# CHAPTER III

# Representation of binary odor mixtures in the output neurons of the honeybee antennal lobe reveals odor-specific interglomerular computation


## ABSTRACT

The representation of binary odor mixtures in the output neurons of the first olfactory processing center of the honeybee, the antennal lobe (AL), were investigated using calcium-imaging techniques. We found that binary mixtures generally evoked patterns that correspond to simple combinations of the constituent odorants. However, the response intensities of the most responsive glomeruli revealed inhibitory mixture interactions in most animals. These interactions were strongest for glomeruli with overlapping response profiles. Blocking the GABAergic inhibitory network within the AL with the chloride channel blocker picrotoxin enhanced these mixture suppressions. Therefore the observed effects emerge from interglomerular computation within the AL. These inhibitory connections are non-GABAergic and glomerulus-specific. As a corollary their action is also odor-specific. The observed mechanisms may ensure efficient coding even for flower fragrances containing a high number of volatile components.


## INTRODUCTION

Most naturally occurring odors are complex blends consisting of a large number of volatile compounds (Knudsen et al. 1993; Pichersky and Gershenzon 2002). It seems unlikely that the olfactory system encodes mixtures simply as the sum of the individual responses to the single components, since the olfactory code would be saturated quickly and mixtures could not be discriminated. This notion is supported by the physiological response properties of the responsible neurons involved in olfactory coding. For example, olfactory receptor neurons (RN) typically respond not only to one but to a variety of odorant molecules (Vareschi 1971; Sicard and Holley 1984; Akers and Getz 1993; Firestein et al. 1993; Sato et al. 1994; Duchamp-Viret et al. 1999; Malnic et al. 1999; Araneda et al. 2000), although these responses usually occur to a restricted set of molecular features (Akers and Getz 1993; Sato

et al. 1994; Araneda et al. 2000; but see de Bruyne et al. (2001) for examples of RNs responding to separate chemical classes). Thus, molecularly similar odorants are encoded by overlapping sets of RNs. This overlap may serve as a requirement for different kinds of non-additive effects that appear when two or more odors occur in a mixture. Electrophysiological studies of single RNs have shown several kinds of mixture interactions. It has been found that the presence of an odor B can interfere with the normally strong response to odor A, which is termed mixture suppression (Gleeson and Ache 1985; Derby et al. 1991; Daniel et al. 1996; Steulett and Derby 1997). In spiny lobsters and catfish it has been shown that this kind of interaction commonly happens when one component of a binary mixture elicits an excitatory RN response and the other either an inhibitory or neutral response (Daniel et al. 1996; Kang and Caprio 1997). Conversely, synergism is the case when a sensory neuron responds to a binary mixture in a way that exceeds the summed response to the single components. This kind of mixture interaction has been reported in catfish RNs (Kang and Caprio 1991) and in single sensilla placodea of honeybees (Akers and Getz 1993).

The RN responses which already feature a notable number of mixture interactions are further transformed and modulated by interglomerular computation within the primary olfactory brain center, the olfactory bulb of vertebrates or the antennal lobe (AL) of insects (Schild 1988; Linster et al. 1994; Mori et al. 1999; Sachse and Galizia 2002). Thus, the coding capacity of mixture responses increases. Different odors are encoded as spatially organized glomerular activity patterns in a combinatorial manner in the olfactory bulb (Friedrich and Korsching 1997; Friedrich and Korsching 1998; Johnson et al. 1998; Rubin and Katz 1999; Uchida et al. 2000; Meister and Bonhoeffer 2001; Wachowiak and Cohen 2001) and the AL (Joerges et al. 1997; Galizia et al. 1999b; Galizia et al. 1999c; Sachse et al. 1999; Galizia et al. 2000). Calcium imaging studies of the honeybee AL which emphasize RN responses (Galizia et al. 1998) have reported that responses to binary mixtures were moderately reduced compared to the single odorant's response (Joerges et al. 1997; Rappert et al. 1998), whereas increasing the number of components in the mixture blend enhanced inhibitory interglomerular interactions (Joerges et al. 1997).

The glomeruli within the honeybee AL are interconnected by inhibitory local interneurons (LNs) (Schäfer and Bicker 1986; Flanagan and Mercer 1989; Fonta et al. 1993) providing the basis for the presumed inhibitory interglomerular processing (Sun et al. 1993). In our previous study, we have shown the presence of two separate inhibitory networks (Sachse and Galizia 2002). A global inhibitory network, accomplished by GABAergic LNs, is thought to affect a global gain control mechanism, whereas putative histaminergic LNs perform inhibitory connections between specific glomeruli to contrast-enhance overlapping glomerular response profiles. Both networks are likely to affect mixture responses, though the latter network would be expected to have more specific effects on the coding of odor mixtures. In order to analyze the mechanisms underlying the processing of odor mixtures,

we have investigated the neural representation of binary mixtures in the projection neurons (PNs) which represents the processed information in the honeybee AL. The PNs of the honeybee integrate the signals of about 60,000 RN (Esslen and Kaissling 1976), which have been modulated by 4000 LNs (Witthöft 1967), resulting in temporally complex excitatory as well as inhibitory PN responses (Müller 1999; Abel et al. 2001; Sachse and Galizia 2002). We investigated the contribution of the specific inhibitory connections to the processing of odor mixtures by using the fast GABA receptor antagonist picrotoxin (PTX) to silence the global inhibitory network. The results confirm that binary mixtures are represented as non-additive combinations of glomerular activities and indicate that glomerulus-specific computations are involved in the processing of odor mixtures.

## MATERIALS AND METHODS

### Preparation and staining

The animal preparation and selective staining of PNs were identical to our previously reported study (Sachse and Galizia 2002). In short, adult worker honeybees were caught at the hive entrance in the morning, immobilized by cooling and fixed in a recording stage. A small window was cut posterior to the antennae in the head capsule and glands and trachea were carefully removed. A glass electrode, coated with crystals of fura-dextran (3000 MW, Molecular Probes, Eugene, OR), dissolved in 2% bovine serum albumin solution, was inserted into the left deutocerebrum lateral to the α-lobe, aiming for the PNs of the lateral antenno-cerebralis tract (ACT). The brain was rinsed with Ringer solution (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7, 500 mOsmol; all chemicals from Sigma) in order to remove extracellular dye. Subsequent to 3 hours of staining the antennae were immobilized with two-component silicon (Kwik-SilTM, WPI) and the abdomen was cut. The preparation was covered with a coverslip and constantly superfused with Ringer during measurements (1 ml/min).

### Calcium measurements

The optophysiological methods applied were identical to those by Sachse and Galizia (2002). Briefly, imaging was done using a T.I.L.L. Photonics imaging system (Germany). Monochromatic excitation light alternated between 340 nm and 380 nm, dicroic: 410 nm, emission: LP 440 nm. Measurements were done with an upright Axioskop microscope (Zeiss), using a Leica 20 x LD NA=0.6 air objective. Pixel image size was 2.4µm x 2.4µm, obtained by 2 x 2 binning on chip. For each measurement, a series of 60 double frames were taken with frequencies of 6 Hz. Light was turned off between frames. Exposure time was on average 60 ms for 340 nm and 15 ms for 380 nm. Interstimulus interval was 40 s.

Odors were delivered to the antennae at frame 12 using a custom-made and computer-controlled olfactometer by switching from a constant air stream to an odor stream in order to eliminate mechanical stimulation (Galizia et al. 1997). Stimulus duration was 2 s. Odors used were 1-hexanol, 1-octanol and 1-nonanol (Sigma-Aldrich, Deisenhofen). Binary mixtures were produced by switching from the constant air stream to two odor streams. For each odor, 6 µl of the odorant dissolved in mineral oil were applied to a filter paper (1 $cm^2$) in a plastic syringe. Dilutions were adjusted to equalize effective vapor pressure for the different odorants (1 : 47 for hexanol, 1 : 3.4 for octanol, and 1 : 0.25 for nonanol). The control stimulus was a syringe plus filter paper with mineral oil.

Solutions of PTX (picrotoxin, Sigma) were first dissolved in Ringer for final concentrations of 1 µM, 10 µM and 100 µM and then bath-applied to the brain. PTX was applied with increasing concentrations for 12 min. each.

**Data processing**

All following calculations were done in IDL (Research Systems, Colorado). Calcium concentration data are given as absolute changes of fluorescence ratio between 340 nm and 380 nm excitation light. We subtracted frames 3-5 from the time traces to superimpose all traces at a value of 0 shortly before stimulus onset. This is necessary, because PNs revealed a high amount of spontaneous activity and each glomerulus had individual background fluorescence. Furthermore, the ratios were median-filtered for shot noise reduction (filter size 3 pixels in two spatial and one temporal dimension) and were corrected for lamp noise by subtracting the median value of each frame from it. We assigned the signals to identified glomeruli by reconstructing the glomerular structure in the fura ratio images and identified the glomeruli on the basis of their morphological borderlines using a digital atlas of the AL (Galizia et al. 1999a). For time courses of identified glomeruli (Fig. 1C) squares of 11 x 11 pixels (corresponding to 26.4 µm side length and always well within the glomerulus chosen) were placed in the center of a glomerulus, their values were averaged and the courses were plotted against time. For the false-color coded images (Fig. 1A, B) we averaged the fluorescence changes between frames 18-24 (i.e. 1 s after stimulus onset until stimulus offset).

We calculated the mean glomerular response to a specific odor (Fig. 1D, E and Fig. 2) by taking the median response of all animals (n = 6). For each animal the response was calculated as the maximum during stimulus application. Repeated stimulations were averaged within one animal. In order to compare animals with different background fluorescence and thus different maximal activities, we defined the strongest glomerular response as 100% either within each animal to each odor (Fig. 1D, E) or within each animal and glomerulus for all odors (Fig. 2) and scaled the other responses accordingly. Calcium decreases during stimulus application were assigned to the category ‘negative responses’ (Fig. 1D, E).

Significant differences (Fig. 2) were determined using a two-tailed paired Wilcoxon's signed-ranks test (SPSS Inc., Chicago, IL).

## RESULTS

We measured the odor representation in the output neurons of the honeybee AL (i.e. PNs) to the three primary alcohols hexanol, octanol, nonanol and their binary mixtures. Since all odors were diluted in mineral oil to adjust equal vapor pressures, the solvent was used as a control stimulus. During mineral oil application no calcium activity above noise level was visible (Fig. 1A), whereas stimulation with odors led to specific spatio-temporal activity patterns of PNs consisting of excitatory as well as inhibitory glomerular responses (Fig. 1B, C). Strong odor-evoked responses occurred in at least three glomeruli, one for each odor. Hexanol elicited a strong and long-lasting calcium increase in glomerulus 28 (red line in Fig. 1C), whereas glomeruli 17 and 33 were not activated above noise limit (green and blue lines). Octanol application showed a strong activation of glomerulus 17, a weak calcium decrease in glomerulus 28 and no calcium changes in glomerulus 33. Nonanol evoked the strongest response in glomerulus 33, an intermediate response in glomerulus 17 and an off-response in glomerulus 28 (i.e. calcium increase after stimulus offset, which is due to a release from inhibitory input during the stimulus). Taken together, these chemically closely related odors elicited opposing responses in these three glomeruli. The binary mixture patterns of the alcohols were combinations of the single odor responses (Fig. 1B). However, the comparison between the temporal responses of these three prominent glomeruli to the single component response and the mixture response reveals some differences (Fig. 1C). The binary mixtures were produced by introducing the two corresponding single components into the continuous air stream (see METHODS). Therefore the concentration of each component in the mixture was approximately equal to the single odorant concentration. Thus, the response of a specific glomerulus to the mixture was expected to be at least as strong as the strongest of the single component's responses. Interestingly, some of the glomerular response intensities were lower than this prediction, revealing inhibitory connections on each of the three glomeruli shown (Fig. 1C). The excitatory response of glomerulus 28 to the binary mixture of hexanol and octanol was half as much as its hexanol response, whereas the mixture of hexanol and nonanol led to only a weak reduction in this glomerulus. The other glomeruli showed similar odor-specific differences in response intensity. The off-response of glomerulus 28 to nonanol remained inhibitory during the stimulation with the octanol-nonanol mixture, but not in the mixture hexanol-nonanol.

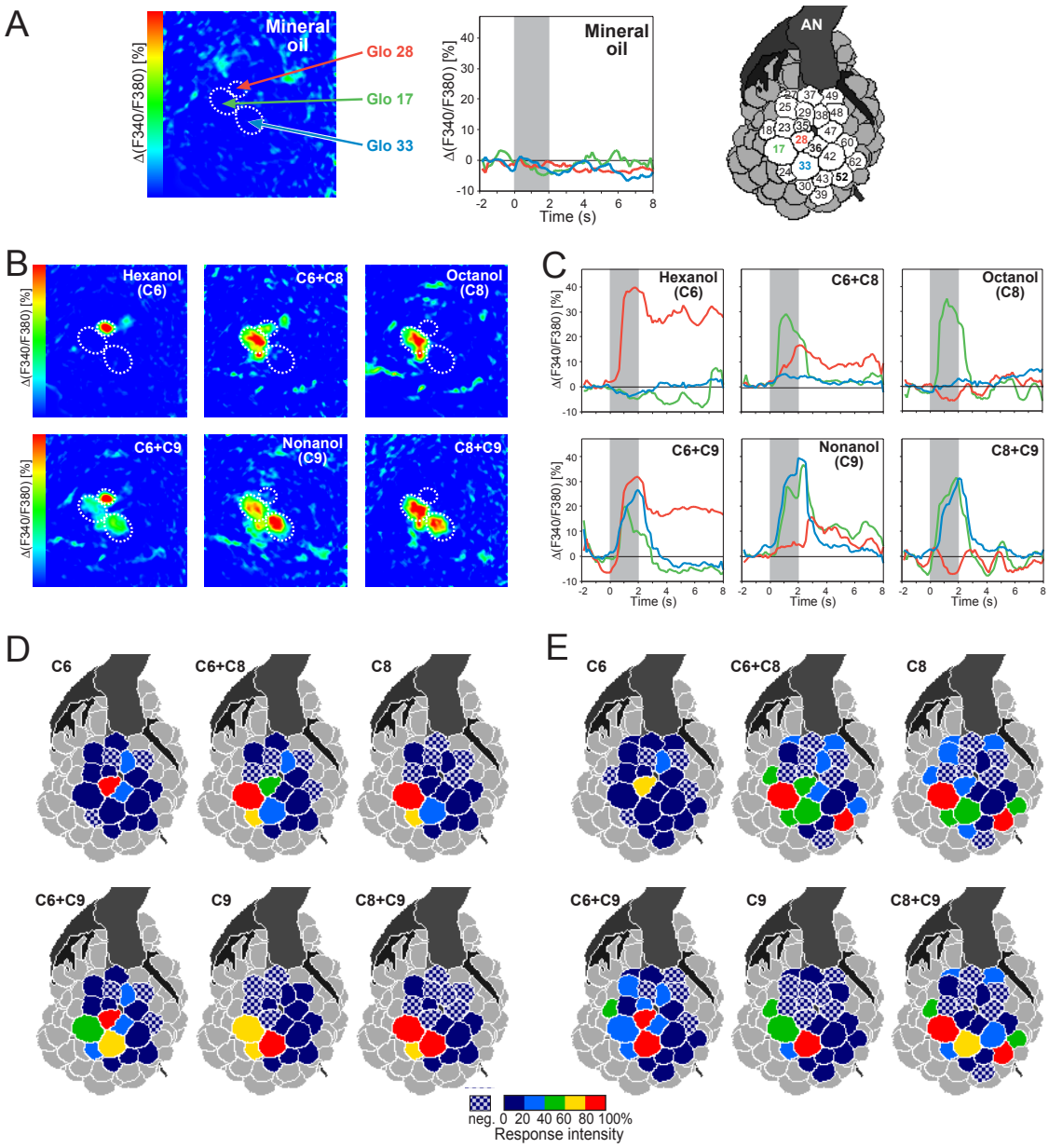


**Figure 1 Glomerular responses of output neurons to primary alcohols and their binary mixtures.** **A**, False-color coded spatial activity pattern (left) and time traces of identified glomeruli to the solvent mineral oil (middle). Positions of the 23 most frequently recognized glomeruli are given by a schematic AL (right); antennal nerve (AN) is to the top. The colors of the time traces correspond to the three glomeruli, whose positions are marked by dotted circles in the activity pattern and colored numbers in a graphical AL. Odor stimulus is marked by a shaded area in the time course. Mineral oil does not evoke any glomerular activities in the AL of this animal. **B**, False-color coded response patterns to the three primary alcohols hexanol, octanol and nonanol and their binary mixtures. Each activity pattern is scaled to its own maximum. Hexanol elicits a strong response in glomerulus 28, octanol in glomerulus 17 and nonanol in glomeruli 17 and 33. The glomerular response patterns to the binary mixtures include the same glomeruli as the single odorant patterns. **C**, Time traces of the glomeruli as in A to the measurements shown in B. Note the temporal properties of the responses, such as the off-response of glomerulus 28 to nonanol and the inhibitory mixture interactions. **D**, **E**, Graphic representations of the spatial activity patterns of the animal shown in A-C (D) and averaged from 6 individuals (E) responding to the three alcohols and their binary mixtures. The color code gives the relative response intensity in five bins. Inhibitory responses are represented by an additional category. The mixture patterns appear as combinations of the single odor responses.

Figure 1D represents the spatial activity patterns of this animal to the alcohols and their binary mixtures in a standardized AL including all measured glomeruli. Besides the three most responsive glomeruli 17, 28 and 33 the remaining glomeruli did not reveal any mixture suppressions. The glomerular response properties observed in this animal were typical for most animals measured. Therefore we averaged the responses of the measurements to the single components and their binary mixtures over all animals (n = 6) and represented them as schematic ALs (Fig. 1E). Comparison of the patterns between the single animal and the mean of all animals reveals some variability (e.g. glomerulus 52 during octanol stimulation), which was higher for glomerular responses of PNs than for the afferent input to the AL (Sachse and Galizia 2002). This may be due to individual experience, which has been shown to affect the glomerular odor representations (Faber et al. 1999). However, the most responsive glomeruli were generally identical for all animals measured, confirming that response properties of PNs are conserved among individuals (Sachse and Galizia 2002). Interestingly, the mixture effects on response intensity as observed in the animal in Fig. 1B-D were attenuated in the mean of all animals. This may indicate that the inhibitory connections between glomeruli, which are responsible for the mixture interactions, are also one site for plasticity in the AL. Therefore their effect is more variable across animals. In our experiments, honeybees were taken as experienced foragers with differing individual backgrounds.

In order to analyze the influence and contribution of inhibitory neurons to the representation of odor mixtures, we applied the fast GABA receptor antagonist picrotoxin (PTX) to the AL. The averaged response intensities of a subset of glomeruli to the tested alcohols and their binary mixtures during Ringer, PTX with increasing concentrations and after wash-out are represented in Fig. 2. The expected mixture responses are given by dotted circles, which correspond to the more effective component response (see Discussion). During Ringer, the response of glomerulus 17 to the hexanol-nonanol mixture was significantly different from the predicted response (marked by a minus sign). Glomerulus 36 revealed the added responses for all mixtures, whereas the other glomeruli showed either an equal response strength or a weak response which was not statistically significant across animals. As has previously been shown (Sachse and Galizia 2002), application of PTX led to more glomeruli responding to an odor (e.g. glomeruli 28 for octanol), whereas individual intermediately-activated glomeruli dropped out of the activity patterns (e.g. glomerulus 17 for nonanol). PTX increased inhibitory mixture interactions and thus led to significant reductions of mixture responses, which were odor- and glomerulus-specific. For example, the responses of glomeruli 17 and 33 to the octanol-nonanol mixture were reduced with increasing PTX concentration compared to the predictions, whereas glomeruli 36 and 52 were not affected and showed equal intensities for the predicted and the measured response.

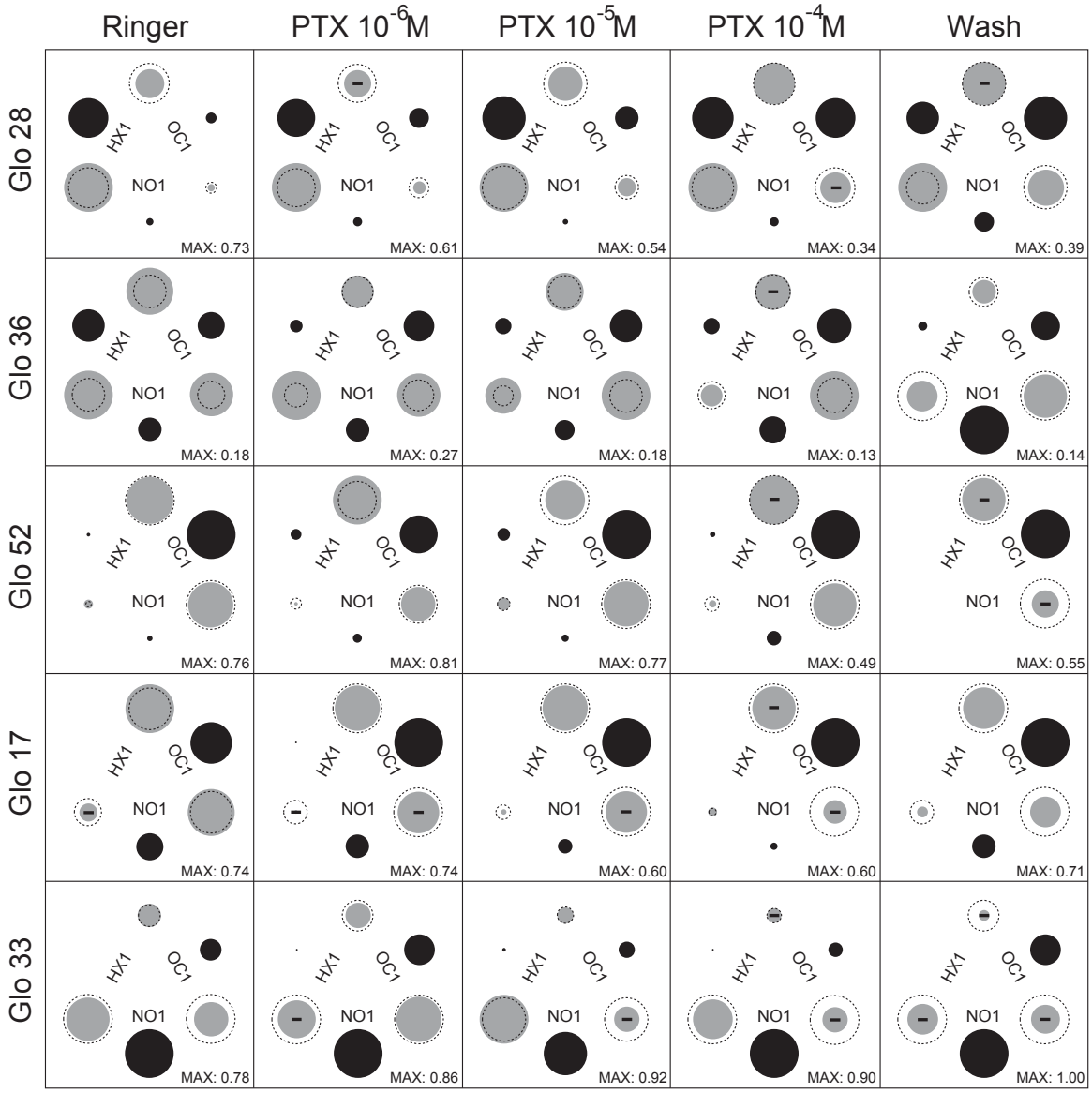


**Figure 2 Glomerular responses to the three alcohols (black circles) and their binary mixtures (gray circles) averaged over all animals (n = 6).**

Each row represents one glomerulus, each column a specific treatment. The circle sizes give the relative response intensity for each circle set, the maximal value is given within each box for comparison. Predicted mixture responses, corresponding to the more effective component response, are indicated by dotted circles for each binary mixture. A minus sign indicates odor-specific mixture suppression, which could be observed in at least 5 of 6 animals ($p < 0.1$, two-tailed paired Wilcoxon's signed ranks test). During Ringer application all mixture responses except one correspond to the predicted responses. PTX application leads to several mixture responses becoming significantly reduced compared to the predictions. The reduction of some mixture responses remained in the wash. No significant synergistic mixture responses were observed.

Moreover, glomeruli 17, 36 and 52 revealed slight reductions in their hexanol-octanol responses during the highest PTX concentration. However, despite long washing periods (> 40 min.) most of these PTX effects persisted and were intensified, even leading to addi-

tional inhibitory mixture interactions during wash, such as the reduced response of glomerulus 52 to the octanol-nonanol mixture.

All remaining glomeruli (n=18) showed no or only weak responses to stimulation with the tested odors (Fig. 1D, E). In all of these no significant differences between the predicted and measured mixture responses were observed: neither at a single-animal level, nor across animals, i.e. the response to the mixture corresponded to the strongest component's response.

## DISCUSSION

In this study we measured the representation of binary mixtures in the output neurons of the honeybee AL using calcium imaging. The identification of mixture interactions is a critical task as it depends on the manner of calculating the predicted mixture response. Moreover, several methods of calculating this predicted response have been proposed (Akers and Getz 1993; Daniel et al. 1996). We used the following simple way of identifying mixture interactions. Since the concentration of each component in the mixture was that for the singly-applied odorants, the response to the mixture should be at least as strong as to the more effective component. Therefore a response significantly below the response to the odorant with the highest response is a secure indication of suppressive interaction. This approach was defined as the more effective component model (MEC) by Daniel et al. (1996). The MEC provides a very conservative measure for mixture suppression. Using a likewise conservative approach we decided to class only responses significantly above the summed response of the two separate odorants as synergism. This ensures that only strong and significant effects were defined as synergism, though it may overlook some less pronounced integrative interactions in mixture processing.

According to the preceding definition, we never observed synergistic responses, whereas reduced and thus inhibited responses were obvious for the most responsive glomeruli (Fig. 1), though not significant across all animals (Fig. 2). This is in line with other calcium imaging studies of glomerular responses in honeybees showing slightly suppressed mixture responses (Joerges et al. 1997; Rappert et al. 1998) but in contrast to recordings from honeybee RNs, which reported that more synergistic than inhibitory responses occurred to binary mixtures (Akers and Getz 1993). However, Akers and Getz used the MEC as a test for synergism which is less conservative than our approach and bears the danger of overestimating synergisms. Their argument was based on the assumption that each RN in a sensillum responds exclusively to one of the tested mixture components, an assumption that is not fulfilled by the functional units investigated in this study, which are the glomeruli.

The inhibitory mixture interactions were intensified by PTX application to a level making them significant across animals (Fig. 2). It may appear odd that PTX, which blocks inhibitory connections, may lead to more inhibitory effects within the AL. In a previous study we have proposed a wiring model of the honeybee AL, which includes two independent inhibitory networks (Sachse and Galizia 2002). On the basis of that model, PTX blocks the GABAergic global inhibitions, in which all glomeruli are involved, but not inhibitory connections between specific glomeruli, which are possibly driven by histamine. While in the intact AL the action of the glomerulus-specific network may be barely visible due to the concurrent GABAergic inhibitory effects, their action is unmasked when PTX is applied. Thus, when the global network is eliminated by PTX, the specific inhibitory network is enhanced. The increased mixture interactions following PTX application may indicate that these interactions emerge from interglomerular computation accomplished by inhibitory connections between specific glomeruli rather than from mixture interactions at a more peripheral level. However, since PTX was bath-applied, it may have diffused up within the antenna. Therefore measurements of RNs including PTX application must be carried out to verify this assumption. Moreover, pharmacological experiments blocking the glomerulus-specific inhibitory connections are necessary to investigate the origin of the mixture interactions.

The reduced mixture responses due to PTX remained in the wash (Fig. 2). Interestingly, other PTX effects, such as the significant reduction of glomerulus 17 during nonanol stimulation (Fig. 2) (Sachse and Galizia 2002), were reversible after long washing periods. These differential effects may suggest that at least two different PTX-sensitive receptors are present in the AL, one with a persistent PTX-binding pharmacology, and the other for which PTX is easily washable. Different types of PTX-sensitive chloride channels have been reported in the single DUM neuron in cockroaches (Raymond et al. 2000), but their presence in the honeybee AL is still unknown.

Optical recording studies of the olfactory bulb and the AL have shown that odors with strong overlap in molecular structure show also stronger overlap in their glomerular response profiles (Friedrich and Korsching 1997; Rubin and Katz 1999; Sachse et al. 1999; Uchida et al. 2000; Fuss and Korsching 2001; Meister and Bonhoeffer 2001; Wachowiak and Cohen 2001; Fried et al. 2002). Hence mixtures of chemically closely related odors should show stronger patterns of interactions than mixtures of molecules that are less similar in structure (Smith 1998). Indeed, the data presented here show that inhibitory interactions were strongest for binary mixtures of odor molecules with neighboring chain length. For example, the strong response of glomerulus 28 to hexanol was specifically inhibited when octanol was added, whereas nonanol did not lead to any reduction in this glomerulus (Fig. 2). Conversely, glomerulus 17 showed a strongly reduced response to the mixture of octanol and nonanol compared to its octanol response, but only slight mixture interactions

when hexanol was added. Since these mixture interactions emerge from non-GABAergic and thus probably glomerulus-specific inhibitory connections, it appears that the network is wired in a way ensuring a 'functional' lateral inhibition. Opto-physiological measurements of mouse olfactory bulbs reported that activity patterns evoked by binary mixtures of chemically closely related odors could be predicted by the sum of the two constituent odors (Belluscio and Katz 2001). In our measurements, a mixture response corresponding to the sum of the components responses is an indication of either separate cellular compartments, so that the optical effects summate, or of synergistic coding effects. The latter is indicated by the fact that even increasing the concentration of a stimulus two-fold does not increase the response two-fold, as all odor-response curves comply to a sigmoidal curve over the logarithm of stimulus intensity (Friedrich and Korsching 1997; Fuss and Korsching 2001; Meister and Bonhoeffer 2001; Wachowiak and Cohen 2001; Fried et al. 2002), and not over a linear scaling of odor intensity. These contrary findings may either reflect basic differences between the AL and the olfactory bulb in terms of mixture processing or could be due to the different processing levels investigated. Belluscio and Katz (2001) used intrinsic signal imaging, which probably emphasize the afferent input to the olfactory bulb, whereas the odor representation in the output neurons, as measured in the present study, underwent glomerular computation. Thus, it is conceivable that the output of the olfactory bulb, i.e. responses of mitral cells, would reveal mixture interactions similar to those in the honeybee. This assumption is supported by the presence of lateral inhibitory connections in the olfactory bulb, which sharpen the responses of mitral cell to a specific odor by inhibiting responses to molecularly related odors (Yokoi et al. 1995), in a similar way to the glomerulus-specific inhibitory network of the honeybee AL (Sachse and Galizia 2002).

In summary, we found odor- and glomerulus-specific mixture interactions in glomeruli with overlapping response profiles, emerging from specific interglomerular computation within the AL. However, the observed mixture suppressions led to strong reductions of some odor-evoked responses, but never pushed them below baseline. Thus, the activity patterns of mixtures still included components of the single odorant patterns. These findings are consistent with several behavioral experiments showing that honeybees are able to relate mixtures to their component parts (Getz and Smith 1987; Smith and Cobey 1994; Smith and Getz 1994). Still, we do not know whether the olfactory system is able to filter out the single odorant or whether these behavioral observations are caused by generalization due to the high similarity between the mixture and its components. Interestingly, when honeybees were differentially trained to an odor mixture and its constituent odorants, the animals could specifically attend to unique qualities of the mixture (Chandra and Smith 1998). Thus, the glomerular activity patterns may comprise both the unique qualities of a mixture as well as information about its single components. Dependent on the behavioral task higher processing centers as the mushroom bodies and the lateral protocerebrum may have access to both

and could read-out the crucial information. Further experiments using a higher number of mixture components which lead to stronger mixture interactions (Joerges et al. 1997), will help to understand the basic principles involved in the processing of odor mixtures.

## ACKNOWLEDGMENTS

We thank Randolf Menzel for valuable discussions and support, Philipp Peele and Mary Wurm for helpful comments on the manuscript and Astrid Klawitter for technical support. This work was supported by the Volkswagenstiftung (VW 1/75-399) and HFSP.

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