differences in the four attractant-responsive glomeruli were significantly reduced after CGP54626 treatment compared to the controls. Interestingly, after blocking GABAergic and GluCl receptors, only glomeruli DM1 and DM4 showed a significant reduction in their inhibition to MIX(−) (Fig. 6d–f). Picrotoxin could not be washed out, as shown previously15. When we applied both antagonists simultaneously, the MIX(−)–induced inhibition was totally abolished in all four attractant-responsive glomeruli (Fig. 6g–i), while we did not observe any obvious effects on the repellent-responsive glomeruli (Supplementary Fig. 6).

Our pharmacological approach has two weak points: first, picrotoxin at the used concentration blocks both, the GABAergic and GluCl receptors. Second, the antagonists act on the pre- and postsynaptic sites and do not allow pinpointing where the inhibition takes place. To overcome these issues we used RNA interference to target either GABAergic or glutamatergic receptors selectively at the pre- and postsynaptic sites of AL input and output neurons. We employed UAS-Rdl RNAi against compared to the control flies (Fig. 5g). This supports the notion that these repellent-responsive glomeruli function as aversive input channels and belong to a special subset of valence-specific glomeruli. This assumption is further substantiated by the fact that the PN axons of DL1 and DL5 in the lateral horn reveal very similar and overlapping axonal arborizations as other aversive-specific PNs38.
the Rdl subunit of GABA_40, UAS-GBi against the GABA_B2 subunit^{28}, UAS-glutamate RNAi against the GluCl^{39} and UAS-empty-RNAi as a control. We confirmed the efficiency of the RNAi lines by RT-PCR (Supplementary Fig. 7a). First, we expressed these RNAi lines separately at the postsynaptic sites (i.e. in PNs), while visualizing odor-evoked calcium signals in PNs (Fig. 7a). Interestingly, blocking GABA_A receptors significantly reduced the inhibition to MIX(−) in two out of four attractant-responsive glomeruli (DM1 and DM4) (Fig. 7b, c). Neither silencing GABA_B receptors nor GluCl a did affect the inhibition induced by MIX(−) in any of the attractant-responsive glomeruli. The repellent-responsive glomeruli revealed a linear mixture response independent of the RNAi line expressed (Supplementary Fig. 7b). As expected, the representation of MIX(+) was also not influenced by any RNAi expression (Supplementary Fig. 7c). These findings indicate that GABA_A...
receptors mediate the MIX(−)-specific inhibition at the postsynaptic site in two out of four attractant-responsive glomeruli, which is well in line with our results deriving from the pharmacological treatment with picrotoxin (Fig. 6d–f).

Although our results show that pharmacological blocking of GABA_B receptors significantly reduced the MIX(−)-induced inhibition in all four attractant-responsive glomeruli (Fig. 6a–c), genetic silencing of GABA_B via RNAi selectively in PNs did not affect the mixture inhibition (Fig. 7a–c). We therefore wondered whether part of the observed GABA_B-dependent inhibition derives from inhibition in ORNs. Indeed, GABA_B-mediated inhibition at the presynaptic sites has already been well characterized27,28. To selectively block GABA_B receptors at the presynaptic site, we expressed the same RNAi lines in ORNs with MIX(−) induced a strong and significant inhibition at the postsynaptic densities of those LNs vary between the attractant-responsive and repellent-responsive glomeruli, we expressed synaptotagmin- fused with GCaMP3 as a postsynaptic marker43 selectively in LNs innervating glomerulus DL1 that target the corresponding lines, we performed neural tracing by reconstructing and annotating their innervation in our glomeruli (Fig. 8e, f). To investigate whether the pre- and postsynaptic densities of those LNs vary between the attractant- and repellent-responsive glomeruli, we expressed synaptotagmin-hemagglutinin (Syt-HA) as a presynaptic marker and DII fused with GCaMP3 as a postsynaptic marker43 selectively in sites, while other glomeruli are inhibited solely at the presynaptic locus.

Defined subsets of GABAergic LNs mediate mixture inhibition. Finally, we aimed at identifying the LN population underlying the mixture-induced GABAergic inhibition. We selected four different enhancer trap lines that label various types of GABAergic LNs ranging from pan-glomerular, continuous, and regional to patchy LN populations13. To selectively silence LN-mediated inhibition we expressed an RNAi construct against glutamic acid decarboxylase (Gad) in conjunction with UAS-Dicer2 to knock-down GABA synthesis41 in each of the different LN subsets, while we monitored calcium responses to MIX(−) and the individual odors in PNs. We confirmed the reduction in GABA production via immunostaining (Fig. 8a–d). Interestingly, silencing GABA release in two out of the four LN lines, that label many patchy LNs13, significantly reduced the mixture inhibition in the attractant-responsive glomeruli. The mixture inhibition in DM3 was reduced by silencing GABAergic LNs using NP3056-Gal4 (Fig. 8b and Supplementary Fig. 9a), while knocking-down GABA release in HB4-93-Gal4 abolished the mixture inhibition in DM1 and DM4 (Fig. 8d and Supplementary Fig. 9a). In contrast, GABAergic LNs labeled by the mostly pan-glomerular LN lines GH298-Gal4 and H24-Gal413 seem not to contribute to the inhibition of the attractant-responsive glomeruli (Fig. 8a, c). Moreover, we observed that the response to ethyl acetate was increased in flies where the GABA release was silenced in GH298-Gal4 and NP3056-Gal4 (Supplementary Fig. 9a), which might be due to the absence of gain control. We attempted to monitor the behavioral consequences regarding mixture processing of flies with impaired lateral inhibition. However, such broad manipulations led to unexpected behavioral responses to single odors which prevented us from employing these RNAi lines for further behavioral experiments.

In sum, our results demonstrate that defined LN types are involved in a glomerulus-specific lateral inhibition induced by odor mixtures. Furthermore, our data suggest that HB4-93-Gal4 should comprise LNs innervating glomerulus DL1 that target glomeruli DM1/DM4, while NP3056-Gal4 should include LNs that innervate DL5 and inhibit DM3. To confirm that the aforementioned glomeruli are connected by patchy LNs from the corresponding lines, we performed neural tracing by expressing photoactivatable GFP (PA-GFP)42. We illuminated PA-GFP in single somata to selectively label individual LNs, reconstructed and annotated their innervation in our glomeruli of interest (Fig. 8e, f). To investigate whether the pre- and postsynaptic densities of those LNs vary between the attractant- and repellent-responsive glomeruli, we expressed synaptotagmin-hemagglutinin (Syt-HA) as a presynaptic marker and DII fused with GCaMP3 as a postsynaptic marker43 selectively in...
quantification of the fluorescent signal of both markers reveals that LNs labeled by NP3056-Gal4 and HB4-93-Gal4 possess a significantly higher density of postsynapses in the repellent-responsive glomeruli (DL5 and DL1), while presynapses are stronger pronounced in the attractant-responsive glomeruli (DM3 and DM1/DM4; Fig. 8g, h). This data suggests that pre- and postsynapses of inhibitory LNs are not uniformly distributed among different glomeruli which might cause the observed heterogeneity of the lateral inhibition.
**Discussion**

In this study, we analyzed the integration of binary odor mixtures of opposing hedonic valences and demonstrate how glomerular-specific inhibition and crosstalk results in an appropriate behavioral output. We show that glomeruli that strongly respond to the attractive odor are inhibited by the repellent odor in the mixture, which is mediated by defined subsets of GABAergic LNs (Fig. 9). Heterogeneity in responses to mixtures has been shown in previous studies where excitation of some glomeruli by one of the mixture components can inhibit the glomeruli activated by the other component. Similar to invertebrates, evidence for non-linearity of mixture interactions has been reported in individual mitral/tufted cells (PNs analogs) in the olfactory bulb of vertebrates. As an alternative scenario it is also conceivable that instead of inhibiting the attractant-coding pathway to shift the behavior towards aversion, the response of the repellent-responsive glomeruli could be boosted via lateral excitation. Lateral excitation has been described to drive synergistic interaction between the binary mixture of cis-vaccenyl acetate and vinegar. Although odors representing sex and food are mutually reinforcing, a binary mixture of odors with opposing valences means a conflicting input. We therefore postulate that, in contrast to reinforcing input, conflicting sensory input is processed via lateral inhibition in the fly AL. An assumption that would be intriguing to be tested in the future.

We did not observe any inhibition of the attractant-responsive glomeruli when we stimulated with MIX(+). This lack of inhibition is probably due to the strong ORN input leading to high presynaptic firing rates in the attractant-responsive glomeruli. Consequently, lateral inhibition deriving from the aversive circuit has only a low impact and does not decrease the excitation of the attractant-responsive glomeruli.

Obviously not all glomeruli that are activated by an attractive odor are inhibited by a repellent in a mixture and might not contribute to the attractiveness of an odor. This observation makes sense in the light of accumulating evidence suggesting that the innate behavioral output is correlated either to the summed weights of specific activated glomeruli or to the activity of single processing channels. The latter argument is supported by the finding that only very few, special glomeruli seem to be valence-specific and induce clear attraction or aversion behavior upon artificial activation.

It is important to mention that our subset of repellent-responsive glomeruli does also respond to non-aversive and even partly attractive odors, such as E2-hexenal and ethyl benzoate. However, an attractive odorant may indeed activate some aversive input channels beside their main activation of the attractive circuitry (or the other way around). What actually matters is the behavioral output that is consequently elicited when a specific glomerulus becomes activated. For example, ORNs that respond to CO2 are also activated by ethyl benzoate and E2-hexanal. However, the CO2 circuit has been clearly demonstrated to mediate behavioral aversion. Following this argument, artificial activation of glomeruli DL1 and/or DL5 leads to aversive behavior, while silencing DM1 and DM4 abolished attraction to the attractant. These experiments provide evidence that activation of the repellent- and attractant-responsive glomeruli causes a valence-specific behavior, and can therefore be defined as attractive or aversive input channels, respectively.

Interestingly, we observed one exception in our data set: although the repellent odor geosmin reduced the attraction to balsamic vinegar in the mixture, we did not observe any mixture inhibition. The detection of geosmin is one of the rare cases, where an odor is detected by only one receptor type and consequently activates only one glomerulus. Similar specialized pathways have been described for the detection of sex pheromones and CO2. Glomeruli processing these ecologically labeled lines differ from broadly tuned glomeruli with regard to their neuronal composition. Hence, it is conceivable that the narrowly tuned geosmin-responsive glomerulus does not exhibit strong interglomerular interactions and has therefore a different impact on the attractant-responsive glomeruli. Mixture interactions between geosmin and attractive odors might be implemented in higher processing centers which contain circuit elements mediating interactions between odors.

Lateral inhibition, which is believed to enhance contrast and to facilitate discrimination of similar stimuli, is an important motif throughout the nervous system. In mice, dense centersurround inhibition refines mitral cell representation of a glomerular map, while other evidence showed that lateral inhibition can be rather selective and biased between different mitral cells. In accordance with the olfactory bulb, the AL exhibits broad, selective or even both forms of lateral inhibition, whereby certain glomeruli can show different sensitivities towards an inhibitory input. Lateral inhibition in the *Drosophila* AL is largely mediated through GABAA receptors. Most of the GABAergic inhibition in the *Drosophila* AL has been shown to take place predominantly on the presynaptic site mediated through GABA_\text{A}_{\text{L}}_1 and GABA_\text{A}_{\text{L}}_2 receptors. In addition, PNs also receive GABAergic inhibition via GABA_\text{A}_{\text{L}}_1 and/or GABA_\text{B}_{\text{L}}_1 receptors from LNs. Notably, we found that two out of four attractant-responsive glomeruli are inhibited on the pre- and postsynaptic levels (via GABA_\text{B}_{\text{L}}_1 receptors), while the other two glomeruli are inhibited only presynaptically through GABA_\text{B}_{\text{L}}_1-type receptors. Previous results have shown that GABA_\text{B}_{\text{L}}_1 receptors can either mediate mixture-induced inhibition during the full period of the odor presentation which is reminiscent to tonic inhibition in the mammalian system.

We show that mixture-induced lateral inhibition of the attractant-responsive glomeruli was abolished when we silenced GABA synthesis in mostly patchy LNs. Hence our data suggest, in consistency with previous studies, that LNs with more selective...
innervations mediate glomerulus-specific interactions and rather contribute to mixture processing, while pan-glomerular LNs (e.g. GH298-Gal4 and H24-Gal4), that globally release GABA, might be involved in gain control\(^{10,12,13,16}\).

Interestingly, the repellent-responsive glomeruli DL1 and DL5 did not show any mixture interaction, but mediate the lateral inhibition of the attractant-responsive glomeruli. We can think of two possible scenarios that would provide the neuronal
circuits offer insights into the principle of sensory integration. It illustrates the selective lateral inhibition in different glomeruli. Both scenarios could either occur separately or reinforce each other. Moreover, it might be ecological relevant not to inhibit the input of the aversive substrate for this mechanism dependent on either the donor (i.e. LN) or the receiver (i.e. glomerulus) side. First, since glomeruli vary dramatically in their GABA sensitivity and consequently their sensitivity to LN activation, lateral inhibition is heterogeneous across different glomeruli. Second, lateral inhibition is biased among different glomeruli due to a glomerulus-specific synaptic distribution of pre- and postsynapses of GABAergic LNs, i.e. the GABA release is not uniform. This assumption is supported by our data revealing that GABAergic LNs possess a higher density of postsynapses in DL1 and DL5 than in the attractant-responsive glomeruli. In line with our findings, EM based data from the larvae AL describe GABAergic, oligoglomerular ‘choisy’ LNs with a clear polarity contributing to postsynaptic inhibition for most glomeruli, while they receive inputs from only a small glomerular subset. Hence, there is strong evidence that some glomeruli can drive lateral inhibition in other glomeruli. Both scenarios could either occur separately or reinforce each other. Moreover, it might be ecological relevant not to inhibit the input of the aversive pathways since these are associated with life-threatening situations that should be coded reliably and rather override an attractive input.

In contrast to our expectation, sole photocapitulation of DL1 or DL5 with stimulation of the repellent alone did not induce inhibition in the attractant-responsive glomeruli. This might be due to the low spontaneous activity of ORNs innervating the attractant-responsive glomeruli, which correlates with spontaneous fluctuations in the membrane potential of the postsynaptic PN. Consequently, inhibitory responses (i.e. hyperpolarizations) are difficult to capture with calcium imaging.

In other sensory systems, lateral inhibitory connections of neuronal subsets involved in sensory processing have been elucidated in great detail, such as in the retina of mice or the rat visual cortex. Also for the Drosophila AL, previous studies suggested that glomerular subgroups are connected via inhibitory LNS. However, these studies could neither pinpoint the precise connections nor their significance for behavioral perception.

Our data provide evidence for a specific inhibitory crosstalk between identified glomeruli and substantiate the existence of selective lateral inhibition in the fly AL. Our postulated network circuits offer insights into the principle of sensory integration. It will be intriguing to see whether neuron-specific crosstalk represents a general phenomenon to integrate multiple and rather conflicting input channels in other sensory modalities.

**Methods**

**Fly stocks.** Flies were reared on conventional cornmeal agar medium under 12 h/12 h light/dark cycle at 25 °C (except for the experiment involving PCR, which were reared at 18 °C). All experiments were performed on adult females. Genotypes used in each figure are listed in Supplementary Table 1. The following stocks were used: Canton-S wildtype flies, GHH16-Gal4 (from Leslie Vosshall’s lab.), 20XUAS-IVS-GCaMP6s (gift from Christo-pher Potter), UAS-RdRed-G and UAS-CgI-EGFP (both are gifts from Mani Ramaswami), UAS-icer2 (BDSC 24644), Or22b-Gal4 (BDSC 9972), Or59b-Gal4 (BDSC 23898), UAS-Kir2.1 (P[w+]; +/+, UAS-Hspa1k;NC2997EGP11 BDSC 6596), UAS- Syt::HA; UAS-mCD8-GFP (gift from Hiromu Tanimoto), UAS-Shn::GCaMP3.0 (gift from André Fiala), GHH16-QF, UAS-NdTomato (BDSC 30037), UAS-CG3PA (gift from Sandeep Datta), nos-Gal4 (BDSC 51635), tubP-GAL80 (BDSC 7018), UAS-GluCl RNAi (BDSC 33336), Or10a-Gal4 (BDSC 9944), Or7a-Gal4 (BDSC 23908), UAS-empty RNAi (attP40, BDSC 36304), 20XUAS-CsChrinson-mCherry-trafficked VK00005 and 20XUAS-CsChrinson-mCherry-trafficked (attpS) (both are gifts from Vivek Jayaraman), Orco-Gal4 (gift from André Fiala), Orco-Gal4 on (BDSC 26818), UAS-GAD RNAi (BDSC 31794), and GHH16-QF, UAS-GuMP3.0 (gift from Hokoto Kozama’s lab.).

**FlyWalk assay.** FlyWalk experiments were performed adapting previous protocols. In brief, we tested 15 (24 h) female flies in 15 parallel glass tubes (inner diameter 0.8 cm). The flies were continuously exposed to a humidified airstream with a velocity of 20 cm s⁻¹ (20 °C, 70% relative humidity). All experiments were performed under red light background conditions (λ = 630 nm) generated by a LED cluster. Flies were monitored during the whole experiment using an overhead camera (HD Webcam C615, Logitech, Switzerland). Odor stimulation was done using a multicomponent stimulus device, where flies were repeatedly presented 1 s pulses of single odors or mixtures with an interstimulus interval of 90 s. Despite the well-controlled production of the odor stimulus, this stimulus temporarily broadens while moving through the tubing system of the setup. At the same time the stimulus has been shown to decrease in concentration only by <10% along its travel through the system resulting in a ca. Two seconds stimulus of well-defined concentration arriving at the tested flies. At the same time the flies’ XY-position was recorded for each pulse. The stimulus protocol consisted of 2 single odors, their mixture, a negative control (mineral oil, MOL) (Cal Roth) and clean air pulses, which were presented for 50 times each in a pseudorandomized sequence.

Odors were presented to the mixing chamber made of polyethyetherketone (PEEK) via ball-stop check valves. Basically there are two airflows – continuous and IVS. The continuous airflow stands for the “no odor”-condition, where the clean airflow passes through empty and clean odor vials. While presenting an odor, the clean airflow is redirected through the vials containing the odor dilution, picking up...
the saturated headspace. Thus, odors are presented to the flies with minimal disturbances in the total airflow.

FlyWalk data analysis. Since flies are allowed to move freely in the glass tubes, the individuals to may show different meeting times and the same odor pulse, depending on whether they sit on a tube end or downwind. We corrected this by calculating the encounter for each single fly for each stimulus based on its position, the delay of the odor traveling through the system and the wind speed within the system using a custom-written script in R (https://www.r-project.org/). A second custom-written script was used to calculate the response of the flies toward an odor. On one hand we calculated the mean movement speed of the flies from 1 s before the odor until 7 s after the odor pulse (Fig. 1b, d). Therefore, we analyzed first the average speed within each fly and in the next step we calculated the mean of all flies from the individual averages. When analyzing the upwind displacement (Fig 1c, e) the distance the flies walking upwind after the odor pulse we used the same approach, but only in 4 s after the odor pulse. Analysis scripts are available at https://github.com/michathoma/flywalk.

2-photon calcium imaging. All calcium imaging experiments were performed on starved (24 h) female flies aged 4–6 days post-emergence unless otherwise mentioned. Flies were briefly cold-anesthetized on ice and fixed with the neck onto a custom-made Plexiglas mounting stage with copper plate (Athene Grids, Plano) and a glass bottle (50 ml, Duran Group, Mainz, Germany), with two sealed openings fixed with the neck onto a custom-made Plexiglas mounting stage with copper plate (Athene Grids, Plano) and a glass bottle (50 ml, Duran Group, Mainz, Germany), with two sealed openings in case of balsamic vinegar. Two milliliters of the diluted odors were added to the saturated headspace. Thus, odors are presented to the flies with minimal disturbances in the total airflow.

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**Single sensillum recordings (SSR).** Three-days-old female adult flies were immobilized in pipette tips, and the third antennal segment or palps were positioned onto a glass coverslip. The tungsten wire electrode (recording electrode) was inserted into extracellular to the base of a sensillum (using a motorized, piezo-translator-equipped micromanipulator (Märzhäuser DC-3K/PM-10; http://www.marrhauser.de)) to measure the extracellular signals originating from the ORNs, while the reference electrode was inserted into the eye. Both electrodes were positioned under a microscope (Olympus BX51WI; http://www.olympus.com). Signals were amplified (Syntech Universal AC/DC Probe; www.syntech.nl), sampled (10,667 samples/s), and filtered (100–300 Hz with 50/60-Hz suppression) via a USBIDAC connection to a computer (Syn-tech). Action potentials were visualized and analyzed using Syntech Auto Spike 32 software. Each measurement was for 10 s, starting 2 s before a stimulation period of 1 s. Responses from individual neurons were calculated as the increase/decrease in the action potential frequency (spikes/s) relative to the prestimulus frequency.

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Author contributions
A.A.M.M. performed all functional imaging recordings, T-maze experiments, immunohistochemistry, molecular genetics, and optogenetics. T.R. performed FlyWalk and SSR experiments. S.D.C. performed immunohistochemistry. B.F. performed photoactivation experiments and neuronal reconstructions. A.A.M.M., B.S.H., M.K. and S.S. together conceived the project. A.A.M.M., M.K. and S.S. designed research, interpreted the results, and wrote the manuscript with input from all authors.

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