Discussion

We tested the hypothesis that biogenic amines are modulating the peripheral olfactory system of *Drosophila melanogaster*. We detected expression of receptors for the biogenic amines serotonin, dopamine, octopamine and tyramine in the antenna, and showed that loss of the tyramine receptor in the mutants $TyrR^{hono}$ and $TyrR^{hono}/TyrR^{neo30}$ results in altered odor responses. Specifically, the sensillum potentials are elevated in one class of sensilla, while the spike frequencies and temporal patterns are unaltered. This "*hono* effect" is specific for ab2 sensilla and stable for a range of low odor concentrations. In contrast the mutant *ebony* with higher levels of biogenic amines displays lower odor responses in EAGs. However, octopamine deficiency (Tbh^{M18}) and elevated dopamine concentrations (Dat) respectively do not alter odor responses. As the loss of tyramine receptor leads to changed odor responses we searched for the source of tyramine in the antenna. We found two putatively tyraminergic or octopaminergic cells or cell groups. Firstly we visualized a neuron or neurons projecting into the 3rd antennal segment and into the arista. The soma or somata are not located in the antenna. But ablation of this neuron or these neurons does not change odor responses under the tested conditions. Secondly we revealed that one of the glia like accessory cells, the thecogen cell, is able to synthesize tyramine in a subset of sensilla. We assume that the thecogen cells are the source of tyramine in the antenna that affects the tyramine receptor and if lost gives rise to the "*hono* effect". Further experiments excluded the possibility that our findings just describe a developmental role for tyramine and the thecogen cells. Additionally, preliminary calcium imaging data suggest that the thecogen cells actually respond to odor stimuli. The data is summarized in the following model (Fig.1)

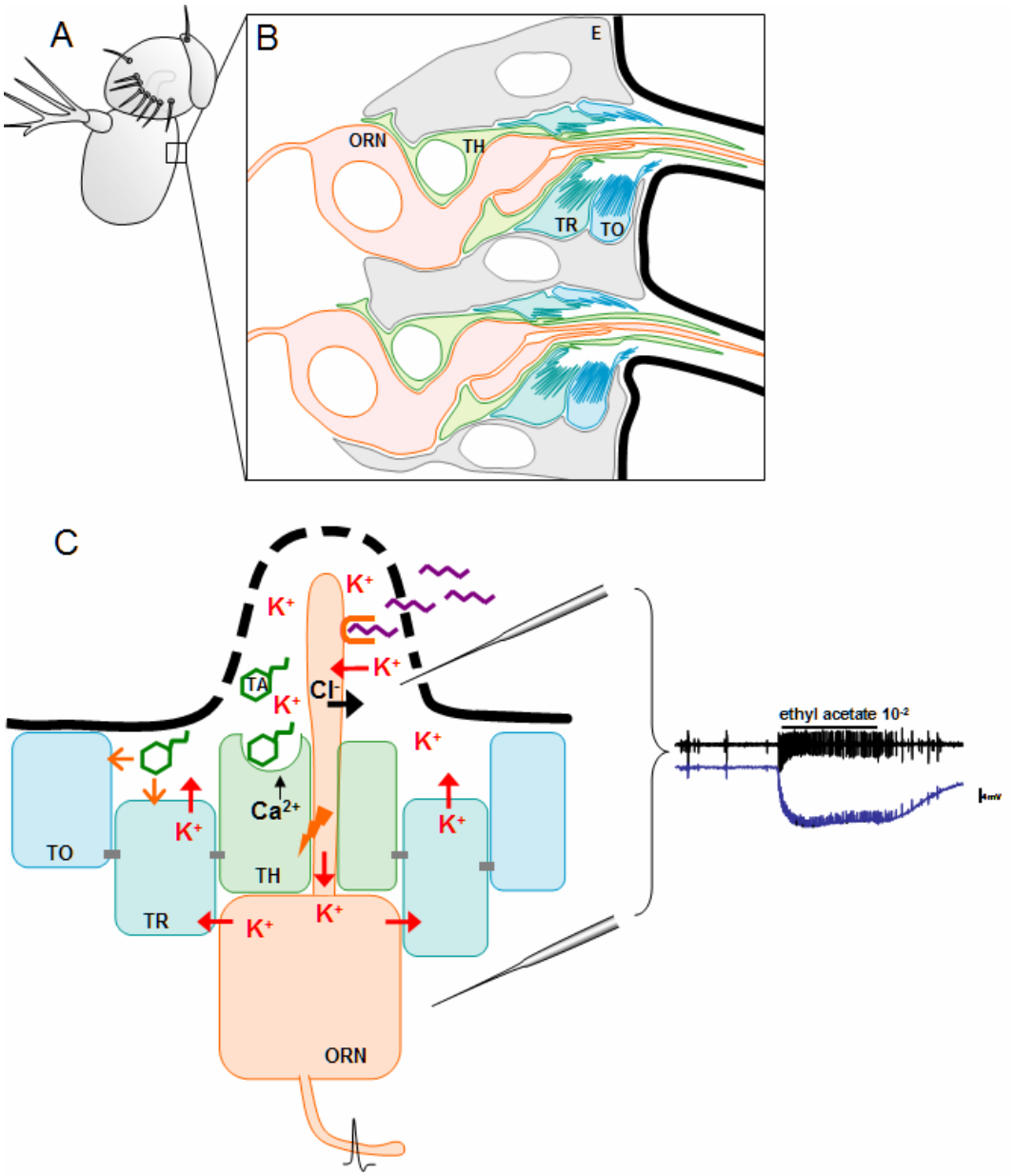

Figure 1: What happens in a sensillum during odor stimulation?

A) schematic drawing of the Drosophila antenna. B) organization of the olfactory epithelium in the 3rd antennal segment with two neighbouring sensilla. E epidermis cell, TH thecogen cell, TO tormogen cell, TR trichogen cell. Colors indicate the cell types in C). Schematic drawing of sensillar organization. An odor binds to an olfactory receptor and leads to depolarization in the dendrite. Positive ions are pumped back continuously into the sensillum lymph by TO and TR. In SSR we record the change in sensillum potential against the hemolymph. The traces in the left show the ab2A cell responding to a stimulus of 500ms ethyl acetate. The accessory cells are tightly connected by septate junctions indicated by small grey bars. My results suggest that the TH cell detects the depolarization leading to calcium concentration increase and release of tyramine that putatively acts on TO and TR.

Biogenic amine receptor expression in the antenna

We found receptors for four biogenic amines in the antenna. As the further localization by in situ hybridization did not work, we can not say in which cells in the antenna they are expressed. However Kutsukake *et al*. (2000) have shown by X-gal staining that the tyramine receptor is expressed in some cells at the base of the 3rd antennal segment that bears only olfactory sensilla on its surface, although some hygro- and thermoreceptors are located on the arista in the sacculus respectively (Shanbhag *et al*., 1999). Kutsukake *et al*. show only one picture where it is not possible to identify the cell type or even the sensillum type. Also the distribution on the antenna is only vaguely visible. But it still suggests that indeed the tyramine receptor is located on the 3rd antennal segment and the distribution could possibly match that of ab1 or ab2 sensilla, although these are more cells than shown by Kutsukake *et al*. But this matter is more complex because the X-gal staining was done with the *hono* mutant, which could have fewer cells expressing the tyramine receptor (TyrR) than wildtype flies. It was shown by Northern Blot analysis that the *hono* mutant expresses less TyrR in head and body (Kutsukake *et al*., 2000). From this analysis we can not decide, whether the expression of TyrR is less in all cells or if some cells express no TyrR at all and some still do so in normal concentrations. In the second case we would also see a reduction of TyrR mRNA in the Northern Blot but not all the TyrR expressing cells in the X-gal staining compared to wildtype flies. It is therefore possible that there are more TyrR expressing cells present in the antenna than shown by a staining of *hono* mutants. This is even more plausible taking into account that the *hono* mutation was created by insertion of a P- element in the regulatory region of the *TyrR* gene (Kutsukake *et al*., 2000). Usually the regulatory region of a gene consists of a promoter and many enhancers, each driving expression in different cell types or developmental stages. The insertion in the *hono* mutant could affect only one or some of these enhancers. It is therefore possible that TyrR expression is normal in some sensilla whereas it is impaired in others. We will have to investigate that matter further.

The *Drosophila* tyramine receptor was described independently by two groups as octopamine receptor and as tyramine receptor respectively (Arakawa *et al*., 1990;Saudou *et al*., 1990b). Later on several homologue tyramine receptors from other species were cloned (Gerhardt et al., 1997b;Blenau et al., 2000;Rex and Komuniecki, 2002;Ohta et al., 2003;Miller et al., 2005). To date a discussion is going on about the nature of these receptors. They all have in common that they cross-react in varying degrees with octopamine. For the *Drosophila* tyramine receptor two studies show that octopamine and tyramine are equally well agonists (Reale *et al*., 1997; Robb *et al*.; 1994) if it is expressed in ovary cells. A recent study describes a new family of tyramine receptors that are fully specific for tyramine and not related to the group of tyramine/octopamine receptors mentioned above (Cazzamali *et al*., 2005). Two other members of this group could be the putative octopamine receptors expressed in the antenna of two moth species at the base of olfactory sensilla (von Nickisch-Rosenegk *et al*., 1996). These receptors show great similarity in amino acid sequence with the *Drosophila* tyramine receptor TyrR and the *Locusta* tyramine receptors (Roeder, 2002). Interestingly also the effectuation of these four receptors is similar. They all are coupled to a reduction of cAMP levels (Roeder, 2002). Therefore it has to be re-evaluated if these receptors actually form a group of tyramine receptors or a group of tyramine/octopamine receptors specifically expressed in peripheral organs. Nevertheless these findings together with our results strongly suggest that tyramine receptors play a role in olfactory sensilla of *Drosophila* and other insects such as moths and locusts.

The *Drosophila* octopamine receptor OAMB shares sequence similarities with octopamine receptors of two other locust species (Roeder, 2002). These three and also a honeybee octopamine receptor are found in high concentrations in the mushroom bodies and the optical lobes. Our own results show that the octopamine receptor OAMB is expressed in the antenna, head and body. In various moth species injection of octopamine changes the activity of pheromone receptor cells (Pophof,

2000;Pophof, 2002;Dolzer et al., 2001b;Grosmaitre et al., 2001b). However, these effects are rather due to activation of the octopamine and/or tyramine receptors described above.

The dopamine receptor DAMB (=DopR2) was detected in the *Drosophila* antenna and/or leg. In a sample with antennae and legs Feng *et al*. (1996) found the DAMB mRNA by Northern blotting. From these data it is not possible to decide if the DAMB mRNA is present in antenna and leg, only leg or only antenna. The authors do also not distinguish between males and females and the two different mRNAs that are transcribed from the DAMB gene. From our results we conclude that one mRNA indeed is expressed in the antenna whereas the other mRNA is expressed in the antenna only of males.

Serotonin has physiological effects on most peripheral organs studied so far, for instance on photoreceptor cells (Hevers and Hardie, 1995). However it has not been shown yet if any serotonin receptors are expressed in the peripheral olfactory system. We demonstrate that at least one serotonin receptor is expressed in the *Drosophila* antenna.

Electrophysiological changes due to tyramine receptor mutation

Our EAG results show that a loss of tyramine receptors changes the response to ethyl acetate in the antenna. If we look a bit closer by single sensillum recordings we observe that this change is restricted to an elevated sensillum potential (SP) and does not show in the spike frequency. The elevated sensillum potential is specific for ab2 sensilla and is seen for both ORNs located in the sensillum that respond to different odors (henceforth called "*hono*-effect"). There could be two explanations for the change in odor responses in *hono* mutants.

Firstly the cuticle could be thicker or thinner or have a different composition in the mutant. This would change its resistance and therefore show differences in EAGs.

But then we would not expect the effect to be specific for one odor and we should not see any change in single unit recordings.

Secondly the properties of the epithelium could be changed and therefore the transepithelial potential is altered. The SP we measure in SSR shows a change in TEP over the olfactory epithelium (Stengl et al., 1992;Kaissling, 1995). The spikes we measure visualize a change in the transmembrane potential of the ORN (Kaissling, 1995). The receptor potential contributes to the TEP as spikes contribute to the SP. It has been shown that the SP can be predicted from the spike frequency (Haehnel, 2005). Obviously in the tyramine receptor mutant *hono* the coupling of SP and spike frequency is impaired and therefore the coupling of TEP and receptor potential.

Assuming a permanently altered TEP in the mutant, we should be able to measure this under stimulus free conditions as well as for all concentrations of odors that elicit a response within this sensillum. We did not systematically analyze if the TEPs of non-stimulated *hono* flies differ from control flies, but preliminary experiments show that there is no difference (Syed and de Bruyne, unpublished). Additionally only the lower concentrations of odors resulted in elevated SPs in *hono* flies whereas the highest concentrations did not. Thus the "*hono* effect" is only visible during odor stimulation with low odor concentrations or in other words when the ORN is below saturation. It is a change in the dynamic properties of the sensillum.

The TEP is maintained by ion pumps in the elaborately folded apical membranes of the tormogen and trichogen cells that create a high extracellular potassium concentration in the sensillum lymph (Thurm and Küppers, 1980;Klein and Zimmermann, 1991). The sensillum lymph also has a higher concentration of sodium and lower concentration of chloride compared to hemolymph (Kaissling, 1986). It is thought that during odor stimulation an inward current of potassium and outward current of chloride depolarize the dendrite in the sensillum lymph (Shanbhag *et al*., 1999). The TEP in moths shifts during odor stimulation from +30 mV to ~0 mV (Kaissling, 1986). As the change in TEP (the SP) is bigger in *hono* mutants we can assume that we have a higher chloride outward current. This correlates nicely with

the findings of Blumenthal (2003), Beyenbach *et al*. (2000) and Zack (Zack, 1979) that tyramine modulates transepithelial chloride conductance in the malpighian tubules of *Drosophila* and other species and moth pheromone sensilla respectively. A higher chloride conductance could be caused by a stronger outward chloride current. But this should affect the spike frequency, which we do not observe. There is another possibility: The potassium that flows into the dendrite is the pumped back into the sensillum lymph via the accessory cells. A higher sensillum potential could therefore also be caused by less pumping of potassium from the trichogen and tormogen cells. In this case the spike frequency would not change but the dynamic of the SP would be altered, if the ion pumps simply work less. There is one possible explanation: The constantly working ion pumps must be upregulated during odor stimulation and this takes longer in the *hono* mutants. This could result initially in a higher SP with a later on normal decline.

If indeed tyramine modulates chloride conductance in the olfactory sensilla and this mechanism is impaired in the *hono* mutant, to questions arise:

Where is the source of the tyramine?

When and for which purpose is the chloride conductance modulated?

We will answer the first question in the next paragraph, and introduce two hypothetic models to answer the second.

Thecogen cells are able to synthesize tyramine

The thecogen cell is one of three accessory cells that ensheath the ORNs in a sensillum and that isolate the sensillum lymph from the hemolymph in the antenna. The accessory cells play an important role in the formation of the sensillum during development and later on in the composition of the sensillum lymph as they constantly pump potassium ions into the lymph and produce the odor binding proteins (Pikielny *et al*., 1994) (Steinbrecht, 1999). The thecogen cell is the closest to the ORN and derives from the same precursor cell (Keil, 1992). We show that thecogen cells express Tdc1 and are therefore putatively able to produce tyramine

and secrete it into the sensillum lymph or into the hemolymph. However this is restricted to mainly basiconic sensilla that represent more than half of antennal olfactory sensilla and some of the trichoid sensilla. These two sensillum classes, basiconic and trichoid s., are both classified as multiporous single-walled wall pored sensilla. The other sensillum type on the 3rd antennal segment, coeloconic s., belongs to the multiporous double-walled wall pored sensilla, a structurally different sensillum type. Single- and double-walled sensilla display a different internal organization. In double-walled sensilla (coeloconic) the thecogen cell has many features of a secretory cell, whereas in single-walled sensilla (basiconic and trichoid) it is reduced to a thin glia-like envelope around the sensory cell (Steinbrecht, 1999). This suggests that the thecogen cell has a different function in coeloconic sensilla than in basiconic and trichoid sensilla. Therefore it is possible that only the latter express Tdc1.

The thecogen cells of a subset of single-walled sensilla may be the source of tyramine that modulates chloride currents in the sensillum that is impaired in the *hono* mutant. As we showed that the "*hono* effect" is only occurring during odor stimulation we must assume that the thecogen cells are able to detect odor stimulation and possibly respond to it.

Thecogen cells respond to odors

Our preliminary calcium imaging data suggest that the thecogen cells detect odors and respond by calcium concentration increase. Calcium is a common regulator for exocytosis and it has been shown that tyramine is acting in a calcium dependent manner in the malpighian tubule (Blumenthal, 2003). Therefore it is likely that the thecogen cell secretes tyramine into the sensillum lymph upon odor stimulation.

How do thecogen cells detect odor stimulation? Detection could take place indirectly via the ORNs. An indirect mechanism could be realized with gap junctions, but there are no gap junctions connecting the ORNs and the thecogen cells (Steinbrecht,

1999;Shanbhag et al., 1999). Nevertheless it is possible that the depolarization of the ORN is transferred to the thecogen cell, for instance passively by induction.

Our data show responses only to one or two odors. Following stimulations with different or the same odor do not elicit a calcium increase. Therefore one could conclude that the thecogen cell signals only when an unknown odor or rather an odor that is not present in the background. But then the thecogen cell would have to know not only which odors are present in the background but also that a different odor elicited the depolarization the ORN. Another possibility is that the odor concentrations used in the imaging were too high. We know from electrophysiology that the tested concentration elicit maximum spike frequencies. Also the "*hono* effect" is not visible with the highest concentration. Possibly the thecogen cell is overstrung by these high concentrations and is not able to recover or does so very slowly. A similar effect was observed in transfected HEK cells stimulated with high doses of octopamine (Grohmann *et al*., 2003). In this case our model may be a way to detect very high odor concentrations and transduct the information to other sensilla in order to desensitize them. But the fact that we can only measure one or two odors in the calcium imaging could also be due to photo damage. This could mean that thecogen cells respond to every odor concentration for instance under certain conditions or for certain odors.

Having identified the source of tyramine we can come back to the assumption that tyramine modulates transepithelial chloride conductance in *Drosophila* olfactory sensilla. We do not know which cells possess the tyramine receptor to receive the input from the thecogen cells. However if our hypothesis is correct it would be very likely to find the tyramine receptor in the trichogen and tormogen cells, because they are actually responsible for the maintenance of the TEP. We developed two different models that could explain our results and suggest what the role of the ominous tyramine signal in the antenna could be.

Model 1: Plastic modulation of electrical properties of sensilla

Putatively tyramine modulates electrical properties of olfactory sensilla under certain conditions.

A possible mechanism could be that the sensitivity of ORNs is reduced during rest in order to save energy. The supply of metabolic energy constrains the function of organs including the CNS and sensory organs (Laughlin, 2001). The brain and sensory organs have a very high metabolic rate due to neural activity. For instance the human brain takes 20% of the resting metabolic rate in adults (Laughlin, 2001). Most of the neuronal energy consumption is associated with signalling and the signalling cost is dominated by action potential propagation and postsynaptic current (Laughlin and Attwell, 2000). The signalling cost of recycling second messengers and neurotransmitters is less than 10% (for review see Laughlin, 2001). Therefore a mechanism to reduce the cost of the olfactory system could underlie our observations.

Another possibility could be adaptation to long term stimulation with high odor concentrations. It has been described that the size of glomeruli decreases due to synapse loss as result of long term odor incubation (Devaud *et al*., 2001).

Some evidence comes from our observation that the "*hono* effect" is slightly different between EAG experiments. In one experiment responses to ethyl acetate and paraffin oil were elevated, in another one responses to ethyl acetate and methyl salicylate. We have additional datasets were only the response to ethyl acetate is elevated. The mutation in the TyrR gene could make the flies more susceptible to changes in their odor environment. As the two experiments were performed at different time points, it is likely that some culture conditions have changed, e.g. a different provider of food contents or even a new incubator, that influence the odorant space the flies experience throughout their life. As both alleles show the same effect for ethyl acetate, it is possible that the circumstance that influences this response is always present in the fly culture. This would suggest that the tyramine signal in wildtype flies keeps odor responses stable although the odor environment is changing.

Model 2: An active mechanism of intersensillum insulation

The sensilla on the antenna lie very close together with only one or sometimes no epidermis cell in between (Shanbhag *et al*., 2000). It is therefore possible that a sensillum induces a current in its neighbouring sensillum. The sensillum potential would leak to the sensilla in its vicinity. A leakage like this was described for *Drosophila* big basiconic sensilla (Haehnel, 2005). For instance ab1 and ab2 sensilla display almost the same SP values in response to ethyl acetate but the spike frequency is much higher in ab1 sensilla. However, it does not hold true that ab2 sensilla generally display higher SP values in response to all odors than ab1 sensilla. A leakage was also shown for other odors and for ab3 sensilla.

In our hypothesis the thecogen cell signals during odor stimulation via tyramine that the trichogen and tormogen cells pump more ions into the sensillum lymph in order not only to re-establish sensillum lymph composition but also to prevent such leakage. However this seems not to be functioning perfectly as the leakage is observed in wildtype flies (Haehnel, 2005). If the tyramine signalling is impaired, like it is the case in the *hono* mutant, we would expect more leakage. The "*hono* effect" was observed in ab2 sensilla while stimulated with ethyl acetate. But the ORN that responds best to ethyl acetate is in ab1 sensilla. The ORN in ab2 sensilla responds less strongly (de Bruyne et al., 2001a) and in the *hono* mutant only the SP is elevated not the spike frequency. Ab1 and ab2 sensilla are tightly packed in the same region of the antenna (de Bruyne et al., 2001a). It could be that the sensillum potential from the ab1 sensillum is leaking into the ab2 sensillum and more in the *hono* mutant.

Tyraminergic or octopaminergic processes innervate the antenna

In our models we did not include the finding of a central nervous innervation of the 3rd antennal segment. We discuss this observation separately because it might be functionally separated and does not affect odor responses under experimental conditions.

We could show that the 3rd antennal segment and the arista are innervated by neurons or a neuron that expresses Tdc2 (in the following called Tdc2 neuron) and is therefore tyraminergic or octopaminergic. The soma of the Tdc2 neuron is not located in the antenna and the processes follow the axons of the ORNs into the antennal lobe. Genetic ablation of the Tdc2 neuron did not change odor response profiles in EAGs. Therefore the Tdc2 neuron can not be the source of tyramine that acts at the tyramine receptor in the antenna and is therefore more likely to be octopaminergic. Yet we must assume that the processes are innervating some cells in the antenna. We could not detect an innervatation of ORNs, but it is nevertheless possible that it is present. Does the Tdc2 neuron give input to the ORNs? Meola *et al*. ((Meola et al., 1998;Meola and Sittertz-Bhatkar, 2002)) showed that neuroendocrine neurons in the antennae of *A. aegypti* and of *C. salinarius* form synapses with the dendrites of ORNs. But these neurons are located in the antenna which is not the case for the Tdc2 neuron. Furthermore they secrete tachykinin-like peptide and not a biogenic amine. U. Schroeter (Schröter, 2002) found in the honeybee brain two putatively octopaminergic VUM (ventral unpaired median) neurons that innervate the antennal nerve. These are the VUMmx3 (maxillary 3) and VUMmd3 (mandibular 3) neurons. Both did not respond to stimulation by odors or sucrose or to mechanical stimuli applied to the bee in this preparation. This corresponds to our EAG results with Tdc2-rpr flies. Ablation of the Tdc2 neuron did not alter odor responses.

Interestingly the VUMmd3 showed a constant firing frequency of 4Hz, which could be of functional significance.
The Tdc2 neuron is a central neuron that could modulate ORNs.

Outlook

Many questions remain unanswered. Which cells express the tyramine receptor? Can we induce a change in sensillum potential in *hono* mutants in other sensilla by changing conditions? When does a leakage of sensillum potential occur and is it stronger in *hono* mutants? What happens if we inject tyramine into sensilla?
We will further analyze the role of tyramine in the antenna. Some experiments are suggested in the following paragraph.

Localization of the tyramine receptor

The first and most important step is to find out where the tyramine receptor is located and therefore which cells receive a signal from the thecogen cell. We could not find any antibodies against this receptor and it would take to much time to generate them ourselves. Therefore it is necessary to do *in situ* hybridizations. This would best be done in collaboration with group that already does successful *in situ* hybridizations.

Functional analysis

It is relatively easy to test the hypothesis of a leakage between sensilla described as Model 2. Single sensillum recordings of sensilla stimulated with odors they do not respond to should show the phenomenon. It can be analysed if this happens between all the sensillum classes and for all odors. Ablation of one ORN should show that leakage for particular odors is reduced. For this we could use the Gr21a-Gal4 line that is expressed by the ab1C cell. This cell responds exclusively to CO2 and is the only cell to do so. Ablation of this cell should result in missing leakage in response to CO2.

It is also necessary to describe the "*hono* effect" further. We will test if a change in odor environment establishes the "*hono* effect" in other sensillum types than ab2.
We could also try to mimic the "*hono* effect" pharmacologically. It is possible to add tyramine or a tyramine antagonist to the saline in the recording electrode during measurements that is then in contact with the sensillum lymph.
We will also analyze the odor response of thecogen cells to odors better. We could do calcium imaging with much lower odor concentration and different odors in changing order and proper controls. To find prove model 2 we will do electrophysiology with flies reared under different conditions, after long term exposure to odors or with altered circadian rhythm.…