Discussion

Methodological aspects

Intact animal approach

Understanding how odors are coded within the olfactory system requires knowledge about its input. The input to the olfactory system is determined by the molecular receptive ranges (MRRs) of its entire ORN population. The MRR of an ORN is largely dictated by the receptor protein expressed in it (Hallem et al., 2004). Therefore, MRRs have been characterized by looking solely at the OR by either employing heterologous expression systems (Gaillard et al., 2002;Hatt et al., 1999;Kajiya et al., 2001;Krautwurst et al., 1998;Raming et al., 1993;Wetzel et al., 1999;Wetzel et al., 2001) or *in vitro* sytems (Bozza et al., 2002;Hamana et al., 2003;Malnic et al., 1999;Murrell and Hunter, 1999;Rawson et al., 1997;Sato et al., 1994;Touhara et al., 1999). In other studies ORs, have been overexpressed (Araneda et al., 2000;Stortkuhl and Kettler, 2001;Zhao et al., 1998) or misexpressed (Hallem et al., 2004) in order to characterize their MRR. A different approach is to consider ORNs as the input channels of the olfactory system and characterize their MRRs *in vivo*, i.e. within the living animal which has been realized for example by single sensillum recordings in *Drosophila* (de Bruyne et al., 1999;de Bruyne et al., 2001;Yao et al., 2005). This approach has the advantage of having all auxillary mechanisms and cells in place. Therefore, MRRs based on ORN physiology include the possible influences of: olfactory binding proteins (Pophof, 2004;Xu et al., 2005), ORNs housed within the same sensillum (Dobritsa et al., 2003), co-receptors (Dobritsa et al., 2003;Larsson et al., 2004;Neuhaus et al., 2005), and receptor associated G-proteins (Shirokova et al., 2005). The link between the ORN physiology and the OR expressed by the respective ORNs is however indirect. The MRR presented in this study is based on the physiology of genetically identified ORNs, thus combining the advantages of an *in vivo* approach with the known identity of an OR.

Automation of experiments

Establishing all MRRs of the entire *Drosophila* ORN population requires the screening of a large number of flies with an even larger number of odors at various concentrations. Thus automating the experiments would make the task more feasible for the experimenter. Additionally, automation has the advantage of a high reproducibility of the experimental conditions. In the experiments presented here, the stimulus-application system was directly linked to the imaging set-up enabling the experimenter theoretically to start an experiment, leave the laboratory and return for saving the data collected in the mean time. However, as the experimental subject was a living fly, this did not prove to be practical. Despite taking great care during the preparation of the flies to avoid movement of the head and/or the brain, movements remained a problem and quite often required readjusting the focal plane during experiments. Additionally, there was a risk of air bubble formation or the fly dying half-way through the experiment. Hence the automation of the experimental set-up was only used for short periods of 15 to 30 minutes with a check of the fly's condition thereafter followed by further measurements if possible.

Calcium imaging – signal sources

Calcium is a secondary reporter of neuronal activity thus one has to be aware of possible Ca^{2+} sources contributing to the signal when interpreting the results. The signal measured on the antenna originated in the dendrites and the cell bodies. Thus Ca^{2+} contributing to it could stem from processes related to sensory transduction like Ca^{2+} entering the cell through IP_3 dependent Ca^{2+} channels, Ca^{2+} dependent cation channels and voltage operated Ca^{2+} channels which are opened following the transduction cascade (Augustine et al., 2003;Stengl, 1994). A second source could be Ca^{2+} release from the ER in the soma triggered by the activation of ryanodine or IP_3 receptors (Augustine et al., 2003) as has been shown for salamander ORNs in culture (Zufall et al., 2000). In the AL, the Ca^{2+} signal was imaged at the presynaptic terminals of the ORNs where the primary Ca^{2+} source is thought to be the influx through voltage-gated Ca^{2+} channels (Augustine et al., 2003). For mitral cells it has been shown that Ca^{2+} fluctuations follow subthreshold changes in membrane potential additionally to transient changes triggered by sodium AP that invade the entire dendritic tree (Charpak et al., 2001). A close relationship between changes of the

membrane potential and calcium influx was also shown for (non-spiking) motion-sensitive visual neurons of the blowfly (Kurtz et al., 2001;Single and Borst, 2002) as well as for local and projection neurons of the honeybee AL (Galizia and Kimmerle, 2004). Thus, the Ca^{2+} signal on the antenna probably reflects the transduction cascade and the resulting receptor potential while the AL signal most likely reflects the change in AP frequency.

Comparison to previously published studies

The MRR of an OR or an ORN should be independent of the method used to establish it. The MRR of ORNs expressing Or22a in this study was based on Ca^{2+} imaging. Calcium is however only a secondary reporter of neuronal activity (see above). All other studies working on ORNs expressing Or22a or Or47b employed electrophysiology (de Bruyne et al., 2001;Dobritsa et al., 2003;Hallem et al., 2004;Stensmyr et al., 2003a;Wilson et al., 2004). Overall, comparing the results presented here to previous studies shows that Ca^{2+} imaging is a valid and reliable method for establishing an ORN population's MRR.

Our results for both receptors are in fairly good agreement with the already published data. Concerning ORNs expressing Or22a, ethyl and methyl hexanoate were also previously identified as, by far, the best ligands (Stensmyr et al., 2003a). The results presented here also agree with previous studies for odors eliciting large and medium sized responses (de Bruyne et al., 2001;Dobritsa et al., 2003;Hallem et al., 2004;Stensmyr et al., 2003a;Wilson et al., 2004). Another similarity between our and previous results are the dynamic ranges of the dose-response curves: just like the dose-response curves shown in Figure 8 those measured with electrophysiology cover between three and four log steps (Dobritsa et al., 2003;Hallem et al., 2004). A comparison of non-activating odors also shows a good agreement with previous studies. However, de Bruyne et al. (2001) indicate no response to four substances: 1-butanol (to which Wilson and co-workers (2004) also did not record a response), iso-amyl alcohol, 4-methylcyclohexanol, cyclohexanone to which we reliably recorded responses. These discrepancies could be due to several reasons, e.g. the different methods employed, i.e. Ca^{2+} imaging and electrophysiology. Differences could also be due to the olfactometers and the stimulation parameters used. Temporal properties

of a stimulus, like velocity, massflow and duration are all likely to influence the ORNs response. Firestein et al. (1993) for example showed in isolated salamander ORNs that the amplitude of the odor-evoked response not only depends on concentration but also on stimulus duration. Furthermore, actual odor concentrations reaching the fly are difficult to compare even if the same dilutions were used because of differences in presentation, e.g. head space samples of odor diluted in mineral oil as done in this study or head space samples from odor laden filter papers as done by de Bruyne et al. (2001). Another reason for the discrepancies could simply be interstudy variations as can also be found between earlier published papers. Ethyl acetate for example was found to elicit a medium to large response in the studies by Dobritsa et al. (2003) as well as by Hallem et al. (2004) while de Bruyne et al. (2001) did not find a response to ethyl acetate.

There are only two papers which present data on the physiology of Or47b (Hallem et al., 2004;Wang et al., 2003). The odors tested by Wang et al. (2003) were all different from those employed here, thus a comparison to our data is not possible. However, two of the odors tested by Hallem et al. (2004) were used in this study: 1-hexanol also elicited a negative response (compare to Figure 18) and E2-hexenal increased the AP frequency in wild-type ORNs, although only slightly (compare to Figure 18). However, Hallem et al. (2003) used a dilution of 10^{-2} [vol/vol], which is the same as used in this study, but further diluted the odor stimulus by a factor of 4 in the constant air stream. Accordingly the difference in response amplitude evoked by E2-hexenal could be due to a difference in stimulus concentration.

ORNs expressing Or22a

Comparison between antennal and AL measurements

The antennal and the AL MRR were virtually identical as the same odors were found to belong to both (Figure 9). Thus in future experiments one can weigh the advantages and disadvantages of measuring in either place against each other. The antennal preparation has the advantage of leaving the fly completely intact, thus making it possible to record for longer times (maximal recording time on the antenna

160 minutes compared to 100 minutes in the AL). Additionally, the preparation is less time consuming and easier to learn in comparison to the AL preparation. On the other hand, the AL preparation resulted in a better sensitivity at low odor concentrations. Furthermore, if one is interested in following the olfactory information from the periphery to higher brain centers, i.e. measuring from 2nd and 3rd order neurons within the olfactory system, it might be advantageous to measure within the AL. In any case the AL MRR will reflect the antennal MRR and vice versa.

Both, antennal and AL dose-response curves had Hill coefficients below 1 (Table 2) which indicates independent interaction of individual odor molecules with the receptor. Furthermore the Hill coefficient is a measure of the dose-response curves' slope thus indicating the dynamic range of concentrations at which the receptor is activated. The dose-response curves measured for ORNs expressing Or22a covered between three and four log units. Similar dynamic ranges have been found for turtle ORNs (Wachowiak et al., 2002b), zebrafish ORNs (Friedrich and Korsching, 1997;Fuss and Korsching, 2001), mouse ORNs (Wachowiak and Cohen, 2001) and moth ORNs (Stranden et al., 2002).

As shown in Figure 8 the antennal dose-response curves were shifted to higher concentrations in comparison to the AL dose-response curves. One possible explanation for the difference in sensitivity between the antennal and the AL results could be a lower signal to noise ratio on the antenna. One source for a higher antennal noise level could be the cuticle through which the antennal signal was imaged. Furthermore, ORNs expressing Or22a on the antenna are not concentrated in one spot but they are interspersed with sensilla housing other ORN types. As the antennal signal was calculated from an area of 35 × 35 µm^2, it is most likely 'diluted' by these interspersed sensilla. In the antennal lobe, on the other hand all ORNs expressing Or22a converge within the same glomerulus, so that the signal measured there is more 'concentrated'. Additionally, the ORNs send a collateral branch to the contralateral side of the AL (Figure 7A right) where they add to the signal measured. All these factors probably contribute to a better sensitivity of the AL signal in comparison to the antennal signal. Another possible explanation could be a difference in the resting Ca^{2+} levels. If the antennal resting Ca^{2+} level was higher than the AL resting Ca^{2+} level a similar change in neuronal activity would probably lead to a smaller relative

change in fluorescence intensity. A difference between the antennal and the AL Ca^{2+} level is supported by the difference in response to 4-methoxybenzene and benzaldehyde (Figure 11E). On the antenna both odors evoked a decrease in fluorescence intensity while in the AL under standard conditions they did not evoke a response. However, stimulation with an odor evoking a long-lasting response raised the resting Ca^{2+} level within the AL. Under such conditions subsequent stimulation with either 4-methoxybenzene or benzaldehyde also lead to decrease in fluorescence intensity within the AL. Finally, the observed differences between antennal and AL signals could be explained by the different Ca^{2+} sources contributing to either signal (see above).

Specialist versus generalist

ORNs have been classified as specialists which respond to a few key substances needing only low concentrations to elicit a response, or as generalists, which respond to a large number of odors (Hildebrand and Shepherd, 1997). Of 104 substances tested initially, graded responses were found to 39 of them. Two of those, ethyl and methyl hexanoate, needed by far the lowest concentration to elicit a halfmaximal response within the AL (Figure 9). The MRR of ORNs expressing Or22a presented here was established under laboratory conditions with concentrations chosen by the experimenter. If the initial screening had been done at a concentration of 10^{-6} [vol/vol] rather than at 10^{-2} [vol/vol] the MRR would have consisted of only 4 substances: ethyl and methyl hexanoate, ethyl and ethyl-2-methyl butanoate. Whether Or22a respectively DM2 could be considered a specialist for ethyl and methyl hexanoate or rather a generalist ultimately depends on the naturally occurring concentrations of the respective odors.

Establishing such data was beyond the scope of this study. However, I found data published on the amount of odor substances in banana, pink guava, yellow passion fruit, muskmelon, charentais melon, strawberries, and mango (Aubert and Bourger, 2004;Jordan et al., 2001b;Jordan et al., 2001a;Jordan et al., 2002;Jordan et al., 2003;Loughrin and Kasperbauer, 2002;Pino et al., 2005). From all the substances detected in these fruit few occurred in amounts which should elicit at least a medium sized response in ORNs expressing Or22a (Table 3). This is in agreement with a study

by Stensmyr and co-workers (2003) where natural odor sources were tested on *Drosophila* ORNs by combining gas chromatography with single sensillum recordings. They also found that each single ORN only responded to a few of the GC-separated extract compounds. The fruit odors found in the literature potentially eliciting a response in ORNs expressing Or22a at natural concentrations were ethyl

Table 3 Natural sources of Or22a activating odors

odors activating Or22a	amount at EC50 [mg/kg]	mango (1)[a]	melon (1)[b]	guava (1)[c]	straw berry (1)[d]	ppm at EC50	passion fruit (2)[e]	musk melon (2)[f]	banana (2)[g]
methyl hexanoate	0.13	**0.15**			**0.67**	0.0042			
ethyl hexanoate	0.15	**0.24**	**0.31**	**1.25**	**0.79**	0.0016	**5.08**	**0.05**	
ethyl butanoate	1.40	**4.89**	**2.22**	*1.13*	0.57	0.3323	**3.86**	**0.44**	0.15
ethyl 2-methyl butanoate	5.83	0.03	1.56		0.03	na			
2-methyl butyl acetate	13.75		3.40		0.05	na			
heptanal	15.10	0.03				0.6842			
pentyl acetate	30.57		0.09			1.1985			
butyl acetate	48.78	0.51	2.01		0.07	12.8830		0.38	1.32
ethyl propionate	70.99	0.13				42.0086		0.45	
iso-amyl acetate	72.82					2.6052			**4.85**
3-penten-2-one	323.79	0.18				na			
E2-hexenyl acetate	452.93				0.19	na			
ethyl-(R)- 3 hydroxy butanoate	562.44	2.67				na	6.62		
1-octen-3-ol	580.54	0.04				1.8056			
ethyl 3-hydroxy hexanoate	621.32	0.06				na	2.63		
1-butanol	766.02	4.26				64.6270		0.42	1.06
2-heptanone	806.44	0.03				8.6482			
hexanal	1465.28	0.22			0.06	313.7591		0.25	21.47
butyl butanoate	1478.34	0.19			0.04	na			0.83
ethyl S 3 hydroxy butanoate	1737.55	2.67				na	6.62		
1-hexanol	2184.64	0.25	0.28		0.03	20.5361	1.56	1.09	1.17
hexyl acetate	2554.32		2.51		0.21	na	0.98	0.62	0.57
3-hydroxy 2-butanone	3805.09	1.32	10.59	22.63		196.6834	27.87		
cyclohexanone	4447.40	0.05				na			
1-heptanol	5042.29	0.01				8.52934			
ethyl aceate	10398.30	4.9				28214.8984			
Z3-hexen-1-ol	10943.39	1.02	0.50	6.79	0.50	na		2.00	

List of odors activating Or22a identified in various fruit. Odors are sorted by increasing amount of odor at EC50. Amount at EC50 indicates mg odor substance per kg mineral oil. ppm at EC50 were estimated as described. Bold names indicate odors which were found in amounts sufficient to elicit at least a half-maximal response. (1) odors quantified as mg per kilogram fruit; (2) odors quantified as ppm; [a] Pino et al. 2005, mango (*Mangifera indica* L.), 372 substances identified, 26 belonging to the Or22a MRR; [b] Aubert and Bourger, 2004, Charentais Cantaloupe Melons (*Cucumis melo* Var. cantalupensis), 28 substances identifies, 10 belonging to the Or22a MRR; [c] Jordan et al. 2003, pink guava (*Psidium guajava* L.); 51 substances identified, 4 belonging to the Or22a MRR; [d] Loughrin and Kasperbauer 2002, Strawberry (*Fragaria ananassa* Duch), 23 substances identified, 14 belonging to the Or22a MRR; [e] Jordan et al. 2002, yellow passion fruit (*Passiflora edulis* Sims F. Flavicarpa degner), 34 substances identified, 8 belonging to the Or22a MRR; [f] Jordan et al. 2001a, muskmelon (*Cucumbis melo* cv Athena), 38 substances identified, 10 out of which belong to the Or22a MRR; [g] Jordan et al. 2001b, banana (*Musa sapientum* L.var. Cavendis), 26 substances identified, 9 belonging to the Or22a MRR.

and methyl hexanoate, ethyl butanoate, and iso-amyl acetate. These are all among the odors with the 10 lowest EC50 found in this study. For all fruit ethyl hexanaote was the substance which would have elicited the largest response. The only exception was banana where ethyl hexanoate was not detected by the authors (Jordan et al., 2001b). However, it is known from other publications that ethyl hexanaote is part of banana odor (Tressl et al., 1969), unfortunately no amounts could be found. Thus, it could not be established whether banana odor contains ethyl hexanoate at concentrations sufficient to elicit a response in ORNs expressing Or22a. However, according to Jordan et al. (2001b) banana contained sufficient amounts of iso-amyl acetate to elicit at least a half-maximal response in DM2. Indeed, the head space above pieces of banana of varying degrees of ripeness (with and without peel) evoked responses in ORNs expressing Or22a slightly above the halfmaximal response.

Although at naturally occuring concentrations ethyl hexanoate might be the main odor eliciting a response in ORNs expressing Or22a, it is not the only activating substance. Therefore it seems unlikely that Or22a is simply an ethyl and methyl hexanoate detector. However, the ecological and ultimately behavioral relevance of the numerous other odors of the MRR remains open. Interestingly, the dose-response curves of the odors which were found to evoke responses in ORNs expressing Or22a at natural concentrations (Table 3) were among the 10 odors belonging to the Or22a MRR (Figure 8) whose dose-response curves reached saturation. No natural source containing sufficient amounts for eliciting a response in ORNs expressing Or22a was found for odors whose dose-response curve did not reach saturation. Responses to these odors might simply reflect the tolerance of the binding site. Dose-response curves reaching saturation could be a good indication for odors with an ecological relevance for the fly.

Role of ORNs expressing Or22a in odor coding

If Or22a with its broad (yet selective) response spectrum is a special case among the family of *Drosophila* basiconic ORNs remains to be determined. Judging from the already published literature on ligands of *Drosophila* ORs, there seem to be several ORs with a broad response spectrum. For example, the ab1a cell responded to almost all of the esters tested (de Bruyne et al., 2001). The ab3b cell (expressing Or

85b) and the ab7a cell (expressing Or98a) responded to a large number of different odors (de Bruyne et al., 2001;Hallem et al., 2004). The ab6a cell responded to a large number of alcohols and esters, preferring alcohols and in this regard mirroring the ab3a cell which also responded to a large number of alcohols and esters but preferring esters instead (de Bruyne et al., 2001). Whether all of these also have a few specific ligands, as Or22a has with ethyl and methyl hexanoate, will have to be shown by testing more odors and determining the respective dose-response curves. Another open question is if all of the very selective ORs (ab5a expressing Or82a e.g. only responded to geranyl acetate) are so because of a special binding site or because of the limited number of odors tested on them so far. In terms of odor coding receptors responding to multiple odors enable a combinatorial code and thereby enlarge the number of odors which can be recognized by the olfactory system.

Odotopes of odors activating Or22a

A largely unanswered question in olfaction is what properties an odor molecule needs to have in order to interact with a given olfactory receptor. So far no olfactory receptor has been crystallized, thus nothing is known about their structure and their binding site/s. Therefore, the only available information on interaction sites between an odor and its receptor comes from the odor molecules belonging to an olfactory receptor's MRR. Understanding the interactions between an odor and its receptor offers large opportunities to learn generally about the interactions between receptors and their ligands. A rigorous analysis of Or22a's binding site was beyond the scope of this study. Nonetheless, some odotopes can still be postulated. The preferred functional group was an ester. This was however not a strict prerequisite as also aldehydes, ketones, alcohols and oxygen heteroaromates were included in the MRR. Common to these functional groups was the occurrence of an oxygen atom which might act as hydrogen acceptor. In the mouse eugenol receptor a serine was suggested to be responsible for hydrogen boding to an oxygen residue of activating odors (Katada et al., 2005). Thus the requirement of an oxygen atom in activating odors could be indicative of the presence of a hydrogen donor within the binding site of Or22a.

Analysis of the mouse eugenol receptor binding site, i.e. the part of the receptor molecule putatively interacting with the odor, identified an isoleucine to be presumably responsible for defining its spatial configuration (Katada et al., 2005). For Or22a the spatial configuration of the binding site also seems to impose some molecular constraints on activating esters. On both sides of the esters group, molecules which were too long were far less effective in eliciting a response than molecules which were too short (Figure 16). A possible mechanism explaining this could be that molecules which are too small can interact with the receptor, yet can not reside stably within the binding site, thus dissociating faster from the receptor. Higher odor concentrations could compensate for this by increasing the probability of an odor molecule binding to the receptor thereby increasing the number of incidents of an odor molecule activating the ORN during stimulus presentation. It seemed that for odors which were too small increased concentrations could compensate reduced potency.

For ORNs expressing Or22a the preferred carboxylic acid moiety of esters is C6 – hexanoate (Figure 16). Esters with longer carboxylic acid moieties hardly elicited a response, indicating that repulsive forces may become important at longer molecular lengths or that the binding pocket imposes steric constraints. Esters with shorter carboxylic acid moieties could elicit responses provided that the alcohol moiety was not too long. The preferred alcohol moiety was a C2 – ethyl group. Esters with a C3 – propyl or larger group were not efficient as ligands and could only elicit a response if the carboxylic acid was short enough as was the case for butyl butanoate or propyl propanoate. The preference for an ethyl over a methyl group only became apparent if the carboxylic acid moiety was not optimal i.e. if the ester was shorter than a hexanoate. Thus the binding pocket seems to require several odotopes which are not independent from each other, an observation which has also been made for other ORs (Araneda et al., 2000;Spehr et al., 2003). Further understanding of the interaction between the receptor and the odors activating it could be gained by predictive modeling followed by testing additional substances experimentally.

As a first step into this direction we started a cooperation with Daniel Baum from the Zuse Institute Berlin (ZIB) who developed a semi-flexible algorithm for super-positioning of drug-sized or smaller molecules (Baum, 2005). The algorithm is

based on the notion that the multiple odors activating a given OR, although different in many respects must share certain odotopes which are recognized by the receptor. Daniel Baum's work is still in progress but we were able to achieve first results. They extend the conclusions drawn about the odotopes of odors activating Or22a from their two-dimensional molecular formulas to their three dimensional conformers, thereby aptly illustrating the size limits of the putative binding pocket (Figure 17). Furthermore, the algorithm made it possible to find a substructure common to all 39 molecules included in the Or22a MRR, thereby showing a putative minimal requirement for activating odors. This included three carbon atoms and one oxygen atom which would enable hydrophobic and hydrogen bond interactions.

Superpositioning of the esters included in Figure 16 allowed us to make a prediction about an atom position presumably distinguishing between activating and non-activating esters. Esters whose carboxylic acid moiety consisted of at least five C atoms (i.e. pentanoates, hexanotes, and heptanoates) were predicted to be activating. However, there were two exceptions. One ester was activating despite being too short (false negative), the other ester was non-activating despite being long enough (false positive). The explanation for these exceptions could be the length of the alcohol moiety, which in case of the false negative was optimal (ethyl) and in case of the false positive was too long (propyl). Thus, the false predictions showed that it is not single atoms which render an odor activating or non-activating but that one has to look for interactions between atoms or groups of atoms in order to fully explain the properties of an activating odor. Including such interactions within the algorithm might eventually enable us to fully understand the subtle differences between odors with a low and a high EC50 and thus increase our understanding about the interactions between an odor and its receptor.

The characterization of ORNs expressing Or22a presented here has extended the knowledge about their response spectrum to the concentration ranges at which individual odors activate the ORNs. This knowledge enabled me to draw a more differentiated picture about Or22a than simply that of a broadly tuned receptor. Despite reacting to a large number of odors very specific ligands exist and thus the concept of generalist versus specialist receptors might not tell the whole story. This data set made it possible to deduce a common binding motif from the responsive

odors and hence contributed to the search for rules of receptor-ligand interactions (Katada et al., 2005;Luu et al., 2004). Finally, I have developed a procedure which will make it feasible to fully characterize the entire input to a model olfactory system thus laying the foundation for further advances in understanding the olfactory code.

ORNs expressing Or47b

A preliminary molecular receptive range

Knowledge about the physiology of *Drosophila* ORNs is already quite considerable: following the characterization of the basiconic sensilla on the maxillary palps (de Bruyne et al., 1999) and on the antenna (de Bruyne et al., 2001;Elmore et al., 2003) a paper was recently published describing the physiology of coeloconic sensilla (Yao et al., 2005). However, there is still one ORN class about which very little is known: the ORNs housed in trichoid sensilla (Clyne et al., 1997;Hallem et al., 2004). Or47b is expressed in trichoid sensilla (Hallem et al., 2004) and I was able to identify a few ligands (activating and inactivating) of this OR.

Of the C6 odors tested on ORNs expressing Or47b, those evoking a response (increase or decrease in fluorescence intensity) were all straight chain molecules with either an alcohol or an aldehyde group (Figure 19). Neither 2-hexanone (a straight chain saturated C6 ketone) nor 4-methylcyclohexanol (a C6 cyclic alcohol with an additional methyl group) nor benzaldehyde (a C6 aromatic with an aldehyde group) evoked a change in fluorescence intensity.

Judging by the absolute magnitude of the responses elicited by the odors at a concentration of 10^{-2} [vol/vol] straight chain alcohols were more potent ligands than straight chain aldehydes. The two aldehydes evoking a response form an interesting pair: hexanal, a saturated C6 molecule evokes a decrease in fluorescence intensity after stimulus offset, whereas E2-hexenal, its 2-cis unsaturated counterpart, evokes an increase in fluorescence intensity (Figure 18). Thus, it is the occurrence of a C-C double bond which turns a ligand from inactivating into activating. This observation however does not hold true for C6 alcohols: E2-hexen-1-ol (2-cis unsaturated C6)

elicited the largest decrease in fluorescence intensity. 1-hexanol (saturated C6) on the other hand elicited the 2nd largest decrease. Hence, just as shown for Or22a, there seem to be several odotopes that make an odor activate or inactivate Or47b. As shown for Or22a and other ORs (Araneda et al., 2000;Spehr et al., 2003) there seems to be an interaction between the odotopes of Or47b. A more detailed prediction about the molecular features that make an odor capable of binding to Or47b can however not be drawn as the number of molecules tested was too small. However, the odors tested so far provide a sound basis for further exploring the Or47b MRR.

'Negative responses'

The odor-evoked responses recorded in ORNs expressing Or47b were mostly decreases in fluorescence intensity. When imaging Ca^{2+}, a decrease in fluorescence intensity upon presentation of a stimulus is thought to reflect a decrease in AP frequency (Charpak et al., 2001;Galizia and Kimmerle, 2004;Single and Borst, 2002). In case of ORNs this would correspond to a suppression of spontaneous activity. A suppression of ORN spontaneous activity upon presentation of an odor has been observed for many different species: in honeybees (Vareschi, 1971), tobacco hawk moths (Shields and Hildebrand, 2000), rats (Duchamp-Viret et al., 1999), toads (Morales et al., 1994), and fruitflies (de Bruyne et al., 1999;de Bruyne et al., 2001;Hallem et al., 2004;Yao et al., 2005). Inhibitory responses only occur rarely, usually in ORNs with an elevated spontaneous activity (de Bruyne et al., 1999;Shields and Hildebrand, 2000) which is the case for ORNs expressing Or47b (Hallem et al., 2004).

Several studies have shown that a given odor can excite some cells and inhibit others and individual ORNs can either be excited or inhibited by different odors (de Bruyne et al., 1999;Duchamp-Viret et al., 1999;Shields and Hildebrand, 2001). There are different models of how these opposing effects can be brought about within the same cell upon odor presentation. One is the possibility of two different transduction cascades being involved. For lobsters it has been shown that both IP_3 and cAMP levels can rise within ORNs upon odor presentation, with IP_3 leading to a depolarization and cAMP to a hyperpolarization (Boekhoff et al., 1994;Fadool and Ache, 1992;Michel and Ache, 1992). For toads a different mechanism has been

proposed where both excitation and inhibition are mediated via cAMP with two different channels being opened, one leading to depolarization, the other to hyperpolarization of the cells (Madrid et al., 2005). Hallem et al. (2004) proposed another model for fruitflies where receptors can either exist in an activated state or an inactivated state. An increase in AP frequency above the spontaneous firing frequency upon odor presentation would result from an odor stabilizing the receptor in the activated state. A decrease in AP frequency below the spontaneous firing frequency upon odor presentation would result from an odor stabilizing the receptor in the inactivated state. For most studies on ORNs a distinction between these different models is not possible as they are based on extracellular recordings where hyperpolarization can not be observed. Such a distinction can also not be done with Ca^{2+} imaging experiments where a decrease in fluorescence intensity reflects a decrease in intracellular Ca^{2+} concentration. The underlying cellular activity leading to this decrease, e.g. opening of ion channels leading to hyperpolarization or cessation of the AP generation caused by interruption of the transduction cascade however remains elusive.

As described above there are different mechanisms by which the decrease of the AP frequency could be brought about. If the binding of an inactivating odor to a receptor would lead to a transduction cascade, which in the end would open a channel leading to hyperpolarization, the inactivating odor would be an agonist. If on the other hand binding of an inactivating odor to a receptor would lead to stabilization of an inactive receptor state these odors could be termed inverse agonists. Inverse agonists are defined as decreasing the activity of a receptor which is active in the absence of an agonist (Strange, 2002). This is in contrast to antagonists which block the response to an agonist when applied simultaneously, like e.g. methyl isoeugenol for the mouse eugenol receptor (Oka et al., 2004;Silverman, 1992). To date only little is known about the transduction machinery in insects and *Drosophila* specifically (Rutzler and Zwiebel, 2005), thus it is currently not possible to decide whether inactivating ligands of Or47b (and Or22a) are true agonists or rather inverse agonists.

Another explanation for the odor-evoked decreases in fluorescence intensity measured in Or47b ORNs could be the influence of the inhibitory AL network (Buchner, 1991;Jackson et al., 1990;Ng et al., 2002). This explanation, however,

seems rather unlikely as a decrease in the action potential frequency upon presentation of 1-hexanol has been measured in single-sensillum recordings (Hallem et al., 2004). Definite proof for an inhibitory response independent of the AL network would require measuring odor-evoked Ca^{2+} responses on the antenna.

Different aspects of odor coding

Excitatory and inhibitory input channels

ORNs expressing either Or22a or Or47b are in many regards different from each other. They are housed in different sensillum classes, they are differently sexually dimorphic, show different levels in spontaneous activity and they also differ in their response properties. While the MRR of ORNs expressing Or22a almost exclusively consists of activating odors that of ORNs expressing Or47b is largely comprised of inactivating odors. This notion is not only based on the odor set tested in this study but also on studies by Wang et al. (2003) and Hallem et al. (2004). Wang et al. (2003) tested 16 odors which were all different from those tested in this study. None of the odors was activating. Hallem et al. (2004) tested 14 odors three of which were also tested by Wang et al. (2003) and two of which were also tested in this study. Again, none of the odors tested was activating but several odors were inactivating. Among those was 1-hexanol which was also inactivating in our study. Thus out of 35 odors tested on ORNs expressing Or47b so far, there is only one activating odor and at least four inactivating odors have been found.

Comparing the odor evoked responses in ORNs expressing Or47b to the odor evoked responses in ORNs expressing Or22a in more detail shows that they are almost always different from each other: E2-hexenal, the activating odor of Or47b is a non-activating odor for Or22a. Benzaldehyde, one of the inactivating odors of ORNs expressing Or22a is non-activating for Or47b ORNs. Hexyl acetate is an example for an odor activating Or22a and non-activating Or47b whereas 1-hexanol is an example of an odor activating Or22a and inactivating Or47b. These examples show the advantage of an inhibitory signaling mode. If ‘increase’ or ‘unchanged’ were the only coding possibilities, two different channels (i.e. two different ORN

populations/glomeruli) would provide three different signaling combinations (a+b+, a+b0, a0b+). Adding the coding possibility 'decrease' raises the signaling combinations of two different ORN populations/glomeruli to eight. De Bruyne et al. (2001) suggested that inhibition might enhance signal recognition by contrast enhancement between an activated and an inactivated ORN population. Furthermore, the information provided by silencing of a normally active channel could principally mean something completely different for postsynaptic neurons than the activation of a normally quiet channel. Therefore an inhibitory input channel increases the coding complexity of the olfactory system already at the sensory level.

Temporal coding aspects

The time courses of the odor-evoked Ca^{2+} responses were odor and concentration dependent. Although three distinct response types (Figure 11C and D) were found in ORNs expressing Or22a there were also all possible intermediate response types. Therefore, it was not possible to classify the odors based on their time courses. Yet, when talking about temporal properties of the odor responses one always has to be aware of the fact that Ca^{2+} is only a secondary reporter of neuronal activity and that the indicator itself influences the temporal dynamics of the signal through its association and dissociation constants. These may have blurred the signals to a considerable extent such that it was not possible to classify the responses based on temporal response properties. Furthermore, the measurements were acquired at a frequency of 3Hz, so that the temporal resolution would have been too low even if the Ca^{2+} indicator was perfect. However, an odor stimulus is characterized by several parameters: its chemical identity, its concentration and its temporal structure, i.e. length and velocity. While the temporal structure was kept constant, the chemical identity and the concentration of the odors tested varied. Despite the confounding factors listed above the kinetics of the odor-evoked responses did not vary randomly but reproducibly across animals. Moreover, there was a predictable shift in the time course of the odor-evoked response upon introduction of an additional hydroxyl group at a certain position of three activating esters. Electrophysiological recordings have also shown that different odors elicit different kinetics within the same ORN (de Bruyne et al., 2001;Hallem et al., 2004;Yao et al., 2005). Thus it seems possible that

temporal aspects of the odor response carry information about the odor's concentration and/or identity.

Presynaptic inhibition of ORNs

A difference between the antenna and the AL is that the axonal endings of the ORNs in the glomerulus form the AL network together with the projection neurons, which transmit the olfactory information to higher brain centers, and neurons local to the AL many of which have been shown to be GABAergic and therefore inhibitory (Wilson and Laurent, 2005). Presynaptic inhibition of ORNs has been shown for lobsters (Wachowiak and Cohen, 1998;Wachowiak and Cohen, 1999), turtles (Wachowiak and Cohen, 1999), rats (Aroniadou-Anderjaska et al., 2000;Ennis et al., 2001) and mice (Ennis et al., 2001;Murphy et al., 2005;Wachowiak et al., 2005). Although these examples include vertebrates and invertebrates, so far nothing is known about a similar phenomenon in insects. Only for the cockroach *Periplaneta americana* there is anatomical evidence for synapses of GABA-immunoreactive cells onto ORNs suggesting a centrifugal control of ORNs (Boeckh and Tolbert, 1993;Distler, 1990). Furthermore, Wilson et al. (2004) found that long inhibitory epochs in PN odor responses were associated with a decrease in EPSP frequency which they interpret as suggestive of presynaptic inhibition.

The pharmacological experiments presented in this study provide the first physiological evidence for presynaptic inhibition in *Drosophila*. Application of muscimol resulted in a decrease of the odor-evoked responses (Figure 12B). Muscimol presumably activates all fast GABA receptors on the presynaptic terminals thus hyperpolarizing the ORNs. This notion is supported by the decrease in the prestimuls fluorescence intensity (Figure 12D), which is indicative of the resting Ca^{2+} level, upon muscimol application. Presenting an activating odor leads to a depolarization of the ORNs. The resulting action potentials reach the axon terminals. The axon terminals are hyperpolarized due to an increased Cl^- conductance induced by muscimol thus the odor-evoked response is reduced.

Picrotoxin (PTX) blocks Cl^- channels, like ionotropic GABA receptors. Hence application of PTX presumably prevented presynaptic inhibition via Cl^- channels

normally occurring during stimulus presentation. As there was no increase in the response to ethyl hexanoate (Figure 12B) I conclude that there is no presynaptic inhibition in Or22a ORNs upon presentation of ethyl hexanoate. When stimulating with ethyl hexanoate (10^{-7} [vol/vol]) only a single glomerulus was active in the entire ORN population (Figure 12A). Based on its dorsomedial position within the AL and its physiological response properties this glomerulus was tentatively identified as DM2, the glomerulus innervated by ORNs expressing Or22a. Thus DM2 was the only glomerulus providing input to the inhibitory AL network. Supposing that activation of the AL network does not lead to feedback inhibition of DM2, the sparse activity pattern within the ORN population upon presentation of ethyl hexanoate (10^{-7} [vol/vol]) could explain the lack of presynaptic inhibition under these circumstances. On the other hand 1-butanol at a concentration of 10^{-3} [vol/vol] elicited a broad activity pattern within the ORN population. Thus I postulate that presentation of 1-butanol (10^{-3} [vol/vol]) leads to activation of the inhibitory AL network resulting in presynaptic inhibition of ORNs expressing Or22a through opening of Cl^- channels, among them ionotropic GABA receptors. However, the application of PTX to the AL increased the response to 1-butanol only by approximately 20% (Figure 12B) thus the effect of the PTX sensitive inhibitory network was rather subtle.

The difference found between 1-butanol and ethyl hexanoate under PTX influence could also explain the difference between both odors under muscimol influence. Muscimol application led to a slightly larger suppression of the ethyl hexanoate response in comparison to the 1-butanol response (Figure 12B). If muscimol activated all ionotropic GABA receptors on the presynaptic ORN terminals, presentation of an odor after muscimol application would show the effect of the receptors activated in addition to those activated by the inhibitory AL network upon presentation of this odor under normal conditions. Supposing that presentation of 1-butanol (10^{-3} [vol/vol]) under normal conditions already results in presynaptic inhibition, application of muscimol can only reveal the additional effect of the normally not activated ionotropic GABA receptors. If presentation of ethyl hexanote (10^{-7} [vol/vol]) on the other hand did normally not lead to presynaptic inhibition, application of muscimol would reveal the effect of all the ionotropic GABA receptors present in the presynaptic terminals. Thus the stronger effect of muscimol on the ethyl hexanote (10^{-7} [vol/vol]) evoked response could result from muscimol activating more

ionotropic GABA receptors than under normal conditions in comparison to the 1-butanol evoked response (10^{-3} [vol/vol]).

Based on the results of the pharmacological experiments one can postulate a model for the role of the inhibitory AL network at the level of the ORNs where responses to odors with a high specificity for a particular ORN class are relayed to the PN without further processing while responses to odors which are less specific for a particular ORN class are reduced through presynaptic inhibition. This presynaptic inhibition would be caused by those ORN classes which are more specific for this odor through activation of local neurons (LN). In case of 1-butanol this could be e.g. the ab4a cells which express Or7a and project to glomerulus DL5 (Couto et al., 2005;de Bruyne et al., 2001;Hallem et al., 2004). Such a model would require spatially structured odor-evoked activation of LNs which has been shown at population and single cell resolution (Ng et al., 2002;Wilson and Laurent, 2005). As a consequence there would be a refinement of the spatial pattern of odor-evoked glomerular responses, a function which has also been suggested by Aroniadou-Anderjaska et al. (2000) for presynaptic ORN inhibition in rat and by Wachowiak et al. (2005) for mice. Interestingly, although GABAergic presynaptic inhibition occurs both in vertebrates and in invertebrates it seems to be mediated by slightly different mechanisms: in vertebrates GABAergic presynaptic inhibition is mediated by $GABA_B$ receptors (Aroniadou-Anderjaska et al., 2000;Murphy et al., 2005;Wachowiak et al., 2005;Wachowiak and Cohen, 1999) whereas in invertebrates it seems to be mediated by $GABA_A$ receptors (Wachowiak and Cohen, 1998). If GABAergic presynaptic inhibition is possibly also mediated by $GABA_B$ receptors in *Drosophila* remains to be determined. In any case presynaptic inhibition of ORN responses seems to be a widespread principle in odor coding.