Material and Methods

Fly rearing and stocks

Flies were reared in an incubator at 25°C, 60% humidity and 12:12 hours LD cycle on treacle-cornmeal medium (1l = water 918ml, treacle 118g, cornmeal 95g, brewers yeast 11g, agar 4,1g, propionic acid 2,4 ml). Crosses were performed with 6-8 virgin females and 3-4 males.

The two tyramine receptor mutants *hono* (w^{1118}; + ;P{lacW}$TyrR^{hono}$) and *neo30* (+; +; mwh P{hsneo}$TyrR^{neo30}$ red e/TM3,ry^{RK} SB Ser) and the corresponding white control line (w^{1118}; +; +) were obtained from Dr. Kutsukake (Kutsukake *et al*., 2000). They both have a mutation in the regulatory sequence of the tyramine receptor gene.

The two Gal4 expression lines *dTdc1-GAL4* (w; +; P{Tdc1-Gal4}/TM3 Ser) and *dTdc2-GAL4* (w; P{Tdc2-Gal4}; +) (Cole *et al*., 2005) and the tyramine-β-hydroxylase mutant *TβH^{M18}* ($wTbh^{M18}$/C(1) DX y w f; +; +) (Monastirioti *et al*., 1996) were provided by the Dr. J. Hirsh.

The dopamine-N-acetyltransferase mutant *Dat* (+; bw Dat^{lo}; +) and the β-alanyl-biogenic amine synthetase mutant *ebony* (+; +; e^{11}) were purchased from the *Drosophila* Stock Center (Bloomington, Indiana, USA) as well as the flies carrying UAS constructs for the apoptosis gene *rpr* (w; + ; P{UAS-rpr}/TM3 Sb) and the membrane bound GFP (y w P{UAS-mCD8::GFP.L}; Pin/CyO).

The Canton-S wildtype flies were obtained from the lab of Dr. J.R. Carlson (New Haven).

TM3, C(1), FM7c and CyO are balancer chromosomes.

w^{1118}, mwh, red, e, ry^{RK}, SB, Ser, DX, y, f, bw, Pin are mutations that are used as markers.

All mutants were backcrossed with *white* or wildtype Canton-S flies carrying balancer chromosomes according to their genetic background. The heterozygous siblings were then used as controls if not indicated otherwise.

Electroantennograms (EAGs)

In *Drosophila* EAG we measure changes in an electrical potential between hemolymph and antennal surface, the transepithelial potential (TEP). The odor dependent signals are thought to consist of the summation of the receptor potentials of ORNs close to the recording electrode (Ayer and Carlson, 1992). All the EAG measurements in this work were recorded from the proximo-medial side, which contains the large basiconic sensilla (de Bruyne et al., 2001a). Changing the position of the recording electrode causes different EAG amplitudes in response to the same odors (Ayer and Carlson, 1992). Therefore it is likely that our EAG signals represent mainly responses from large basiconic sensilla. But M. Hähnel (Haehnel, 2005) showed in her diploma thesis that also small basiconic sensilla and coeloconic sensilla contribute to the EAG amplitudes and that EAG amplitudes cannot be predicted by just summing the sensillum potentials measured in single sensillum recordings (method see below), but that the sensillar distribution has to be taken into account. However it is a convenient method to identify olfactory *Drosophila* mutants (Ayer and Carlson, 1991).

Flies were immobilized in a plastic pipette tip and a glass electrode was placed on the proximo-medial side of the antenna. The reference electrode was inserted at the base of the proboscis (Fig.1). We used a 10x DC amplifier, AD converter and analysis software from Syntech (Hilversum, Netherlands). See Ayer and Carlson (Ayer and Carlson, 1992) for more details.

We recorded EAGs from the following genotypes:

hono (w^{1118}; + ;P{lacW}$TyrR^{hono}$) and *hono/neo30* (w^{1118}/+; +; P{lacW}$TyrR^{hono}$/mwh P{hsneo}$TyrR^{neo30}$ red e/TM3,ry^{RK} SB Ser) and the corresponding white control line (w^{1118}; +; +).

Dat (+; bw Dat^{lo}; +) and control (+; bw Dat^{lo}/CyO; +)

TβH^{M18} (wTbh^{M18}) and control (w Tbh^{M18}/FM7c)

ebony (+; +; e^{11}) and control Canton-S (+; +; +)

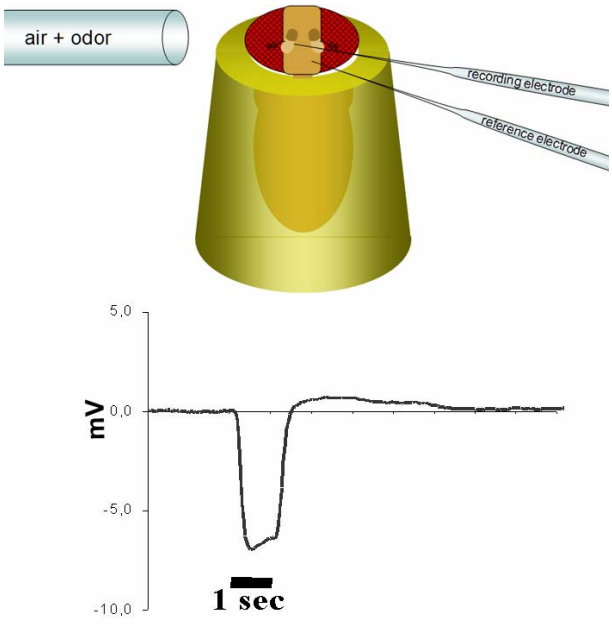

Figure 1: The fly EAG. *The fly is fixed in a plastic pipette tip and a constant air flow blows directly over the head and is exhausted on the other side. Electrodes are placed with micromanipulators. Bottom: a typical EAG signal elicited by a 1 second odor stimulus (ethyl acetate). The temporal pattern is specific for each odor. The trace shown has a steep decline and rise and a small overshoot in the end. Other odors elicit signals with gentler rise and without overshoot.*

Feeding of flies with tyramine

Hono (w^{1118}; +; P{lacW}$TyrR^{hono}$) and control (w^{1118}; +; +) flies were starved over night in vials with 2% agar and directly before the experiment for 1-2 hours without agar (humidity). Then they were immobilized in a plastic pipette tip and fed 0,1µl of Ringer (111,2mM NaCl, 5,5mM KCl, 0,08mM NaH_2PO_4-$2H_2O$, 1,2mM $NaHCO_3$, 1,8mM $CaCl_2$-$2H_2O$, 0,8mM $CaCl_2$-$6H_2O$, 5mM Hepes) or tyramine (2g/l in Ringer) with a Hamilton syringe (Hamilton Modified Microliter 7001 1µl). In total each fly was fed 0.2 µg of tyramine. Flies were then released into culture vials with 2% agar. EAG experiments were performed 1 hour after feeding.

Single sensillum recordings (SSR)

In SSR we measure the potential of sensillum lymph versus hemolymph. We visualize voltage differences in the sensillum lymph during odor stimulation. Signals

consist of slow sensillum potentials that most likely represent membrane potentials of the ORNs in the sensillum (Stengl *et al*., 1992;Kaissling, 1995) and spikes that represent action potentials (Fig. 3). Spikes can be sorted and allocated the corresponding ORNs in the sensillum according to their amplitude (Kaissling, 1995). Flies were immobilized in plastic pipette tips. The antenna was lifted, placed on a cover slip and fixed with a micropipette (Clyne *et al*., 1997). The recording electrode (glass electrode filled with 0.015M potassium chloride and 0.1% polyvinylpirolidon) was inserted at the base of the sensillum and the reference electrode at the base of the proboscis (Fig. 2). Spikes and sensillum potential were measured extracellularly in the sensillum lymph. Signals were amplified (1000x), digitalized and recorded using an IDAC interface and analysed with Autospike software (Syntech, Hilversum, Netherlands). Spike sorting was performed manually as described in de Bruyne *et al*. (de Bruyne et al., 2001b).

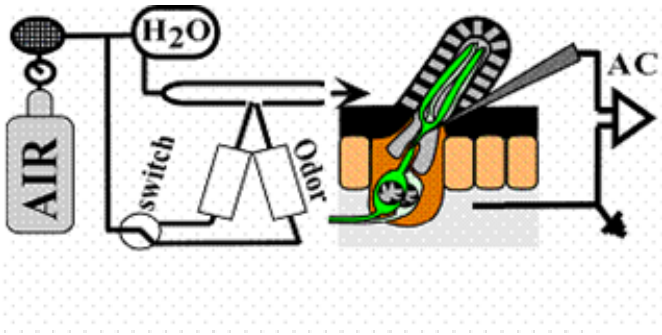

Figure 2: Single sensillum recording. *Picture from M. de Bruyne showing a sensillum with two ORNs, the two electrodes and a schema of the odor stimulation apparatus.*

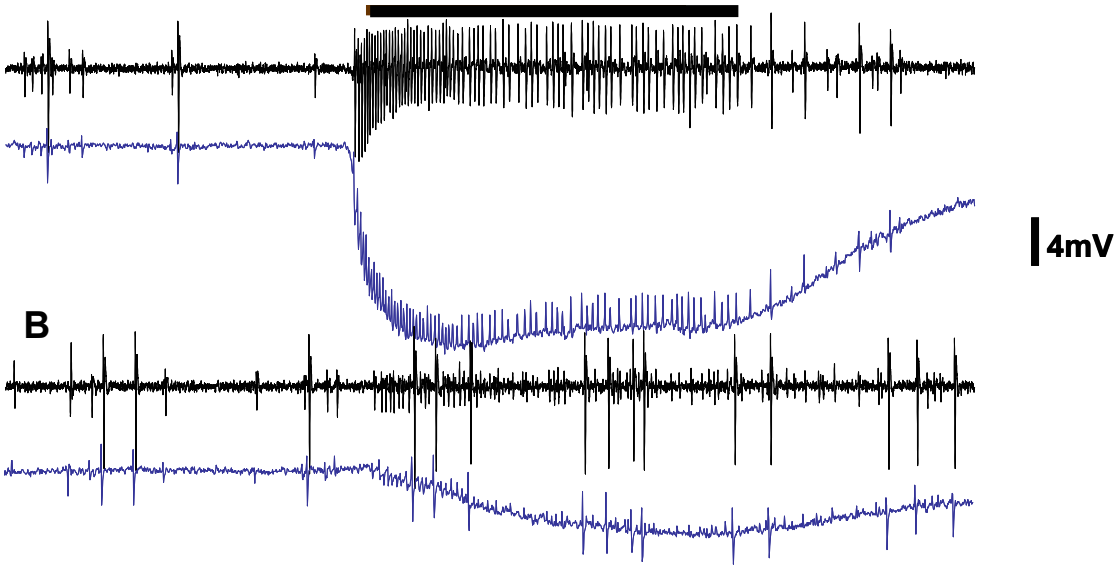

Figure 3: SSR traces of an ab2 sensillum with two ORNs. *Shown in blue is the actually measured trace from where the sensillum potential and the spikes of the two ORNs in the sensillum can be filtered. Shown in black is the filtered spike trace. We can distinguish spikes of two cells (small ab2B and large ab2A) according to their amplitude. The ab2A cell responds to stimulation with ethyl acetate (A), the ab2B cell responds to ethyl,3-hydroxybutyrate (B). Black bar on top indicates odor stimulus of 500ms.*

We recorded from the following genotypes:

hono/neo30 (w^{1118}/+; +; P{lacW}$TyrR^{hono}$/mwh P{hsneo}$TyrR^{neo30}$ red e) and the heterozygous siblings (w^{1118}; +; P{lacW}$TyrR^{hono}$ /TM3,ry^{RK} SB Ser)

TβH^{M18} (w Tbh^{M18}) and its control (w Tbh^{M18}/FM7c)

All SSR recordings were done by Dr. Z. Syed.

Calcium Imaging

Calcium signals from cells in the antenna that express the calcium sensor cameleon were recorded from intact flies. Male flies, 7-10 days old, were immobilized in a plastic pipette tip. The head was fixed at the tube with n-eicosan (Sigma, Germany). One of the antennae was pulled backwards by a fine metal wire (Rediohm-800, HP Reid Inc, Palm Coast, FL, USA), such that the dorso-medial surface was facing upward. The preparation was covered with a cover slip allowing such that the antennal surface was touching it. A drop of aqua dest. was placed on top of the coverslip. Preparations was imaged using two CCD cameras on an Olympus BX 50 microscope with a 20x W NA 0.5 objective as described in Fiala *et al.* (Fiala *et al.*,

2002). Optics and software were from TILL photonics. The cameleon protein is excited with 440 nm light and shifts emission between 480 nm at low calcium and 550 nm at high calcium. Binning on chip was set to give 2µm/pixel (76 x 53 pixels). The fluorescence ratio, $F^R = F^{550nm} / F^{480nm}$, is used as a measure for calcium concentration. For each recording (40 frames, 3 frames/s, exposure time 60-100 ms) the baseline F^R was calculated as a mean over the 6 frames before stimulation. Response was quantified as the difference in fluorescence ratio during four frames following odor stimulation onset relative to the baseline ($\Delta F^R/F^R$ [%]). The responses were quantified in small regions (11 x 11 pixel bins) on the antenna. Data analysis was done using custom written scripts for the IDL software package (Research Systems, USA).
Dr. D. Pelz did the preparation and assisted with the set-up and data analysis.

Odor stimulation in EAG, SSR and Imaging

Odors were injected automatically for 1s into a continuous air flow (160 cm/s) of synthetic air (80% N_2, 20% O_2) from 5ml disposable syringes (Omnifix, Braun), in which a filter paper with 20µl of odor solution was placed. Artefacts caused by mechanical stimulation were avoided by switching between two syringes, one without odor and one with odor and not just adding air to the continuos air flow. All odors were purchased from Aldrich (Milwaukee, WI) at highest available purity (>97%), dissolved at 1% v/v in paraffin oil (Fluka, Buchs, Switzerland). CO_2 was obtained from ultra pure grade pressurized gas tanks (Messer Griesheim, Krefeld, Germany) and filled manually into the syringes used for injection. Odors sets were chosen in order to test responses of all ORNs in basiconic sensilla. Odor stimulation is further described in de Bruyne *et al*. (1999, 2001).

Laser Scanning confocal microscopy

Antennae were removed from flies under CO_2 anesthetisation, mounted in slowfade solution (Molecular Probes, Eugene, OR) and directly viewed under a Leica DMR SP2 confocal microscope (Leica, Bensheim, Germany) with 40x or 63x oil objectives

(Leica, Bensheim). GFP was excited with the 488 nm band of the Ar/Kr-Laser. Fluorescent emission light was collected in the range of 500-550 nm. We used reflection of 633 nm laser-light on cuticular structures such as sensilla and tracheae and transmission in a second and third channel respectively. Stacks of optical sections (~1 μm) were created for each channel, and image analysis was done with AMIRA software (TGS, San Diego, CA) and ImageJ (NIH, Bethesda, ML).

We acquired and evaluated anatomical data from the following genotypes:

Tdc1-GFP (y w P{UAS-mCD8::GFP.L}/w; CyO/+; P{Tdc1-Gal4}/+) males,

Tdc2-GFP (y w P{UAS-mCD8::GFP.L}/w; P{Tdc2-Gal4}/CyO; +) males and

Tdc2-GFP-rpr (y w P{UAS-mCD8::GFP.L}/w; P{Tdc2-Gal4}/CyO; rpr/+) males.

Isolation of total RNA from *Drosophila* Antennae, Heads and Bodies

Canton-S wildtype flies of both sexes were anaesthetised and sorted on ice. To isolate antennae, heads and bodies, flies were picked with tweezers and dipped into liquid nitrogen. The antennae of the frozen flies were then touched in order to fall in an RNase-free eppendorf tube placed in liquid nitrogen. The head and body were also collected in different eppendorf tubes. A number of ~1000 antennae were collected for one sample of cDNA. The tissue was homogenized with Trizol (Invitrogen), and the RNA was extracted with chloroform by standard procedure. The concentration of RNA was calculated from its optical density at 260nm using the formula:

Conc. (μg/μl) = OD_{260} x dilution factor x 40. The isolated RNA was then used for the synthesis of cDNA.

RT-PCR

cDNA synthesis

The reverse transcription was performed in a thermocycler (Mastercycler Gradient, eppendorf) using 1μg of RNA isolated from antennae, heads or bodies with solutions

and enzymes (M-Mu-LV RT) from Fermentas International Inc (Gregson, 1973) by standard procedure. cDNA was used to perform RT-PCR and to synthesize DIG-labelled riboprobes for in situ hybridization.

PCR

Drosophila RNA was isolated and cDNA synthesized. To control for the amount of cDNA PCR for standard proteins was performed. For head and body cDNA I used synaptotagmin and for the antenna is used Or47a. These tests show if there is enough cDNA in the sample, and allows adjusting the amounts used in the following PCRs to the same level. Then PCRs for the biogenic amine receptors were done. In each data set a negative control without DNA was included.

All PCRs were performed under standard conditions with enzymes and solutions from Promega (Madison, WI). Primers were designed with Lasergene software (DNASTAR, Madison, WI) and synthesized by TIBMolbiol (Heimbeck *et al*., 1999). The samples were analyzed on 1.8% agarose gels in TAE buffer and viewed with ethidium bromide under UV light.

Primer sequences:

5-HT1A: 5′-CGTGTGCGGCGTTGGATGGT-3′ 5′-TTCGGCTTTTGGCTTTCACTTTTC-3′

5-HT1B: 5′-TGCCGCTGCTGGTGATTCTCTGTTT-3′ 5′-CCGCGTGGCTTTTGTTGTCGTAG-3′

5-HT2: 5′-TGCCACGCCCACCCTCAA-3′ 5′-GCGTCGCATTAGCCGTAGA-3′

5-HT7: 5′-TTTCCAACGCCAGTGCTATTATTA-3′ 5′-CCACCGTGCCGCGTTTTACTTC-3′

DD2R: 5′-GTGGCGGAGCGGGCAAGAAAG-3′ 5′-GCGGTTGGGCGTGTTGTTGAG-3′

DopR2_1: 5′-TGCTCCGCCAACTGCCCAAAAT-3′ 5′-CCGCACTAGCCGTTCCCGACTG-3′

DopR2_2: 5′-GGCTGCCCTTCTTCGTGGTCAA-3′ 5′-GAGGCGTCCGGTCGATCTGGTTCA-3′

DopR: 5′-CCATCGGCGGGAATCACACC-3′ 5′-CACCAGCGAGGCCACGAAGAG-3′

Oamb: 5′-TCGAACGAGCGGAGAAAACTGG-3′ 5′-GAGATTAGCGAGAGCCCGTCACC-3′

Ocr-1: 5′-ATCGCCTGCCTCCTCCCATACACG-3′ 5′-AAAAACCGACGACCAACCGATAGC-3′

Tdc1: 5′-TCGCAAATACGGCAAGGAAGTGA-3′ 5′-GGGCGCGGGCTGTGATTAGA-3′

Tdc2: 5′-TCCACTCAGTCGGCGTTTTCGTTC-3′ 5′-CTTGCGGTTCATGATCTCGGACAG-3′

TyrR: 5′-GGCGGGGATGGCGGTAGC-3′ 5′-CGGCGGTAGTCCAGGTTGAAGATG-3′

Or47a: 5′-CGATTTCAAGCGACTGATTG-3′ 5′-AGCACTGATTCAGATCGTTG-3′

Syt: 5′-CGGATCCCTAATGTCAAGGTG-3′ 5′-TCTGGTCGTGCTTCGAGAAG-3′

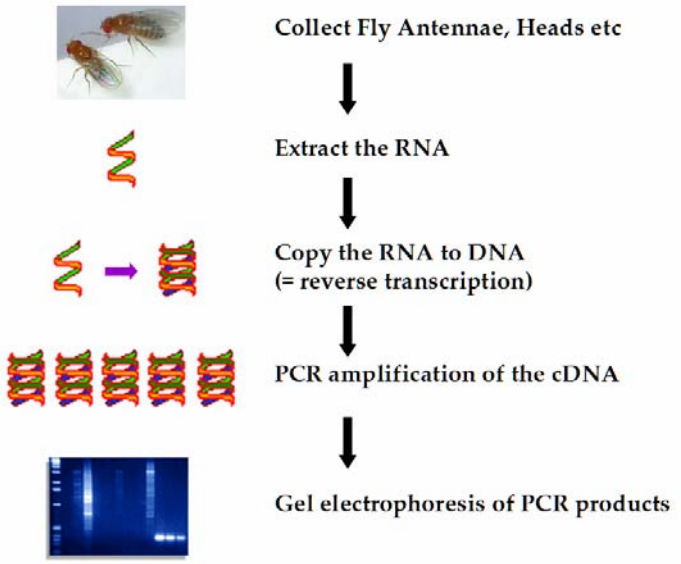

Figure 3: The RT-PCR steps.

Isolation of nucleic acids from agarose gels

PCR products are in a buffer with excess nucleotides and primers, template DNA, enzymes and an ionic composition that is suitable for the activity of DNA polymerase. To use a PCR product in further enzymatic reactions it has to be purified. Additionally from RT-PCR you sometimes get an extra product from contaminations with genomic DNA during RNA isolation. This can be easily distinguished from the mRNA product because the primers are designed accordingly. To get rid of such extra bands and the PCR buffer, the PCR products used later on for *in situ*-hybridaztion were purified by isolation from agarose gels. PCR products were loaded on a gel and separated. The band with the desired nucleic acid was quickly cut from the gel with a scalpel under UV-light and transferred into an eppendorf tube. The nucleic acid was recovered from the gel with the qiaquick nucleic acid isolation kit (Qiagen).

Preparation of DIG-labelled riboprobes for *in situ*-Hybridization

RNA was isolated from whole flies and a RT-PCR was performed and products isolated from an agarose gel as described above.

Syntesis of DIG labelled Riboprobes was performed:

10.5 µl of template in dH2O (~ 1µg template DNA)

4 µl 5X Transcription Buffer (Promega, Germany)

2 µl BMB 10X DIG RNA labeling mix (Roche Diagnostics, Germany)

1 µl 0.1M DTT (Promega, Germany)

0.5 µl RNAsin (Promega, Germany)

2 µl RNA polymerase (T3 or T7 from Promega, Germany)

Total volume=20ul

incubated at 37 degrees for 2 hours.

RT-PCR primers for *in situ* probes included the sequence of the T3 and the T7 promotor respectively. Therefore RT-PCR products were flanked by both promoter sequences. It is then possible to synthesize a sense and an antisense probe from each product simply by using T3 or T7 polymerase. The antisense probe is able to bind the mRNA in the tissue; the sense probe should not bind and is therefore used as negative control. Additionally a short clamp sequence was added at the 5 prime end of each primer to facilitate RNA polymerase binding.

In situ Upper-Primers (clamp – T7 promotor – gene specific sequence):

DopR2_1: 5´- CAGAGATGCA – TAATACGACTCACTATAGGGAGA-TGCTCCGCCAACTGCCCAAAAT

DopR2_2: 5´- CAGAGATGCA – TAATACGACTCACTATAGGGAGA – GGCTGCCCTTCTTCGTGGTCAA

TyrR: 5´- CAGAGATGCA – TAATACGACTCACTATAGGGAGA – GGAACGGAGGCGGTGGCTAACA

Or47a: 5´- CAGAGATGCA – TAATACGACTCACTATAGGGAGA – GCGGAACAGGGCGAGGAATAC

In situ Lower-Primers (clamp – T3 promotor – gene specific sequence):

DopR2_1: 5′- CCAAGCCTTC – ATTAACCCTCACTAAAGGGAGA – CCGCACTAGCCGTTCCCGACTG

DopR2_2: 5′- CCAAGCCTTC – ATTAACCCTCACTAAAGGGAGA – GAGCGTCCGGTCGATCTGGTTCA

TyrR: 5′- CCAAGCCTTC – ATTAACCCTCACTAAAGGGAGA – CCGGTCGAGGGCTATGGCACAC

Or47a: 5′- CCAAGCCTTC – ATTAACCCTCACTAAAGGGAGA – GGGCGCAGATAATCGGTTGGT

Probes were then passed over G50 ProbeQuant columns and volume of eluate brought to a final volume of 100 µl with hybridization buffer. 10µl-20µl/ml were used for in situ hybridization. A DIG labelling test (with solutions and protocol from Roche Diagnostics, Germany) was done to assure proper labelling of the probes.

In situ-Hybridization on *Drosophila* antennae

Flies were embedded in Tissue Tek OCT compound (Sakura Finetech, Japan), frozen at -25°C and cut in 14µm slices in a cryostat (Pelz, 2005). Slices were collected on Superfrost slides (Menzel) dried for 2-3 hours, fixed in 4% PFA (450ml dH_2O, 50ml 10xPBS, 20g paraformaldehyde, 50µl 10N NaOH) and washed in PBS for 3x3 min. After 10 min acetylation (25g triethanolamine HCl, 1,12 ml 10N NaOH, 1,25 ml acetic anhydride in 500 ml total volume), washing in PBS for 3x5 min and 1 h prehybridization, slices were incubated over night with DIG-labelled riboprobes in hybridization buffer (50% formamide, 5x SSC, 5x Denhardts, 250 µg/ml yeast tRNA, 500 µg/ml herring sperm DNA, 50 µg/ml Heparin, 2,5 mM EDTA, 0,1% Tween-20, 0,25% CHAPS) at 65°C and covered with Hybrislip plastic cover slips (Grace Biolabs). Coverslips were removed by soaking in 5xSSC and slices washed with 0,2xSSC for 3x20 min and in PBS-T for 10 min. We blocked with B2 Goat for 1hour and incubated with antibody solution (anti-DIG-Alkaline Phosphatase antibody (BMB Cat. #1 093 274) 1:1000 in B2 Goat) at 4°C over night. After washing in PBS-T for 3x30 min and equilibration in B3 (0,1M Tris pH 9,5, 0,1M NaCl, 50 mM MgCl2,

0,1% Tween-20) for 10 min, slides were developed inverted on parafilm in 300 µl drops of B4 (45 µl of NBT, 35 µl of BCIP, 100 µl of 100mM Levamisole, 10 ml B3) at room temperature in the dark for 10 minutes to 3 days, depending on abundance of transcript. Reaction was stopped by washing for 1x5 minutes in 1xPBS-T and slices rinsed with distilled water and mounted in Glycergel (Georgopapadakou and Walsh, 1994).

Only RNase-free material was used. Glass ware was incubated with 1N NaOH for 30 min and rinsed with RNase-free distilled water.

Some of the *in situ*-hybridization experiments were done by two students (Manja Wendt and Marion Westphal) under my supervision.

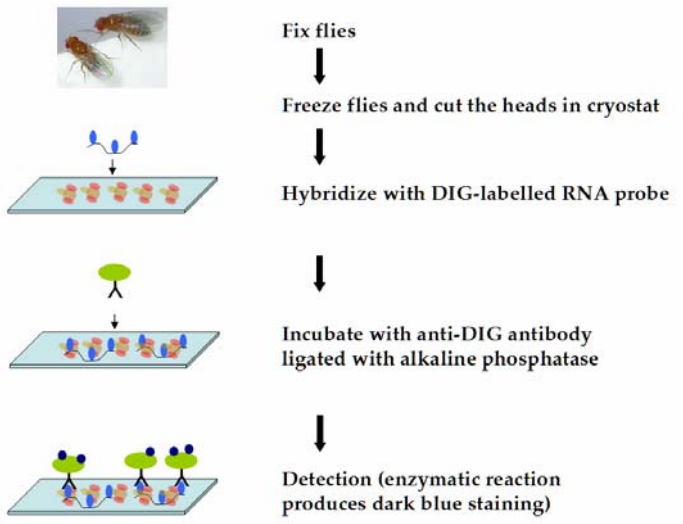

Figure 4: The In situ-Hybridization Steps.