# Discussion

In this work we examined how olfactory PNs encode odors presented at different concentrations. We show that glomerular PN responses exhibit different temporal dynamics and propose a general classification. Using a linear discriminant analysis, we show that chemically similar odors elicit similar activity patterns. In most cases, increasing odor concentrations lead to an increase in glomerular activity and the recruitment of additional glomeruli to the patterns. Nevertheless odor identity cannot simply be encoded in the relative glomerular activity, as this may change over concentrations. Approximating the olfactory space with a PCA, we show that odor patterns separate according to their chemical properties, as described previously for behavioral data based on the same odors (Guerrieri *et al.*, 2005). The correlation between our data and this behavioral data was high, indicating that the similarity between glomerular PN activity is maintained during further processing steps. As a last result, we show that the redundancy of the olfactory information increases with concentration.

### Methodological aspects

Whether the observed changes in intracellular calcium reflect different spike frequencies cannot be resolved. It seems parsimonious to assume that for example the decrease in intracellular calcium indicative of a type III response is caused by inhibitory input into the cells. Nevertheless, we did not record electrophysiologically from these neurons, and therefore are limited to speculation. Several studies suggest the intracellular calcium concentration to be a function of the membrane potential (Egelhaaf and Borst, 1995; Single and Borst, 2002; Galizia and Kimmerle, 2004). While two of these studies were conducted in another animal, specifically the bowfly, and were examining different neurons, the third was based on honeybee PNs (Galizia and Kimmerle, 2004). Subsequent to intracellular recording, these neurons were ionophoretically loaded with a reporter and imaged. In general the resulting calcium signals reflected the electrophysiological responses well, though there was not always a one to one correspondence. In any case, the highly significant correlation between the calcium

imaging data and the behavior shows that the intracellular calcium concentration indeed reflects behaviorally relevant neural activity.

**Different types of glomerular PN responses exist**

The PN responses could be classified by their shape into four different response types. These types were not indicative for the glomerulus, as different odors, or in some cases even different concentrations of the same odor could change the shape of the response. Three possible explanations for these response types exist: First, the different response types are a result of an inhibitory LN network shaping the temporal characteristics of the odor representation. Second, the response types directly reflect the temporal dynamics of the underlying OSN activity. Third, the response types are influenced by both OSNs and the inhibitory network. Recordings form honeybee sensilla placodea (Akers and Getz, 1992) revealed OSN responses which were comparable to our type I responses. In recordings from Drosophila basiconic sensilla OSN responses could be observed which were comparable to the first three of our four response types (de Bruyne *et al.*, 2001). Here some OSNs also showed a delayed response onset and in some neuons the response could outlast the odor offset considerably. Only the phasic response type IV could not be observed, OSN responses always lasted throughout the stimulus. This suggests at least some effect of the inhibitory network on the temporal response characteristics.

A direct comparison between calcium response characteristics of OSNs and PNs is not yet possible. Though imaging data based on the same set of odors recorded with bath-applied calcium green AM exists and has been published (Sachse *et al.*, 1999), the measured responses are a compound signal representing the activity of all major neuron types (though a major contribution of the numerically dominant OSNs has been suggested (Galizia *et al.*, 1998)). In the compound signal, odor responses are significantly slower and do show none of our response types. This difference may be caused by a different compartmentalization of the used calcium reporter (Thomas *et al.*, 2000). The development of genetically encoded calcium reporters and their selective introduction into specific Drosophila neuron populations may help to further elucidate this question.

Though up to now, the used calcium reporters lack the necessary temporal resolution, new faster calcium reporters may be available soon (Griesbeck, 2004).

**Variability of the PN responses across animals**

Though PN odor representations are conserved across animals, individual differences do exist. As, except for the controls, odors were not measured repetitively, we cannot further investigate this effect, for example by systematically comparing within and between group variance. In many cases this might be attributable to variations in the experimental parameters. Due to the staining method the distribution and overall amount of the calcium reporter in the PNs may vary. Additionally, slight variations of the recording angle and the three dimensional structure of the AL resulted in different glomeruli being differently strong in focus. Nevertheless, this cannot explain those cases in which glomeruli generally active in an odor were not active at all. It has been suggested that such differences might be experience dependant (Sachse and Galizia, 2002). Though different studies have demonstrated that learning induced changes in the glomerular activity patterns in the honeybee and drosophila AL (Faber *et al.*, 1999; Sandoz, 2003; Yu *et al.*, 2004) exist, recent results show that this is not the case for the l-ACT PNs examined in this work (Peele, personal communication).

Also when comparing the our dose response curves, which are averaged across animals, to those published previously (Sachse and Galizia, 2003), we find many differences. Especially the activity in glomeruli 17 and 33 to 1-hexanol is not as strong in our data. This effect might be caused by the different stimulus devices used or by differences in the way the data was processed. Another possibility is that other aspects, like daytime or season might influence the recorded glomerular activity patterns. And indeed, in the silkmoth *Bombyx mori* serotonin mediated changes in olfactory sensitivity have been shown to correlate with the circadian rhythm (Gatellier *et al.*, 2004).

**Glomerular activity increases with increasing odor concentrations**

A general observation was that with increasing concentrations, glomeruli already active at lower odor concentrations got stronger activated. At the same time, additional glomeruli became active.

In some cases, weak signals present at low concentrations could disappear at higher odor concentrations. Though persistent across animals, this effect was very weak and might not be behaviorally significant. Nevertheless, it could reflect the inhibitory circuitry of the AL and therefore could be used to further investigate how single glomeruli are interconnected.

In general, our results confirm and extend the work from Sachse et. al.(Sachse and Galizia, 2003), who examined changes in concentration for three of the 16 odorants tested here.

In OSNs, an increase in activity and the recruitment of new glomeruli as response to increasing odor concentrations have been observed on several occasions (Ng *et al.*, 2002; Wang *et al.*, 2003; Cinelli *et al.*, 1995; Friedrich and Korsching, 1997; Duchamp-Viret *et al.*, 1999; Rubin and Katz, 1999; Wachowiak *et al.*, 2004; Sachse and Galizia, 2003; Spors and Grinvald, 2002a). Other studies have examined the effect of different odor concentrations in the insect PNs and in the vertebrate mitral cells (Ng *et al.*, 2002; Sachse and Galizia, 2003; Wang *et al.*, 2003; Stopfer *et al.*, 2003; Chalansonnet and Chaput, 1998). While in most studies, PNs responded with a general increase of activity, *Stopfer et al*. could not observe this in their multi-electrode recordings in the locust AL (Stopfer *et al.*, 2003). In turn, they postulated that different odor concentrations are encoded by continuously changing ensembles of PN activity. This may be due to differences in the experimental animals. Unlike the honeybee with its ~160 glomeruli, the locust has a microglomerular AL consisting of ~1000 glomeruli. In the locust PNs have been shown to be multiglomerular with each PN innervating many different glomeruli (Leitch and Laurent, 1996). In contrast, the lACT PNs recorded in this study have been shown to innervate only one glomerulus (Müller *et al.*, 2002; Abel *et al.*, 2001a).

We show that the PNs, which are active at low concentrations, are active at higher concentrations, too. As in many cases the relative activity between glomeruli changes with concentrations, we found no linear rule according to which odor identity is encoded

across concentrations. Therefore odor identity cannot be encoded by the relative glomerular activity, as had previously been suggested (Sachse and Galizia, 2003). This result seems to contradict the concentration invariant correlation between our physiological data and the perceptual similarity matrix. One has to keep in mind, though, that these correlations are not based on odor identity but on the relative similarity between glomerular activity patterns. So while the odor patterns may change across concentrations, the relative similarity between them remains unchanged.

Except for some cases when odors were presented at low concentrations, all PN activity patterns evoked by the 16 odors and 4 concentrations were unique. The question arises how a putative readout mechanism in the higher centers as the mushroom bodies (MB) or the lateral horn (LH) might extract the encoded odor information; and more concrete, how they could mediate the seemingly opposing tasks of odor discrimination and generalization? MB Kenyon cells (KCs) have been shown to respond to odor stimulation with sparse, non overlapping activity (Perez-Orive *et al.*, 2002) A situation, in which the distinct patterns of PN activity excited different, non-overlapping sets of KCs (Perez-Orive *et al.*, 2002), would result in as many different representations in the MB, as there are odor/concentration combinations. Though this mechanism could perfectly well mediate odor discrimination, the information of odor similarity, and therefore the capacity to generalize, would be lost. Several hypothesis can be established how generalization might occur nevertheless.

We show that the identity of the odor irrespective of concentration is contained in the PN activity. This, in theory, allows for a generalization across concentrations (Bhagavan and Smith, 1997; Pelz *et al.*, 1997). This point can be illustrated with a simple example. One can imagine the transformations to the linear discriminants (LDs) shown as bar plots in Figure 7 to represent the strength of the synaptic connectivity between the 14 glomeruli and as many KCs as there are LDs. As 13 LDs were needed to separate the 16 odors, irrespective of their concentration, odor identity irrespective of concentration could be encoded by the graded ensemble activity of 13 KCs. That this example is only of a theoretical nature can be seen in the fact that KC odor responses have been shown to be very short, sparse and not gradual (Perez-Orive *et al.*, 2002; Wang *et al.*, 2004b).

Nevertheless it shows, that the divergent connectivity between the PNs and the KCs allows for almost any computation.

Generalization might also take place in another neuropil, where the degree of similarity between glomerular PN patterns is maintained. Indeed several studies have shown the existence of an odor map in the lateral horn (Marin *et al.*, 2002; Wong *et al.*, 2002; Tanaka *et al.*, 2004), and at least one study (Sivan and Kopell, 2004) has suggested its role in odor generalization. Future studies focusing on the LH output neurons will elucidate their role in odor generalization.

In insects most PN innervating the MBs have been shown to be cholinergic (Bicker, 1999; Yasuyama *et al.*, 2002), and therefore give excitatory input to the KCs. As those PNs which are activating a specific KC at one concentration are also active at higher concentrations the global input to the KC should increase with. Some KCs, in turn, have been reported to respond to odors, presented at specific concentrations only, while remaining unresponsive at lower or higher concentrations of the same odors (Stopfer *et al.*, 2003; Wang *et al.*, 2004a). This concentration specificity of KCs cannot be accomplished by the PN input to KCs alone. In the lip neuropil of the honeybee MB, GABAergic profiles have been shown to form microcircuits with PN boutons and postsynaptic KC profiles (Ganeshina and Menzel, 2001). These microcircuits have been proposed as causal to presynaptic sparsening observed in the calcium activity of l-ACT PN boutons (Szyszka, personal communication). Increased PN activity at higher odor concentrations, for example the activity of newly recruited glomeruli, could lead to a form of “lateral inhibition” via these GABAergic profiles, thereby preventing the KCs from firing. Following our theory, blocking chloride channels would disable this “lateral inhibition”. Overlapping sets of PNs would then excite overlapping sets KCs and fine odor or concentration discrimination would be impaired.

**Chemical characteristics of the odorants constitute the inner dimensions of the olfactory space**

Statistical methods for dimension reduction are helpful for extracting general features hidden in complex multidimensional data. When instructing a linear discriminant analysis to predict the odor on the basis of the glomerular activity patterns, we observed

many false predictions. In the majority of all cases, these consisted of glomerular activity patterns elicited by an odor, which were falsely attributed to a chemically similar odor. This shows that the information about chemical odor similarity is contained in the glomerular PN activity patterns. When subsequently calculating a principal component analysis, we reduced the 14 dimensions of glomerular activity to three dimensions, representing the first three principle components (PCs).

These first three PCs separated the odors according to their chemical properties, like chain length and functional group. This separation could be observed at all odor concentrations. This result is very similar to what *Guerrieri et al* (Guerrieri *et al.*, 2005) found when applying the same method to their behavioral generalization matrix. There, too, the first PC separated odors according to chain length and the third PC separated aldehydes from ketones. The separation of alcohols from ketones and aldehydes we observed in PC2 was visible in the behavior, too, though not as pronounced. We found the specific tuning of AL PNs to be responsible for this separation. While some glomeruli, for example 28 and 36, responded strongly for odors with shorter carbon chain length only, others did the inverse (17). The same differential PN activity separated odors according to their functional groups.

The time point when this separation was optimal differed for different odor concentrations. For the two higher concentrations, this optimal separation was already achieved after 400ms and got less pronounced during the remaining stimulus duration. For the lower two concentrations it was achieved after 600 ms and remained strong during the whole stimulus duration. When observing the correlations between our physiological data with the behavioral similarity matrix, we found that it was high whenever the degree of separation was high, too. This would suggest that the role of the antennal lobe consists in separating the odor representations and that a read out occurs, when the degree of separations is optimal. The described time points of optimal correlation, 400 ms for the higher two concentrations and 600 ms for the lower two odor concentrations, could suggest that higher concentrated odors are processed faster by the AL network, which in turn could lead to a faster behavioral response. In a behavioral study free flying honeybees discriminated odors after 690 ms (Ditzen *et al.*, 2003). Subtracting the time needed for the execution of the measured behavioral response this

discrimination time fits well the latencies recorded in our data. Nevertheless, in the behavioral study higher concentrated odors were not discriminated faster than odors of lower concentration.

**Odors with similar glomerular activity patterns are perceived as similar by the animals**

When comparing our optophysiological measurements with behavior (Guerrieri *et al.*, 2005), we found a high degree of correlation. When including all odors, the maximal correlation values ranged around r=0.7.

Already *Guerrieri et al*. (Guerrieri *et al.*, 2005) had calculated the correlation between their perceptual similarity matrix and optophysiological measurements based on the same odors presented as pure substances. These imaging measurements had been obtained by *Sachse et al. (Sachse et al., 1999)*, using bath applied Calcium green-AM. When bath applied, this membrane permeable dye unselectively stains all antennal lobe neurons and the recorded changes in calcium concentration are a compound signal. Due to the superior number of receptor neurons in the AL, signals obtained with this method are interpreted as reflecting the AL input (Galizia *et al.*, 1998).

We observed that the correlation between our PN data and the behavior was higher than the published correlation value between the compound signal and the behavior(r=0.51). Two major differences between the compound data and our data make a direct comparison of the correlations impossible. First, the compound signal was calculated as the integral of the response over time, while we correlated each time point separately. Second, *Giurfa et al.* did not use continuous values, but derived the glomerular activity of the compound signal from a figure in the publication of *Sachse et al*. In this figure, the response strength was ranked into 5 groups, ranging from 0-100% by steps of 20% and 100% was the activity of the glomerulus which showed the strongest activation at this odor. Additionally, instead of using the five groups of the figure, they used four, combining the two groups showing the lowest activity (0-40%).

In order to base their comparison on a more exact measure of glomerular activity, they then used the cross correlation values between a subset of the 16 odors, namely the primary and secondary alcohols, published by *Sachse et al*. When comparing this subset

to the same subset of their behavioral matrix, they found a much higher correlation, resulting in a value of r=0.81. We, in turn, observed an even higher degree of correlation when using the same subset of our PN data and comparing it to the behavior. The maximal correlation values ranged around r=0.88 for all four concentrations and even reached r=0.92 for the highest odor concentration of $10^{-1}$. But these high correlation values have to be put into the perspective of the high correlation values obtained by the Monte Carlo analysis. These ranged from -0.7 to 0.7. This result is not surprising, as the principal component analysis revealed a high degree of similarity between the odor presentations of primary and secondary alcohols, inevitably resulting in very small Euclidean distances between the odor presentations. These small distances will in turn minimize the effect of randomly permuting them. Therefore the high correlation values of the primary and secondary alcohols with the behavior have to be treated with caution.

Two major conclusions can be drawn from the high correlation between optophysiological and behavioral data:

First, in honeybees, perceived odor similarity is a result of chemical similarity. Chemically similar odors excite overlapping sets of glomeruli, which in turn are perceived as more similar than non overlapping glomerular activity patterns. This has also been shown at the level of olfactory receptors in rats (Linster *et al.*, 2001). Rats were differentially conditioned to pairs of odorant enantiomers and their OSN activity was recorded with 2-deoxyglucose. The animals could easily differentiate between odors eliciting significantly different OSNs, while they were unable to do so with odors activating very similar sets of OSNs. Here we show a very similar result at the level of the PNs.

Second, the high degree of correlation between our PN data and the behavior shows that further processing of olfactory information beyond the PNs does not significantly change the relative similarity between odors. This suggests an exclusive role of the AL in the processing of odor similarity and again suggests that odor generalization is not mediated by the MB KCs, where such information is lost.

**Redundancy in the olfactory system**

On many occasions olfactory receptor neurons have been shown to be broadly tuned, responding to a varying range of different odors (Duchamp-Viret *et al.*, 1999; Hallem *et al.*, 2004; Sachse *et al.*, 1998). This broad tuning results in a redundancy of the olfactory system, making it particularly resistant to damage or a loss of receptors caused by mutations (Slotnick *et al.*, 1997; Vedin *et al.*, 2004; Slotnick and Bisulco, 2003). Animals having lost specific receptors are still able to perceive odors which strongly activate these receptors, though with a higher detection threshold (Vedin *et al.*, 2004). This is in line with our findings. At low odor concentrations, only a small number of glomeruli are activated. These glomeruli are therefore necessary for odor detection at these particular concentrations, as shown in the collapse of the correlation values in Figures 10c and 11. With increasing odor concentrations, other glomeruli become active, and the code becomes more resistant to the loss of one or several of them. It is noteworthy that there were no two glomeruli having the same response spectrum. Therefore, the redundancy is not simply implemented by sending the same information to several glomeruli, but by several glomeruli, having overlapping but distinct odor response profiles. Even the relative similarity between odors is maintained, when some glomeruli are missing; as is visible in the high correlation between this “ablated” glomerular patterns and the behavior.

Nevertheless these results have to be interpreted with caution. As glomeruli are interconnected, the absence one glomerulus might influence the responses of other glomeruli in a way that the overall glomerular patterns change. We could not observe such changes in the few animals, where a glomerulus was missing in the pattern. Nevertheless further evidence is needed to prove that the overall glomerular patterns remain unchanged, when some glomeruli are quiescent as result of damage or mutation. In Drosophila, such evidence could be acquired by selectively silencing all OSNs carrying a specific receptor and subsequently imaging the resulting activity patterns.