Results

Several biogenic amine receptors are expressed in the *Drosophila* antenna

In order to test the hypothesis that biogenic amines (BAs) modulate the periphery of the olfactory system of *Drosophila* we wanted to find the candidate BAs. Therefore we checked the expression of specific amine receptors in the antenna. In *Drosophila melanogaster* 21 biogenic amine receptors are known through sequence analysis. We created mRNA specific PCR primers and performed RT-PCR for the eleven ones that are also pharmacologically analyzed. The other 10 are only annotated by their sequence similarities to other biogenic amine receptors and no ligands are known. Therefore they were not of much use for our study. We collected separately antennae, heads without antennae and bodies from male and female flies. The results are summarized in table 1.

We found ubiquitary expression of a DA receptor (Feng *et al*., 1996), one OA receptor (Han *et al*., 1998) and one 5-HT receptor (Gerhardt and van Heerikhuizen, 1997) and of the TA receptor (TyrR). The receptor 5-HT1A can also be considered as ubiquitary expressed, although one result remains unclear (two of four experiments showed positive results and two showed negative results). This could just mean that the concentration is close to detection threshold.

Some receptors show organ specific expression. The receptor 5-HT7 was found in head and body but not in the antenna. The DA receptor DopR2 can be transcribed into two different mRNAs, so we designed specific primers for both mRNAs. We found that one DopR2 mRNA is present in head and antenna and not in the body of both sexes, whereas the other one is expressed in heads of both sexes but only in the body and antenna of males. If we assume that the receptor is expressed in the brain and in non-neuronal cells in the body and periphery, there could be a sexual

dimorphism concerning only expression of this dopamine receptor in non-neuronal cells. This is of course only one possible explanation.

The olfactory receptor Or47a was used as positive control and is known to be exclusively expressed in a subset of ORNs in the antenna (Vosshall *et al*., 2000). We could clearly show expression of Or47a only in the antenna, assuring the sensitivity and reliability of the method. Expression of two other biogenic amine receptors was tested (Ocr-1 for OA and D2R for DA) but we could not detect expression in any tissue and assume that these PCRs did not work.

These results show that all four biogenic amines are candidate modulators in the peripheral olfactory system.

Table1: Expression of biogenic amine receptors in Drosophila Canton-S wildtype.

Ligand	**Receptor**	**Head** ♂	**Head** ♀	**Body** ♂	**Body** ♀	**Antenna** ♂	**Antenna** ♀
Dopamine	DopR	+	+	+	+	+	+
	DopR2_1	+	+	?	-	+	-
	DopR2_2	+	+	-	-	+	+
Octopamine	Oamb	+	+	+	+	+	+
Serotonin	5-HT1A	+	+	+	+	+	?
	5-HT1B	-	+	+	?	?	-
	5-HT2	+	+	+	+	+	+
	5-HT7	+	+	+	+	-	-
Tyramine	TyrR	+	+	+	+	+	+
Odors	Or47a	-	-	-	-	+	+

All RT-PCR experiments were performed three to four times. + expression detected, - no expression detected, ? unclear because in two of four experiments expression was detected and in two experiments not.

Octopamine deficiency has no effect on odor response profiles in EAGs

From the expression analysis we did not get just one candidate BA, so they could all be modulating the peripheral olfactory system of *Drosophila*. But we know that OA is modulating properties of pheromone receptor neurons in some moth species (Pophof, 2000;Grosmaitre et al., 2001a;Dolzer et al., 2001a). Therefore we tested the only available *Drosophila* mutant concerning OA for changes in its odor response profile as represented by electroantennograms (EAGs). The mutant exhibits a completely impaired biosynthesis of OA due to a mutation in the tyramine-β-hydroxylase gene (Monastirioti *et al*., 1996). We see no significant effect of this mutation in the odor responses in EAGs compared to the control flies (Fig. 1) but there is a tendency that $T\beta h^{M18}$ shows lower responses to all the tested odors but CO_2. However loss of OA activity is not significantly influencing odor responses on the level of the receptor neurons in the antenna. As the reduction seems to be rather general it is possible to be an effect that influences the odor responses indirectly, e.g. change in cuticle resistance.

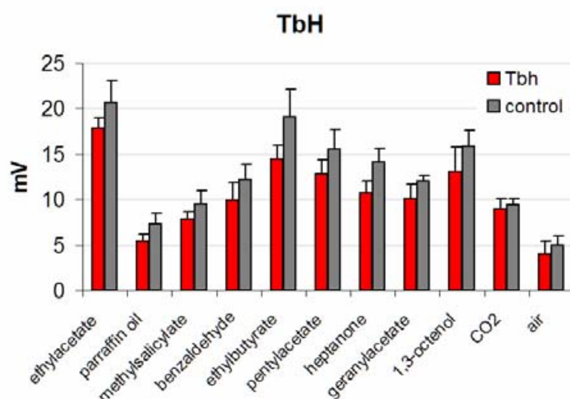

Figure 1: EAG odor response profiles of Tbh^{M18} ***flies measured by EAG.***
Mean amplitudes of 10 animals during 1 second of odor stimulation are shown. Error bars are SEM. Student t test, $p < 0.05$*. The mutation Tbh shows no effect in the EAG. Controls are heterozygous siblings Tbh/FM7c.*

Higher dopamine concentrations do not change odor responses

Another putative modulator of the olfactory periphery is DA. It was shown that a DA receptor is expressed in the olfactory epithelium of fish (Vacher *et al*., 2003), and that DA inhibits currents in rat olfactory receptor cells in vitro (Okada *et al*., 2003). Hence we used the mutant *Dat* to check how DA affects odor responses in EAGs. The *Dat* mutants are deficient for dopamine-N-acetyltransferase, that is involved in DA catabolism and therefore show higher levels of DA concentration. Here we also see no effect in the odor response profile in EAGs (Fig. 2). A higher DA concentration does not change odor responses of ORNs.

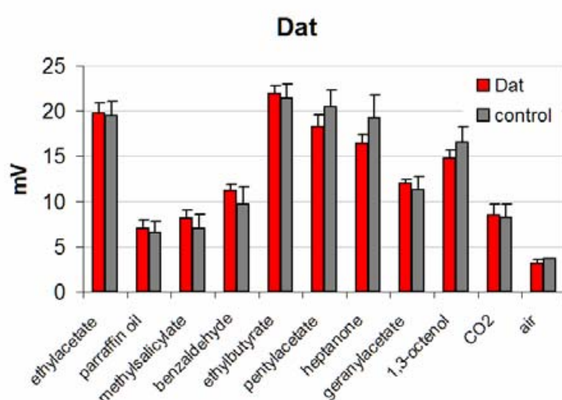

Figure 2: EAG odor response profiles of Dat flies measured by EAG.
Mean amplitudes of 10 animals during 1 second of odor stimulation are shown. Error bars are SEM. Student t test, $p < 0.05$. The mutation Dat shows no effect in the EAG. Controls are heterozygous siblings Dat/CyO.

Higher concentrations of all biogenic amines cause lower odor responses

To investigate further, we tried to find effects on odor responses for any BA and used the mutant *ebony*. The ebony protein acts as β-alanyl-biogenic amine synthetase. It plays a role in BA deactivation and recycling (Richardt *et al*., 2003). The mutation causes increased concentrations of all biogenic amines. EAG recordings revealed a tendency of lower responses to all the tested odors in *ebony* flies (Fig. 3). Significantly lower responses were found for methyl salicylate, pentyl acetate, heptanone and 1-octen-3-ol. We conclude that a higher concentration of at least one BA alters the odor

response profile in EAG. This result encouraged us to test the remaining biogenic amines for effects on odor responses.

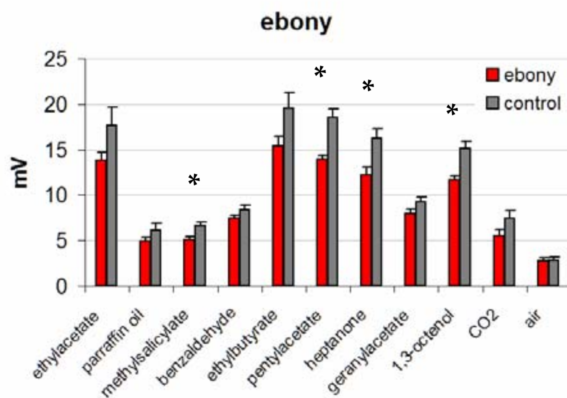

Figure 3: EAG odor response profiles of ebony flies measured by EAG.
Mean amplitudes of 10 animals during 1 second of odor stimulation are shown. Error bars are SEM. Student t test, $p < 0.05$. The mutation ebony shows no effect in the EAG. Controls are heterozygous siblings e/+.

Flies lacking the tyramine receptor show an altered odor response profile

We looked at the only existent biogenic amine receptor mutant, *TyrR*^{hono} (*hono*). *Hono* flies have a ~ 80% reduced expression of tyramine receptor due to a mutation in the regulatory region of the *TyrR* gene and display a reduced response in olfactory avoidance behavior (Kutsukake *et al.*, 2000). Another allele of the *TyrR* gene is *TyrR*^{neo30} (neo30). The homozygous neo30 flies are lethal but heterozygous *TyrR*^{hono}/*TyrR*^{neo30} (*hono/neo30*) flies are viable and show a more severe phenotype than the homozygous *hono* flies (Kutsukake *et al.*, 2000). Compared to control flies (*white*) *hono* flies show normal EAG responses to all tested odors but an elevated response exclusively to ethyl acetate and the solvent paraffin oil (Fig. 4). The *hono/neo30* flies show a stronger increase in the ethyl acetate response. *Hono/neo30* flies do not show an elevated response to paraffin oil but instead to methyl salicylate. This can be due to variety among the flies with different genetic background. It is possible that a combination of the *white* marker in the genetic background of the *hono* flies and the mutation in *TyrR* causes changes in EAG responses different from the ones in the heterozygous *hono/neo30* flies, because the *neo30* flies have a wildtype background

without the *white* mutation. Additionally the *hono* mutants carry a P element with mini-*white* as marker gene, which could also show an effect in EAGs. However, we can be sure that the elevated ethyl acetate response is really due to the loss of tyramine receptor and not caused by an interaction between marker genes and the *hono* mutation. In this case we would expect the effect in *hono/neo30* flies to be much smaller, because *neo30* flies do not carry the marker *white* and mini-*white*. Another possibility for the differences between the two mutants is that the mutation in the TyrR gene makes 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. We should consider this possibility because tyramine could modulate ORNs during changing circumstances.

The robust and reproducible phenotype in EAGs is an elevated response to ethyl acetate. From previous studies we know that the ORNs responding to ethyl acetate are the ab1A and ab2A cells (de Bruyne et al., 2001a). The ab1A ORN also responds to the tested odor pentyl acetate that shows normal responses in the mutant *hono* EAGs. Therefore we expect the mutation to affect the ab2A neuron.

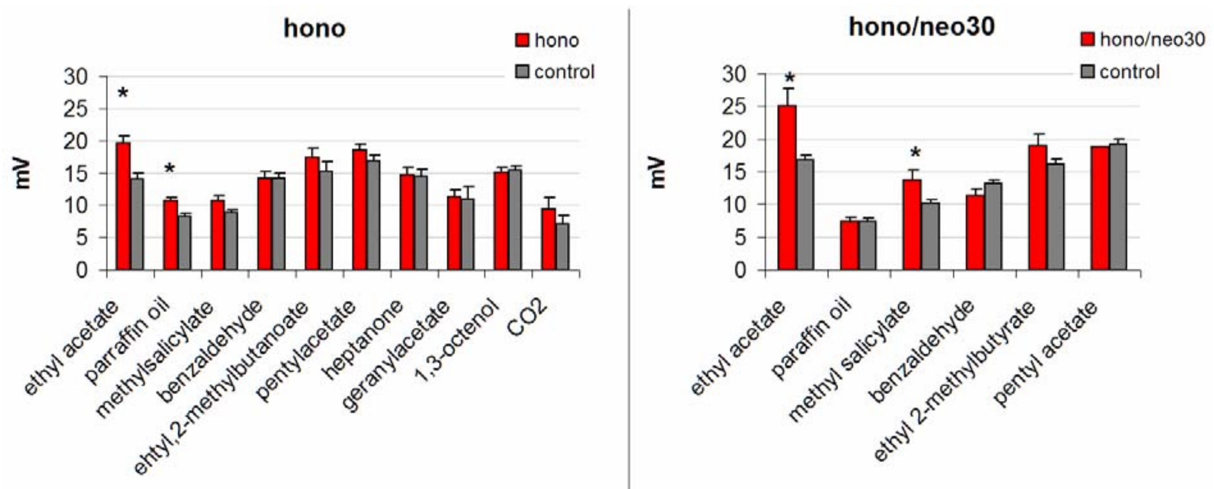

Figure 4: EAG odor response profile of hono and hono/neo30 flies.
Mean amplitudes of 7-12 animals during 1 second of odor stimulation are shown. Error bars are SEM. Student t test, $p < 0.05$. In hono mutants the responses to ethyl acetate and to paraffin oil are significantly elevated (experiment performed 07/03) and in hono/neo30 flies the responses to ethyl acteate and methyl salicylate are elevated (experiment performed 03/03). Controls= corresponding hono white control.

Sensillum potentials of ab2 sensilla are elevated in tyramine receptor mutants but spike frequencies are not changed

In order to further examine the effect of reduced tyramine receptor levels on odor responses, we recorded sensillum potentials (SPs) and spike frequencies (APs) from large basiconic sensilla (ab1, 2 and 3) by single sensillum recordings. We used the *hono/neo30* flies because they display a stronger phenotype. As suggested only ab2A neurons in *hono/neo30* flies show altered responses to ethyl acetate and paraffin oil, whereas the responses of the ab1A and ab3A cells remain normal. But unexpectedly the spike frequency remains unaltered over a range of doses tested, while only the SPs are elevated in the mutant in ab2A cells (Fig. 5). Therefore the correlation of spike frequency and SP is shifted in the mutant (Fig. 6A). Obviously in *hono/neo30* mutants the same odor concentration leads to a higher SP but the same spike frequency, or in other words, a higher SP is required to evoke the same spike frequency. However, the temporal patterns of the SP of the spike train are unaltered (Fig. 6B, C). Interestingly SPs evoked by the highest concentration of ethyl acetate do

not differ between *hono/neo30* and control flies. The SP values do not reach saturation, but it is possible that the concentration is unphysiologically high.
We observe a sensillum specific effect in tyramine mutants. Is this effect also cell and/or odor specific? Therefore we recorded from ab2 sensilla and stimulated the other ORN, the ab2B cell with the odor ethyl, 3-hydroxybutyrate. We observe similar effects as in ab2A cells (Fig.7). The sensillum potential is elevated in *hono* mutants, whereas the spike frequency is the same as in control flies. Consequently the correlation of SP and spike frequency is shifted. Again this is only observed for the lower odor concentrations. For higher concentrations SPs of *hono* flies are the same as that of control flies. As a control ab2 sensilla of the OA deficient $T\beta h^{M18}$ flies were also recorded. Here, just as in EAGs, no difference between mutants and control could be detected (Fig. 8).
From our data we conclude that tyramine plays a role in specific olfactory sensilla through the tyramine receptor TyrR. This raises two questions:

1. Which is the source of tyramine?
2. What is modulated by tyramine?

In the following experiments we tried to answer these questions.

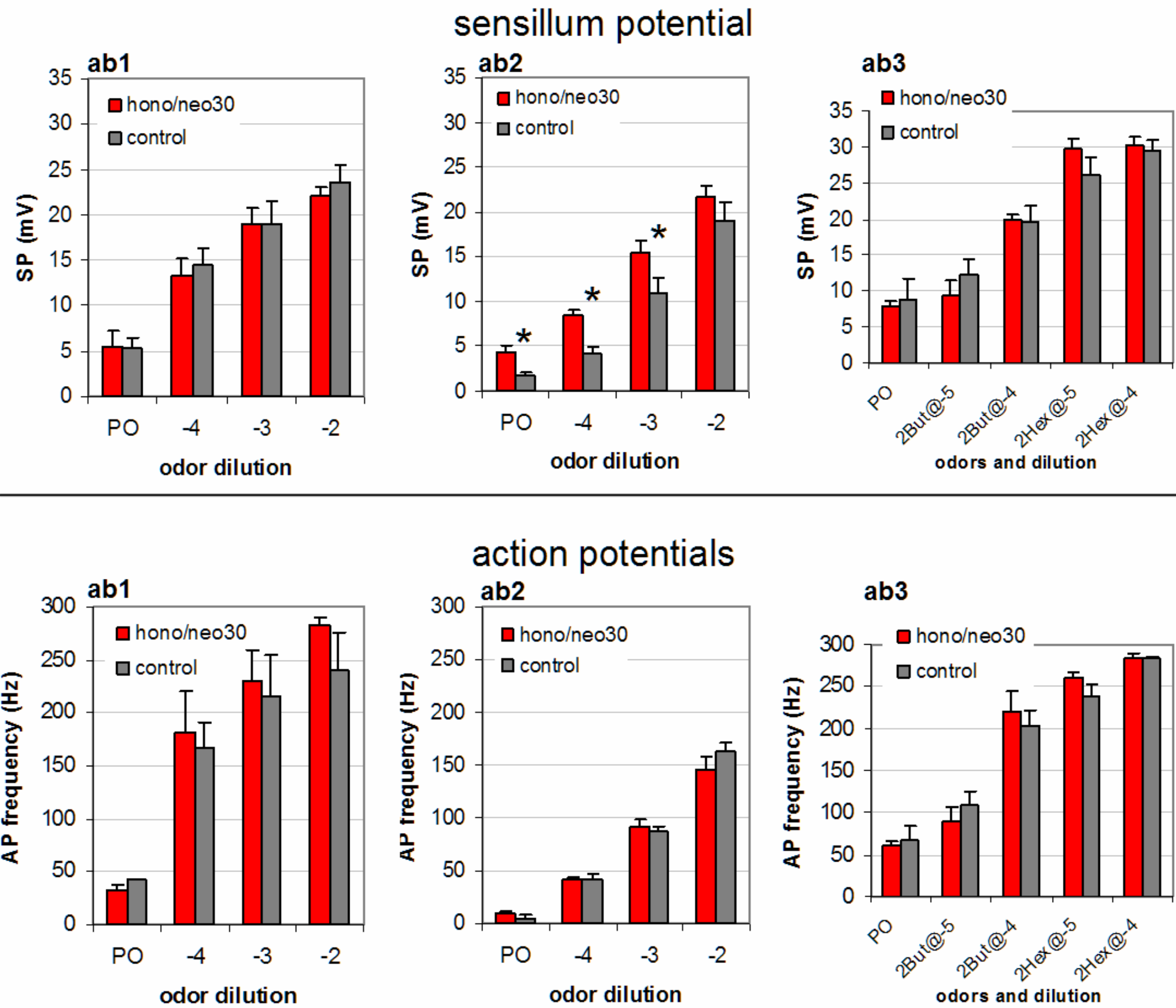

Figure 5: Extracellular recordings from large basiconic sensilla of hono/neo30 flies.
Dose response relations for spikes and corresponding sensillum potential of large basiconic sensilla stimulated during 500ms with increasing concentrations of ethyl acetate for ab1 and ab2. For ab3 sensilla two different odors were used to test the complete dynamic range of the neuron. SP in first row, AP in second row. First column ab1 sensilla, second column ab2 sensilla, third column ab3 sensilla. Only in ab2 sensilla we see an elevated SP, significant for the lower doses. Mann-Whitney, $p < 0.5$. Control = hono/TM3. All single sensillum recordings were kindly performed by Z. Syed. N=4-7.

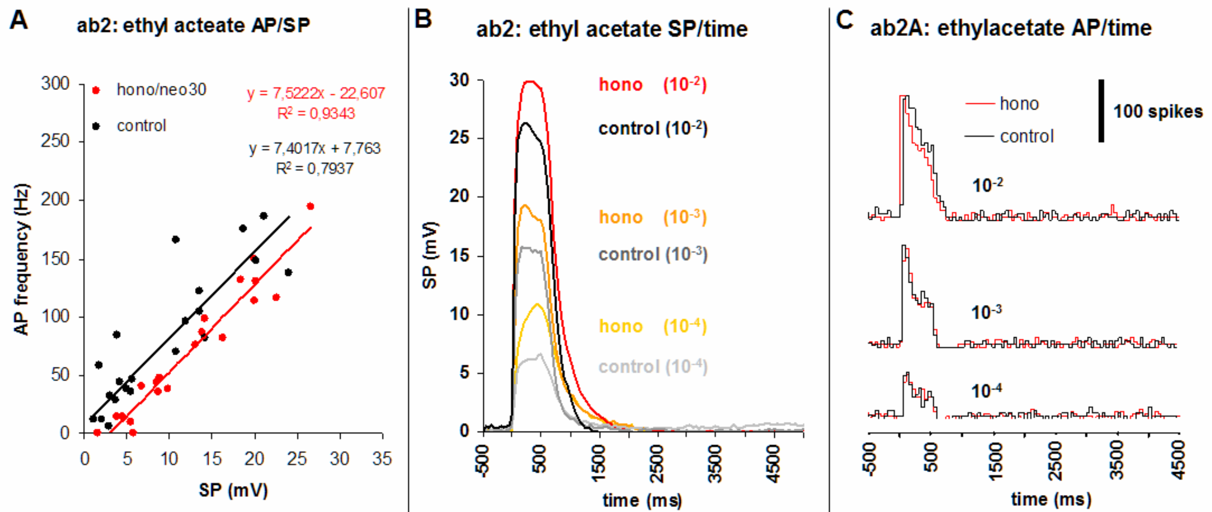

Figure 6: Correlation of APs and SP and temporal patterns of APs and SP from ab2A neurons.
A) Correlation of spike frequency and sensillum potential in hono/neo30 flies is shifted but the slope remains the same. B) Sensillum potential over time for different odor concentrations. The SP in hono/neo30 flies is always higher than the corresponding one in control flies but the temporal pattern remains unaltered. Odor onset is at 0ms. C) Temporal pattern of AP is not altered in hono/neo30 flies. Average of 5 recordings, spikes are pooled into 50 ms bins. Control=hono/TM3

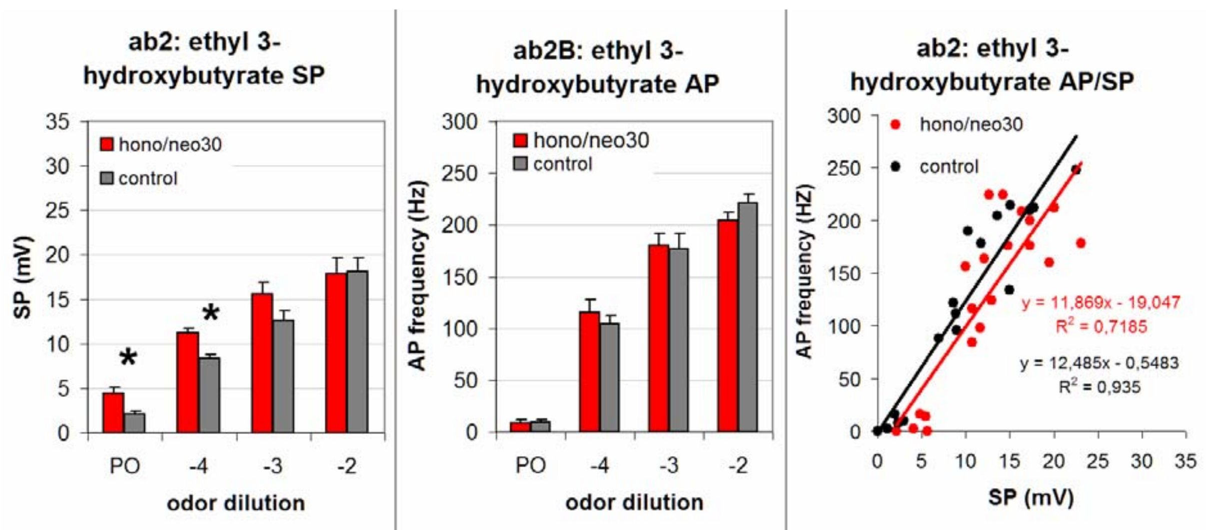

Figure 7: Extracellular recordings from ab2B ORNs of hono flies.
Dose response relations for sensillum potentials (left) and corresponding spikes of ab2 sensilla (middle) and ab2B cell respectively stimulated during 500ms with increasing concentrations of ethyl, 3-hydroxybutyrate. Correlation of spike frequency and sensillum potential (right). Control = hono/TM3. All single sensillum recordings were kindly performed by Z. Syed. N=5.

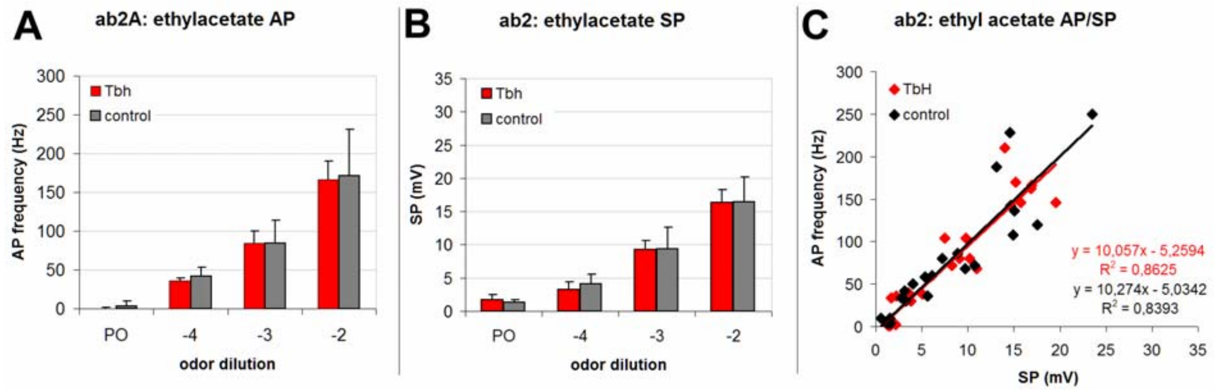

Figure 8: Extracellular recordings from ab2 sensilla of Tbh flies.
Dose response relations for spikes (A) and corresponding sensillum potential (B) of large basiconic sensilla stimulated during 500ms with increasing concentrations of ethyl acetate. C) Correlation of spike frequency and sensillum potential. Control = Tbh/FM7c. All single sensillum recordings were kindly performed by Z. Syed. N=6

Putative tyraminergic or octopaminergic neurons project into the antenna

We want to know what is the source of tyramine in the antenna. Cells that secrete tyramine should express the enzyme to synthesize tyramine from tyrosine. This enzyme is Tdc (tyrosine decarboxylase). It catalyses the synthesis of TA of the amino acid tyrosine. Because TA is the precursor of OA, Tdc is also expressed in octopaminergic cells. *Drosophila* possesses two different genes for this enzyme. One is expressed exclusively in neurons (*Tdc2*) the other one in non-neuronal cells (*Tdc1*). Some insects possess neuroendocrine neurons that project into the antenna, e.g. the VUM-mx1 in honeybees and axo-dendritic projections in mosquitoes (Hammer, 1993;Kreissl *et al.*, 1994;Meola and Sittertz-Bhatkar, 2002). Therefore we started looking for a neuronal tyramine source. With the aid of the Gal4-UAS system we expressed membrane bound green fluorescent protein (GFP) in the *Tdc2* cells and viewed the antennae under the confocal microscope.

The confocal pictures show three branches of processes that arborize in the antenna and into the arista (Fig. 9A, B). The processes end in the middle of the antenna not reaching very close to the cuticle, which is clearly seen in the depth encoded view

(Fig. 9B). In this view the optic sections are colored according to their depth in the stack as indicated by the color table. The *Tdc2* positive processes are coded in green, yellow and orange indicating the middle 10µm of 21µm shown in total. A male *Drosophila* antenna measures around 60 µm in diameter but is jolted in our preparation. We also removed 10 slices of 1µm each on top and at the bottom that showed no staining. However, it is possible that we are not able to see where the processes end, because of too low resolution. So we cannot exclude that the *Tdc2* positive neuron or neurons innervate the ORN dendrites. It seems the *Tdc2* positive processes travel along the three distinct fascicles of olfactory axons as they exit the antenna. We stained one of the three fascicles by the ORN driver line *Gr21a-Gal4* to compare the *Tdc2* localization with the position of ORNs. In Fig. 9C we show an antenna with both *Gr21a-GFP* and *Tdc2-GFP* staining. Unfortunately we cannot distinguish between the two different signals because they are both stained with GFP, but we see no additional fourth branch. So they could be overlapping. It is also obvious that the processes indicated by red arrows belong to the *Tdc2* signal as they do not end at with the Gr21a expressing cells. These processes are parallel to the axons of the Gr21a expressing cells and we do not see if they reach the region where the ORN dendrites are located. One branch extends into the arista where hygroreceptors were found (Sayeed and Benzer, 1996).

We conclude that the *Drosophila* antenna is innervated by neural processes that are *Tdc2* positive. Therefore they are able to produce tyramine and putatively also octopamine, because tyramine is its precursor. As we could not see the cell body in the 3rd antennal segment it could be located somewhere else in the antenna or in the brain. In the next step we need to find out, if these processes are indeed the source of tyramine that is related to the phenotype of *hono*.

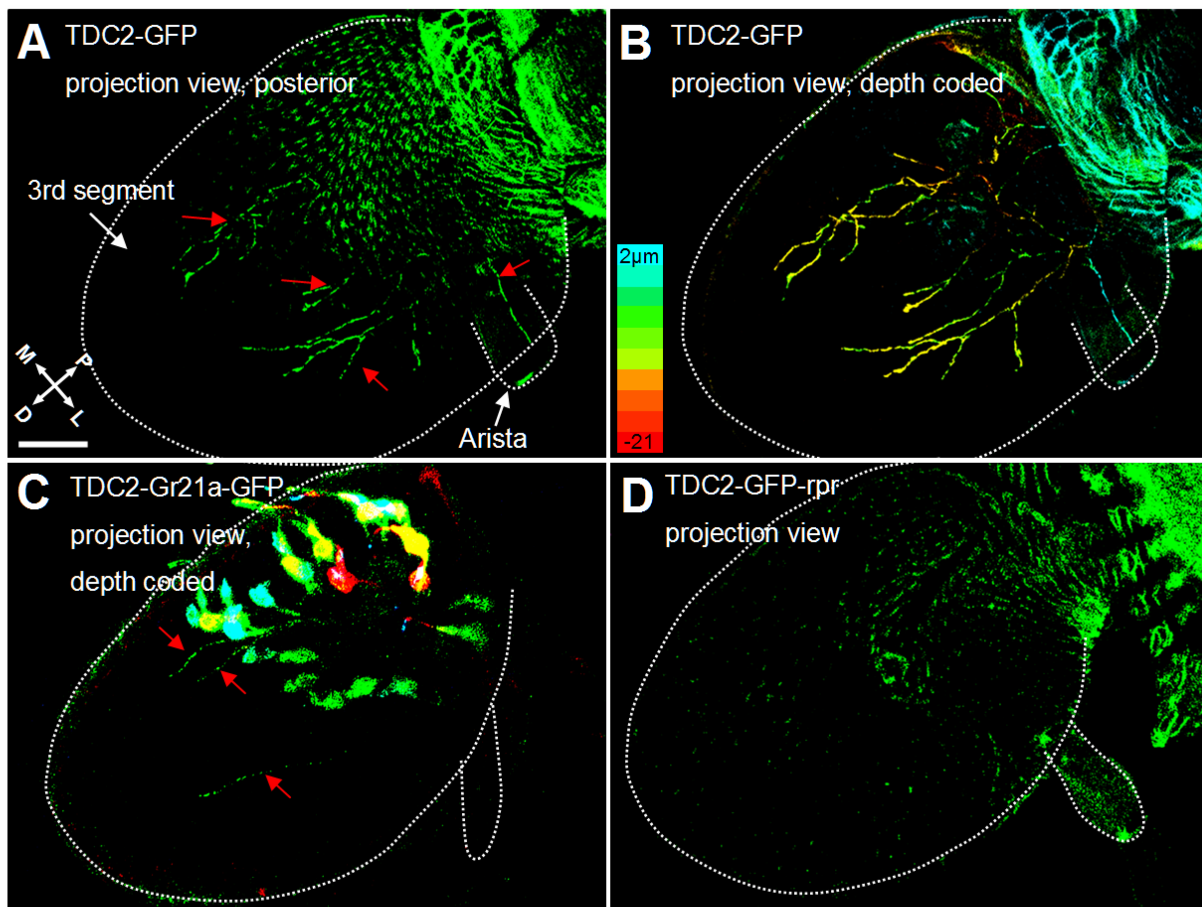

Figure 9: Neuronal Tdc2 is expressed in cells that innervate the 3rd antennal segment and the arista. *Confocal pictures of freshly removed unfixed antennae stained by membrane bound GFP expressed in Tdc2 cells. Green, emission of GFP. The dotted line indicates the outline of the 3rd antennal segment and the arista. Scale bar, 20µm. A) Projection of Tdc2 signal. Red arrows point at neuronal processes of Tdc2 expressing cells. B) Depth coded projection of the Tdc2 signal, same antenna as shown in A). C) Depth coded projection of the Tdc2 signal plus Gr21a signal. The Gr21a ORNs send their axons along the same fascicles to the antennal nerve as the Tdc2 processes. D) Tdc2 cells were deleted by expression of the apoptosis gene rpr*

Ablation of the *Tdc2* positive processes does not change odor responses

To test whether the *Tdc2* positive neural processes described above are the source of tyramine that modulates the sensillum potential, we ablated all *Tdc2* cells by expression of the apoptosis gene *reaper* (*rpr*). We showed the effectiveness of this method before by ablating CO_2 sensitive ORNs (see Chapter II). If our hypothesis is proven we would expect to see a similar effect on odor responses as in *hono* and

hono/neo30 flies. In both cases the TA signaling is impaired, in *hono* mutants the receptor is not present and in *Tdc2-rpr* flies no TA should be synthesized. It is also possible that we see much stronger or additional effects in the *Tdc2-rpr* flies as they do not just carry a mutation but actually miss a whole cell population.

First we controlled the loss of *Tdc2* positive processes under the microscope (Fig. 9D). No *Tdc2* specific staining is present. We then performed electroantennograms (EAGs) on antenna of these flies. The *Tdc2-rpr* flies show absolutely normal responses to all tested odors in comparison with the control group (Fig. 10). Obviously the *Tdc2* cells are not the source of tyramine that is related to the elevated sensillum potential in *hono* mutants. It is however possible that the *Tdc2* neuron or neurons affect/s odor responses under certain circumstances, e.g. day/night. As Tdc is also expressed in OA syntheszing neurons it is possible that this Tdc2 expressing neuron or the Tdc2 expressing neurons are actually octopaminergic.

We now have to look for another source of antennal tyramine.

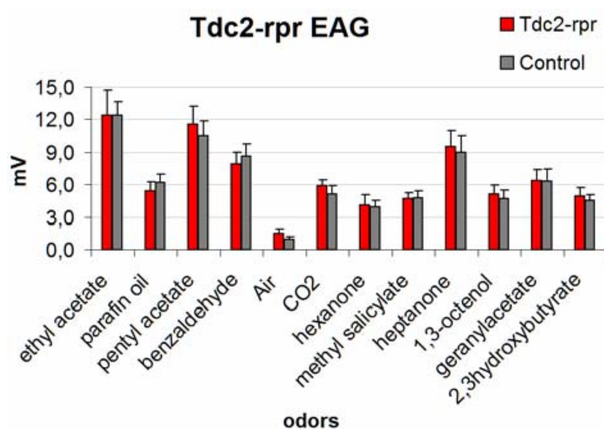

Figure 10: Electroantennograms of Tdc2-rpr flies. *Controls are siblings without rpr (Tdc2/TM3). Error bars are SEM. No differences between neuronal tyramine ablated animals and controls were found. N=8.*

Non-neuronal *Tdc1* cells are a possible source for tyramine in the antenna

We can exclude *Tdc2* neurons as source for tyramine from the result in the previous experiment. Looking for another possible source for tyramine we examined the expression of the non-neuronal *Tdc1* in the antenna. First we tested expression of *Tdc1* and *Tdc2* in the antenna by RT-PCR. Specific primers for both mRNAs were designed and antennae and heads of male and female flies were collected. The experiment reveals that *Tdc1* mRNA is present in the antenna and in the head (Fig. 11). Thus there must be non-neuronal cells in the antenna that are able to synthesize tyramine. *Tdc2* mRNA was not detected in the antenna. This means that the cell bodies of the *Tdc2* positive processes, that contain the mRNA, are not located in the antenna but rather in the brain.

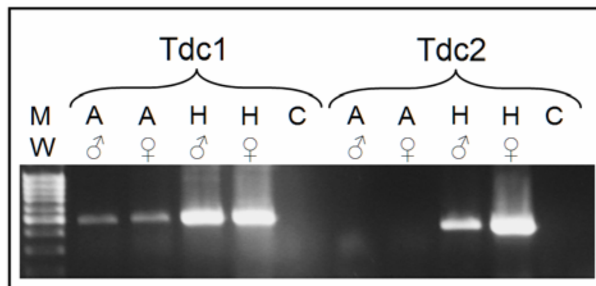

Figure 11: RT-PCR products of Tdc1 and Tdc2. *A antenna, H head, C negative control, MW molecular weight marker.*

Accessory cells of a subset of olfactory sensilla are able to synthesize tyramine

From our RT-PCR experiment we know that tdc1 is expressed in the antenna. To further examine the expression of *Tdc1* in the antenna we took again advantage of the UAS-Gal4 system. Expression of *Tdc1* driven GFP is detected in the third antennal segment, in the olfactory sensilla (Figure 12). In detail, staining does not reach the tip of the sensilla but only about half of the sensillum length. The stained cells are

located directly under the cuticucle (Fig. 12D). In contrast, ORN somata are located more proximally (Shanbhag *et al*., 2000). Optical cross sections of the *Tdc1* cells show circles with small diameter, where they are wrapped around the inner dendrite. Each sensillum contains only one stained cell, because we see only one nucleus per stained structure (Fig. 12C, F). From our detailed morphological analysis and comparisons with electron microscopic pictures and data shown in Shanbhag *et al*. (1999, 2000; see Fig. 14A) we conclude that the staining derives from one of the three accessory cells in each sensillum, the thecogen cell. This cell is wrapped around the outer dendrite and reaches into the sensillum. The cell body is located close to the cuticle whereas the cell bodies of the other two accessory cells are about halfway between cuticle and basal lamina. Our results suggest that the thecogen cells are able to produce the biogenic amine TA. As TA is the precursor of OA it is also possible that the cells produce OA.

GFP expressing cells were counted automatically with the software ImageJ. We count an average of 125 stained sensilla (Fig. 12E and 15I). Many of the basiconic sensilla (including all the subtypes small, thin, large basiconic and intermediate sensilla) show GFP expression (Fig. 13C-F). Male *Drosophila* flies, which were used in the experiment, possess around 200 basiconic and intermediate sensilla from a total number of ~380 sensilla on the surface 3rd antennal segment (Shanbhag *et al*., 1999). From the location and number of stained cells we conclude that all three of the large basiconic sensillum classes (ab1, 2, 3) and one of the small basiconic sensillum classes are *Tdc1* positive. Also 1-3 (from a total number of ~115) trichoid sensilla, consistently located close to the base of the arista, are stained (Fig. 13E). We could not find any stained coeloconic sensilla (total number ~60).

We conclude that most basiconic sensilla and a small subset of trichoid sensilla express *Tdc1* and are theoretically able to produce TA and or OA.

We ablated the *Tdc1* cells by expression of the apoptosis gene reaper (rpr) in order to test the effect on odor response profiles. Unfortunately the *Tdc1-rpr* flies are embryonic lethal just as the mutants (Cole *et al*., 2005).

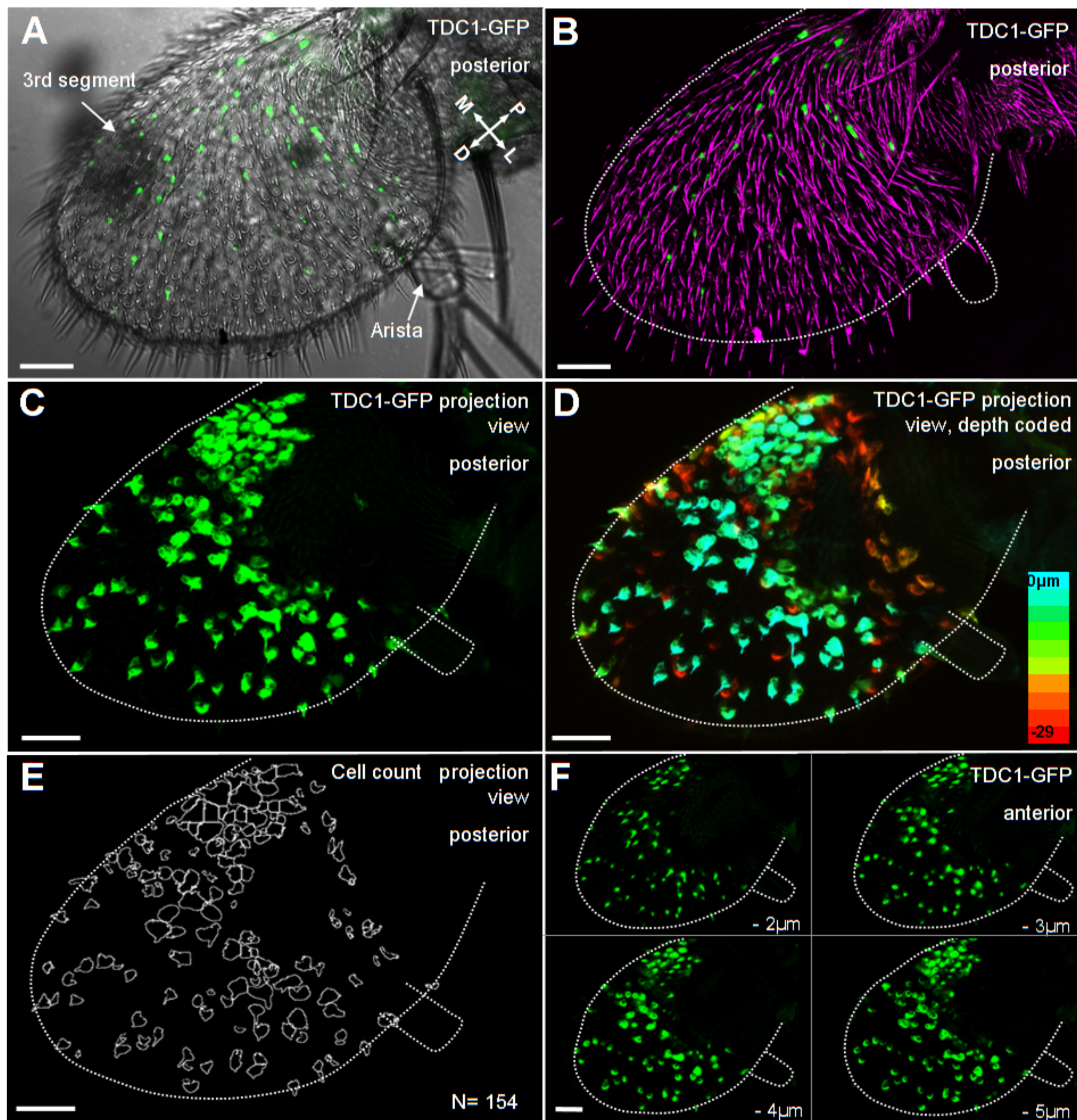

Figure 12: Expression of Tdc1 in Drosophila antenna. *Confocal pictures of freshly removed unfixed antennae stained with membrane bound GFP expressed in Tdc1 cells. Green = GFP emission, magenta = reflection of cuticle showing shape of sensilla, grey = transmission. The dotted line indicates the outline of the 3rd antennal segment and arista. Scale bars 20µm, P proximal; D distal; M medial; L lateral.*

A, B) Overview of Tdc1 signal on antennal surface.

C) Projection view of Tdc1 signal showing distribution and cellular shape.

D) Depth encoded 3D stack. Cyan (0µm) is up most, warmer colours are deeper. In this preparation the Drosophila antenna is ~ 30µm thick.

E) Drawing of automatic cell counting with ImageJ software..

F) Tdc1 signal in succeeding pictures of 3D stack.

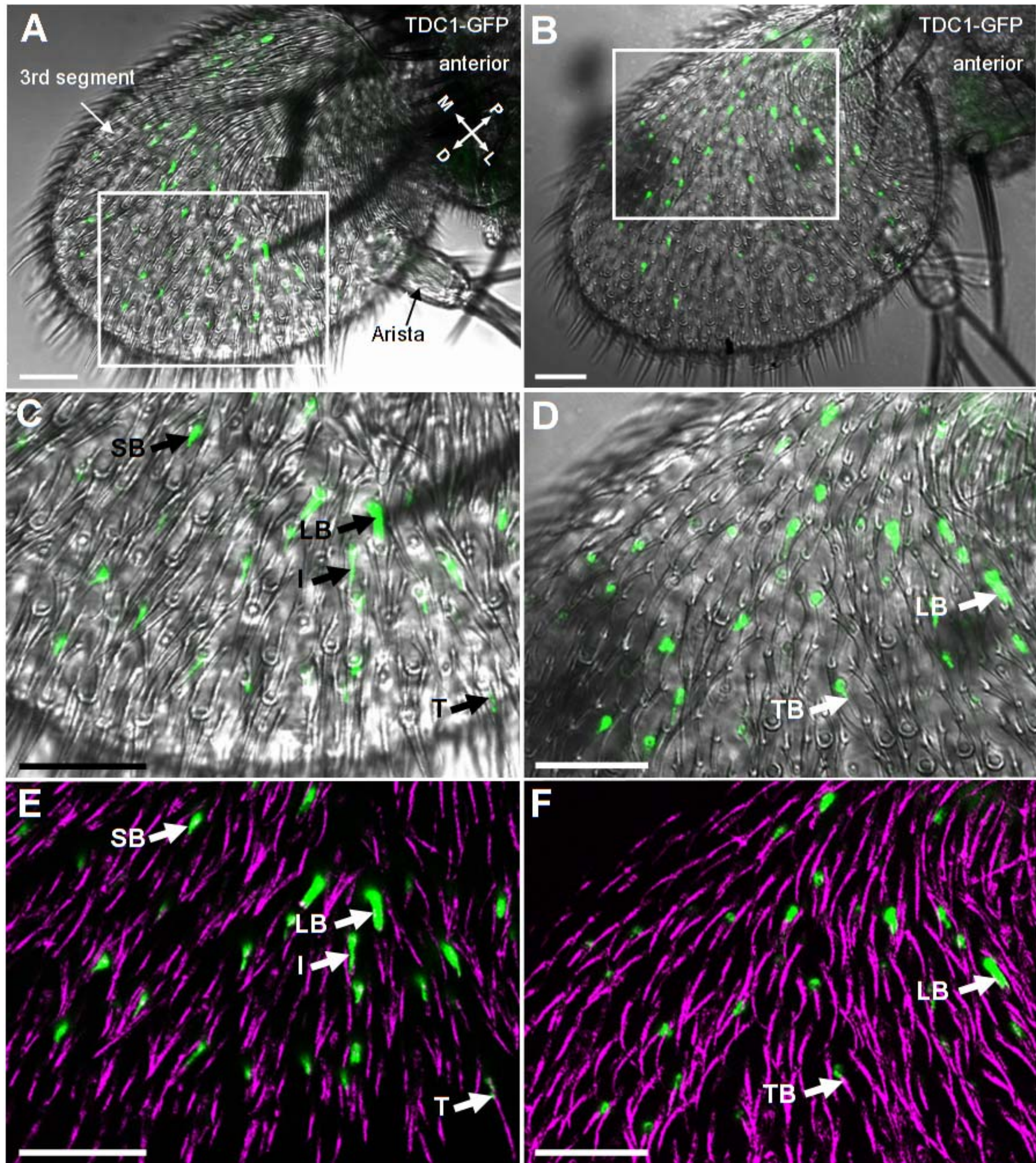

Figure 13: Tdc1 is expressed in different morphological sensillum types.

Confocal pictures of freshly removed unfixed antennae. Green = GFP emission, magenta = reflection (cuticle with sensilla), grey = transmission picture. Scale bars, 20µm. P, proximal; D, distal; M, medial; L, lateral.

A, B) Overview of TDC1 signal on antennal surface of two different animals. White box indicates area of inset shown in higher magnification below.

C-F) Different morphological sensillum types can be distinguished with help of autofluorescence pictures. TDC1 signals are found in different basiconic sensilla as well as in some of the trichoid sensilla. SB, small basiconic; LB large basiconic; TB, thin basiconic; I, intermediate; T, trichoid.

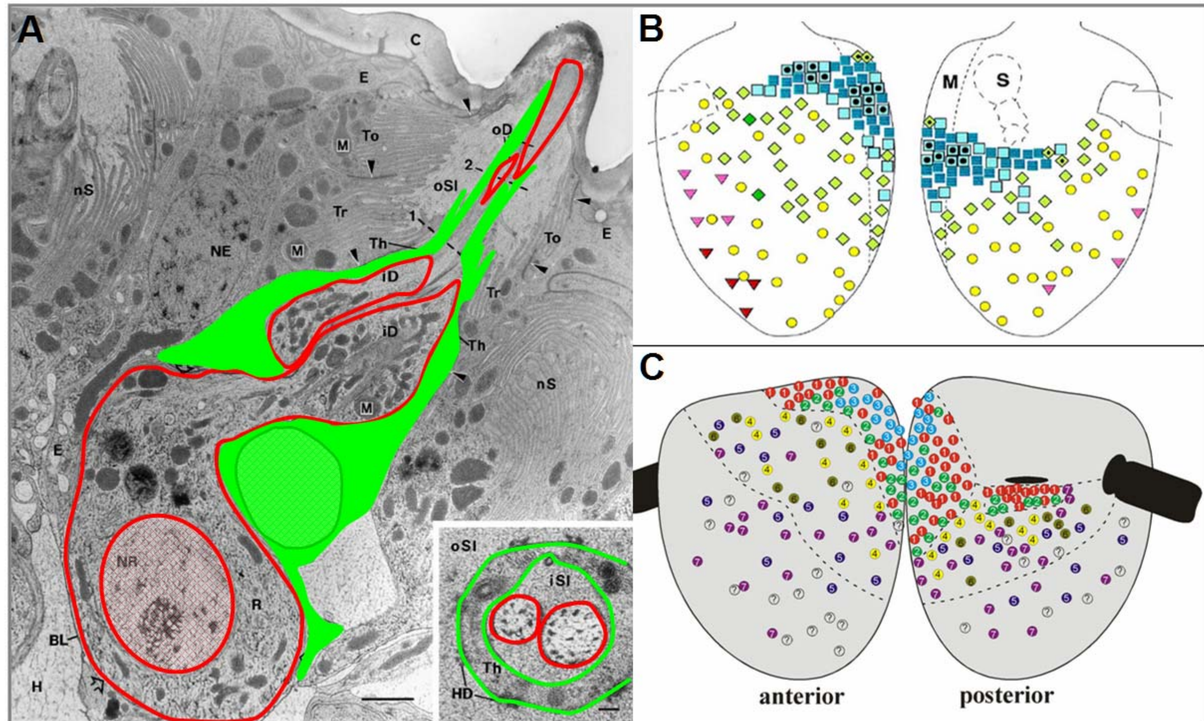

Figure 14: Location of the tdc1 cell in the sensillum and distribution of basiconic sensilla.
A) Internal organization of a large basiconic sensillum, modified from Shanbhag et al., 2000. Shown in red is the ORN (R) and in green the thecogen cell (Th). The inset shows the outer dendritic segments in cross-section (indicated by the dashed line 2). Two dendrites are enclosed by a ring-shaped profile of the thecogen cell. iSI inner sensillum lymph cavitiy, oSI outer sensillum lymph cavitiy, C cuticle, nS neighbouring sensillum, M mitochondria, N nucleus, HD hemidesmosome-like structures, BL basal lamina, epidermis cell (E), scale bar 1µm, inset 100nm. B) Distribution of basiconic and intermediate sensilla on the 3rd antennal segment from Shanbhag et al., 1999. Blue, large basiconic sensilla, green thick basiconic s., yellow small basiconic s., red intermediate s., M medial surface, S sacculus C) Distribution of basicionic sensilla according to their odor response profile from de Bruyne et al., 2001. red ab1, green ab2, blue ab3, yellow ab4, purple ab5, orange ab6, pink ab7.

Tdc1 expression in the antenna does not occur during metamorphosis

Biogenic amines often play a role in development, e.g. DA is important for the development of sensory tissue, e.g. retina (Neckameyer *et al*., 2001). In addition the tormogen and trichogen cells form the sensillum structure during development (Hartenstein and Posakony, 1989). Therefore it is obvious that tyramine could be a regulator of the genesis of olfactory sensilla. To investigate the potential role of TA during development of the peripheral olfactory system, we studied the flies under the microscope (as described above) at different time points. We collected 3rd instar

larvae, freshly emerged flies with still soft cuticle and folded wings and adult flies 4, 15 and 24 hours post eclosion.

In freshly emerged flies we could not detect any *Tdc1* expression (Fig. 15A), although the sensilla with ORNs and olfactory receptors are fully developed and functional at this time (Ayer and Carlson, 1992). Since Tdc1 is expressed before pupation but not after, we conclude that *Tdc1* is not expressed in the antenna during metamorphosis. The number of stained cells increases during the first 24 hours but does not reach the same number counted in 1 week old flies (Fig. 15B-D and I). However the kinetics of GFP expression via the UAS-Gal4 system might not necessarily reflect the kinetics of the intrinsic *Tdc1* expression. It is possible that Tdc1 expression occurs earlier and faster than GFP expression. But we can be sure that there is a time point in metamorphosis when *Tdc1* is not expressed.

In larvae we found Tdc1 expression in the dorsal organ (Fig. 15 E-G). The dorsal organ, being the larval olfactory organ, houses the larval ORNs. Or83b was used as positive control, as it is an olfactory receptor present in most ORNs of adults and larvae and therefore also in the larval dorsal organ (Larsson *et al*., 2004). Gut and trachea are also stained in larvae.

The results suggest that TA deriving from the thecogen cells in the sensilla does not play a role in the development of the adult antenna, but rather in the ORNs of mature flies and of 3rd instar larvae.

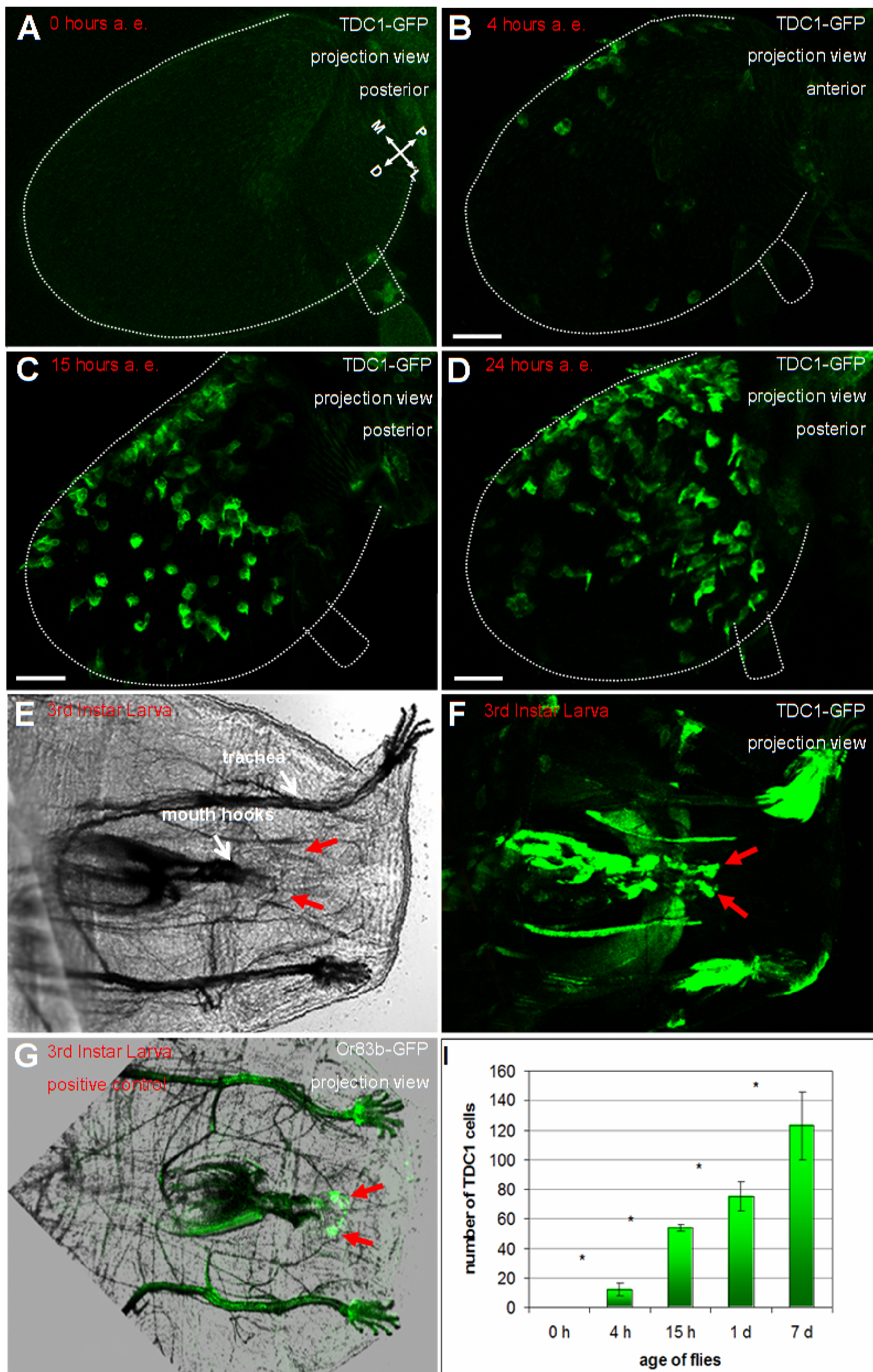
A
0 hours a. e.
TDC1-GFP
projection view
posterior
M
P
D
L
B
4 hours a. e.
TDC1-GFP
projection view
anterior
C
15 hours a. e.
TDC1-GFP
projection view
posterior
D
24 hours a. e.
TDC1-GFP
projection view
posterior
E
3rd Instar Larva
trachea
mouth hooks
F
3rd Instar Larva
TDC1-GFP
projection view
G
3rd Instar Larva
positive control
Or83b-GFP
projection view
I
number of TDC1 cells
0
20
40
60
80
100
120
140
160
0 h
4 h
15 h
1 d
7 d
age of flies

Figure 15: Tdc1 is not expressed during metamorphosis.
Confocal pictures of freshly removed unfixed antennae and 3rd instar larvae. Green = GFP emission, grey = transmission picture. Scale bars, 20µm. P, proximal; D, distal; M, medial; L, lateral.
A-D) Projection view of Tdc1 signal in antennae from animals of different age. 0 hour (A), 4 hours (B), 15 hours and 1 day post eclosion.
E-H) 3rd instar larvae. The dorsal organ domes are marked with red arrows.
E) transmission picture.
F) Same larva as shown in E) Tdc1 signal is clearly visible in the dorsal organ.
G) Or83b-GFP signal in the dorsal organ
I) Number of Tdc1 positive cells in flies of different ages. Error bars are SD. N = 5-9. Student t-test $p < 0.03$.

Accessory cells expressing *Tdc1* respond to odor stimuli

The thecogen cells in the antenna express *Tdc1* and are putatively the source of a TA signal that modulates odor responses of ORNs as reflected in the *hono* mutants. We then addressed the question whether thecogen cells respond to odors. To answer this question we expressed the calcium sensing dye *cameleon* (Fiala *et al*., 2002) in the *Tdc1* cells, and performed calcium imaging with the antennae of these flies. In preliminary experiments with two animals we could show that odor stimulation elicits a calcium concentration increase in *Tdc1* cells in the antenna (Fig. 16). Two odors ethyl acetate and pentyl acetate evoked calcium concentration changes. A response could only be observed during the first (first animal, first odor was ethyl acetate) or the first two stimulations (second animal, ethyl acetate and pentyl acetate, see fig. 16). Odors given afterwards did not evoke calcium concentration changes high enough to be sensed by our method, neither different odor nor the same odors. The temporal pattern of the calcium signal is very different from that evoked by ORNs (compare with Chapter II). The calcium signal begins during the 1 second odor stimulation, which is the same in ORNs. However the rise is very flat and keeps rising for at least 13 seconds. Calcium signals of ORNs typically rise much steeper and decline within 10 seconds after onset of even longer odor stimuli of comparable concentrations ((Pelz, 2005); Chapter II of this thesis). It is possible that the calcium signal in the accessory cells shows a different temporal pattern than in neurons.

We conclude that the *Tdc1* expressing accessory cells in the antenna are able to sense odor stimuli and respond by calcium concentration increase. As we only have limited data we are not sure if the restriction of responses to only a few odors is an artifact caused by photo damage. However, this effect could also be due to an active mechanism, e.g. for the detection of odor stimuli with a certain quality.

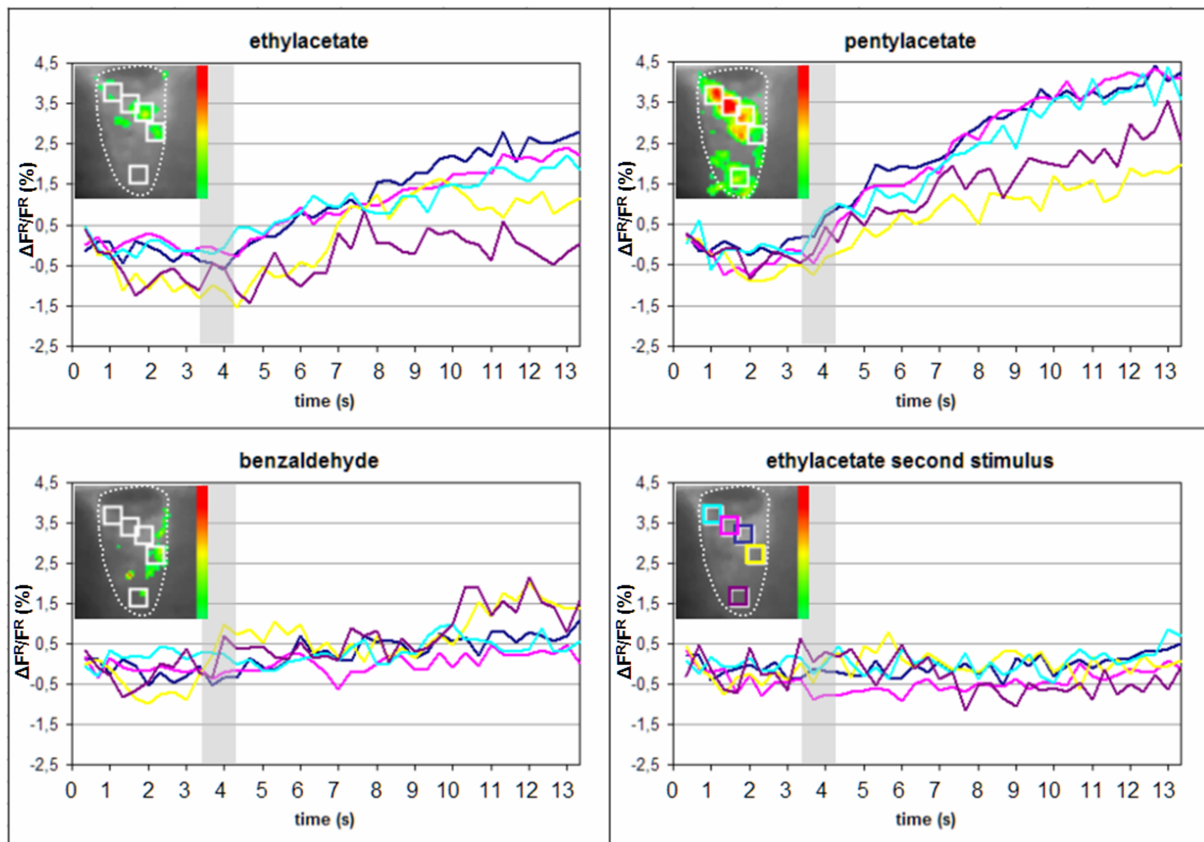

Figure 16: Calcium Imaging of Antenna.
Shown are four succeeding 1 second odor stimulations of one antenna of the same living animal. The lines are colored according to the area on the antenna as indicated in the lower right graph. The small pictures show an overlay of an anatomical of the antenna with the signal in false colors. A high signal all over the antenna is seen for ethyl acetate and pentyl acetate whereas benzaldehyde elicits only a small signal and the forth stimulus, a repetition of ethyl acetate shows no signal any more. The inter stimulus interval was ~5 minutes.

Localization of biogenic amine receptor expressing cells in the antenna

We could localize and identify the cells that detect odors and putatively produce a tyramine signal. Now we wanted to find the cells that possess the tyramine receptor to respond to such a tyramine signal. Our RT-PCR experiments prove expression of the tyramine receptor in the antenna. The antenna includes sensory cells of different modalities (temperature, humidity, odors) and a variety of non-neuronal cell types (epidermal cells, glial cells, accessory cells). With *in situ*-hybridization it is possible to visualize expression of a gene in single cells by labeling the mRNA. A specific labeled RNA probe binds to the mRNA in the cell and can be localized by antibodies and visualized under a microscope. Unfortunately this method could not be established. Therefore we can only show one of few results for the odorant receptor *Or47a* that we used as positive control. The labeled cells in Fig. 17 show the distribution of the ORN ab5 in which *Or47a* is expressed (Störtkuhl and Kettler, 2001).

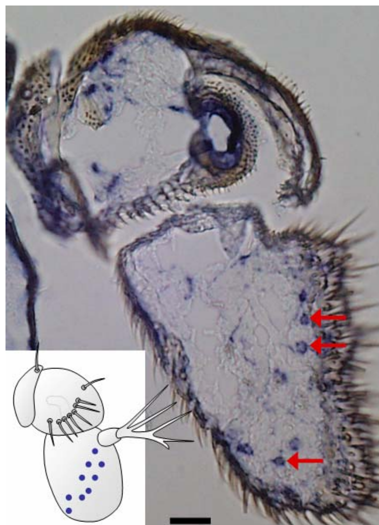

Figure 17: Localization of Or47a by In situ- Hybridization. *Cryosection of the antenna labelled with RNA probes specific for Or47a. Arrows indicate Or47a positive cells. Scale bar, 20µm. The inset shows the expected distribution of ab5 sensilla that matches the distribution of labelled cells.*

Feeding of tyramine does not rescue the *hono* phenotype

To prove that the observations we make in electrophysiology with *hono* flies are indeed due to the tyramine receptor loss, we attempted to rescue this phenotype. A phenotype of *hono* flies displayed in the neuromuscular junction of larva could be rescued by feeding tyramine (Saraswati *et al.*, 2004). Another effect of the *hono* mutation is impaired odor learning in a special paradigm. This effect could also be rescued by tyramine feeding one hour before experiments (Ozaki, personal communication). Thus we fed *hono* flies with 0.2µg of tyramine using the same protocol and concentration and measured EAGs one hour later.

The elevated response for ethyl acetate could not be rescued (Fig. 18). This could be due to seclusion of sensillum lymph against antennal lymph. It is possible that tyramine is not or only slowly transported into the sensillum lymph.

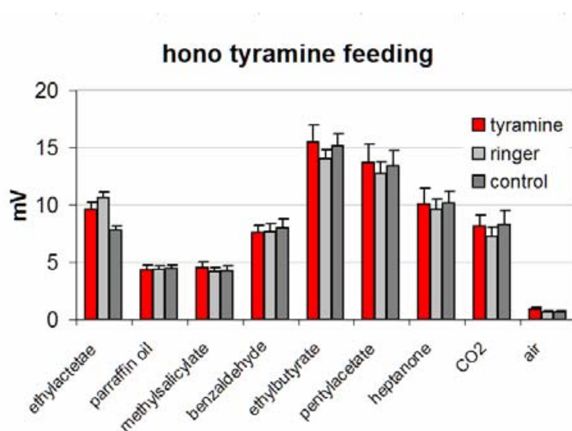

Figure 18: EAG odor response profile of hono flies fed with tyramine solution.
Mean amplitudes of 8 animals during 1 second of odor stimulation are shown. Error bars are SEM. Student t test, $p < 0.05$. Hono flies fed with 0.1µl tyramine solution (2µg/µl) show the same odor responses as the hono flies fed with 0.1µl saline. Both show elevated responses to ethyl acetate compared to control flies = white.