Odor Intensity Learning in *Drosophila*

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von
Pavel Mašek
aus Ústí nad Labem

Würzburg, 2005

Eingereicht am: 13.9.2005.....

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. U. Scheer.....

Gutachter: Prof. Dr. Martin Heisenberg.....

Gutachter: Prof. Dr. Wolfgang Rössler.....

Tag des Promotionskolloquiums: 9.11.2005.....

Doktorurkunde ausgehändigt am: .....

To Eva

Acknowledgments:

First I want to thank my parents for letting me do what I wanted and for supporting me every time I needed it.

I want to thank Prof. Heisenberg for being my supervisor and for his great attitude to science and to people.

I want to thank Martin Schwaerzel for introducing me to the T-maze paradigm and helping me with its caveats. And Hans and Konrad for building the machine and keeping it running.

My thanks goes to all members of the Department of Genetics and Neurobiology for creating such a friendly and stimulating atmosphere in the lab. For a lot of help and endless discussions over the science and meaning of life, I want to thank namely Jens, Dhananjay, Markus, Franco, Suse, Isa, Ron, Roland, Reinhard and many others.

I want to thank Eva for being with me here for so many years.

I also thank to all computers and other machines that participated on my work; without them I would be lost.

The last thanks goes to all those nameless millions of flies, which sacrificed their lives in name of science.

Table of content

1. Introduction... .....**7**
1.1. Olfactory system of *Drosophila*.....8
1.2. Dose response curve.....12
1.3. Olfactory conditioning.....14
1.4. Genetic dissection of olfactory memory.....15
1.5. Mushroom bodies.....19
1.6. Genetic tools in *Drosophila*.....22
1.7. Motivation.....23
1.8. Terminology of olfaction and olfactory memories 25

2. Material and Methods.....**26**
2.1. Fly care.....26
2.2. Genotypes.....26
2.3. Behavioral paradigm.....27

3. Results.....**31**
3.1. Behavioral characterization of olfactory learning in the T-maze 31
3.1.1. Non-associative effects.....31
3.1.2. Significance of CS^+ and CS^-.....33
3.1.3. Two-odor learning is symmetrical 35
3.2. Odor intensity learning.....35
3.2.1. Odor intensity learning is symmetrical.....37
3.2.2. The CS^- is dispensable in odor intensity learning.....37
3.2.3. Increasing the concentration ratio in the test leads to higher learning scores 38
3.2.4. Memory decay after odor intensity learning 39
3.2.5. Influence of the test on memory readout 39
3.2.6. Flies store multiple olfactory memories. 40
3.2.7. Flies learn the absolute odor concentration. 42
3.3. Flies generalize olfactory cues.....43
3.3.1. Two ways of testing olfactory generalization.....43
3.3.2. Generalization of concentration is limited.....44
3.3.3. Cross-generalization is dependent on odorant and concentration.....46
3.4. *Rutabaga*-independent memory.....47
3.4.1. Odor intensity and two-odor learning differ in the cAMP pathway 47
3.4.2. Two-odor learning with cross-generalized odors is *rutabaga*-independent . .48
3.4.3. Memory readout in *rutabaga* is not improved in a one-odor test 49
3.5. Localizing *rutabaga*-independent memory.....50

4. Discussion.....**53**
4.1. Properties of odor learning.....53
4.2. Why two olfactory STMs?.....56
4.3. Generalization.....59
4.4. Odor-quality and intensity learning do not rely the same on cAMP signaling.....61
4.5. Localization of the memory trace in odor intensity learning.....64
4.6. Evolutionary perspective.....65
4.7. Possible model.....65

5. Summary.....**67**

6. Zusammenfassung.....**69**

7. References.....**71**

8. List of abbreviations.....**82**

1. Introduction

Chemosensation may be the oldest of all senses. Even bacteria have it. Olfaction is extremely sensitive, especially for sexual pheromones (Christensen and Hildebrand, 2002), food (Takken and Kline, 1989; Hartlieb and Anderson et al., 1999; Dicke, 1999), and prey (Hartlieb and Anderson et al., 1999; Dekker et al., 2001). It has to handle a near to infinite number of possible odors that cannot be sorted yet based on few simple physical properties, like wavelength and intensity in vision. In fact, a systematic classification of scents is still missing. There is no clear relation between chemical structure of the odorant and its smell. Odorants that are structurally very distinct still may smell very similar (Malnic et al., 1999).

Detection of odor quality is not sufficient for finding one's way towards a food source or away from a dangerous chemical. Unlike light or sound waves, odors do not propagate from their sources in a straight line, but are distributed, in most instances, by air movements in the form of so-called odor plumes (Vickers, 2000; Vickers et al., 2001). Under these conditions, the odor alone gives no information about its origin. Wind direction in addition guides the animal to the source (e.g. female emanating a pheromone).

In still air, on the other hand, odors diffuse from their source forming concentration gradients, which, then, define the direction to the source. This kind of distribution rarely happens in the natural habitat except at short distance and in enclosed spaces. Sensing and discriminating intensity differences is necessary also for orienting within odor plumes, where the animal needs to analyze the structure of the plume (Vickers, 2000). Gradient detection is therefore very important for any kind of odor orientation. The fly *Drosophila melanogaster* is able to orient in respect to different odor intensities (Borst and Heisenberg, 1982). Whether osmotaxis is positive or negative depends on odor intensity but it is also context dependent (Rodrigues and Siddiqi, 1978; Heisenberg, 1980; Borst and Heisenberg, 1982; and unpublished data).

Once the odor is airborne it is independent of its source odorant and can persist for a long time after the source is gone. Therefore, the presence of an odor does not necessarily indicate the presence of the odor source.

Except for specific cases it cannot be discriminated whether the smell is a product of a single chemical or a blend. One of such special case is the perception of pheromones. It is well known that the central pathways processing pheromone and non-pheromone information in the insect brain are largely separated (Hildebrand, 1996).

1.1. Olfactory system of *Drosophila*

Insects perceive olfactory stimuli with receptors placed on antennae, on maxillary and labial palps, and on other parts of their bodies (Tuccini et al., 1996). In *Drosophila*, the main olfactory organs are the third segment of the antennae and the last segment of the maxillary palps (Fig. 1.1A, B). Odors are received on olfactory sensillae (Shanbhag et al., 1999), which contain two or four olfactory receptor neurons (ORN - Fig. 1.1C, D; Shanbhag et al., 2000). Single neurons are specific to subsets of odors (DeBruyne et al., 1999; 2001; Hallem et al., 2004). This specificity is due to exclusive expression of only one specific olfactory receptor gene in one type of ORN (Wang J. et al., 2003; Hallem et al., 2004; Larsson et al. 2004). ORNs from both, the antennae and the maxillary palps, send their axons directly to the antennal lobes (ALs - Fig. 1.1E, Strausfeld, 1976; Strausfeld et al., 1998; Stocker, 1994). Additionally, it seems that most neurons expressing a particular olfactory receptor (OR) project to the same glomerulus (Gao et al., 2000; Vosshall et al., 2000; Bhalearo et al., 2003; Komiyama et al., 2004). From there on, the information is carried via projection neurons (PNs) to other brain regions (Fig. 1.1F). The majority of PNs receive dendritic input from one glomerulus (Jefferis et al., 2001) and send their axons via the inner antennocerebral tract (iACT, Stocker et al., 1990) to the calyx of the mushroom body (MB - Fig. 1.1G) and to the lateral protocerebrum (LPC, Stocker et al., 1990; Stocker, 1994; Heimbeck et al., 1997; Fiala et al., 2002; Wong et al., 2002; Marin et al., 2002; Wang Y. et al. 2004). A second group of PNs is oligo- and multiglomerular. Most of these send their axons via the medial antennocerebral tract (mACT) directly to the LPC (Stocker et al. 1990; Jefferis et al., 2002). In the AL, PNs have been shown to also maintain reciprocal synapses with local interneurons (Yu et al., 2004; Ng et al., 2002).

Local interneurons (LNs - Fig. 1.1F) terminate in many (if not all) glomeruli of the AL (Stocker, 1994). A large fraction of them are GABAergic inhibitory neurons (Laissue et al., 1999; Stocker, 1994). No data on their function are available so far but it was proposed that they could play a role in concentration invariance (Mason, 1977 cited in Borst, 1983). In their recent work on learning plasticity in the AL, Yu et al. (2004) proposed the possibility that some local interneurons may convey the CS information from other glomeruli by synapsing on PNs innervating the recruited glomeruli.

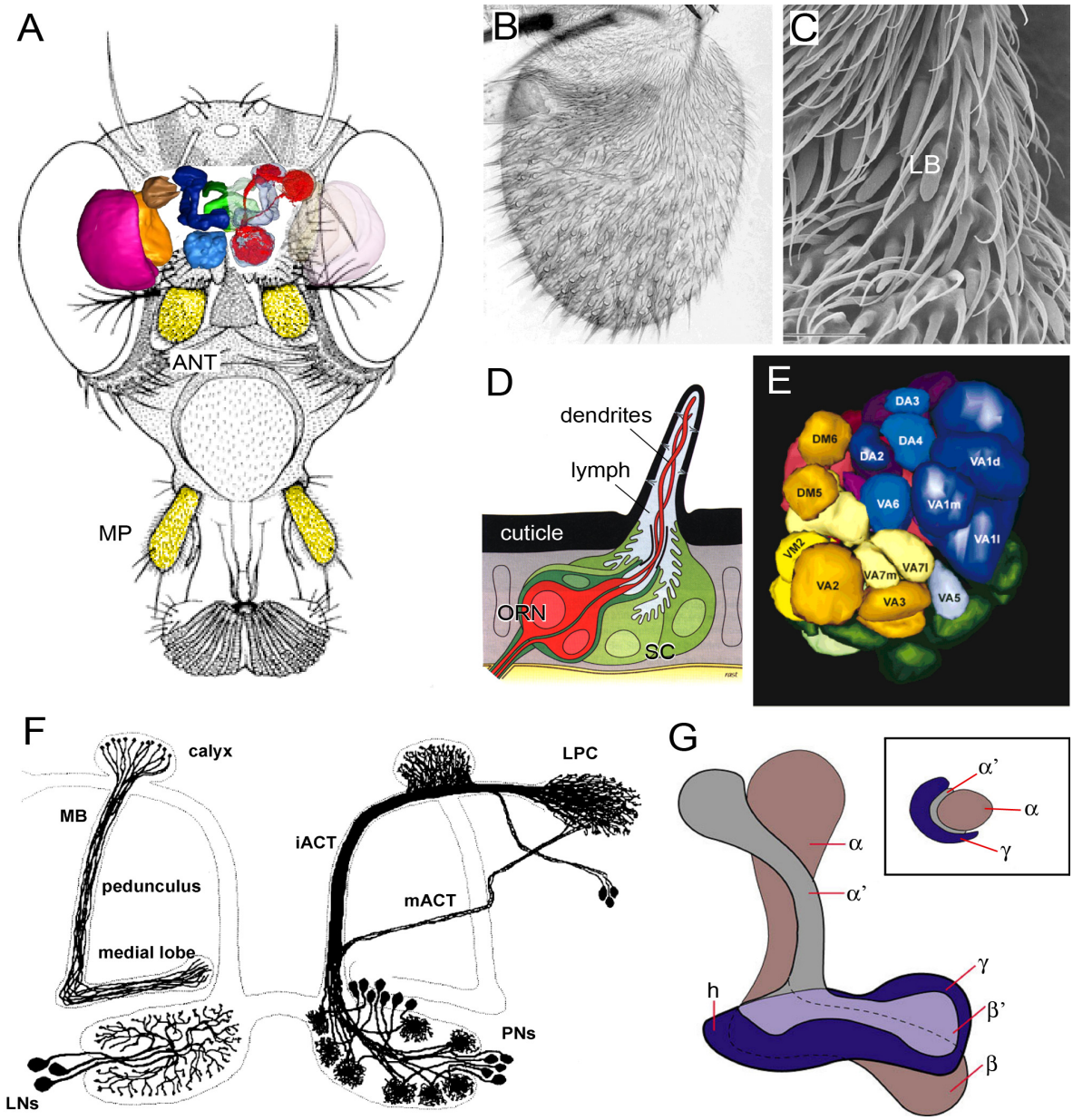

Figure 1.1. Organization of olfactory system in *Drosophila*. **(A)** Frontal view of *Drosophila* head with the two main olfactory organs highlighted in yellow - the third segment of antennae (ANT) and last segment of maxillary palps (MP). Picture shows extended proboscis. With retracted proboscis, the palps are partially hidden. Overlaid is 3-D reconstruction of the *Drosophila* brain based on MAB nc82 immunostaining. Central are the mushroom bodies (dark blue) and the antennal lobes (light blue). These are connected with GH146 positive projection neurons (red). These neurons also project to lateral protocerebrum (brown). On sides are optical lobes, medulla (magenta) and lobula (orange). In the back is the central complex (green). **(B)** Third antennal segment (funiculus) bearing The three principal types of olfactory sensilla. **(C)** Detail of antennal surface with several types of olfactory sensilla. The most common are large basiconic (LB). **(D)** Cross section of basiconic sensilla. Olfactory receptor neurons (ORNs) sending their dendrites into the cavity of cuticular sensilla. (SC - supporting cells). Axon project to the antennal lobes (AL) **(E)**. AL is divided into glomeruli consisting of synapses of ORNs, lateral interneurons (LNs - **F**) and projection neurons (PNs - **F**). **(F)** PNs send the information via inner- and medial-antennocerebral tracts (iACT and mACT) to calyx of the mushroom bodies (MB) and to lateral protocerbrum (LPC). **(G)** Kenyon cell organization of the MBs. Medial lobe consists of neurons of β, β' and γ subsystems and vertical lobe contain α and α' neurons. Inlet - cross section schematic of the pedunculus, showing the layered/concentric organization of axons from calycal zones. (A - Composed images, from http://www.flybrain.org and A. Jenett, A. Fiala and T. Riemensperger; B - modified from Stocker, 2001; C, D - modified from internet; E - from Laissue et al., 1999; F - modified from Stocker et al., 1997; G - modified from Crittender et al., 1998)

Olfactory coding

How can animals with a relatively small number of olfactory receptors discriminate hundreds of odors? It was reported first from mammals that the sense of smell is based on a combinatorial approach to recognize and process odors (Malnic et al., 1999). The mechanism of odor coding is based on three important points: 1) a single receptor recognizes multiple odorants, 2) a single odorant is recognized by multiple receptors and 3) different odorants are recognized by distinct combinations of receptors (Malnic et al., 1999). An additional point could be that at different concentrations, an odor stimulates different combinations of ORs. Hence, different concentrations are represented by different receptor code and this could lead to a change in odor quality (Malnic et al., 1999). High specificity of ORs for certain molecular features, but low specificity for others, enables the olfactory system to be both highly discriminative and still be able to recognize numerous odors (Araneda et al., 2000).

Early work on mammals showed that each olfactory neuron expresses only one OR gene (Strotman et al., 1992). Also in *Drosophila*, a single OR gene is expressed in discrete subpopulations of olfactory receptor neurons which in turn all end in one glomerulus (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999; 2000; Jefferis et al., 2001; Marin et al., 2002; Dobritsa et al., 2003). Since then there were some exceptions found. In most olfactory receptor neurons (ORNs) two OR genes are expressed, one coding for the respective receptor itself and the other is always the same, Or83b (Vosshall et al., 1999; 2000; Pitts et al., 2004; Larsson et al., 2004). Or83b is coexpressed in large portion of ORNs but does not have function in odor specificity but instead acts as an essential cofactor for localizing conventional ORs in chemosensory dendrites (Larsson et al., 2004). Another exception is that one subclass of neurons expresses two OR genes - Or22a and Or22b. Or22a functions independently of Or22b but no contribution of Or22b to odor processing was found (Dobritsa et al., 2003). In addition, recently coexpression of two fully functional ORs - *Or33c* and *Or85e* in the pb2A neuron was reported (Goldman et al., 2005). This is apparently not a transition state during the evolution of ORs as this situation is conserved over 45 milion years (Goldman et al., 2005). It was proposed (Spehr et al., 2005) that this finding could have important implications for the logic of olfactory coding based on the combinatorial approach (Malnic et al., 1999).

Molecular mechanisms of olfaction

Fundamental aspects of olfactory signal transduction are shared across animal phyla and similar molecules are involved in olfactory signaling pathways (Hildebrand and Shepherd, 1997; Prasad and Reed, 1999). Two main messenger systems [Inositol trisphosphate (IP3) and cyclic adenosine monophosphate (cAMP) cascades] were associated with olfactory reception in vertebrates as well as invertebrates (Breer and Boekhoff, 1992; Mori and Shepard, 1994; Schild and Restrepo, 1998; Dubin et al., 1998).

The interaction of odor molecules with olfactory receptors on the surface of sensory neurons triggers intracellular G proteins, which in turn activate key enzymes of the respective signalling cascades (Fig. 1.2). Adenylyl cyclase catalyzes the formation of cAMP from adenosine triphosphate (ATP), whereas phospholipase C (PLC) hydrolyzes membrane phosphatidylinositol, liberating IP3 and diacylglycerol (DAG). An involvement of PLC in odor perception is indicated by impaired olfaction in *Drosophila norpA* mutants, suggesting that odorant responses require an intact *norpA* (PLC) gene (Riesgo-Escovar et al., 1995). The rapid increase in the concentration of intracellular mediators activates ion channels in the plasma membrane, thereby generating a receptor potential. As for cAMP pathway, olfactory responses of *Drosophila* ORNs are influenced by mutations in the genes involved in this cascade, like *rutabaga* and *dunce* (Martin et al., 2001; Gomez-Diaz et al., 2004).

Certain odorants appear to increase cAMP, whereas others have been shown to elicit IP3 increase (Breer and Boekhoff, 1992; Schild and Restrepo, 1998). These results indicate that

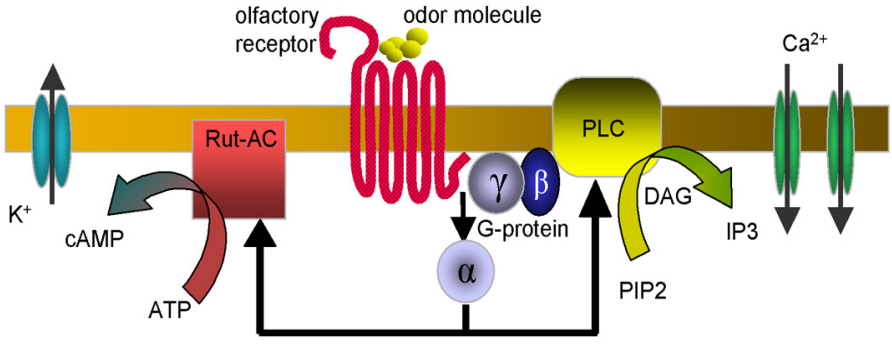

Figure 1.2. The molecular components of olfactory signaling pathways in insects. The proteins that are graphically represented here are present on the inside surface of the dendritic membrane on olfactory receptor neurons. The signaling starts when odor molecule binds to G-protein coupled olfactory receptor (OR)(either alone or in complex with odorant binding protein). This binding causes a conformational change to the OR that release the Gα-subunit from heterotrimeric G-protein (αβγ). The Gα-subunit activates downstream effector enzymes like rutabaga-adenyl cyclase (Rut-AC) or phospholipaseC (PLC). AC converts ATP to the second messenger cAMP while PLC converts PIP2 to the second messengers IP3 + DAG. Both cAMP and IP3 are capable of opening potassium or calcium channels. (modified from http://www.cas.vanderbilt.edu/zwiebel)

different subsets of odorants selectively trigger distinct reaction cascades and provide evidence that the cAMP and the IP3/DAG appear to operate as two alternative pathways (Breer and Boekhoff, 1992; Breer et al., 1994; Schild and Restrepo, 1998).

The cAMP pathway results in the activation of potassium channels and hyperpolarization (Boekhoff et al., 1994), whereas the IP3 pathway opens cation channels leading to depolarization (Fadool and Ache, 1992). In mammals, it was demonstrated that cyclic nucleotide and IP3-gated ion channels could occur in the same cell (Hatt and Ache, 1994). It has been proposed that chemosensory information is not only transduced but also processed on the level of the sensory cell. Natural odors are usually complex blends of chemicals. They probably activate both second-messenger systems and opposing membrane conductance in an individual neuron; thus, a sensory cell indeed would function as a complex integrating unit (Krieger and Breer, 1999).

Odorant-binding proteins (OBPs) are small, soluble proteins present in the aqueous medium surrounding olfactory receptor neurons. They are produced by nonneuronal cells and secreted to the lymph around ORN dendrites (Fig. 1.1D). Their function in olfaction is unknown: they have been proposed to facilitate the transit of hydrophobic molecules to olfactory receptors, to deactivate the odorant stimulus, and/or to play a role in chemosensory coding (Pelosi, 1994, Hekmat-Scafe et al., 1998). In *Drosophila*, at least 35 genes encodes OBPs (Galindo and Smith, 2001; Hekmat-Scafe et al., 2002). The only mutant defective in OBP expression is lush (Kim et al., 1998; Xu et al., 2005)

Drosophila TRP channel mutants are defective in adaptation to odorants (Stoertkuhl et al., 1998) whereas IP_3-receptor mutants are defective in maintaining olfactory adaptation (Deshpande et al., 2000)

1.2. Dose response curves

One of the characteristics of olfactory responses in *Drosophila* is that they can change dramatically when concentrations of the stimulus vary. The most obvious is the shift between attraction and avoidance, when animals are faced with rather small concentration changes. Indeed, olfactory avoidance and attraction appear to be much more a matter of intensity of the stimulus rather than its composition. Hence, early classifications of odorants as either attractants or repellents (Rodrigues, 1980; Ayyub et al., 1990) seem not to be justified. For instance, ‘strong repellents’ such as benzaldehyde (Rodrigues and Siddiqi, 1978; Rodrigues, 1980; Ayyub et al., 1990) can yield mild attraction over a narrow concentration range, at least

in some assays (Devaud et al., 2001; West, 1961; Kim and Smith, 2001 and unpublished data).

A simple increase or decrease in sensitivity to a given odorant inducing a shift in the dose-response curve may, at some concentrations, result in a shift from attraction to avoidance or the reverse. At other concentrations, it may shift from attraction or avoidance to a loss of response. It was proposed that such a switch between attraction and avoidance at a given concentration does not necessarily mean that the animal perceives the odor as different but it just perceives it with a different intensity (Devaud, 2003). This may happen, for example, if sensitivity is decreased after ablation of olfactory organs (Charro and Alcorta, 1994). Indeed, most mutant phenotypes observed so far on a small range of concentrations could be due to such shifts in sensitivity (Rodrigues and Siddiqi, 1978; Ayyub et al., 1990; Balakrishnan and Rodrigues, 1991; Inamdar et al., 1993; Dubin et al., 1998).

An alternative to a shift in sensitivity would be a reduced behavioral responsiveness. Reduced responses to odors have been observed after electric shocks (Préat, 1998). Possibly, in this case the odor response curve is not shifted but rather flattened. This would imply that all responses over the concentration range would be more alike - less aversive in high concentration and less attractive in lower concentration. The opposite effect can be seen in the mutant *gigas*: Due to an increased number of synapses in the antennal lobe the amplitude of its responses is increased both at attractive and repulsive odorant concentrations (Acebes and Ferrus, 2001).

It is still not known where and how the balance between attraction and avoidance is controlled at the neural level. Recent experiments proposed that attraction but not repulsion is processed via MBs (Wang Y. et al., 2003). Yet other experiments suggest that flies without MBs are still attracted to various odors (unpublished data). It is well established that increasing the stimulus concentration increases activity in the antennal lobes (Fiala et al., 2002; Ng et al., 2002; Silbering et al., 2003), probably as more sensory neurons are activated (DeBruyne et al., 1999; 2001). Increasing concentration of an odor increases activity in the respective glomerulus and new glomeruli are activated in addition (Silbering et al., 2003; Fiala et al., 2005). As a result, the internal representation of the odor should change. The combination of activated glomeruli is assumed to represent the quality (scent) of an odor. This would imply that different intensities of one odor result in qualitatively different subsets of activated glomeruli and hence in different scents.

In humans, some odorants presented at different concentrations are perceived as different qualities. For example, indole smells putrid in high concentration but floral when diluted and thioterpineol is described as "tropical fruit" at a low concentration, and as "stench" at high (R. Boden, cited in Malnic et al., 1999). This perception of (vastly) different concentrations of one odor as different scents is likely to be the consequence of the specific design of the olfactory system. This would not be possible with analytic sense (McBurney, 1975) as is for example taste (Wang Z. et al., 2004).

At least to some extent, generalization of different odor concentrations (concentration invariance) is necessary. Otherwise, orientation tasks in odor gradients would be impossible. Concentration invariance, albeit limited, was found in *Drosophila* (Borst, 1983) and also in *Musca* (Fukushi, 1973). On the contrary, Kramer (1976) proposed a model in which odor discrimination is based only on differences of spike frequency of respective receptors. In this concept, there is no distinction between intensity and quality, and hence also no concentration invariance.

1.3. Olfactory conditioning

In *Drosophila*, olfaction has been most intensely studied by olfactory discriminative conditioning (Quinn et al., 1974; Dudai et al., 1977; Waddell and Quinn, 2001, for review see Davis, 2004). This classical learning and memory task can be used with electric shock and other noxious stimuli as aversive reinforcers (Quinn et al., 1974; Tully and Quinn, 1985) or with sucrose (Tempel et al., 1983; Borst, 1983) as appetitive unconditioned stimulus (US). This paradigm originates from an earlier operant version (Quinn et al., 1974; Dudai et al., 1977). In later studies the operant training was combined with the test of the present-day version (Borst, 1983; Dudai, 1983), before Tully & Quinn (1985) finally added the current purely classical training procedure. Using positive or negative reinforcement can lead to comparably strong (high) learning scores (Borst, 1983; Dudai, 1983; Tully and Quinn, 1985) but the temporal properties of those memories are different as positively reinforced memories last longer than those reinforced negatively (Tempel et al., 1983).

The training consists of two olfactory cues (conditioned stimuli - CSs), which are sequentially presented to the flies, the first accompanied with the US (marked as CS^+), the second without the US (marked as CS^-). In a subsequent test trial, the animals choose between the two olfactory cues (CS^+ and CS^-) in a forced choice maze (for details see Material and Methods). After the training in the old setup version (Dudai et al., 1977) it was

reported that flies repeatedly run during the test between the tubes (Borst, 1983). Making the correct choice in the choice point of the T-maze during the test is only a matter of probability and not of the different levels of learning of the single flies. Flies that made the correct response in the first test had the same PI as flies from the incorrect group when retested (Tully et al., 1994).

As stated before, *Drosophila* can associate odors with electric shock as a negative, as well as sugar as a positive reinforcer. Memory traces for both of these learning tasks have been localized to the Kenyon cells of the mushroom bodies (MBs) (Zars et al., 2000a; Schwaerzel et al., 2002). Electric shock is commonly used as aversive reinforcer during olfactory conditioning in *Drosophila* (Tully & Quinn, 1985). Yet, until recently no neuronal correlate for processing information about electric shock had been identified (Yu et al, 2004; Fiala et al. 2005). *Drosophila* can be trained to discriminate not only between different odorants but also between different concentrations of the same odor. Concentration differences as small as 1:2 can be discriminatively learned (Dudai et al., 1977; Borst, 1983).

1.4. Genetic dissection of olfactory memory

Learning and memory as everything else in animal life have genetic components. The discovery of such components in mammals and later in flies (Tryon, 1940; McGuire and Hirsch, 1977) allowed for searching and isolating behavioral mutants in *Drosophila* (Benzer, 1973). Using an aversive olfactory conditioning paradigm (Quinn et al., 1974), Dudai et al. (1976) isolated the first learning mutants. The very first one, $dunce^{1}$ (dnc^{1}) is deficient in a cAMP phosphodiesterase normally degrading cAMP (Byers et al., 1981). *Rutabaga* (rut^{1}, Livingston et al., 1984) is deficient in a type I Ca^{2+}/calmodulin dependent adenylyl cyclase (AC) synthesizing cAMP (Levin et al., 1992). Both are part of the cAMP second messenger pathway (Fig. 1.3). The cyclase is homologous to the mammalian type-1 adenylyl cyclase and is responsive to both, G-protein and $Ca2^{+}$/CAM dependent stimulation (Dudai et al., 1988). This co-activation property suggested that the adenylyl cyclase could be a molecular detector of coincidence between the conditioned stimulus (odor) and the reinforcer during classical (Pavlovian) learning (Dudai et al., 1988; Abrams and Kandel, 1988; Anholt 1994).

Both the *dnc* and *rut* gene products are highly expressed in the intrinsic cells of the MBs, the Kenyon cells (Nighorn et al., 1991; Han et al., 1992; Crittenden et al., 1998). As they receive massive olfactory input via the calyx, the Kenyon cells of the MBs have been proposed to represent the anatomical level of coincidence detection between the olfactory impulses (CSs)

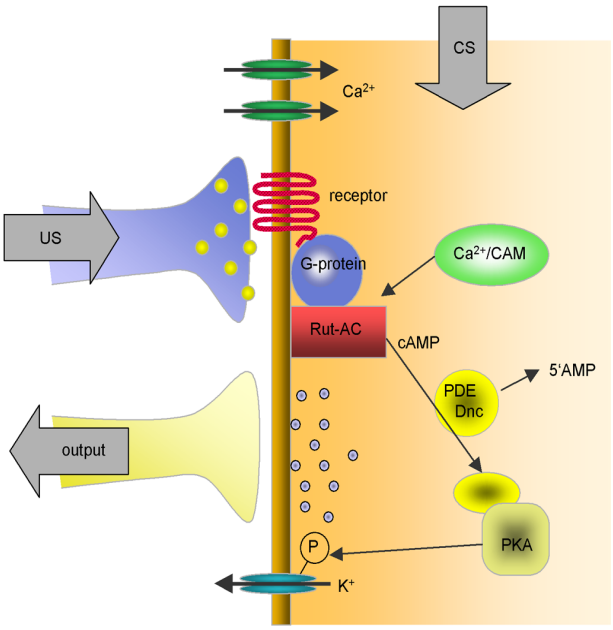

Figure 1.3. The cellular model of olfactory learning. Presynaptic modulation of transmission at Kenyon cell synapse to output neuron is thought to underly olfactory short and middle term memory in Drosophila. Simultaneous arrival of two unrelated stimuli, olfactory via Kenyon cells (CS) and reinforcing via modulating neurons (US) activate adenylyl cyclase A (rut-AC), via calcium/calmodulin increase in Kenyon cells, caused by CS and activation of G-protein coupled receptor for neuromodulator released from US-delivering neuron. Rut-AC activation leads to increase of cAMP level. Elevated level of cAMP lead to activated protain kinase A (PKA), which may phosporylate target proteins at the synapse. This coincident neural activity results in altered synaptic responses to subsequent CS presentation and modification of motor output driven by follower neurons. Phosphodiaesterase (PDE) degrades cAMP back to 5'AMP, and brings the increased level back to the original state. (modified from Heisenberg 2003)

and the reinforcement (US) during olfactory conditioning (Fig. 1.2). Indeed, those cells have been shown to be necessary for olfactory learning by several experimental approaches (Heisenberg et al., 1985; deBelle and Heisenberg, 1994). In addition, disrupting normal cAMP signaling in the MBs by expressing a constitutively activate $G\alpha_s$ subunit abolishes olfactory learning (Connolly et al., 1996). The biogenic amines dopamine and octopamine are suggested to be molecular representations of the reinforcing capacities of electric shock and sugar, respectively (Schwaerzel et al., 2002). Receptors for these biogenic amines (DAMB; dDA1 for dopamine and OAMB for octopamine) have been found to be coupled via G-proteins to AC and were found to be expressed at elevated concentrations in the MB lobes (Han et al., 1996; Crittenden et al., 1998; Han et al., 1998; Kim et al., 2003). All this evidence suggests *rut*-AC to be a coincidence detector underlying the convergence of pathways from the odor and the electric shock (or sugar, respectively) reinforcement. Expressing *rut* exclusively in subsets of Kenyon cells in otherwise *rut*-deficient flies, Zars et al. (2000) showed that *rut* was not only necessary but also sufficient in these neurons to allow for olfactory learning. Finally, it could be shown that this function is required during adulthood and not during development (McGuire et al., 2003; Mao et al., 2004).

Expression of the protein kinase A (PKA) catalytic and regulatory subunits is also elevated in the MBs (Crittenden et al., 1998; Skoulakis et al., 1993). Disrupting PKA activity globally with inducible inhibitory transgenes acutely reduces olfactory learning (Drain et al., 1991). Furthermore, flies mutated in the genes for the catalytic or regulatory subunits of PKA are

deficient in learning and memory (Skoulakis et al., 1993; Goodwin et al., 1997; Li et al., 1996).

Amnesiac (*amn*) is a memory mutant which affects a very early stage of olfactory memory but not the initial learning (Quinn et al., 1979). The *amn* gene encodes a homolog of vertebrate pituitary adenylyl cyclase-activating peptide (PACAP), and it is strongly expressed in dorsal paired medial (DPM) neurons (Keene et al., 2004). They project to all lobes of the MBs and seem to have modulatory function. Expressing the intact *amn* gene in DPM cells restores normal olfactory memory to *amnesiac* flies. Blocking synaptic transmission from the DPM neurons blocks one-hour memory, but leaves 3 min memory intact (Waddell et al., 2000). Moreover, the output from DPM neurons is only required during consolidation phase of the MTM but is dispensable during acquisition and retrieval (Keene et al., 2004). It is proposed that the *amn* neuropeptide, released onto the Kenyon cells in the MB lobes, triggers a prolonged activation of the cAMP pathway, which would lead to the consolidation of STM into more permanent memory (Faeny and Quinn, 1995). Duration of cAMP-dependent PKA activation is believed to determine whether only the short (STM) or also middle (MTM) or long-term memory (LTM) is formed (Li et al., 1996).

In *Drosophila*, several types of odor memories were proposed (Fig. 1.4, for review see Dubnau et al., 2003). Immediately after learning, short term memory (STM) is created, which last presumably only one hour and is succeeded by middle term memory (MTM). Two types of consolidated memory of conditioned odor avoidance one day after extended training have been described. They are genetically distinct and functionally independent memory components: anesthesia-resistant memory (ARM), a shorter-lived form, and stabilized long-term memory (LTM). ARM decays within four days, is resistant to cold shock, is insensitive to the protein synthesis inhibitor cycloheximide (CXM), and is disrupted by the *radish*^{1} mutation (Chiang et al., 2004). LTM shows no appreciable decay over 7 days, is sensitive to CXM, and is not disrupted by the *radish*^{1} mutation (Tully et al., 1994). It was shown that these two memories do not coexist but that LTM formation leads to the extinction of ARM (Isabel et al., 2004). The α-lobes of the mushroom bodies are specifically necessary for an olfactory LTM, whereas they are not necessary for the shorter forms (Pascual and Preat, 2001) including ARM (Isabel et al., 2004).

The transcription factor cAMP response element binding protein (CREB) is a major target of PKA phosphorylation. Studies of flies with inducible CREB transgenes have shown that

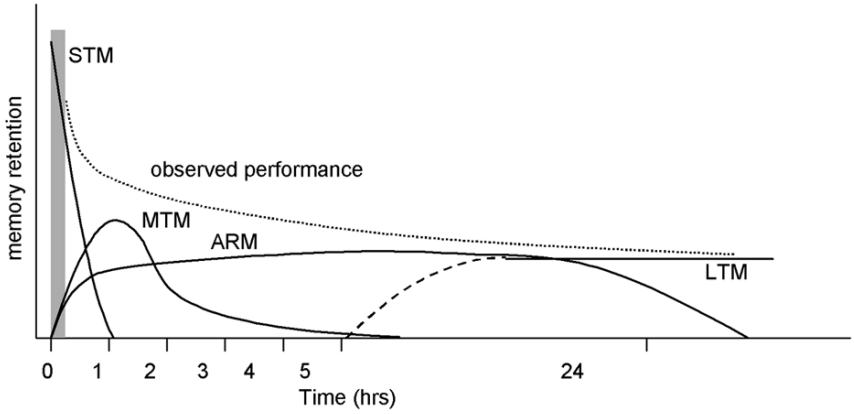

Figure 1.4. The behavioral model of memory formation. The decay of memory observed over time appears relatively seamless. Experimental disruptions, however, reveal several temporally, mechanistically, and anatomically distinct memory phases underlying memory retention, including short-term (STM), middle-term (MTM), anesthesia resistant (ARM), and long-term (LTM) memory. (modified from Dubnau et al., 2003)

CREB is crucial for protein-synthesis-dependent LTM formation (Yin et al., 1994; 1995). Experiments at the Drosophila larval neuromuscular junction suggested that CREB-dependent transcription is crucial for new gene expression that increases synaptic efficacy (Davis et al., 1996). Yet, the role of CREB in the formation of LTM in *Drosophila* is far from being understood as was shown by new experiments which revisited the previous findings (Perazzona et al., 2004). A simpler model where only the repressor isoform of CREB suppress formation of an LTM was proposed (Perazzona et al., 2004).

The dissection of memory into temporally distinct phases is commonly accepted and convergent with findings from vertebrates and invertebrates (Quinn and Dudai, 1976; Davis and Squire, 1984; Allweis, 1991; Squire, 1992; Folkers et al., 1993; Tully et al., 1994; Xia et al., 1997; Frankland et al., 1998; Milner, 1972; Scotville and Milner, 2000). In mammals, crosstalk between different regions of the brain is required to consolidate a memory from the short lived into more stable forms. These forms can be separated by pharmacological means and require different anatomical structures (Day and Morris, 2001).

In olfactory classical conditioning, *rut* mutants have significantly reduced memory even if measured in less than one minute after training (Dudai et al., 1988). Longer-lasting forms of memory than MTM do not seem to be affected in *rutabaga* (Dudai et al., 1983; Tully and Quinn 1985) and the residual memory consolidates into ARM (Dudai et al., 1988). At least ARM was confirmed to be *rutabaga*-independent (Isabel et al., 2004).

Increasing the intensity of the US does not improve immediate memory of *rutabaga* flies but slows down memory decay within the first hours (Dudai et al., 1983; Dudai et al., 1988). This suggests that other mechanisms that do not involve Ca^{2+}/calmodulin-activated AC cannot compensate for the absence of AC during acquisition, but can in the later phases of memory retention (Dudai et al., 1988). Even the process of consolidation to ARM may already start within the first fraction of a second of acquisition (Dudai et al., 1988). Also other learning mutants (*dunce*, *amnesiac*, *turnip*) that are impaired in STM but seems to be normal in LTM. This also supports the idea that there are two separate memory mechanisms and these mutations affect only the first one (Ferrus, 1992). Interestingly, saving experiments (*reversal training*) suggested the presence of a "mute" shorter form of memory in *rutabaga* flies and thus, defective retrieval (Dudai, 1983). In any case, a distinction should be made between acquisition measured by behavioral assays and the actual process of acquisition of new information by a molecular learning apparatus (Dudai et al., 1988).

1.5. Mushroom bodies

Mushroom bodies of *Drosophila* consist of about 2500 intrinsic neurons (Kenyon cells - KCs, see Technau and Heisenberg, 1982). The axons of these neurons form characteristically shaped structures extending from the back of the brain frontally and bifurcating into a medial and vertical lobe (Fig. 1.1G). Dendrites form the calyx as the main olfactory input region of the MBs (Strausfeld et al., 2003). The input pathways of other sensory modalities have not yet been identified.

Several classes of KCs are defined by antibodies against transmitters and neuropeptides, as well as by the expression of genes and by enhancer trap lines. In *Drosophila,* until now, five subdivisions of the MBs have been described (Strausfeld at al., 2003). Crittenden et al., (1998) and Lee et al. (1999) had already described three of them (α/β, α`/β` and γ). All adult KCs originate from four neuroblasts during larval and pupal development (Ito et al., 1998). Efferent neurons leave the MBs from lobes and from the distal peduncles and project to various anterior neuropil regions, contra and ipsilateral MB lobes and the lateral protocerebrum (Ito et al., 1998; Schürmann, 1987).

Function of mushroom bodies

The best-studied function of MBs is olfactory learning and memory (for review, see Davis, 2004; 2005) and MB-model of olfactory learning and memory was proposed (Fig. 1.5; Heisenberg, 2003). Already Dujardin (1850) who was the first to describe MBs observed that the largest and most elaborate MBs occur in social insects. Vertical lobes of the MB were assumed to play a crucial role in olfactory associations (Erber et al., 1980) and medial lobes and pedunculi were shown to be necessary for place memory (Mizunami et al., 1998).

In *Drosophila* several independent techniques were developed to ablate or functionally block MBs. First, two structural brain mutants with deranged or reduced MBs were reported to be defective in olfactory learning and memory (Heisenberg et al., 1985). Flies with strongly reduced MBs could also be obtained by applying a cytostatic drug, hydroxyurea (HU) to the first larval instar (Prokop and Technau, 1994). These flies were also impaired in olfactory learning (de Belle and Heisenberg, 1994). Connolly et al. (1996) introduced the two-component GAL4 system (Brand and Perrimon, 1993) to MB research. Using MB specific driver lines and a constitutively active $G\alpha_s$ protein subunit presumably interfering with a neuromodulatory function involved in learning/memory, they blocked odor discrimination learning. Subsequently, M. Schwaerzel (personal communication) found reduced learning scores in this paradigm using different MB driver lines and, as effector, the bacterial gene for

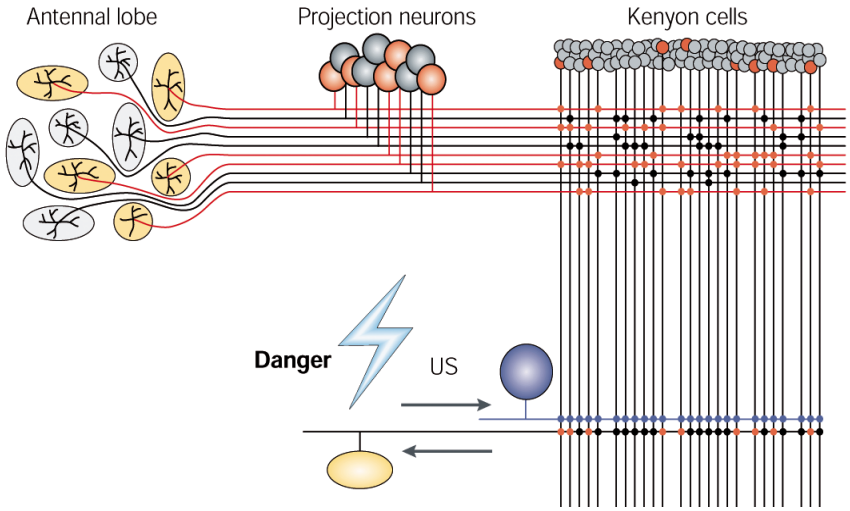

Figure 1.5. Mushroom body model of olfactory learning and memory. Particular olfactory stimuli activate distinct sets of projection neurons which differ in number of involved neurons and in level of activation based on the stimulus and its intensity. This activation of projection neurons is transformed into an activation pattern of a subset of Kenyon cells. These subsets are only combinatorial and lack the information about activation intensity of projection neurons. During training, a US-delivering neuron (dopaminergic neuron in this case) fires on every Kenyon cell. Only in those cells which are simultaneously stimulated by the olfactory stimulus, the output is strengthened. This altered output neuron(s) are supposed to cause the change in subsequent behavior in the test, where only the odor but not the reinforcer is presented (picture taken with permission from Heisenberg, 2003).

tetanus toxin light chain (TNT) blocking neuronal output. More recently, Kitamoto (2001) designed an effector gene carrying a dominant negative temperature sensitive allele of dynamin (UAS-*shi*^{ts1}). With this conditional effector it was shown that the output from MB neurons is necessary only during retrieval of memory but not during acquisition (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002). Other learning experiments involving olfactory cues as conditioned stimuli have been sensitive to MB ablation as well (see Wolf et al., 1998). Armstrong et al. (1998) proposed that the γ lobe stores information of relevance to both developmental stages, whereas the α and β lobes have uniquely adult roles. Recently, it was also proposed that MBs should be specifically needed for a naïve appetitive response towards odors but not for avoidance (Wang Y. et al., 2003).
Ablation of MBs also showed that they were necessary for context-dependent memory of heat avoidance in flight (Liu et al., 1999). WT-flies tolerate moderate context changes whereas in MB-less flies these already interfere with visual memory. In flight, flies lacking MBs had also difficulties with “conflict” situations (Tang and Guo et al., 2001). Flies with blocked or missing MBs show prolonged bout phases in spontaneous walking activity (Martin et al., 1998). Also, when flies reached a water barrier, those without MBs had difficulties to stop their attempt to reach an inaccessible landmark ("perseverance"; M. Mronz, personal communication).

As olfactory learning and memory are treated as the “main function” of the MBs, calyces are considered the main and primary input neuropiles to the MBs (reviewed in Davis, 1993; Heisenberg, 1998; 2003). However, phylogenetic studies suggest that the MBs evolved long before the origin of the calyces and antennal lobes (Strausfeld, 1998) suggesting that the whole airborne olfactory perception is evolved after the evolution of MBs itself. The most primitive ectognathan insect, Archeognatha, lacks MBs entirely. Calyces are found only in neopteran insects. But secondarily, also some neopteran aquatic insects lost their antennal lobes and calyces but still retained vertical and medial lobes (Strausfeld et al., 1998). EM studies show that Kenyon cell axons are both pre- and postsynaptic in the lobes, where they provide local circuits between afferent processes ending in the lobes and dendrites of efferent neurons leaving them (Strausfeld and Li, 1999).

1.6. Genetic tools in *Drosophila* behavioral studies

The beauty of studying the behavior in *Drosophila* lies in the well-developed genetic methods and in the easiness of using them to alter this behavior.

Several systems are available for regulating transgene expression in the fly. The heat-shock promoter (hsp-70 system, see Pirrota, 1988) provides temporal control by inducing expression with exposure to elevated temperature. The GAL4/UAS-system (Brand and Perrimon, 1993) provides spatial control. The GAL4/UAS-system consists of two transgenic components, each inserted separately into the genomes of either the GAL4-fly or the UAS-effector-fly allowing expression of any transgenic effector in a spatially restricted pattern (Brand and Perrimon, 1993). For this purpose a construct carrying the gene of the yeast transcription factor GAL4 is inserted into the *Drosophila* genome (GAL4-driver line). Depending on the insertion site in the genome, GAL4 expression is driven in a spatial pattern, controlled by endogenous enhancer elements in the vicinity of the place of insertion. The UAS-effector line carries a second construct with the GAL4 binding sequence (UAS) and the effector gene. After crossing the respective GAL4 line with the UAS-effector line, in the progeny, the effector is expressed only in those cells in which GAL4 is present. This system allows gathering the desired flies by a simple cross in large numbers as is necessary for behavioral studies. In combination with temporal control the system avoids developmental effects that could influence the flies' behavior.

Probably the most powerful effector gene for studying the relation between behavior and neuronal structures in the *Drosophila* brain is UAS-*shi*^{ts1} transgene that is ambient-temperature sensitive (Kitamoto, 2001). Together with the GAL4 lines, which provide the spatial control over the affected neurons, UAS-*shi*^{ts1} allows temporal control over synaptic transmission in these neurons (Waddell et al., 2000; Dubnau et al., 2001; McGuire et al., 2001; Kitamoto, 2002; Schwaerzel et al., 2002). S*hibire* (*shi*) is a dominant negative allele of the *Drosophila* dynamin gene (Chen et al., 1991), which is involved in endocytosis and is essential for synaptic vesicle recycling and for release of vesicles (Kosaka and Ikeda, 1983). The temperature-sensitive allele *shi*^{ts1} is defective at restrictive temperatures (> 29°C) and results in rapid (~1 min) and reversible inhibition of synaptic transmission (Koenig et al., 1983). Neurons expressing the *shi*^{ts1} allele are inhibited in neurotransmission at the restrictive temperature (Waddell et al., 2000; Dubnau et al., 2001; McGuire et al., 2001; Kitamoto 2001, 2002; Schwaerzel et al., 2002).

The newly developed GAL80 system is able to increase the precision of the GAL4 system by allowing transgene expression in only a subset of neuron that express the GAL4 (Lee and Luo, 1999). This system also allows for both temporal and spatial control of gene expression using the conventional GAL4/UAS system and a temperature-sensitive version of the GAL80 protein ($GAL80^{ts}$) (McGuire et al., 2003). As an alternative, the GAL4-based Gene-Switch system has been engineered to regulate transgene expression in *Drosophila* in both time and space using the pharmacological ligand RU486 (Mao et al., 2003).

1.7. Motivation to investigate intensity dependent behavior and neuronal plasticity

Drosophila can learn and discriminate different odorants when paired with either reward or punishment (e.g. electric shock as a negative or sugar as a positive reinforcement). Both of these memories are localized to the intrinsic neurons of the MBs (Zars et al., 2000; Schwaerzel et al., 2002). Many genes involved in the formation of olfactory short-term memories have been described and have demonstrated the cAMP signaling pathway as the central process governing the plasticity (Tully and Quinn, 1985; Connolly et al., 1996; Han et al., 1992; Levin et al., 1992; Zars et al., 2000) Short-term memory mutants *rutabaga* and *dunce* are well established examples of genes that belong to the cAMP pathway. Their learning score is about 50% of that of the wild type flies. Based on this research, the MB-model of olfactory learning and memory was suggested (Heisenberg, 2003; Gerber et al., 2004). This model is in agreement with recent findings and could also be effectively used to predict unexplored aspects of olfactory STM.

Many interesting questions about concentration learning have arisen in recent time. The MB-model predicts that learning of different concentrations either is not possible as was previously demonstrated in other model systems (e.g. honeybee, Pelz et al., 1997), or is possible (Borst, 1983) only to a limited extent. The ability of the fly to discriminate between similar concentrations of the same odor, in comparison to the discrete stimuli in two-odor learning demands a detailed investigation of a possible concentration dependent memory. In addition, also the more general question of representation of odor intensity in the *Drosophila* brain needs consideration. This representation must allow the same two concentrations to be generalized and discriminated in the same task.

With the help of a forced T-maze assay developed by Tully and Quinn (1985) I have tried to answer some of these question. I studied the behavior underlying concentration dependent

plasticity. I wanted to find out whether concentration learning is associative or non-associative. Being capable of learning concentrations, flies may learn relative or an absolute concentration. As concentrations change gradually, the fly needs some kind of concentration invariance to recognize an odor as the same despite of different concentrations (Borst, 1983). I dissected the range of possible concentrations into the concentration invariant part and the part where two concentrations are qualitatively different. I separated them by their system properties and by showing that only learning of odor quality is *rut*-dependent.

Finally, I propose the existence of a novel STM called odor-intensity memory, which has different properties from the previously established olfactory STM and is independent of the *rutabaga*. The possible neuronal substrate underlaying this memory and the potential storage site will be discussed.

The existence of more than one STM suggests that usage of the particular memory depends on the information available to the fly during the test. The learning performance of the fly is not necessarily a faithful measure of the memory(ies) established during conditioning.

1.8. Terminology of olfaction and olfactory memories

Odorant - chemical that produces cue (an odor) perceivable by olfactory system

Odor - air-born part of odorant

Scent or Smell - subjective percept of an odor

Concentration - amount of a substance (odorant) in a solvent (can be given in molar concentration or as weight/volume

Intensity - subjective perception of the odor concentration

Olfactory learning - any associative learning in which an odor is the conditioned stimulus (CS)

Two-odor learning - olfactory learning in which two odors are presented as CS^+ (paired) and CS^- (unpaired) in the memory test. (contains odor-quality as well as odor-intensity learning; see below)

Concentration learning - olfactory learning in which the two CSs are represented by two concentrations of one odor (contains odor-quality as well as odor-intensity learning; see below)

Odor learning (smell or scent learning) - olfactory learning in which the two CSs are qualitatively different (the stimuli are not generalized)

Odor-intensity learning - olfactory learning in which the two CSs are qualitatively the same and differ only in their intensity (the stimuli can be generalized)

Odor-quality learning - olfactory learning in which the two CSs differ only in their quality but not their intensity (has not been realized experimentally so far)

2. Material and Methods

2.1. Fly care

All flies were raised on corn-meal food (Guo et al., 1996) in 14:10 hours light:dark cycle at 25°C and 60% relative humidity. Experimental flies were fed on fresh food vials for up to 48 hours before being tested. For behavioral experiments, I used 3 to 6 day old males and females in mixed groups, either taken from homozygote lines or from progeny of crosses between homozygote parental lines. All behavioral experiments were done at 26°C and 80% relative humidity, under red light (invisible to the flies) during the training phase and in complete darkness during the test

2.2. Genotypes

I used the Canton-S (Wuerzburg) as a wild-type control. For *rutabaga* experiments, null alleles rut^{2080} and rut^{2080}; $^{+}$; UAS-rut^{+} lines were used. Canton-S served as a control for these flies. For *dunce* experiments was used amorphic allele $dunce^{M14}$ (Davis and Kiger, 1981). Behavioral experiments were done with animals from these homozygous lines.

For neurotransmission blocking experiments, I used mushroom bodies specific Gal4-line MB247 (Zars et al., 2000) to drive transgene expression within the Kenyon cells of the MBs. This line drive expression in nearly all subsystems of the MB. The other line was GH146 that drives expression in projection neurons connecting the antennal lobes with the mushroom bodies and the lateral protocerebrum (Stocker et al., 1997; Jefferis et al., 2001; Fiala et al., 2002; Ng et al., 2002). I used GH146 GAL4-line with Cameleo (Fiala et al., 2002)

For temperature-dependent blocking of synaptic transmission, I used progeny of crosses between the homozygous parental lines UAS-shi^{ts1} (as virgin females) and GAL4-lines (as males). The line UAS-Shi1 contains multiple inserts of UAS-shi^{ts1} on the 3rd chromosome, whereas UAS-Shi2 bears inserts on X and 3rd chromosomes (Kitamoto, 2001).

Line	Genotype	Reference
Canton-S	wild-type	from Wuerzburg
rut^{2080}	*rut*-P-element containing wild-type *rosy*-cDNA; X chromosome.	Zars et al., 2000a
rut^{2080}; $^{+}$; UAS-*rut*$^{+}$	*rut*-P-element containing wild-type *rosy*-cDNA; X chromosome. Transgene-insertion containing wild-type *white*-cDNA; 3rd chromosome.	Zars et al., 2000a
dnc^{M14}	EMS-mutation; with FM7a balancer	Meinertzhagen et al. 1998
MB247-GAL4	*White*-, P-element containing wild-type *white*-cDNA; 3rd chromosome.	Zars et al., 2000a
$^{+}$; UAS-*Cameleon 2.1* 82/GH146-GAL4; $^{+}$	*white*-,P-element containing wild-type *white*-cDNA; 2nd chromosome. Transgene-insertion of *Cameleon 2.1* 82 cDNA; 2nd chromosome.	Spall, 2004
UAS-Shi2	*white*-,multiple UAS-*shi*^{ts1} transgene insertions containing wild-type *white*-cDNA; 3rd and X chromosome.	Kitamoto, 2001

2.3. Behavior paradigm

Flies were trained and tested in T-maze paradigm - the classical conditioning procedure as described by Tully and Quinn (1985). All experiments were done with modified version of that machine (description in Schwaerzel et al., 2002). About 50-100 flies were placed into a training tube, the inside of which was covered with an electrifiable copper grid. Flies were then exposed sequentially to two odors, which were carried through the training tube with a current of air. The air stream was created by sucking the air through the machine by a pump on the constant speed (750 ml/minute). During 60 seconds exposure of the first odor (CS^{+}) flies were given 12 times electric shock (US) of 90 V and 1,3 seconds duration. After 45 seconds of fresh air, flies were exposed for another minute to the second odor (CS^{-}) but now without the electric shock, followed by another period of 45 seconds of air. This completed one training cycle. After training, flies were gently tapped into the middle compartment. After next 100 seconds the middle compartment was shifted that way, that flies were transported to the choice point of the T-maze, where they were exposed to two air streams

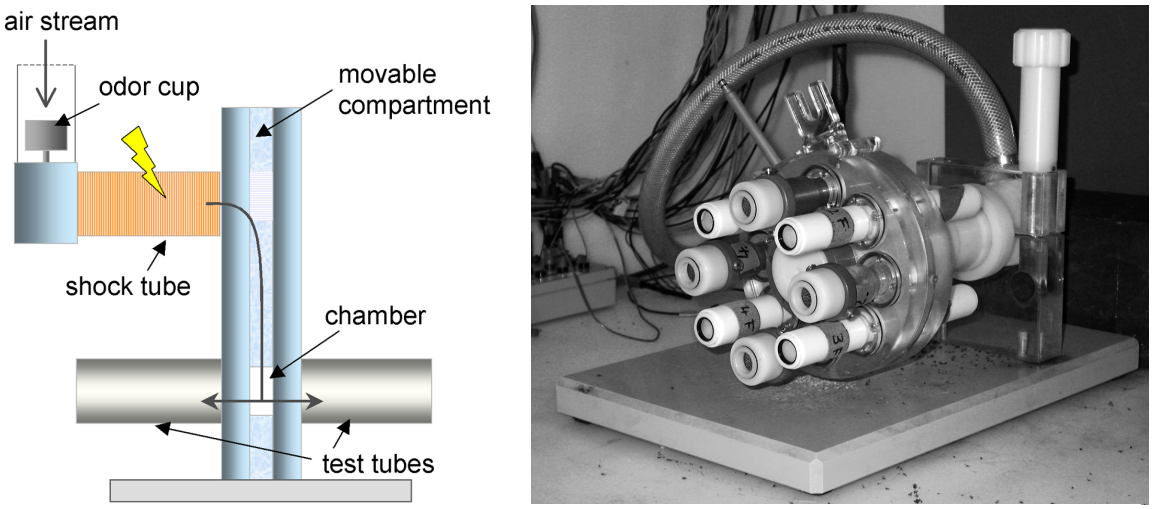

Figure 2.1. T-maze olfactory learning device. (**A**) The principle of the paradigm is based on the T-maze device of Tully & Quinn (1985). At the beginning of an experiment, about 50-100 flies are put into a training tube and from there gently tapped into the chamber. For the test, the movable compartment is pushed down and the chamber is aligned with the two tubes at the choice point. From there, flies can enter one of the two tubes. With no stimuli presented, flies distribute equally between the tubes. The apparatus consists of two horizontal (test) tubes, opened to the space of the chamber in the movable compartment. At the top of the machine is training (shock) tube lined with copper wire connected to electric shock generator. To all of those tubes, a holder with teflon odor cup can be attached. Air is continuously pumped from the machine at a constant flow rate of 750 ml/min. To test a naive response towards an odor, shock tube is used only to collect flies before they are tapped to the chamber and slided to the choice point. No electric shock is given. For learning experiments, flies are given a series of electric shocks while in the shock tube. (**B**) The actually used machine is modified (Schwaerzel et al. 2001) T-maze in that way it consists of four independent T-mazes placed on a rotating disc. This design allows to train and test four independent groups of flies simultaneously.

carrying one of the two odors. One scented with the formerly shocked, the other one with the formerly non-shocked odor. Flies were allowed to choose between them for 120, 60 or 15 seconds, depending on the experiment. In the end of the test, flies were trapped in one of the two tubes, collected, anesthetized, and counted.

Two groups of flies were usually reciprocally trained and tested for one complete experiment. Memory score for one of those groups is a "half score" (Keene et al., 2004). Punished odor (CS^+) from the first experiment became the non-punished one (CS^-) in the second experiment. For each experiment, performance index as $PI_{1,2} = (N_{\text{non punished}} - N_{\text{punished}})/(N_{\text{punished}} + N_{\text{non punished}})$ was calculated and averaged over these two complimentary experiments as $PI = (PI_1 + PI_2)/2$. Averaging of the two reciprocal scores eliminates possible bias related to the machine.

For two-odor learning I used two different sets of odorants. The test odorants were placed at the end of the tubes in small cups of various diameters to adjust spontaneous balance between the two odors. The first set consisted of benzaldehyde (BAL; Fluka) presented in a 4,3 mm diameter cup and 3-octanol (3-OCT; Fluka) in a 15 mm cup as pure substances or diluted in paraffin oil (Fluka). The second set consisted of isoamylacetate (IAA; Aldrich) and

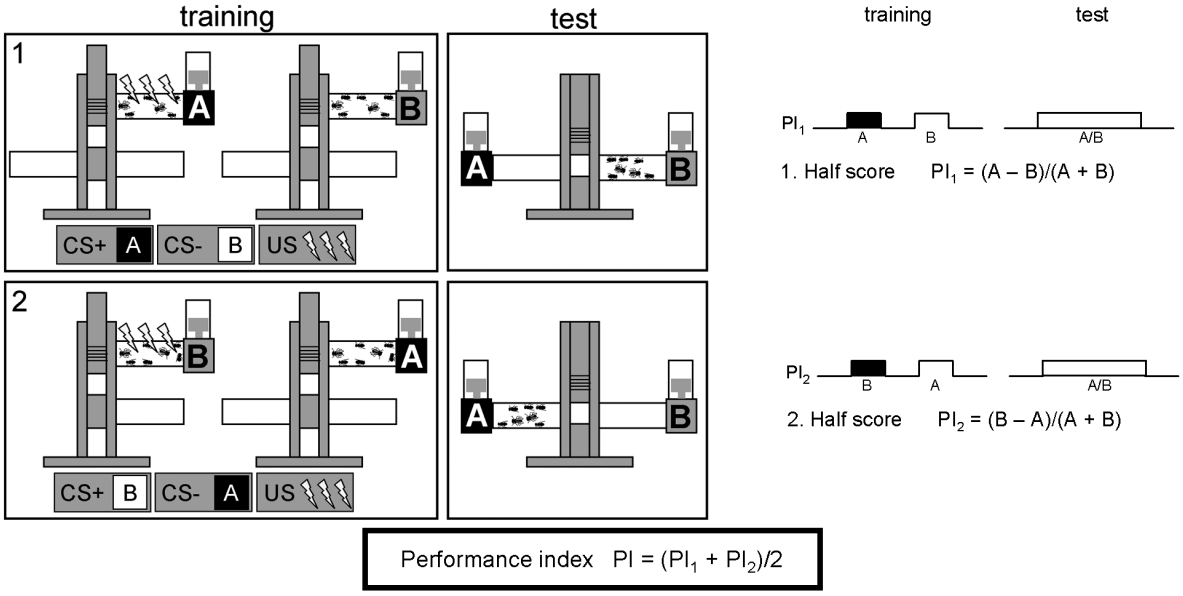

Figure 2.2. Electroshock learning procedure. About 50-100 flies are put in the 'shock' tube. There, they recieve twelve electric shocks within 60 sec. period in presence of olfactory stimulus 'A'. After 45 sec. of air exposure, they are exposed in the same tube for next 60 sec. to the stimulus 'B' but now without accompanying electric shock. After training, flies are tapped into small chamber in a movable compartment and after 100 sec. break, they are tested. During the test, they are exposed, at the choice point, to both stimuli 'A' and 'B', which are placed on the opposite tubes. This procedure makes only half of the final score. Therefore called ‚half score' (Keene et al. 2004). In the other half of the experiment, the previously shocked olfactory stimulus 'A' become the non-shocked and the other way round for the stimulus 'B'. For each half experiment is calculated PI and these PIs are averaged to get rid of a potential bias in the machine or in the stimulus balance.

amylacetate (AM; Merck), both in 15 mm cups. Both odor pairs were set up that way that under described conditions, naive flies showed no overall preference for one of the two odorants over the other. Pure odorants in these cup sizes elicit high spontaneous avoidance against air (PI around -0,7 to -0,9). As the odors are diluted, naive avoidance response decreases and therefore low concentrations of all these odorants leads to the indifferent response when compare to air (PI ~ 0) or, to the preference of the odor over air (PI > 0) .

BAL and 3-OCT are traditionally used odorants for *Drosophila* learning experiments. They are chemically very different from each other. In addition, BAL was reported to be distinct from other odorants in the way how the memory is processed (Keene et al., 2004). IAA and AM were used because they seems to be more natural odorants for *Drosophila*. IAA is a key component of rotting fruits and it signal a food for *Drosophila* (Stensmyr et al., 2003). AM is on of the key part of an overripe mango smell (Zhu et al., 2003).

For concentration learning, instead of two odorants, two different dilutions of one odorant were used the same way as described above for two different odorants. One concentration served as an "odor A" and the other as an "odor B". A choice was provided between two concentrations of IAA, EA or BAL diluted in paraffin oil. For purely practical reasons

odorants were diluted in steps of 6:1 [log6]. These steps are denoted as nx, i.e. a dilution in 3 steps of 6:1 (6^3:1 = 216:1) would be written as 3x.

It should be noted that the absolute concentrations of the odors in the air stream (molecules/volume) is not known. Concentrations of odors in the tubes correspond to the dilution prepared and filled into the odor cups placed on the end of training or test tubes. There was no measurement done regarding concentrations inside of the tubes. These values strongly depend on air flow within the machine and therefore absolute values in this study cannot be directly compare with values in others experiments done in different apparatuses. On the other hand, ratios between different concentrations in our experiments should be constant. The same approach was used in other studies as well (Wang Y. et al., 2003).

3. Results

3.1. Behavioral characterization of olfactory learning in the T-maze

3.1.1. Non-associative effects

The T-maze olfactory paradigm of Tully and Quinn (1985) is commonly used for experiments on olfactory learning and memory and is reported as a pure classical associative learning paradigm. The learning score which is obtained in a choice behavior between two odors, is supposed to be solely due to the pairing of one of the odors (CS^+) with electric shock (US) (Tully and Quinn, 1985). That the learning index is independent of any non-associative effects would be clear, if in the test, the only difference between the two odors were the previous pairing and non-pairing with the shock. This condition cannot be met for the single learning trial but can be approximated by summing all the possible permutations of the training procedure into the final (averaged) score. However, in most protocols (Tully and Quinn, 1985; Zars et al., 2000; Schwaerzel et al., 2003) one permutation is omitted and, therefore, a second difference distinguishes the two odors: It is always the unpaired odor (CS^-) that had been presented last in the training session. Hence, because of this exposure the fly might be differently adapted to the CS^+ and CS^- during the test, and this adaptation might systematically influence the learning score.

Here I show that under standard conditions the learning score, indeed, consists in part of a non-associative component caused by exposure of the flies to the odors during training. I performed a series of experiments with high concentrations of benzaldehyde (BAL) and 3-octanol (3-OCT), presenting one or both odorants with or without electric shock in several combinations. In addition to the standard procedure, I exposed flies only to the CS^+ while the following CS^- was omitted and was substituted by exposure to air. Further training sequences consisted of exposure to air followed by the CS^+ or the CS^-, the CS^- followed by air, and finally, the $CS1^-$ followed by $CS2^-$. In all cases, test conditions were the same as in the standard procedure (Fig. 3.1A).

In the standard experiment (training procedure 1 in Fig. 3.1A,B), flies showed a learning score (performance index) PI= -0,77 (see Fig. 2.2 and Materials and Methods for calculation of PI values). All other scores were significantly reduced. Flies provided with one CS, regardless of being CS^+ or CS^-, performed about 50% lower (PI= -0,33 and 0,35) (training

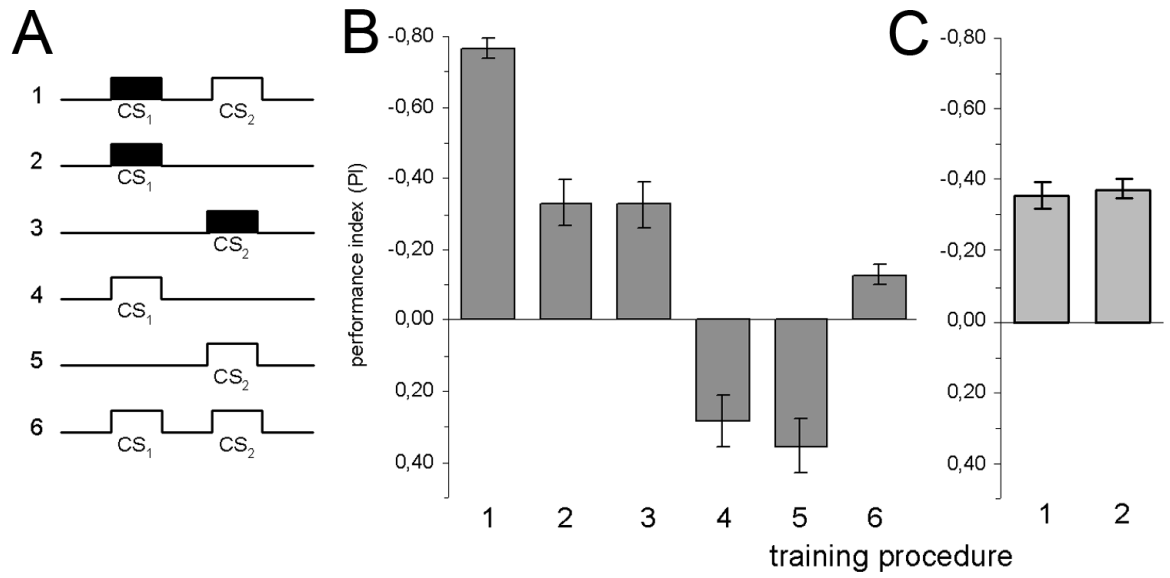

Figure 3.1. Odor adaptation causes non-associative learning effect in the T-maze paradigm. (**A**) Alternation of the presence or the absence of CS^+ or CS^- during the training influence test performance (**B**). When flies are tested with high concentrations of undiluted odorants, they perform very well (PI= -0,77) after the standard training procedure (**1B**). When CS^- odor was omitted during training and flies were instead exposed only to air, their performance in the test dropped to about 50% of the previous score (PI= -0,34) (**2B, 3B**). Similar effect but in the opposite direction was caused by exposure to CS^- odor only. In this case, flies preferred in the test the previously presented odor (**4B, 5B**). Spontaneous response to choice between 3-OCT and BAL with preexposure to both odors without shock caused significant score of avoidance of the first presented odor (PI= -0,13) (**6B**). (**C**) Diluted odorants caused no measurable adaptation effect. Learning scores with two diluted odorants (4x diluted 3-OCT and BAL) from standard training procedure (**1C**) and after omission of CS- (**2C**) are almost identical, showing that presence or absence of CS^- during training does not influence olfactory learning. Spontaneous response to choice between 3-OCT and BAL (pure (**B**) or diluted (**C**)) without any preexposure is balanced between these two odors (PI~ 0).

procedures 2 and 5 in Fig. 3.1A,B). Flies only exposed to the CS^+ during training avoided this previously punished odor in the test. If flies were exposed only to one CS^-, they always preferred this odor in the choice situation during test. As under this conditions flies were not exposed to the US, I assume that the shift in preference between the two odors used in the test was due to the adaptation caused by the exposure to the odors during training. This decrease of avoidance of the odor that was experienced during training occurred irrespective of whether it was followed by exposure to air (training procedures 4 and 5 in Fig. 3.1A,B). The only difference was that the shift was slightly smaller for the sequence CS^-/air than for the inverse sequence air/CS^-. In the second sequence, the odor exposure was closer (in time) to the test, therefore I assume that the length of the period between odor exposure and the memory test caused this difference. If the odor was presented at the beginning of the training phase, the time was 4 minutes from the end of the odor exposure to the start of the test. If the odor was presented after the air exposure, time until test was only 2,5 minutes.

The PI obtained after presenting only the CS^- was equal but in fact opposite to the score obtained by training with CS^+ (and the US) (training procedures 4 and 5 versus 2 and 3 in Fig. 3.1A and B). Adding the negative and positive scores together gave almost the standard learning score (Fig. 3.1A,B). Thus the decrease in the PI after training with CS^+/air and the PI after training with CS^-/air or air/CS^- only was caused by adaptation to the odor presented during the training phase. In the standard procedure (CS^+/CS^-), flies were adapted to both odors and thus those adaptation effects largely canceled out and most of the score could be attributed to the association made between CS and US. The non-associative part, however, still could be measured: exposing flies to both CSs but without US produced a significant score (PI= -0,13). This was the difference in the level of adaptations between the odor to which flies were exposed first (CS1) and the odor they were exposed to later (CS2). Tully and Quinn (1985) have also shown that this small non-associative component is indeed part of the standard learning scores.

The adaptation effect could seriously confound experimentation if only one CS would be presented during training or if different CSs would be used in the training and in the test. Also, due to adaptation, the perceived intensity of an odor would be higher in the training than in the test. Flies would have to generalize between these two stimuli to perform well. Inability to generalize, stronger (or faster) adaptation or longer lasting adaptation as might be observed in mutants, would lead to reduced learning scores even though the learning and memory abilities would be intact. Fortunately, this level of adaptation is concentration dependent (Stoertkuhl et al., 1998) and therefore using diluted odorants abolishes the adaptation effect but keeps the overall learning score at a sufficiently high level comparable to that of undiluted odorants (Fig. 3.1C, 3.2B and 3.4).

3.1.2. Significance of CS^+ and CS^-

In the standard procedure two odorants are used during training and test. During training, one of them (CS^+) is paired with the noxious stimulus (electric shock), the other odor is presented without shock (CS^-). In the simplest case, only the paired odor should be remembered (Borst, 1983). The experiment of Fig. 3.1C, performed with sufficiently diluted odorants that cause no visible adaptation, is in line with this conjecture. There is no difference between the sequences CS^+/CS^- and CS^+/air. The CS^- is dispensable for associative learning in the T-maze paradigm granted that adaptation does not bias the learning score.

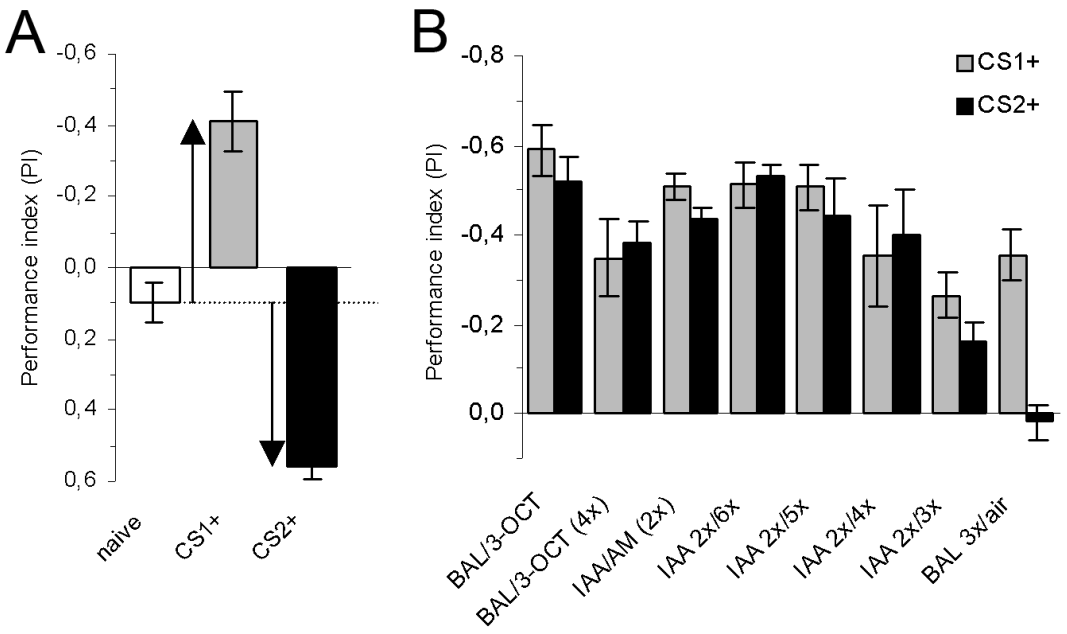

Figure 3.2. Both half scores participate equaly on the final score. (**A**) Flies without any training experience have certain naive preference between presented stimuli during the test. Flies shocked in presence of one CS avoid it in the test later on and run towards the other. Pairing shock with the other CS leads to the avoidance, which is opposite in direction to the first one. (**B**) Performance after training showing half scores after CS1 or CS2 were shocked (the average between them gives standard PI). The avoidances after pairing shock with CS1 or CS2 are equal. The input from the two stimuli to the final learning score is thus symmetrical regardless of type of stimulus. The symmetricity is a characteristic of all measured olfactory discriminative learnings where the two stimuli are two odorants of various dilutions or two concentrations of one odor. The only exception is an odor tested against air, because air cannot be associated with shock.

After training in the new version of the T-maze paradigm (Tully and Quinn, 1985), the decision in the choice point is made very fast and once the respective tube is chosen this choice is never corrected. In contrast, after the training in the old setup version (Dudai et al., 1977), flies were repeatedly running during the test between the tubes (Borst, 1983). My observation performed in red light revealed that flies decided at the choice point once and very quickly (within 5 sec) and then stayed in the chosen tube without moving further. They did not correct their first choice. To support this observation, I carried out two experiments, which differed only in the time devoted for the test. In the first experiment the test lasted for the usual 120 sec (Tully and Quinn, 1985; Schwaerzel et al., 2003) and in the other just for 15 sec. Even such a short time was sufficient to provide the same scores as seen after usual 120 sec (PI= -0,38, ±0,06 and -0,34, ±0,04, respectively).

3.1.3. Two-odor learning is symmetrical

The two odors used for odor discrimination learning are adjusted in their concentrations such that during the test naïve flies distribute about equally between the two tubes (PI ~ 0). During the training, one odor is paired with shock and as a consequence, in the test, it is avoided more than the other one. The amplitude of this shift in avoidance is equal to that of the shift if the other odor is paired with shock (Fig. 3.2A). In the discrimination task, both odors participate equally in generating the distribution of flies between the two tubes. This rule is valid regardless of concentration or the values of the learning scores obtained by testing single odorants against air. The latter means that two odors that would have different learned avoidance after being trained and tested against air would still produce equal shifts if tested against each other (see also Fig. 3.7 and 3.14). The previous statement holds in broad range of concentrations but when the odor is diluted such that the associative strength between this concentration and shock is only incrementally larger than zero, the fly in the discrimination test would give the previously paired odor only a very slight disadvantage. This limitation is caused by the fact that clean air cannot be successfully associated with electric shock (Fig. 3.2B). There can be several reasons. 1) Flies are “adapted” to the air of the laboratory. 2) During "exposure to air" neurons are activated at baseline level. 3) After training with air, continuous airflow without electric shock eventually leads to memory extinction. Therefore, in a learning experiment with one odor only in training and test, half-scores cannot be symmetrical, because all the learning would come from the shock association with the odor and nothing from the shock association with air. I assume that decreasing the concentration of one odor below the detection threshold of the fly would decrease the corresponding half-score to zero. This would also hold for an experiment with two odors of which one could not be detected or memorized (Keene et al., 2004).

3.2. Odor intensity learning

Odor intensity learning should be defined as an association between an odor concentration and a US. This concept implies that flies perceive the intensity of a scent somehow independently of its quality. For flies lacking odor quality learning, a concentration ratio should be found for each pair of odors, for which no learning would be observed, because the two odor percepts would have the same intensity. As shown above, in odor discrimination learning (to be called two-odor learning) flies reveal a memory trace only of the odor that had been paired with the shock.

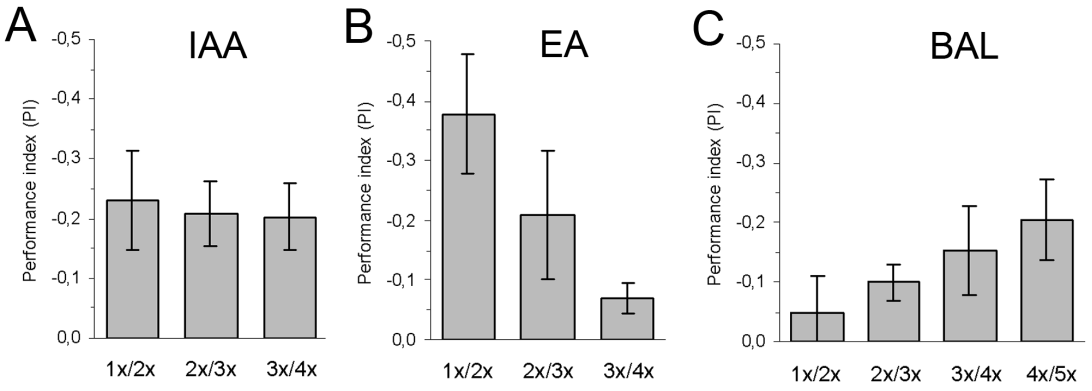

Figure 3.3. Concentration learning with three different odorants is differentialy dependent on overall odor concentration. For each measurement, always the same ratio (1:6) between the two test stimuli was used. But the overall concentration of the odors was different for each experiment. These three odorants show three different ways how flies react in learning performance to changing intensity of the olfactory stimuli. (**A**) With IAA flies do not change their performance. (**B**) For EA, learning performance is highest with high odor intensity and for BAL (**C**), the performance is negatively dependent on the odor intensity.

To investigate odor intensity learning I used the standard procedure and tested three different odorants (BAL, IAA, EA). For purely practical reasons odorants were diluted in steps of 6:1. These steps are denoted as nx, i.e. a dilution in 3 steps of 6:1 (6^3:1 = 216:1) would be written as 3x. In the following sets of experiments, I used dilutions at the relative concentrations of 1:6 keeping this ratio constant but changing the absolute concentrations (i.e. 1x/2x or 2x/3x, etc) (Fig. 3.3). Note that the absolute concentrations of the odors in the air stream (molecules/volume) is not known.

As shown in Fig. 3.3, even for the same ratio of concentrations, scores can vary depending on the overall concentration of the particular odorant (Fig. 3.3). For IAA no dependency on overall concentration was observed (Fig. 3.3A). This odorant has a flat odor response curve in the tested range of concentrations. For EA the learning score went down when the overall concentration went down (Fig. 3.3B). This odorant is very volatile and the effective concentrations of EA in the air stream might decrease during the training. At low overall concentrations flies might have difficulties to detect the concentration difference in the test. For BAL the learning score went down when the overall concentration went up (Fig. 3.3C). Likely, this is due to the very high avoidance (and possibly toxicity) of BAL at these concentrations. Pairing the shock with the high concentration of BAL may not further increase its avoidance. Moreover, the high concentration may cause adaptation and hence a shift in the perceived odor intensity between training and test. This would lead to a situation analogous to the "concentration shift" experiments (see Fig. 3.7).

3.2.1. Odor intensity learning is symmetrical

I have already shown that the two half scores in two-odor learning with two odorants added equally to the final learning score (Fig. 3.2). Certain models of two-odor learning, however, suggest that for odor intensity learning the half-score for the low concentration should not contibute to the final learning score (Heisenberg, 2003). First experiments with honeybees had shown that bees, indeed, appeared unable to solve the task when presented with rewarded low versus unrewarded high concentrations (Bhagavan and Smith, 1997; Pelz et al., 1997). Since then, however, experiments performed in a different way showed that bees could learn high and low concentrations equally well (Ditzen et al., 2003).

For odor intensity learning in flies, the half scores for both concentrations contribute to the learning score. This holds for high and low concentration ratios (Fig. 3.2B). At high concentration ratios the two half-scores clearly contribute equally. Even for IAA at a ratio of 1:6 the two half-scores are not significantly different from each other (and both are significantly different from zero). Evidently, as noted above, at concentrations below the detection threshold no memory trace can be formed.

3.2.2. The CS^- is dispensable in odor intensity learning

Using the training procedures 1 and 2 of Fig. 3.1A (CS^+/CS^- and CS^+/air), I performed analogous experiments for odor intensity learning. The test was always a decision between the two concentrations irrespective of whether one concentration was omitted during training or not. I conducted the experiment twice using the same high concentration in both experiments, but in a small and a large concentration ratio to the other concentration. For the small as well as for the large concentration ratio in the test, flies showed the same learning

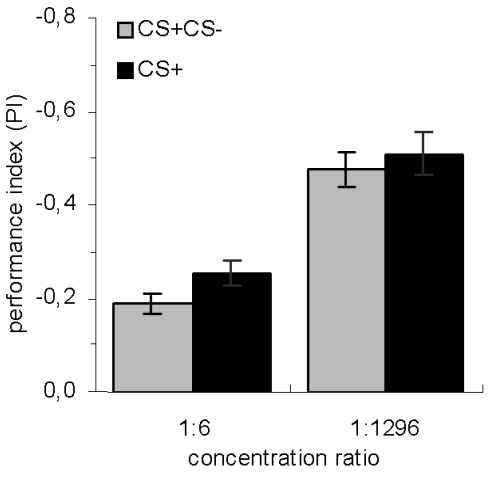

Figure 3.4. Olfactory learning of two concentration of one odor is not influenced by presence or absence of the CS^-. It shows that presence or absence of CS^- during training does not influence flies performance in the test even with odor concentrations. The first experiment was done with large relative dilution ratio 1:1296 (IAA, 2x versus 6x diluted) and the other with small relative dilution ratio 1:6 (IAA, 2x versus 3x diluted). Learning score after standard training procedures (CS^+/CS^-) and with omission of CS^- (CS^+/air) were almost identical (ANOVA; ps > 0,05). The results for both concentration pairs were comparable. This diluted odorant cause no measurable adaptation effect (for details see Fig. 1.1.)

score after both training procedures (Fig. 3.4; PI ~ -0,50 and -0,20, respectively). Apparently, no memory trace of odor intensity is generated for the CS^-. For the decision between two odor concentrations in the test, the fly has only the memory trace of the CS^+.

3.2.3. Increasing the concentration ratio in the test leads to higher learning scores

As shown above, absolute odor intensity can be associated with electric shock and flies can discriminate small differences between two concentrations presented in the test. Concentration ratios as small as 1:2 give low but significant learning scores (Borst, 1983). Learning scores gradually went up with the increase of the concentration ratio (see WT in Fig. 3.5). At ratios of 1:216 or higher learning scores were at the same level as those for two-odor learning with undiluted odorants (Fig. 3.2B).

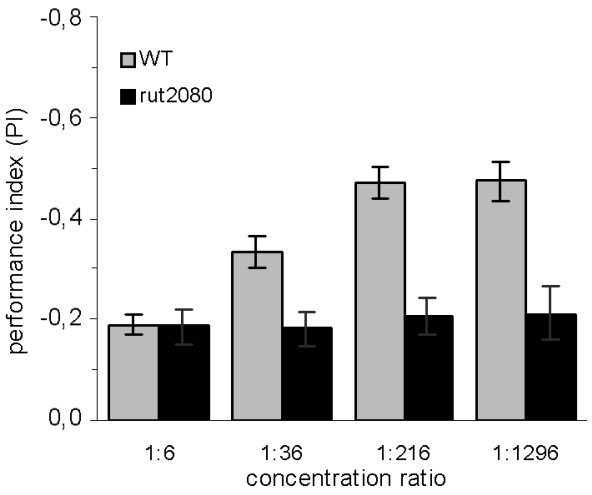

Figure 3.5. Increase of difference between test concentrations of IAA caused increase of learning score in WT flies. In each case, one test concentration was always 2x diluted and the other ranged from 3x to 6x diluted, resulting in the concentration ratios shown in the graph. In contrast to WT-flies, rutabaga (*rut*2080; UAS-*rut*+) flies performed at about the same level through the whole range.

3.2.4. Memory decay after odor intensity learning

Odor memory at 3 min after training was at PI= -0,60. It decreased about 30% in one hour and 50% in 3 hours (PI= -0,45 and -0,33 respectively). In comparison, odor-intensity memory at 3 min had a score of PI= -0,28. It decreased about 18% in 15min (PI = -0,24), about 36% in 1 hour (PI = -0,18) and by 100% in 3 hours (PI = -0,06, n.s.) (Fig. 3.6).

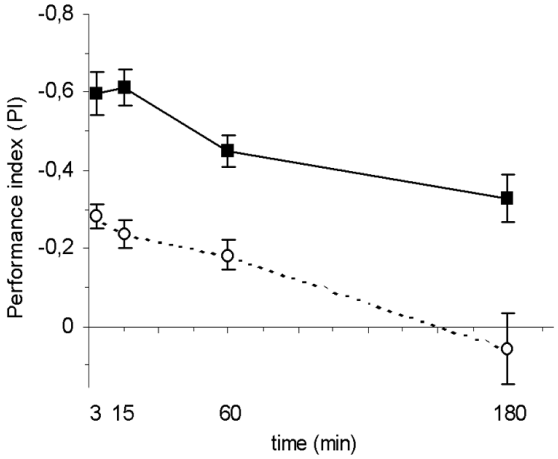

Figure 3.6. Memory decay after odor intensity (○) and two-odor learning (■). WT flies were trained with two concentrations of IAA (dilutions 2x and 3x) and tested after 3, 15, 60 and 180 minutes. Flies' performance decrased over time from PIs = -0,28 at 3 min after training, to score not significantly different from zero (PI= 0,06) at 3 hours. In the two odor learning flies were trained with IAA and AM (2x dilution) and performance overall higher, from PI= -0,60 after 3 min to PI= -0,33 after 3 hours.

3.2.5. Influence of the test on memory readout

The learning score depends on the training as well as on the test. Given that the CS^- stimulus is not necessary to be presented during training (Fig. 3.1C and 3.4) and that the contributions of half-scores for the two CSs to the final learning score mostly are equal (Fig. 3.2), I could train flies with one concentration of one odor only (leaving out the CS^-) and let flies choose

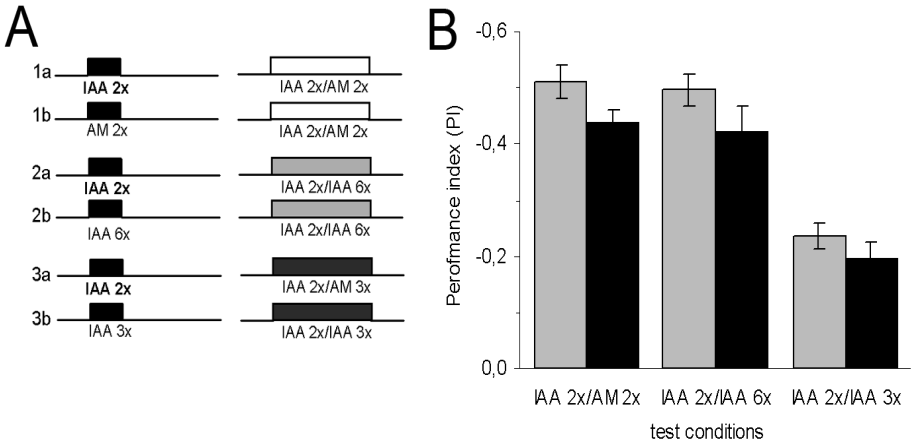

Figure 3.7. Memory score depends on the dificulty of the test. (**A**) Training for each data point can be split into two parts (half scores). For each, only one of the tested olfactory stimuli is paired with an electric shock (for details see Methods and Fig. 3.2.). One part of all three experiments (1a, 2a and 3a lines in the scheme) was done with one concentration of the same odorant (2x diluted IAA). Therefore the created memories after this identical training has to be identical. (**B**) Despite of this fact, learning scores differ in dependence on the particular pair of the olfactory stimuli presented in the test. The lowest performance was achieved with two close concentrations with relative ratio 1:6 (IAA 2x versus 3x diluted) and the highest with two different odorants moderately diluted (IAA versus AM, both 2x diluted). Performance of flies tested with two concentrations of high relative ratio 1:1296 (IAA 2x versus 6x diluted) was comparable to the test done with two odorants. It implies that PI is negatively correlated with the test difficulty and does not reflect only the memory created during a training.

between two different concentrations or two different odors in the test. For one half score, I trained flies with a high concentration of IAA (dilution 2x). For this half score, the training phase was identical for all groups, but the difficulty of the test varied. Flies were tested for 3 min memory in three different test conditions (Fig. 3.7A) - 1) against a different odorant (AM) of the same dilution factor as IAA (dilution 2x), 2) against a lower concentration of IAA (dilution 4x; large concentration ratio 1:1296) and 3) against lower concentration of IAA (dilution 1x; small concentration ratio 1:6). Flies tested against a small concentration ratio performed poorly (PI = -0,24) but with the large concentration ratio or with different odorants flies reached scores two times higher (PI = -0,50 and -0,51, respectively; Fig. 3.7B). Due to exactly the same training, flies had to learn the same and therefore had to build the same memory. The differences in the PIs could therefore be explained only by different difficulty of the test.

3.2.6. Flies store multiple olfactory memories

During odor intensity learning, memories for lower or higher concentration are created, depending on which one of them is paired with the electric shock during training (Fig. 3.2). As I have shown before, the odor concentration presented as the CS^- during training is not important for the final test and therefore can be left out. Looking only on one part of the experiment in which one concentration is trained and two are tested against each other, one observes a significant half-score-avoidance of the previously punished concentration (1 in Fig. 3.8B). A similar result is obtained punishing the complementary concentration in the reciprocal part of the experiment (2 in Fig. 3.8B). As expected, these shifts are about equal but in opposite directions (1 and 2 in Fig. 3.8B and see also Fig. 3.2A). Similar results are obtained using two odorants instead of two concentrations (Fig. 3.2B).

Surprisingly, pairing both concentrations with an electric shock during one training, the effects of punishment did not cancel out. Rather, different shifts depending on the succession of the two concentrations occurred. Flies avoided the concentration that was shocked as the second one rather than the one shocked first (3 in Fig. 3.8B). With the high concentration following the low one, the shift was very obvious and almost identical to the first experiment with only the high concentration paired with shock (2 and 3 in Fig. 3.8B). The opposite high/low sequence resulted in a shift in the opposite direction (4 in Fig. 3.8B). This one was not significantly different from the naïve response but significantly different from the low/high sequence.

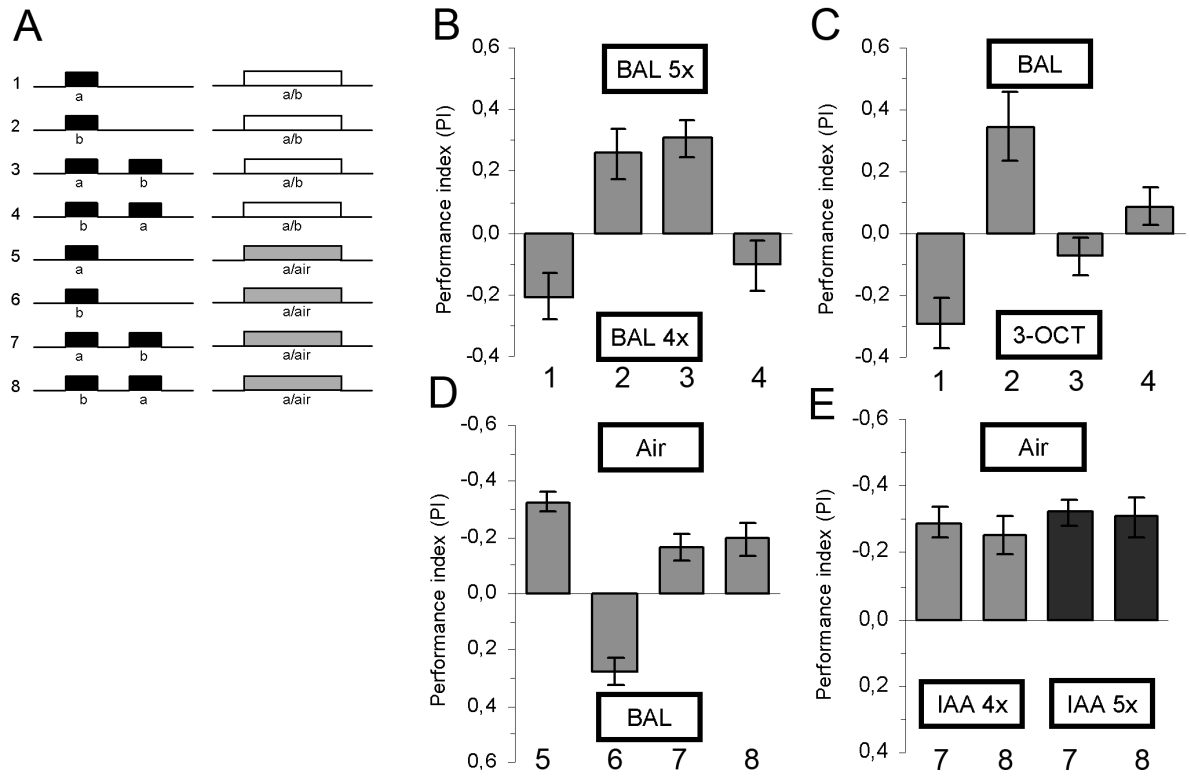

Figure 3.8. Flies can store and reveal multiple olfactory memories. (**A**) Time course of experimental procedure. Used odors represented in the scheme as (**a**) were 5x diluted BAL, 4x and 5x diluted IAA or BAL and as (**b**) were 5x diluted BAL or 3-OCT in concentration learning or two-odor learning respectively. (**B**) When flies are trained with one concentration and tested with two concentrations of the same odorant (BAL, 4x and 5x diluted), they perfomed in a similar way as when they were trained with one and tested with two odorants (BAL and 3-OCT) (**C**). When, during training, two concentrations were (subsequently) paired with the shock, flies avoided more the concentration that was shocked in the sequence as the second one. This shift was not very prominent when the low concentration followed the high one. But the difference between the two sequences showed that the succession did matter. However, with two different odors, the results for the two sequences were not significantly different from each other and also the shift after training was not significantly different from zero. Zero value is naive spontaneous response to the odorants without any previous treatment. During experiment with where both CSs are paired with shock, independent avoidance memory is built for each odorant. Flies underwent the same training as in previous experiment (**B**) but in the test they could chose only between one odor (BAL) and air (**D**). After they were shocked in presence of BAL, they increased their avoidance of BAL in the test. When BAL and 3-OCT were both punished during training, flies afterwards also increased their avoidance of BAL. There is no difference between BAL being the first or the second in the sequence. As a control, other group was shocked in the presence of 3-OCT and these flies did not increse their avoidance of BAL (They rather decreased their avoidance, for reasons explained in the text). These results shows that flies have memory specific for single odor and that they are able to demonstrate this memory in suitable designed test. (E) Seemingly the same results were obtained with two concentrations of one odor. But despite of the correct outcome, with so similar concentrations it was not possible to discriminate in this kind of experimentation between single memory and a generalization of the two concentrations. Pairing one of the two concentrations gave the same results when tested with any of them (see also Fig 3.10). All values were substracted from naive responses to the odors without any previous treatment.

If one of two odorants (BAL and 3-OCT) was paired with shock and later tested against the other, flies avoided the shock-associated odor as was expected (see Fig. 3.1). In contrast, when both those odorants were paired with shock during the same training, there was no significant shift in any direction after the two training sequences (BAL/3-OCT and 3-OCT/BAL; 3 and 4 in Fig. 3.8C). This result was clearly different from the situation with two

concentrations. It could be caused by interference of the two memories in the learning process or by competition of two intact memories during the test.

To find out whether those two memories are created and stored intact I tested only one odor (BAL) against air (Fig. 3.8D). In four different training procedures (5-8 in fig. 3.8A) I either exposed flies only to BAL paired with the shock, BAL followed by 3-OCT (both paired with shock) or 3-OCT followed by BAL (both paired with shocks). As a control I used 3-OCT paired with shock. 3-OCT can serve as a control because there is no generalization between these two odorants (see Fig 3.10 and 3.11). The three training procedures in which BAL was paired with shock all led to a significant avoidance of BAL in the test, compared to naïve flies and to the control with the 3-OCT. Results of the sequences BAL/3-OCT and 3-OCT/BAL did not differ from each other (Fig. 3.8D). The experiment was repeated with 3-OCT as the main odor with equal results (data not shown). The same results are obtained with two concentrations (Fig. 3.8E). Yet, in the case of concentration, a simple conclusion about two independent memories cannot be made as will be discussed later.

3.2.7. Flies learn the absolute odor concentration

In the standard intensity learning experiment, one of the two concentrations was paired with the shock and afterwards was tested against the other concentration. In the next run, the other concentration was paired and flies were subjected to the same test as before. The same two concentrations from the training were later presented in the test (3 and 4 in Fig. 3.9). In the following experiment, training was the same as described above but in the test I exchanged one of the two concentrations with a new one (*shift experiments*). In one experiment. the lower concentration was shifted two dilution steps up after the training, in the other experiment the higher concentration was shifted two dilution steps down. One of the concentrations remained the same between training and test but it changed its status either from low to high (1 and 2 in Fig. 3.9A) or from high to low (5 and 6 in Fig. 3.9A) in comparison to the other presented concentration. If presented in the tests, flies always avoided the previously punished concentration as in the standard experiment. However, given that flies had learned only the paired odor, they experienced in half of the tests after the shift two odors for which they had no memory trace (2 and 5 in Fig. 3.9). Interestingly, irrespective of this situation, in all cases flies avoided the concentration closest to the one paired with the shock. This implies that they avoided the concentration that was presented during training but was not paired with the shock. If flies had learned relative concentrations,

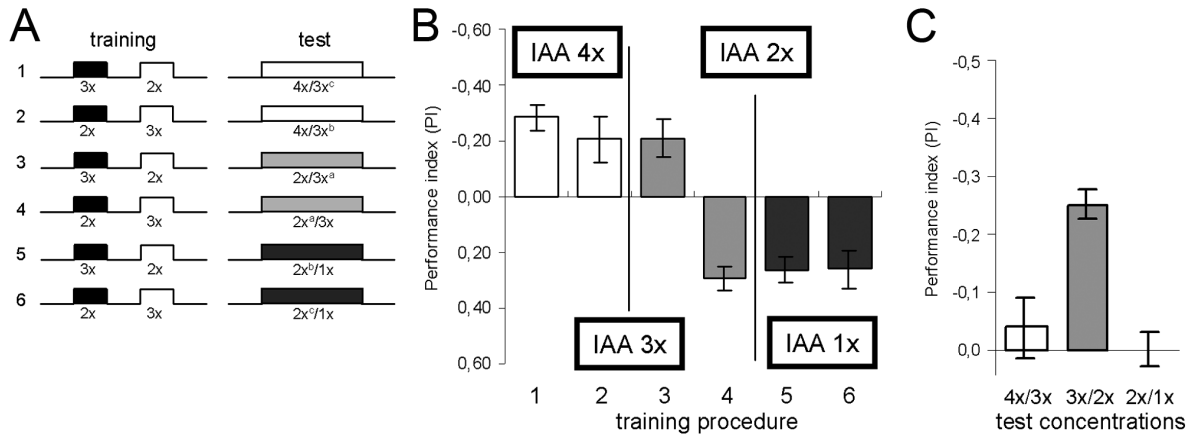

Figure 3.9. Concentration-shift experiment - flies learn absolute, not relative concentration. (**A**) Scheme of the experiments procedure. Two neighboring dilutions of IAA in ratio 1:6 are used in training as CS^+ and CS- (dilution 2x and 3x). In the test, one of them is exchanged with a novel concentration.Stimulus avoided in the test could either (**a**) match with training concentration and with the relative ratio towards the other training stimulus, (**b**) unmatch with training concentration but have the same relative ratio to the other training stimulus or (**c**) match with training concentration but have opposite relative ratio towards the other training stimulus. (**a**) represents the standard training and test procedure. (**b**) and (**c**) represent experiments where one of the concentration used during training is exchanged with the novel concentration for CS^+ or CS^-, respectively. Therefore, the pair of concentrations is shifted for the test up or down compare to the training. (**B**) For the shift up, 1x diluted IAA was added and 4x diluted for the shift down. Therefore the ratio between the trained and the tested concentrations can be either 1:1, 1:6 or 1:36. Flies always avoid the concentration with the lowest possible ratio. In the standard experiment this ratio is always 1:1. Avoidance of one concentration is in all tests significantly different from the naive distribution. (**C**) When shift was applied, flies trained reciprocaly always avoided the same concentration in the test and therefore by calculating learning performance the usual way, these flies show no significant learning score.
Experiments 1 and 2 (white bars) represent exchange of the high concentration with the low (shift down). Experiments 3 and 4 (grey bars) represent standart procedure with the same stimuli in training and test. Experiments 5 and 6 (black bars) represent exchange of the low concentration with the high (shift up).

they should have avoided IAA 4x in (1) and IAA 1x in (6). The experiment unambiguously shows that the concentration was learned as an absolute value and not in relation to the other training stimulus. Learning scores calculated from the shift data in the same way as in the standard procedure (3) and (4) were close to zero (Fig. 3.9C), since in the shift experiment, flies always preferred the concentration introduced by the shift, as this was the least similar to the shock-paired concentration.

3.3. Flies generalize olfactory cues

3.3.1. Two ways of testing olfactory generalization

Two ways to test generalization were explored (Fig. 3.10): In the first one (Fig. 3.10A), I measured responses to different concentrations of an odor (BAL) against air and compared responses of naïve flies to those of flies that had been conditioned with one of these concentrations.

Qualitatively, odor responses of naïve flies change from aversive to attractive with decreasing concentration (Martin et al., 2001; Acebes and Ferrus, 2001). With complete generalization over the whole concentration range, after the training with any single concentration, the odor response curve would shift towards higher or lower aversion (depending on whether the US is positive or negative). Without any generalization, flies would change their response only to the respective trained concentration, whereas for all other concentrations, responses should stay the same. This approach has its limitation in the sense that the naïve responses are very different through the concentration range. Aversive responses to high concentrations are often almost saturated (PI= -1) leaving no room for an additional increases in avoidance.

Plotting the difference between naïve and after-training responses, revealed a decrease of generalization with increasing distance from the shock-paired concentration (Fig. 3.10B). As mentioned above, this result does not allow deciding whether it was caused by an increase in the stimulus dissimilarity or by the fly's naïve odor responses.

In the other approach I tested always only one concentration against air and varied those concentrations that were presented during training (Fig. 3.10C). Therefore, in this case, the test was always the same and the result was compared to the response of naïve flies towards this particular concentration. In this approach, the variable was the concentration of the odor during training. Full generalization would result in the equal increase of aversion after training of any concentration of the same odor. Absence of generalization would lead to an increase of aversion only if exactly the same concentration was used during training as the one used in the test. All other trained concentrations would leave the test responses at the level of naïve animals.

3.3.2. Generalization of concentration is limited

I followed the second scheme and used six different concentrations of IAA during the training. Dilution 2x (1/36) was taken as a reference concentration for the test. Training with this dilution gave the highest score (PI= -0,59). Training concentrations six times more or less diluted than the test concentration (1x; 3x) were similar enough to the test concentration to make flies show higher avoidance than naïve animals (Fig. 3.10C, D). Any concentration diluted 36 times more or less did not lead to such increased avoidance (Fig. 3.10C, D). No change in avoidance was observed when other odorants (BAL, 3-OCT, AM, the last not

shown) were used during training instead of dilution of IAA or when shock alone was given (Fig. 3.10C, D, E).

The results showed that flies could generalize odor concentration only in a narrow range (roughly one order of magnitude up and down, see Fig. 3.10E) and that they responded to larger differences in concentration the same way as to different odor qualities. The

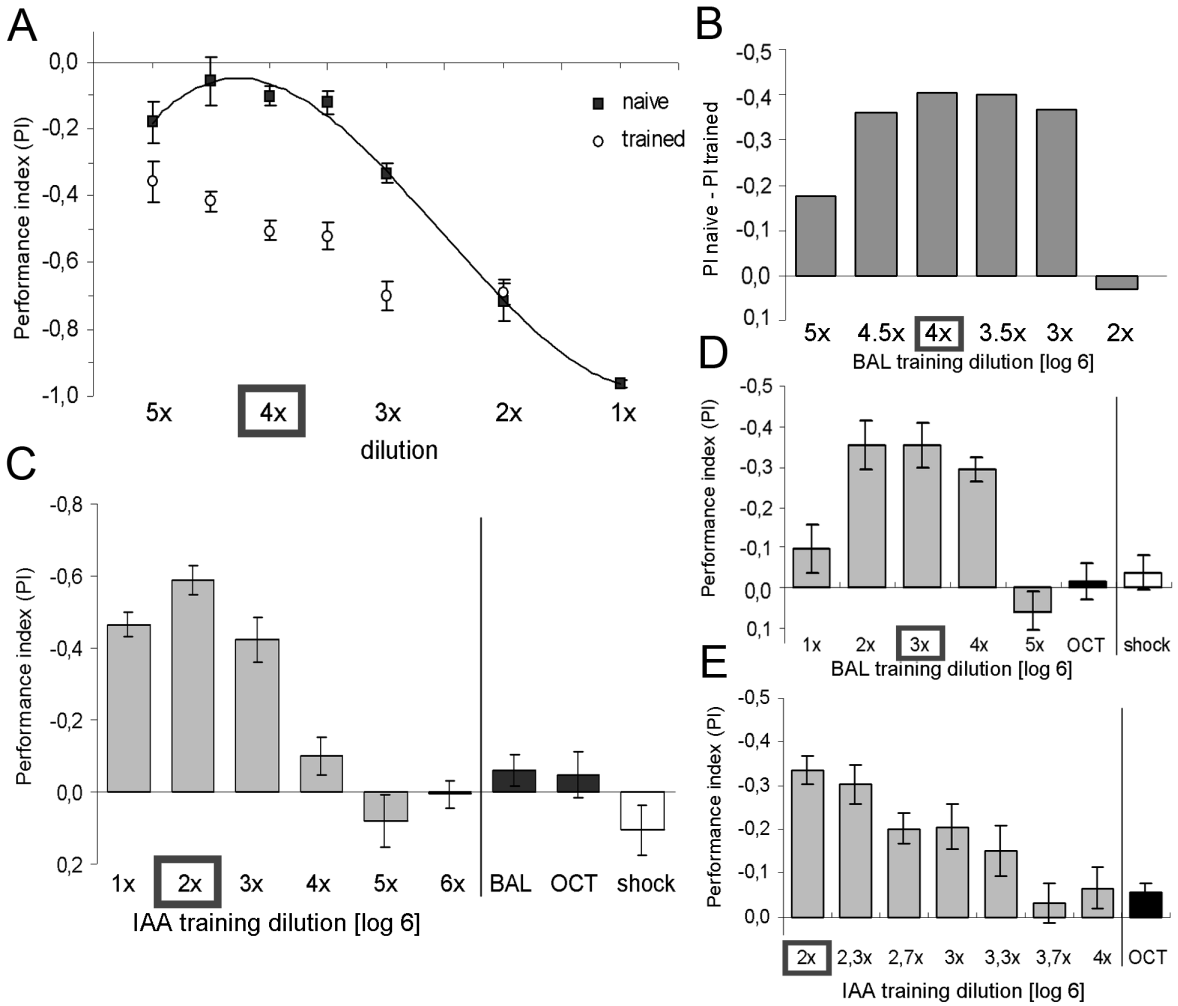

Figure 3.10. Generalization of concentrations can be measured in two different experimental procedures. In the first (**A**), comparison is made between naive response (■) to the respective concentration of the odor and the response after reference concentration (4x dilution) was paired with the electric shock (○). Subtraction of the naive and the trained response (**B**) is learning response when the trained and tested concentration is the same (4x dilution) and it can be taken as a level of generalization when they are different. In the second procedure (**C**), flies are shocked in the presence of a range of IAA concentrations. In addition they were also trained with 2x diluted BAL and 3-OCT and with shock alone. After every of these training procedures, flies were tested with the same concentration of IAA (2x dilution). Flies which were trained with the same concentration which was tested, performed with the best score (PI= -0,59). Trained concentration six times higher or lower gave also high significant score (PI= -0,47 and -0,42). All other trained concentrations as well as the other odorants did not significantly change flies spontaneous responses to the tested concentration of IAA. Similar results were obtained in equivalent experiment done with different odorant (BAL) (**D**). Trained and tested with the same concentration, PI was -0,35 and dilution six times higher or lower were not significantly different from it (PIs= -0,35 and -0,30 respectively). In the next experiment (**E**), IAA was diluted in smaller steps and the level of generalization gradually decreases from full (2,3x dilution) to no generalization (3,7x dilution). Black frames mark reference dilutions used for all trainings (**A,B**) or for all tests (**C,D,E**).

experiment was repeated with BAL giving similar results (Fig. 3.10D). For both odorants I found the same ratios of concentrations that were and were not generalized.

Flies generalized between concentrations of a dilution ratio 1:6 and they did not generalize between concentrations at the ratio 1:36. To find out whether there was a sudden or gradual change between generalization and non-generalization, I diluted IAA in smaller steps than before (Fig. 3.10E). The level of generalization, indeed, decreased gradually from full to no generalization without any sudden change (Fig. 3.10E).

3.3.3. Cross-generalization is dependent on odorant and concentration

From the previous experiments it was apparent that generalization did not occur if one odorant was trained but another one was tested. I also varied concentration of the trained odor (BAL) from undiluted to clean air and tested always one dilution of another odorant (3-OCT). There was no significant difference between results obtained with this range of training concentrations (data not shown). This experiment would imply that generalization between odors is not possible regardless of the concentration.

To test whether this result was generally applicable, I performed a similar experiment with IAA and AM. During training, IAA or AM were paired with shock and in the test flies had

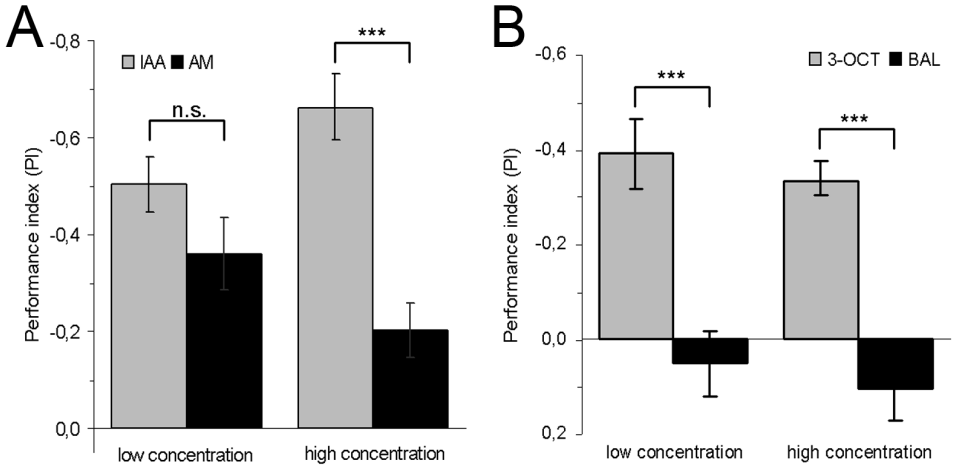

Figure 3.11. Generalization between two odorants. Similar procedure as in the concentration generalization was used in this experiments. Always one odorant of certain dilution was paired with the shock during training. Two pairs of two odorants were used (IAA and AM or 3-OCT and BAL respectively). For high concentrations of both pairs, flies were not able to generalized between the two odorants (**A,B**). Therefore, when they were trained with odorant different from the test they showed significantly lower score (PI= -0,20 and 0,10 respectively) than flies trained with matching trained and tested odorant (PI= -0,66 and -0,34 respectively). For low concentrations of the pair 3-OCT and BAL (**B**), flies performed in similar way and did not show any generalization (PI= -0,40 for matched compare to PI= 0,05 for unmatched odorant). IAA and AM in low concentrations (**A**) were generalized and scores obtained with training either IAA and AM were not significantly different from each other (PI= -0,50 and PI= -0,36 respectively).

always a choice between IAA and air. I picked two concentrations of those odors, one low (dilution 1/1296) and one high (dilution 1/36). After training and testing a high concentration of the same odor (IAA), a high conditioned avoidance score was obtained (PI= -0,66). After training with the other odor (AM) the conditioned score was much lower (PI= -0,20) but still different from zero. Apparently, some generalization across odorants had occurred. In the parallel experiment at low concentrations, training and testing of the same odor (IAA) also led to a high conditioned avoidance score (PI= -0,50). But now, flies trained with AM performed much better than before (PI= -0,36). Their conditioned avoidance was not significantly different from that of flies trained with IAA (Fig. 3.11A). This would imply that generalization between some odorants (IAA and AM) is possible and depends on odor intensity. The same experiment was repeated with two other odorants (BAL and 3-OCT). Between these, flies failed to generalize regardless of concentration (Fig. 3.11B).

3.4. *Rutabaga*-independent memory

3.4.1. Odor intensity and two-odor learning differ in the cAMP pathway

Odor intensity learning with two concentrations of IAA at the ratio 1:6 was tested with cAMP pathway mutants *rutabaga* and *dunce*. These mutants performed as well as WT flies under these conditions (Fig. 3.12A). Tested for two-odor learning with 3-OCT and BAL, they had a learning score of only 50-60% of WT flies (Fig. 3.12B), as reported previously (Dubnau and Tully, 1998; Zars et al., 2000; Schwaerzel et al., 2002).

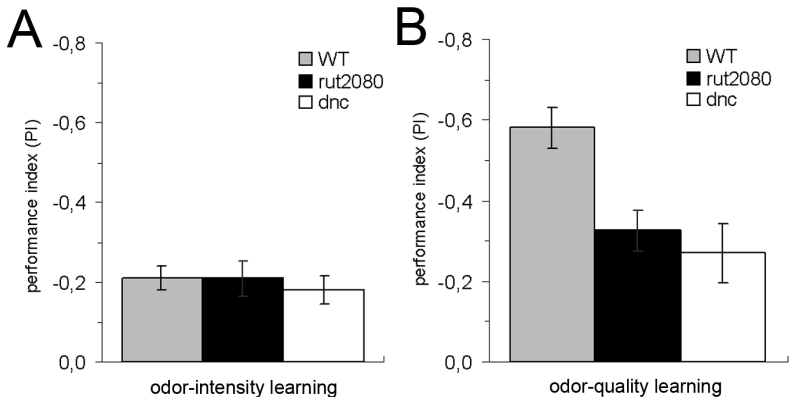

Figure 3.12. Odor-intensity learning is independent of cAMP signaling pathway. (**A**) WT flies performed in 3-min memory in odor-intensity learning at PI around -0,20 over a wide range of absolute concentrations (see Fig. 3.3.). cAMP pathway mutants rutabaga and dunce performed in odor-intensity learning with relative ratio 1:6 at the same level as WT flies. These results obtained for mutant flies suggested that 3-min memory for this type of learning does not critically depend on cAMP concentration. (**B**) Under the same conditions, the odor-quality learning of WT flies gave three times higher score than for odor-intensity learning (PI= -0,60) while rutabaga and dunce mutant flies both showed significantly lower score memory around PI= -0,30, reaching only about 50% of the WT flies score.

3.4.2. Two-odor learning with cross-generalized odors is *rutabaga*-independent

It has been shown in the experiment of Fig. 3.11 that WT flies cross-generalize between IAA and AM at low concentration (4x) but not between BAL and 3-OCT. IAA and AM elicited very similar spontaneous responses throughout the concentration range: In choice experiments flies had no strong preference for one of them if presented at the same dilutions. Testing two-odor learning I presented both odors at three concentrations (2x, 4x and 6x diluted) (Fig. 3.13A). With the highest concentrations flies performed as well as with BAL and 3-OCT (PI= -0,61). Decreasing concentration gradually lowered the learning score (gray bars in Fig. 3.13A). The same dependency of the learning score on overall concentration was observed with BAL and 3-OCT (gray bars in Fig. 3.13B). Learning scores reached about PI= -0,2 in both cases.

Interestingly, *rut* flies behaved differently with these two odor pairs. With BAL and 3-OCT, as expected, they showed an about 40% lower learning score than WT-flies, regardless of concentration (black bars in Fig. 3.13B). In contrast, with IAA and AM the learning impairment of *rut* flies decreased with lower concentrations. At the lowest concentration (6x) they performed as well as WT (black bars in Fig. 3.13A).

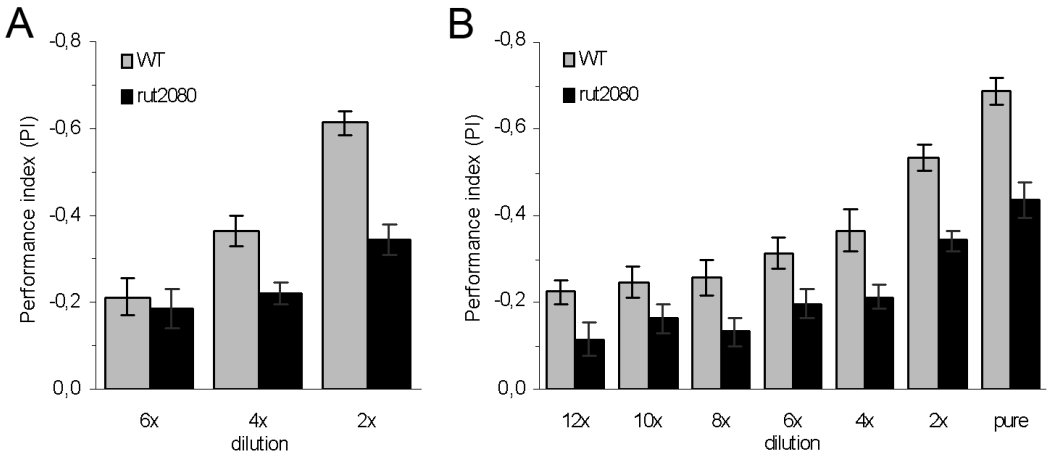

Figure 3.13. Two-odor learning of similar odorants can be rutabaga independent. (**A**) With decreasing concentration of similar odorants (IAA and AM) in two-odor learning, learning performance of WT flies goes gradually down (from PI= -0,61 to -0,21). With high odor concentration rutabaga flies performed on usual 60% level of WT flies score (PI= -0,35). But their performance decreased less than that of WT flies and lined up with it for low concentrations (PI= -0,19 vs. -0,21). (**B**) Dissimilar odorants (BAL and 3-OCT), which were not generalized (see Fig. 3.11.) showed rut-dependent memory over the broad range of concentrations. With decreasing concentration of these odorants, learning performance of WT flies went gradually down (from PI= -0,65 to -0,22). Also rutabaga flies showed similar decrease and performed always at 50-60% of WT flies performance.

3.4.3. Memory readout in *rutabaga* is not improved in a one-odor test

In concentration learning, high concentration ratios (1:216 or larger) lead to learning scores comparable to the scores obtained in two-odor learning (Fig. 3.5 and 3.13). Given that the CS^- has no influence on the learning score, this dependency of the learning score on concentration ratio must reflect the difficulty of choice in the test. In the experiment of Fig. 3.5, the *rut* mutant had been tested under the same conditions as WT. At all concentration ratios the learning score stayed low around PI= -0,20 (black bars in Fig. 3.5). This finding and the result of Chapter 3.4.2. raised the possibility that the *rut* gene might be only necessary during the test phase of the experiment. Following up on this idea, it would appear that for *rut* flies, two odors and high concentration ratios would make the test particularly demanding because under these conditions they were impaired.

To further characterize the mutant behavior I tried to make the test situation as simple as possible. I repeated the experiment of Fig. 3.13A with the three concentrations of IAA but omitted the second odorant (AM) in the test. Therefore the reciprocal training (second half score) could not be measured. The concentration ratio in the test now was infinitely large. I have shown before that the concentration ratio positively influences the memory performance of WT flies (see Fig. 3.7). As expected, learning scores for WT flies increased if the second odor was omitted. This effect was most pronounced at the lowest concentration (6x). For *rut,* omitting the second odor had no measurable effect (compare Figs 3.13A and 3.14). The mutant flies obviously did not profit from the "simpler" test situation. It is therefore rather unlikely that the *rut* gene is only needed during the test.

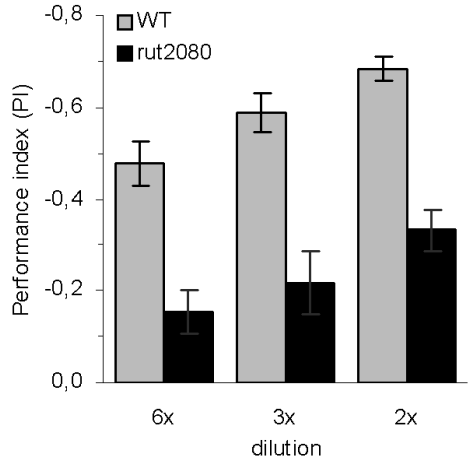

Figure 3.14. One-odor learning does not restore rutabaga dependent memory. Flies were trained and tested only with one odor concentration against air. WT flies as well as rutabaga flies decreased their performance with decreasing concentration of the trained stimulus (PI= -0,68 to -0,48 and -0,33 to -0,15 respectively). Rutabaga performed in all cases significantly less than WT flies ($p<0,001$).

3.5. Localizing *rutabaga*-independent memory

As the experiments above suggested, the remaining olfactory memory in the *rut* mutant has different properties from that of WT flies. I assumed that in *rut*-independent memory, some other molecular mechanisms of synaptic plasticity must be involved and I tried to find out where this memory trace may be located. The experiments localizing two-odor learning, especially the most important experiment - *rut* rescue in the intrinsic neurons of the MBs, can not be applicable here (Zars et al., 2000). Yet, the first candidate place for storing this second memory trace are the MBs. Alternatively, other brain regions would have to be considered.

To address this question, I used GAL4 drivers and the UAS-*shibire*^{ts1} (UAS-*shi*^{ts1}) transgene, which allows for conditional blocking of chemical synapses depending on the ambient temperature (Kitamoto, 2001). Permissive temperature was 26°C and restrictive temperature 32°C (Schwaerzel, 2003). A *Shibire* line (UAS-Shi2) with two copies, one on the X and one on the third chromosome, was used in all experiments (Kitamoto, 2001).

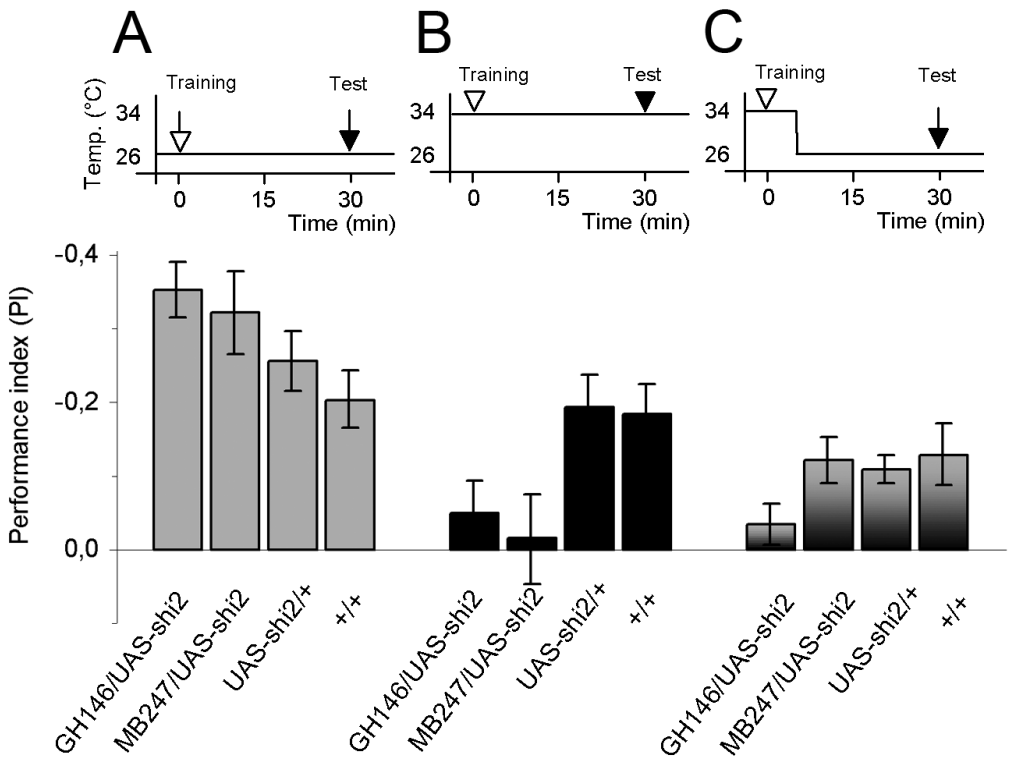

Figure 3.15. 30-minute olfactory memory under different temperature regimes. Flies expressing the UAS-*shits*1 transgene in either olfactory projection neurons (GH146/UAS-Shi2) or Kenyon cells (247/UAS-Shi2) show a severe reduction in performance of olfactory memory at the restrictive temperature (**B**) compared to the permissive temperature (**A**)($p < 0{,}001$). Moreover, they performance is not significantly different from zero ($p > 0{,}05$). The genetic control and WT flies perform at the restrictive temperature equaly well as at permisive temperature. **(C)** When trained under restrictive teperature but tested at the permissive temperature, memory scores of flies expressing the UAS-shits1 transgene in the Kenyon cells of the mushroom bodies were low but still significantly different from zero and comparable with the genetic control and WT-flies. Expression of the UAS-*shi*^{ts1} transgene in the olfactory projection neurons in GH146/UAS-Shi2 totally abolished the memory score ($p > 0{,}05$).

In order to investigate the functional significance of neurons along the olfactory pathway and to test whether odor intensity learning also resides in the MBs or is MB-independent, I expressed the UAS-*shi*^{ts1} transgene in the MBs and the projection neurons (PNs). To express the effector in the PNs I used the GAL4 driver line GH146 and, in the MBs line MB247. In the GAL4-line GH146, two populations of PNs are affected. One connects the antennal lobe (AL) with the calyx of the MB and the lateral protocerebrum (LPC), the other connects the AL directly to the LPC (Heimbeck et al., 2001).

Blocking synaptic transmission at the level of the MBs (MB247-GAL4) abolished 30-minute memory of odor intensity learning if flies were trained and tested at the restrictive temperature (Fig. 3.15B). Expressing the transgene in the projection neurons (GH146-GAL4), which are presynaptic to the MBs and provide them with the olfactory input from the ALs, also resulted in a memory score not significantly different from zero if flies were trained and tested at the restrictive temperature (Fig. 3.15B).

The experiment showed that for 30-minute memory in odor intensity learning the branch of the olfactory pathway through the MBs was required. Both, the output of the PNs labeled in GH146 and the output of the Kenyon cells labeled in MB247 were necessary. It did not show that the memory had to reside within any of these neurons.

To find out whether the memory was localized upstream of the MBs I blocked the input to the MBs (by blocking the output of PNs) or only the output from the MBs during acquisition. Expressing UAS-*shi*^{ts1} in the PNs during the training phase and hence blocking the input to the MBs, fully abolished memory showing that the input to the MBs was necessary during memory acquisition (Fig. 3.15C). Therefore, the memory trace could not be located in the AL or PNs (with the MBs being only required for reading the memory out during the test). Blocking the MBs during acquisition reduced memory compared to the score obtained at the permissive temperature but this decrease was in line with control flies (Fig. 3.15C). This indicates that the memory should be located upstream of the MB output.

All genotypes used in this experiments were tested for electric shock avoidance and for odor sensitivity, and were normal compared to WT flies (Schwaerzel et al., 2002; 2003). Spontaneous olfactory behavior is affected by blocking synaptic transmission in GH146 (expressing tetanus toxin). The defect is concentration dependent. Only responses to low

concentrations are affected (Heimbeck et al., 2001). The olfactory stimuli I used here were in the “high” concentration range (Heimbeck et al., 2001), where GH146/UAS-Shi2 flies at restrictive temperature should still be able to show normal spontaneous olfactory responses.

Taken together, these experiments show, that the memory trace for odor intensity learning could well be located in the MBs, together with the two-odor memory trace. For a more definitive localization no tools were available.

4. Discussion

4.1. Properties of odor learning

Non-associative part of odor learning

Studying olfactory learning, we are interested in the association made between the olfactory stimulus (CS) and the electric shock (US). Any non-associative factors confound the results. One such factor is the effect of the second olfactory stimulus (CS^-) from which the fly should discriminate the shock-associated odor. Tully and Quinn (1985) investigated the effect of leaving out the CS^- during training. Despite a significant effect, the overall impact on the final score was rather low (14% decrease). I have shown that omitting the CS^- during training could reduce learning scores by 50% for WT flies. From the data on WT and *rutabaga* flies I assume that this reduction is caused by adaptation. As the adaptation is non-associative, the strength of the reduction is not proportional to the learning score but to the odor intensity. Therefore, the same level of adaptation reduces learning in the mutant *rutabaga* to zero (data not shown).

Presenting the CS^- during training almost canceled the effect of adaptation to the CS^+ odor. Because the level of adaptation also depends on time (Stoertkuhl et al., 1999), I could see a difference between adaptation to the first and to the second odor presented. This difference was already described by Tully and Quinn (1985) as having a low impact (7% difference) on the learning score. But their experiment was influenced by two factors: time dependent memory and time dependent adaptation. Flies learned the first odor and also adapted to it. Memory as well as the learning-score reducing effect of the adaptation decreased over time. The first reduced, the second increase the learning score. Therefore, the overall effect was quite low. In my experiment, I did not find any difference between presentations of CS^+ on the beginning of the training and at the end when the CS^- was skipped because the effects of fading memory and fading adaptation just canceled out. But when the CS^+ was omitted, I found a difference (yet not significant) between presentations of the CS^- at the beginning or at the end of the training, because in this case the difference between fading adaptations was not compensated by the fading memory. Yet, if I presented both odors without pairing any of them with shock, the difference between the levels of adaptation for the first and second odor caused a significant score (PI= -0,13). This score is entirely non-associative due to the lack of any specific US. Also, the same score was obtained with *rutabaga* flies (data not shown). This value is about 17% of the learning score in WT flies but already 40% of that of the *rutabaga* mutant.

Flies recover from adaptation in the range of minutes (Stoertkuhl et al., 1999). In fact, the two stimuli above that cause the highly significant difference in adaptation are only 1.5 min apart. Therefore I assume that adaptation influences only STM.

Since the level of adaptation linearly depends on the concentration of an odor (Stoertkuhl et al., 1998), this non-associative effect does not influence performance in the intensity learning experiments, in which only sufficiently low odor concentrations were used. This allowed me to carry out experiments such as generalization in which flies were exposed to only one odor and air during training and test.

Préat (1998) has also found that the performance in the T-maze paradigm may be influenced by other variables than the CS/US association. He reported that electric shocks alone reduced the fly's avoidance response to odorants. This avoidance was altered in learning / memory mutants, such as *rutabaga*, *dunce* and *amnesiac,* compared to WT flies. As the learning score depends on the salience of the CSs (Tully and Quinn, 1985), reduction in odor avoidance may lead to a decreased learning score. He therefore proposed that at least part of the learning score reduction in learning mutants is due to their reduced odor avoidance after electric shock. In contrast to the adaptation effect studied here, the electric shock could influence odor-intensity learning at low stimulus intensities.

Olfactory learning is symmetric

I have shown that air cannot be associated with shock and therefore any discriminative learning between air and an odor has to be asymmetrical. Also the combination of two odors which one is diluted so much that it is not easily perceived, should result in asymmetrical learning. The symmetry can be disrupted also due to certain mutations in the memory acquisition process. For example, the memory mutant *amnesiac* is impaired specifically in acquisition of BAL, whereas acquisition of all other tested odorants was normal, as was avoidance of BAL (Keene et al., 2004). With these exceptions, I found that olfactory learning in all tested cases was symmetrical. It could have been expected for equally strong olfactory stimuli but not for two different concentrations that were perceived as different odor intensities. Learned odor avoidance increases with increasing odor concentration if the odor is tested against air (Fig. 3.14). Therefore we could expect that high concentration should give always higher PI (half score) than the low concentration. Despite this expectancy, they give always equal half-scores. Moreover, testing two concentrations against each other gives a higher score for combinations of distinct concentrations than of the two high but similar

concentrations (Fig. 3.5). These experiments suggest that the ratio of CS1/CS2 has a larger impact than the intensity of the CS itself. From this point of view, the test performed with low concentration (CS1) versus air (CS2) has lower ratio of CS1/CS2 than high concentration versus air and therefore lower learning score. There is no indication that (within reasonable range) learning of a low concentration is more difficult than of a high one. These considerations reflect our inability to take apart the level of the fly's memory and the ability of the fly to solve the test.

The role of CS^+ in the learning

I have shown that under controlled condition the CS^- does not play an associative role during training. As a consequence, we cannot draw a line between different kinds of learning, namely intensity and quality learning, already during the training. At the point of presenting the odor (CS^+), flies have no way how to determine what will be important to learn for the test. Already Borst (1983) pointed out that the odor of the CS^- is not necessary for reward learning and that "we cannot tell to the fly what to learn, what to pay attention to". It is obvious that flies learn both, intensity and quality of an odor at the time they are exposed to the CS^+ during the training. Any olfactory learning is thus one-odor learning and only in the test does the fly discriminate between two stimuli. By the way, the MB model of olfactory learning (Heisenberg, 2003; Gerber et al., 2004) generates a memory only for the odor associated with the reinforcement. A distinction should be made between acquisition measured by behavioral assays and the actual process of acquisition of new information by a molecular learning apparatus (Dudai et al., 1988).

In the generalization experiments, I showed that flies learn the quality of an odor and in the concentration-shift experiments I showed that flies also learn absolute intensity of an odor. Therefore, one can conclude that during presentation of the CS^+ a single fly learns a particular scent, which is defined as an odor of a certain intensity. This scent would be represented as a restricted area in a hypothetical multidimensional odor space. Changes in intensity or composition large enough to change the quality of the stimulus would be represented as a shift in the position out of this area in a certain dimension and distance. Small change (in range of 1:10 dilution ratio) in intensity would lead also to shift of the position but the representation would still stay within this area.

Being exposed to CS^+ and CS^- in the test, flies make a decision fast and most of them do not change their choice afterwards. I have shown that a test lasting 15 sec is sufficient compare to

the usual 120 sec test (Tully and Quinn, 1985; Schwaerzel et al., 2001). I did not observed any difference in behavior during the test with two odorants or with two concentrations. Whereas in many studies performed with rats, response time and accuracy were inversely related to each other (Uchida and Mainen, 2003), in free flying honeybees choosing between two odors in an olfactory discrimination task, choice duration was independent of odor similarity (Ditzen et al., 2003). Mean processing time was around 690ms, rarely exceeding 2 seconds (Ditzen et al., 2003), a time range we cannot precisely evaluate in the T-maze. On the other hand, it would fit to my visual observation performed under red light, that flies make their decision in the range of a second.

4.2. Why two olfactory STMs?

With my data I wanted to show that odor intensity and odor quality learning share some common aspects but differ in other important ones. This distinction led me to postulate two independent mechanisms of memory formation.

Flies learn attributes of the CS paired with the US, and in olfactory learning, we consider two such attributes - odor quality and odor intensity (Davis, 2005). This is experimentally revealed by the test with the need of comparing two olfactory stimuli that are equal in quality and differ only in intensity or differ in both (the third possibility - differing only on quality - is unlikely as will be discussed later).

Trained flies in the Tully/Quinn paradigm memorize attributes of a single odor and what is relevant for them is the similarity between this odor and the two stimuli in the test. In the test, flies tend to avoid the stimulus that is more similar to the trained one. They even avoid the same concentration that was presented in the training as the unpunished one and therefore might be treated as “safe”, if the other odor in the choice is even more different from the memorized odor. It also does not matter if the high/low relation between the two concentrations is flipped between training and test. Flies learn only the absolute intensity of the punished stimulus and in the test they avoid the best matching stimulus, regardless of the relation of concentrations of the two odors in the test.

Multiple memories reveal a difference between quality and intensity learning

A major difference between quality and intensity learning at the behavioral level is revealed by the experiment testing for multiple memories. Flies can store more than one memory for different odorants (quality) but not for different concentrations of one odor (Fig. 3.8).

This striking difference is most apparent if one compares training procedure 3 in the two experiments of Fig. 3.8B and C. For the low/high sequence (3 in Fig. 3.8B) it appears that the lower concentration has been "overwritten" by the higher one. This outcome is in line with the MB-model of olfactory learning (Heisenberg, 2003) if the odor representation of the lower concentration is assumed to be fully included in that of the higher concentration. But this model does not account for the basic odor intensity learning (procedure 1 in Fig. 3.8B) because, if low concentration is punished and afterwards tested against a higher concentration, flies should not show any learning score. They should avoid both concentrations equally or they might avoid the higher one even more than the lower, previously punished one. This is what happened in some experiments with honeybees (Pelz et al., 1997). However it is important to mention here that the honeybee experiment is different from the T-maze paradigm in the fact that the test in the bee experiment is not based on a simultaneous discriminative choice but rather subsequent exposures to single odorants. The honeybees should have the possibility to smell and compare two concentrations at the same time. In a recent experiment (Ditzen et al., 2003) bees were able to learn two different concentrations discriminatively. Although they were free-flying, they still sampled odor sources serially. The reason is possibly due to the prolonged training where bees could learn not only that certain stimulus was rewarded but also that the visits to other stimuli were not rewarded. Also, flies can be trained discriminatively to two olfactory stimuli; one being rewarded by sugar and the other punished by electric shock (Tempel et al., 1983). That means that flies as well as bees store one positive and one negative memory connected to different stimuli and during the test they use the one which is relevant for the presented stimuli at that time. Here it would be interesting to see whether the bee is capable to learn one concentration and discriminate it from the other in an equivalent to the force T-maze test.

If in *Drosophila* the first memory is "overwritten", the training procedure 4 (high/low) in Fig. 3.8B should give the same result as the procedure 1. Indeed, the two learning scores are not statistically different from each other, although in the high/low case the score is also not significantly different from the naive response. Yet, the result is clearly distinct from that

obtained after training procedure 3 showing that the order of concentrations in the sequence matters. The first memory is largely overwritten by the second one, regardless of whether it is the high or low concentration.

In contrast, the experiments in which two odors were both paired with shock but only one of them was later tested against air, showed that independent memory for each odor was created. In the two-odor discriminative test, these memories compete against each other resulting in no measurable learning score (3 and 4 in Fig. 3.8C). If testing one odor against air or against a third odor (data not shown), this competition is eliminated and the memory can be seen (7 and 8 in Fig. 3.8D). This way of detecting the single memories cannot be in fact applied to odor intensity learning because the similar concentrations used in this experiment are mutually generalized. Any kind of training with any of those led to the avoidance of both concentrations in the test (Fig. 3.8E).

Tully and Quinn (1985) performed a similar experiment, a reversal training. They trained flies with two standard training cycles, instead of one. During the first cycle one odor was paired with shock and the other odor was presented without shock. During the second cycle the other odor was paired with shock and the first odor not. They found a learning score of PI = 0,26 (compared to their usual PI = 0,90) and interpreted their result to indicate that despite of what flies learned during the first cycle, they avoided the odor more recently paired with the shock. Their 70% reduction in learning score, however, is in accord with my finding. One has to take into account that during their experiment, the two shocked odors were separated in time much more than in mine and between, flies were also exposed to the unpunished odors. These details of the procedure might explain why in their experiment the two memories did not fully cancel out.

I conclude that flies build independent memories for each odor they were trained with. These memories do not interfere with each other on the neuronal level, but they do so on the behavioral level if the two memories demand incompatible actions. In contrast, memories for different concentrations interact already with each other at the neuronal level because the first memory is largely erased by the second training. The findings for multiple odor memories fit into the MB-model of olfactory learning (Heisenberg, 2003), whereas the interference between concentration memories speaks for a different neuronal storage process.

4.3. Generalization

An organism can be said to generalize if he/she treats two stimuli or situations the same although he/she can distinguish them. Animals behaviorally generalize different odorants, even though they are capable to discriminate them. In many cases it is desirable to "(mis)take" one odor for another (Kay, 2004). In this study I have used generalization experiments to show how similar two olfactory stimuli are for flies.

Flies can discriminate odor concentrations as small as 30% (Borst, 1983 and Schwaerzel, personal communication). Hence, my finding that they treat an odor as "same" even at a 6-fold higher or lower concentration if tested appropriately, demonstrates true generalization. It is in this concentration range that odor intensity learning results in low learning scores. Apparently, odor concentration ratios in this range are difficult to discriminate in the Tully/Quinn paradigm. Quite surprisingly, almost the same concentration range for generalization was found in *Musca* (Fukushi, 1973).

Preliminary data from odor evoked activity in the PNs on the level of the ALs suggested that different concentrations of one odor activate different subsets of glomeruli and the amount of overlap was concentration depend (Silbering et al., 2003). Similar results were obtained by measuring calcium activity in PNs on the level of the calyx region of MBs (Fiala et al. 2005).
Linster and coworkers showed that rats fully generalized between two enantiomer pairs (limonene or terpinen-4-ol) in habituation experiments (Linster et al., 2001) while discriminating them in differential reinforcement (Linster et al., 2002). The generalized enantiomer pairs evoked such similar olfactory glomerular activity patterns (Linster et al., 2002) that the differences could have easily been overlooked (Linster et al., 2001). In comparison, enantiomers of carvone were not generalized in habituation experiment and their olfactory glomerular response patterns differed considerably (Linster et al., 2001).
These two sets of experiments suggest that small difference in odor activated patterns are usually neglected by the animals and they react as if all stimuli falling within a certain range of small variability would be the same. On the other hand they are able to clearly discriminate them from each other when trained appropriately.

In my studies, generalization between two odorants was possible only between low concentrations of IAA and AM but not for high concentrations of IAA and AM and not for BAL and 3-OCT at any concentrations. IAA and AM are similar in their chemical structure

(amylacetates). Although they do not smell the same to us they might activate similar subsets of glomeruli in the AL. Two other acetates (IAA and EA) activate very similar subsets of glomeruli where the main glomerulus is common for both and only some of the other glomeruli differ (Silbering et al., 2003). With decreasing odor concentration (tested with hexanal) intensity of odor evoked activity as well as number of activated glomeruli decreases. The weakly firing glomeruli disappear first and the strongest stay as the last (Silbering et al., 2003). The same is observed in the terminals of the PNs in the calyx region of the MBs (Fiala et al., 2005). It could mean that with decreasing concentration similar odors get more similar (see Ng et al., 2002).

My data suggests that flies perceive IAA and AM at low concentrations only as two similar concentrations of the same odor (intensities of the same scent) and that they do not recognize the different qualities of these odors anymore. Comparing these results with my findings on generalization of concentrations (Fig. 3.10) it seems that in certain cases flies treat two odorants as similar as two close concentrations of the same odorant.

The other two odorants, BAL and 3-OCT are very different in chemical structure, they activate different olfactory sensilla (de Bruyne et al., 1999) and differ in the dynamics of electrophysiological responses (de Bruyne et al., 1999). In addition, BAL is reported as an odorant distinct in processing from all other tested odorants (Keene et al., 2004).
Activity patterns for BAL and 3-OCT observed on the level of calyx are clearly distinct over a broad range of concentrations (Fiala et al., 2005).

Even if two odorants would be genetically convey to the same glomerulus it would not necessarily mean that they would be perceived as exactly the same. What we see as patterns of calcium activity could be just a part of the picture. There is no evidence that different odors activating same glomerulus are perceived as of the same odor quality. We have to take in account temporal coding of the receptor neuron firing (Wehr and Laurent, 1996; Laurent, 1999; Laurent et al., 2001) as firing rate, amplitude, response dynamics and the response termination (Hallem et al., 2004). All of these can characterize odors in addition to the activated glomerulus.

4.4. Odor-quality and intensity learning do not rely the same on cAMP signaling

Expression of the *rutabaga* gene was found to be especially abundant in MB Kenyon cells (Han et al., 1992) and was shown to be necessary and sufficient in MB neuropil for a component of odor/electric shock learning (Zars et al., 2000). The component of the learning score remaining in the *rutabaga* mutant was preliminarily called *rutabaga*-independent because it turned out to be fragile lasting little more than an hour (Tempel et al., 1983; Tully et al., 1994; Schwaerzel et al., 2002). The activity of AC in homogenates prepared from *rutabaga* flies is abnormal (lower) than in WT flies (Dudai et al., 1983) and AC also display an altered responsiveness to $Ca2^{+}$ (Dudai and Zvi, 1984). Mutant *rut* flies were demonstrated biochemically to be missing a $CA2^{+}$/calmodulin-sensitive AC but the mutation did not reduce basal cAMP levels (Livingston et al., 1984), suggesting that the enzyme is used primarily for regulation rather than maintenance of steady-state cAMP levels (Davis, 1993). No detectable effect of calcium levels on rutabaga's AC activity (Dudai and Zvi, 1984; Livingston et al., 1984) suggested that the rutabaga allele may be an apomorph with complete loss-of-function (Tully and Quinn, 1985). From an enhancer detector screen, seven different alleles of rutabaga were recovered (Levin et al., 1992; Han et al., 1992). Out of them, at least the most widely used, rut^{2080}, is also reported a loss-of-function allele (Mao et al., 2004). Yet, this allele is not a null at the RNA level (Zars, personal communication).

Mutant strains *dunce* used in this work, $dunce^{M14}$ and also $dunce^{M11}$ and $dunce^{ML}$ appear to be amorphic, because they completely lack the cAMP-specific phosphodiesterase normally present in adult flies (Davis and Kiger, 1981; Salz and Kiger, 1984).
Therefore, we can assume that the *rutabaga*-independent part of the memory score and scores of intensity learning are not caused by residual functions of these genes.

For *rutabaga* flies, there was no difference between testing two concentrations with low or with high concentration differences. In contrast, WT flies decreased their PI as a function of the difficulty of the test (Fig. 3.5). On the one hand, one could say that *rutabaga* flies, unlike WT, perform at the same level despite the increase in the test difficulty. On the other hand, one could look at it from the other side: with increasing easiness of the task, WT flies were able to take advantage of it and improve their learning performance, whereas *rutabaga* flies were not able to do it and their score did not change and stayed low. To obtain a high memory score, flies needed the normal functioning of the *rutabaga* gene and distinct

olfactory cues during the test. Missing one of those resulted in low learning performance. As discussed before, flies learn both intensity and quality at the same time. The mutant results show in addition that only for storing the odor quality information the normal expression of the *rutabaga* gene is required.

Yet, it cannot be excluded that in *rut* flies, the odor memory is the same as in WT flies. Flies might be lacking some requirement for an efficient orientation (choice) behavior in the test. To give an example, with two odors or with high concentration ratios WT flies might be able to use a simultaneous comparison of the signals from the two antennae to efficiently choose their orientation (simultaneous system). At low concentration ratios the distance between the two antennae would be too small for the simultaneous system to work. In this situation the fly would have to sample its environment by locomotion and consecutive measurements of concentration (consecutive system). If only the simultaneous system would require the *rut* gene and would therefore be missing in mutant *rut* flies, these flies would perform like WT under all stimulus conditions that could not be solved by the simultaneous system.

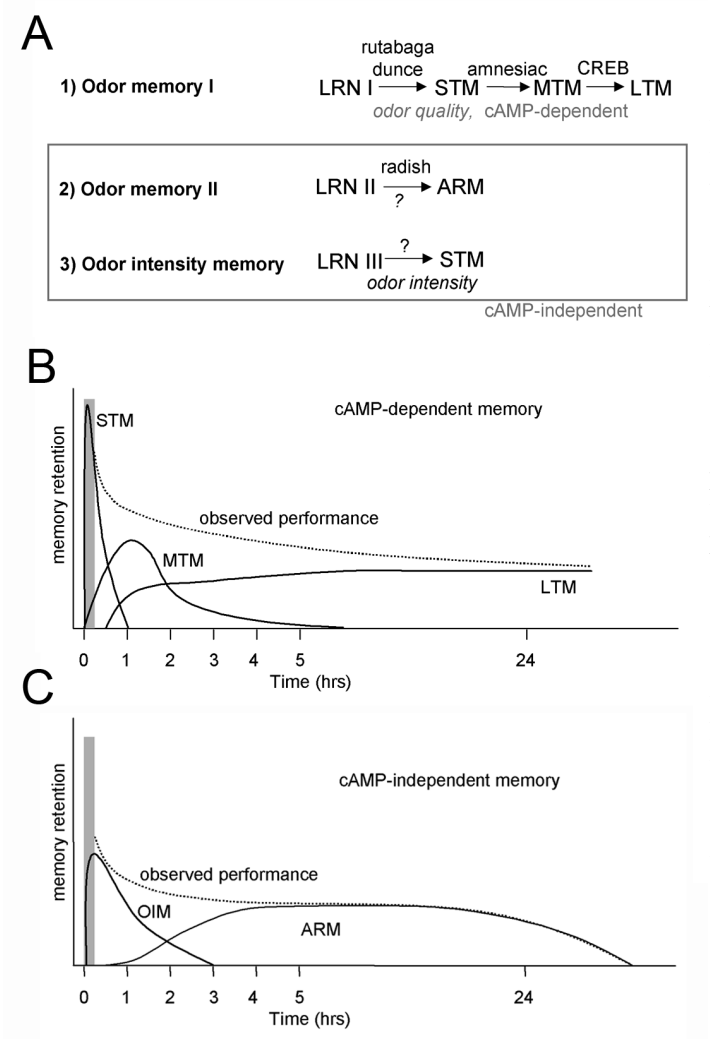

Figure 4.1. Proposed olfactory memories. **(A)** List of three distinct memories and the scheme of their formation. **1)** represents more or less previously described olfactory memory caused by pairing individual odor with electric shock. **2)** is a independent formation of ARM proposed by Isabell et al. (2004). **3)** is the novel odor intensity memory described in this work. **(B)** The behavioral model of cAMP-dependent memories. Model similar to the traditional model, but without ARM. STM and MTM are created after single training session but differ in acquisition speed and in persistence. LTM requires spaced training to build the memory but after this one can persists for several days. **(C)** cAMP-independent memories. OIM is established during the conditioning but is very short-lasting (only one to two hours). On the contrary, ARM is not detectable right after training but emerges gradually withing few hours. Usually, it is established after massed conditioning and can last for several hours. Seemingly, they complement each other. Despite of that, it does not mean they are anyhow connected.

I have argued above that unlike the BAL/OCT pair, IAA and AM at low concentrations loose their distinction in odor quality while still being distinguishable as different intensities (Fig. 3.11 and 3.13). The mutant data warrant the further conclusion that the dissimilarity between two concentrations or between two odorants allowing for generalization between them cannot be exploited by the *rutabaga*-dependent learning behavior. The *rutabaga*-dependent memory for a given odor (scent) is stored in the MBs but during the test, flies are subjected to two olfactory stimuli. When these two perceived scents are too similar, they activate indistinguishable subsets of Kenyon cells and flies cannot use the available memory to solve this test. To distinguish between two very similar scents, flies seem to possess a second behavior less dependent on the expression of the *rutabaga* gene. In the MB-model, the odor representation in the Kenyon cells has to be concentration invariant to some degree. This fits to the present observations as well as to earlier behavioral data (Borst, 1983). Two stimuli that are not generalized allow significant increase (3x) of the learning score but only for WT flies. For *rutabaga* flies, the increase is very low (two odors) or none (two concentrations).

As mentioned above, *rutabaga*'s proposed function is to act during training to create the memory. Both *rutabaga* or *dunce* are reported as STM mutants. Decrease of memory over 3 hours for WT flies and *rutabaga* mutant flies shows that these curves are almost parallel. The *rutabaga*-independent part of two-odor memory decreases from 60% of the WT score at 3 min after training (Tully and Quinn, 1985; Zars et al., 2000) to about zero in 3 hours (Tully and Quinn, 1985; Schwaerzel et al., 2002). The score of the WT flies decreases by about the same absolute amount. It implies that there is almost no decrease of *rutabaga*-dependent memory during this time. Odor-intensity learning lasts at least for one hour but after 3 hours the score is zero. This suggests that odor-intensity learning may indeed be the same as the *rutabaga*-independent memory seen in two-odor learning experiments. The remaining score of PI= 0,10 after 3 hours in *rutabaga* (Tully and Quinn, 1985) is very likely the ARM which was described by Isabel et al. (2004) as a *rutabaga*-independent memory but is not yet formed at 3 minutes.

4.5. Localization of the memory trace in odor intensity learning

With two-odor learning, blocking input to and output from MBs during training or during training and test (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002) gave similar results as in the equivalent experiments carried out here. Output signals from the MBs are required for retrieval of the memory but not for its acquisition, indicating that synaptic plasticity is induced upstream of the MBs' synaptic output. Secondly, blocking the output from the PNs during acquisition shows no memory score for odor intensity learning. These new results are compatible with the notion that *rutabaga*-independent concentration memory resides in the MBs, as does STM (and probably also MTM) for odor quality. Other possible storage places are the LPC or AL. LNs in the AL were already suggested as a possible substrate for concentration invariance (Borst, 1983). Overall, the AL seems to be a more likely candidate out of those two. Creating and maybe also temporally storing olfactory memory in the AL was reported in honeybee (Hammer and Menzel, 1998) and recently, neuronal plasticity in the AL correlated with associative training was reported in *Drosophila* (Yu et al., 2004). But this plasticity was observed on the synapses of PNs onto the LNs in the AL (Yu et al., 2004). PNs do not only project to MB and LPC but also form excitatory synapses onto LNs within the glomerulus of their origin (Ng et al., 2002). Therefore, changing their outputs between the training and the test (by blocking them with *shibire*^{ts} expression) changes the odor signaling in the AL. As a consequence, the representation of the odor in the AL, stored during training would be different from that perceived in the test. If one observes memory reduction, it is possibly not because the memory was not stored in the AL but because the stored memory did not correspond to the odor stimulus presented during the test. For these reasons, blocking the PNs by *shibire*^{ts} expression does not exclude that the *rutabaga*-independent memory might reside in the AL. So far, there is no proof that the *rutabaga*-independent memory is located in the MBs because the crucial experiment that had shown this for odor quality memory was the specific rescue of *rutabaga* in the intrinsic neurons of the MBs (Zars et al., 2000). This experiment, however, localized only *rutabaga*-dependent olfactory STM and left the *rutabaga*-independent memory part unsolved.

One indication that also the odor intensity memory resides in the Kenyon cells of MBs comes from experiment where olfactory memory was disrupted by constitutive expression of G-protein α-subunit (Connolly et al., 1996). MB expression of Gαs* fully abolished associative learning, whereas null alleles of *dunce* and *rutabaga* exhibit only partial impairments (Connolly et al., 1996). This indicates that even odor intensity memory rely in some way on Gαs. In mammals, Gαs stimulates all ACs to some degree (Cooper et al., 1995) but the

rutabaga and *dunce* mutations in flies affect only one class of AC or PDE, respectively. In flies, in addition to *rutabaga*-AC, three other ACs have been identified (Levin et al., 1992). Thus, disruption of all ACs by Gαs* expression could have more drastic effects on signaling than removal of one form of AC (Connolly et al., 1996). Another possibility is that Gαs participates not only on the cAMP pathway but it also directly modulates ion channels (Clapham, 1994; Connolly et al., 1996). In any case, Gαs* seems to interfere with the odor intensity memory in the way the *rutabaga* and *dunce* mutations do not.

For *rutabaga* flies, attractive odors are still attractive and repellent odors repellent. Hence, some quality information is still available. Yet, it is possible that *rutabaga* flies cannot learn odor quality. The test does not reveal whether *rutabaga* flies remember the particular olfactory stimulus as quality and intensity or just as intensity. Only in the unlikely case that two odorants were diluted to the same intensity percept for the flies, *rutabaga* flies should fail in learning to discriminate these stimuli. Unfortunately, this cannot be tested in the mass essay because odor intensities in individual flies most likely are differently tuned (Acebes and Ferrus, 2001). Therefore, intensities in the test could only be balanced for a small fraction of the flies. A single fly paradigm would have to be established.

4.6. Evolutionary perspective

One would like to assume that the *rutabaga*-independent memory is the evolutionarily older form of memory mainly involved in perceiving concentration gradients. A memory used in osmotaxis of flies should be short-lived. Even an hour seems much too long, given the structure of natural odor plumes. We do not know why flies need to learn absolute concentrations of odors.

4.7. A possible model

Intensity memory is characterized by independence to mutations in genes of the cAMP pathway. Only one memory item can be stored at a time. Using this memory, flies perform in the test at a suboptimal level and the memory decay is rather fast. Odor memory requires the cAMP pathway. More than one memory item can be stored, i.e. flies can remember several odors at the same time. This memory provides for optimal avoidance scores and is stable over several hours.

The intensity differences higher than 1:10 are treated like quality differences; a different scent. In other words: a specific scent is the percept of an odorant at a certain (rough)

concentration. Scents stay "the same" within a small concentration range. "Sameness of scent" is an important concept provided by invariance operations in the brain. These scents are defined by the points in a multidimensional odor space. At each point in scent space there is a scale for fine odor intensity. This scale must relate to a certain scent because the fly has this "small-ratios ability" with different scents that differ only in the concentration of an odorant. I propose that the fly memorizes for a particular scent (a) the specific memory trace for the scent and (b) the total neuronal activity of the odor stimulus. This can be at the level of the ALs mediated by LNs or in the MBs or the LPC. The fly would only apply the intensity memory if in the test no information on scent differences would be available. To avoid the expected electric shock, all available information is used. Intensity information would be meaningless if different scents were detected in the test. Only if no useful information on scents is available, will intensity information be used as a last resort.

5. Summary

It has been known for a long time that *Drosophila* can learn to discriminate not only between different odorants but also between different concentrations of the same odor.
Olfactory associative learning has been described as a pairing between odorant and electric shock and since then, most of the experiments conducted in this respect have largely neglected the dual properties of odors: quality and intensity. For odorant-coupled short-term memory, a biochemical model has been proposed that mainly relies on the known cAMP signaling pathway. Mushroom bodies (MB) have been shown to be necessary and sufficient for this type of memory, and the MB-model of odor learning and short-term memory was established. Yet, theoretically, based on the MB-model, flies should not be able to learn concentrations if trained to the lower of the two concentrations in the test.

In this thesis, I investigate the role of concentration-dependent learning, establishment of a concentration-dependent memory and their correlation to the standard two-odor learning as described by the MB-model.

In order to highlight the difference between learning of quality and learning of intensity of the same odor I have tried to characterize the nature of the stimulus that is actually learned by the flies, leading to the conclusion that during the training flies learn all possible cues that are presented at the time. The type of the following test seems to govern the usage of the information available. This revealed a distinction between what flies learned and what is actually measured.

Furthermore, I have shown that learning of concentration is associative and that it is symmetrical between high and low concentrations. I have also shown how the subjective quality perception of an odor changes with changing intensity, suggesting that one odor can have more than one scent. There is no proof that flies perceive a range of concentrations of one odorant as one (odor) quality. Flies display a certain level of concentration invariance that is limited and related to the particular concentration. Learning of concentration is relevant only to a limited range of concentrations within the boundaries of concentration invariance.

Moreover, under certain conditions, two chemically distinct odorants could smell sufficiently similarly such, that they can be generalized between each other like if they would be of the same quality. Therefore, the abilities of the fly to identify the difference in quality or in intensity of the stimuli need to be distinguished. The way how the stimulus is analyzed and processed speaks in favor of a concept postulating the existence of two separated memories.

To follow this concept, I have proposed a new form of memory called odor intensity memory (OIM), characterized it and compared it to other olfactory memories. OIM is independent of some members of the known cAMP signaling pathway and very likely forms the *rutabaga*-independent component of the standard two-odor memory. The *rutabaga*-dependent odor memory requires qualitatively different olfactory stimuli. OIM is revealed within the limits of concentration invariance where the memory test gives only sub-optimal performance for the concentration differences but discrimination of odor quality is not possible at all.

Based on the available experimental tools, OIM seems to require the mushroom bodies the same as odor-quality memory but its properties are different. Flies can memorize the quality of several odorants at a given time but a newly formed memory of one odor interferes with the OIM stored before. In addition, the OIM lasts only 1 to 3 hours - much shorter than the odor-quality memory.

6. Zusammenfassung

Assoziatives olfaktorisches Lernen bei *Drosophila* wurde ursprünglich als die Paarung eines Duftes mit einem elektrischen Bestrafungsreiz beschrieben. Seit langem ist dazu bekannt, daß *Drosophila* nicht nur lernen kann zwei Düfte zu unterscheiden, sondern auch verschiedene Konzentrationen desselben Dufts. Jedoch wird in den meisten auf diese Art durchgeführten Experimenten die Duftintensität weitestgehend ignoriert. - Für das olfaktorische Kurzzeitgedächtnis wurde ein biochemisches Modell vorgeschlagen, welches sich hauptsächlich auf die bekannte cAMP-Signalkaskade stützt. Es wurde gezeigt, dass die Pilzkörper (mushroom bodies, „MB“) notwendig und hinreichend für diese Art der Gedächtnisbildung sind und ein MB-Modell für Duftlernen und Kurzzeitgedächtnis konnte etabliert werden. Interessanterweise sollten Fliegen nach diesem Modell Konzentrationsunterschiede nur in einer Richtung lernen können. Sie würden den gelernten Duft nur gegenüber einer niedrigeren Konzentration wiedererkennen.

In der vorliegenden Doktorarbeit habe ich das konzentrationsabhängige Duftlernen und seine Beziehung zum MB-Modell untersucht. Dabei hat sich gezeigt, dass die Fliege eine Gedächtnisspur für Geruchsintensität anlegt.

Um den Unterschied zwischen dem Lernen einer Qualität und dem einer Intensität des gleichen Duftes hervorzuheben, habe ich versucht, den Reiz, der eigentlich von der Fliege gelernt wird, zu charakterisieren. Dies führte zu der Schlussfolgerung, dass die Fliege während des Trainings alle in diesem Zeitabschnitt präsentierten Reize erlernt. Erst der dem Training folgende Test scheint den Gebrauch der verfügbaren Information festzulegen. Diese Erkenntnis ist eine wesentliche Grundlage um zwischen dem Testergebnis und dem, was die Fliege gelernt hat zu unterscheiden.

Ich habe außerdem gezeigt, daß das Konzentrationslernen eine Form assoziativen Lernens ist und, dass entgegen der Erwartung nach dem MB-Modell eine Symmetrie zwischen den Lernwerten für die hohe und niedrige Konzentration besteht.

Es gibt keinen Beweis dafür, dass Fliegen eine Vielfalt von Konzentrationen desselben Duftes als ein und dieselbe (Duft-)Qualität wahrnehmen. Die Ergebnisse legen vielmehr

nahe, dass sich bei einer größeren Veränderung der Intensität eines Duftes für die Fliege (wie in vielen Fällen auch beim Menschen) seine Qualität verändert. Demzufolge ist mit jedem Geruchsstoff mehr als nur eine Fliegen-subjektive Geruchsqualität verbunden. Fliegen zeigen andererseits in engen Grenzen Konzentrationsinvarianz. Sie generalisieren zwischen Konzentrationen eines Duftes innerhalb einer Konzentrationsdekade. Deshalb ist das Konzept des Konzentrationslernens nur für ein begrenztes Konzentrationsspektrum innerhalb der Grenzen der Konzentrationsinvarianz relevant.

Des weiteren habe ich gezeigt, dass unter besonderen Bedingungen zwei chemisch verschiedene Düfte generalisiert werden können. Möglicherweise haben die beiden Düfte hinreichend "ähnliche" oder gleiche Fliegen-subjektive Qualität und können nur nach der Intensität unterschieden werden. Die Fliege hat die Fähigkeit im Test Unterschiede einerseits in der Qualität und andererseits in der Intensität des Reizes zu ermitteln. Die Art und Weise, wie der Reiz analysiert und verarbeitet wird, erfordern ein Konzept zweier getrennter Gedächtnisse.

Dementsprechend habe ich eine neue Gedächtnisart, ein sogenanntes Duftintensitätsgedächtnis (OIM) vorgeschlagent und versucht dieses neben anderen olfaktorischen Gedächtnissen einzuordnen. Das OIM ist unabhängig bezüglich einiger Bestandteile des bekannten cAMP-Signalwegs und stellt höchstwahrscheinlich den *rutabaga*-unabhängigen Teil des Zwei-Düfte-Lernens dar. Das *rutabaga*-abhängige Duftgedächtnis benötigt qualitativ verschiedene Duftreize. Das OIM reicht lediglich für eine suboptimale Leistung aus, funktioniert aber in den Grenzen der Konzentrationsinvarianz, innerhalb derer die Diskriminierung und damit auch das Lernen der Duftqualität nicht möglich sind.

Das OIM scheint wie die Duftqualitätsgedächtnisse die Pilzkörper zu benötigen. Aber die Art der Speicherung ist von der der Duftqualitätsgedächtnisse verschieden. Fliegen können viele Duftqualitäten zu einem bestimmten Zeitpunkt aus dem Gedächtnis abrufen, jedoch interferiert ein neu gebildetes Gedächtnis eines bestimmten Duftes mit dem bereits gespeicherten OIM. Außerdem ist das OIM für nur 1-3 Stunden stabil, was erheblich kürzer als beim Duftgedächtnis ist.

7. References

Abrams T.W. and Kandel E.R. (1988) Is contiguity detection in classical conditioning a system or a cellular property? Learning in *Aplysia* suggests a possible molecular site. *Trends Neurosci* 11, 128-35.

Acebes A. and Ferrus A. (2001) Increasing the number of synapses modifies olfactory perception in *Drosophila*. *J Neurosci.* 21(16):6264-73.

Allweis C. (1991) The congruity of rat and chick multiphasic memory-consolidation models. In: Andrew RJ, editor. Neural and behavioural plasticity. New York: Oxford University Press, p. 370-393.

Anholt R.R. (1994) Signal integration in the nervous system: adenylate cyclases as molecular coincidence detectors. *Trends Neurosci.* 17(1):37-41.

Araneda R.C., Kini A.D. and Firestein S. (2000) The molecular receptive range of an odorant receptor. *Nat Neurosci.* 3(12):1248-1255.

Armstrong J.D., de Belle J.S., Wang Z., and Kim Kaiser (1998) Metamorphosis of the mushroom bodies; large-scale rearrangements of the neural substrates for associative learning and memory in *Drosophila*. *Learn Mem* 5, 102-114.

Ayyub C., Paranjape J., Rodrigues V. and Siddiqi O. (1990) Genetics of olfactory behavior in *Drosophila melanogaster*. *J Neurogenet.* 6(4):243-62.

Balakrishnan R. and Rodrigues V. (1991) The shaker and shaking-B genes specify elements in the processing of gustatory information in *Drosophila melanogaster*. J Exp Biol. 157:161-81.

Benzer S. (1973) Genetic dissection of behavior. *Sci. Am*., 229:24-37.

Bhagavan S. and Smith B.H. (1997) Olfactory conditioning in the honey bee, *Apis mellifera*: effects of odor intensity. *Physiol Behav.* 61(1):107-17.

Bhalerao S., Sen A., Stocker R., and Rodrigues V. (2003) Olfactory neurons expressing identified receptor genes project to subsets of glomeruli within the antennal lobe of *Drosophila melanogaster*. *J Neurobiol* 54:577-92.

Boekhoff I., Michel W.C., Breer H. and Ache B.W. (1994) Single odors differentially stimulate dual second messenger pathways in lobster olfactory receptor cells. *J Neurosci.* 14(5 Pt 2):3304-9.

Borst A. (1983) Computation of olfactory signal in *Drosophila melanogaster*. *J. Comp. Physiol A.* 152. 373-383

Borst A. and Heisenberg M. (1982) Osmotropotaxis in *Drosophila melanogaster*. *J Comp Physiol* 147:479-484.

Brand A. H. & Perrimon N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401-15.

Breer H. and Boekhoff I. (1992) Second messenger signaling in olfaction. *Curr Opin Neurobiol.* 2(4):439-43.

Breer H., Raming K. and Krieger J. (1994) Signal recognition and transduction in olfactory neurons. *Biochim Biophys Acta.* 1224(2):277-87.

Byers D., Davis R. L. and Kiger J. A. (1981) Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in *Drosophila melanogaster*. *Nature* 289, 79-81.

Charro M.J. and Alcorta E. (1994) Quantifying relative importance of maxillary palp information on the olfactory behavior of *Drosophila melanogaster*. *J Comp Physiol A.* 175(6):761-6.

Chen M.S., Obar R.A., Schroeder C.C., Austin T.W., Poodry C.A., Wadsworth S.C. and Vallee R.B. (1991) Multiple forms of dynamin are encoded by *shibire*, a *Drosophila* gene involved in endocytosis. *Nature* 351(6327):583-6.

Chiang A.S., Blum A., Barditch J., Chen Y.H., Chiu S.L., Regulski M., Armstrong J.D., Tully T. and Dubnau J. (2004) *radish* encodes a phospholipase-A2 and defines a neural circuit involved in anesthesia-resistant memory. *Curr Biol.* 14(4):263-72.

Christensen T.A. and Hildebrand J.G. (2002) Pheromonal and host-odor processing in the insect antennal lobe: how different? *Curr Opin Neurobiol*. 12(4):393-9.

Clapham D.E. (1994) Direct G Protein Activation of Ion Channels? *Ann Review Neurosci.* 17:441-464

Clyne P.J., Warr C.G., Freeman M.R., Lessing D., Kim J. and Carlson J.R. (1999) A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* 22, 327-338.

Connolly J.B., Roberts I.J., Armstrong J.D., Kaiser K., Forte M., Tully T. and O'Kane, C.J. (1996) Associative learning disrupted by impaired Gs signaling in *Drosophila* mushroom bodies. *Science*, 274, 2104-7.

Cooper D.M., Mons N. and Karpen J.W. (1995) Adenylyl cyclases and the interaction between calcium and cAMP signaling. *Nature* 374(6521):421-4.

Crittenden J.R., Skoulakis E.M., Han K.A., Kalderon D. and Davis R.L. (1998) Tripartite mushroom body architecture revealed by antigenic markers. *Learn Mem*, 5, 38-51.

Davis G. W., Schuster C. M. and Goodman C. S. (1996) Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. *Neuron* 17:669-79.

Davis H.P. and Squire L.R. (1984) Protein synthesis and memory: a review. *Psychol Bull* 96:518-59.

Davis R.L. (2004) Olfactory learning, *Neuron.* 44:31-48.

Davis R.L. (2005) Olfactory memory formation in *Drosophila*: from molecular to systems neuro*Science*. *Annu Rev Neurosci.* 28:275-302.

Davis R.L. and Kiger J.A. Jr. (1981) Dunce mutants of *Drosophila melanogaster*: mutants defective in the cyclic AMP phosphodiesterase enzyme system. *J Cell Biol.* 90(1):101-7.

Day M., and Morris R.G. (2001) Memory consolidation and NMDA receptors: discrepancy between genetic and pharmacological approaches. *Science* 293:755.

de Belle J. S., and Heisenberg M. (1994) Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science* 263:692-5.

de Bruyne M., Clyne P.J. and Carlson J.R., (1999) Odor coding in a model olfactory organ: the *Drosophila* maxillary palp. *J. Neurosci.* 19:4520-4532.

de Bruyne M., Foster K. and Carlson J.R., (2001) Odor coding in the *Drosophila* antenna. Neuron 30:537-552.

De Cesare D., Fimia G.M. and Sassone-Corsi P. (1999) Signaling routes to CREM and CREB: plasticity in transcriptional activation. *Trends Biochem Sci* 24:281-285.

Dekker T., Takken W. and Cardea A. (2001) Structure of host-odour plumes influences catch of *Anopheles gambiae* s.s. and *Aedes aegypti* in a dual-choice olfactometer. *Physiological Entomology* 26:124 -134.

Deshpande M., Venkatesh K., Rodrigues V. and Hasan G. (2000) The inositol 1,4,5-trisphosphate receptor is required for maintenance of olfactory adaptation in *Drosophila* antennae. *J Neurobiol*. 43(3):282-8.

Devaud J.M. (2003) Experimental studies of adult *Drosophila* chemosensory behaviour. *Behav Processes.* 64(2):177-196.

Devaud J.M., Acebes A. and Ferrus A. (2001) Odor exposure causes central adaptation and morphological changes in selected olfactory glomeruli in *Drosophila*. *J Neurosci.* 21(16):6274-82.

Dicke M. (1999) Specificity of herbivore-induced plant defences. *Novartis Found Symp.* 223:43-54.

Ditzen M., Evers J.F. and Galizia C.G. (2003) Odor similarity does not influence the time needed for odor processing. *Chem Senses*. 28(9):781-9.

Dobritsa A.A., van der Goes van Naters W., Warr C.G., Steinbrecht R.A. and Carlson, J.R., (2003) Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37:827-841.

Drain P., Folkers E. and Quinn W. G. (1991) cAMP-dependent protein kinase and the disruption of learning in transgenic flies. *Neuron* 6:71-82.

Dubin A.E., Liles M.M. and Harris G.L. (1998) The K^+ channel gene ether a go-go is required for the transduction of a subset of odorants in adult *Drosophila melanogaster*. *J Neurosci.* 18(15):5603-13.

Dubnau J. and Tully T. (1998) Gene discovery in *Drosophila*: new insights for learning and memory. *Annu Rev Neurosci.* 21:407-44.

Dubnau J., Grady L., Kitamoto T. and Tully T. (2001) Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. *Nature* 411:476-80.

Dubnau J., Chiang A.S. and Tully, T. (2003) Neural substrates of memory: From synapse to system. *J Neurobiol*, 54:238-53.

Dudai Y. (1983) Mutations affect storage and use of memory differentially in *Drosophila*. *Proc. Natl. Acad. Sci. USA*. 80:5445-5448.

Dudai Y. and Zvi S. (1984) Adenylate cyclase in the *Drosophila* memory mutant rutabaga displays an altered Ca^{2+} sensitivity. *Neurosci Lett.* 47(2):119-24.

Dudai Y., Corfas G. and Hazvi S. (1988) What is possible contribution of Ca2+-stimulated adenylyl cyclase to acquition, consolidation and retention of an associative olfactory memory in *Drosophila*. *J. Comp. Physiol. A.* 162:101-109.

Dudai Y., Corfas G. and Hazvi S. (1988) What is the possible contribution of $Ca2^+$-stimulated adenylate cyclase to acquisition, consolidation and retention of an associative olfactory memory in *Drosophila*. *J Comp Physiol [A]* 162:101-9.

Dudai Y., Jan Y. N., Byers D., Quinn W. G. and Benzer S. (1976) *dunce*, a mutant of *Drosophila* deficient in learning. *Proc Natl Acad Sci USA 73:*1684-8.

Dudai Y., Uzzan A. and Zvi S. (1983) Abnormal activity of adenylate cyclase in the *Drosophila* memory mutant rutabaga. *Neurosci Lett.* 42(2):207-12.

Dujardin F. (1850) Memoire sur le systeme nerveux des insectes. *Ann Sci Natl Zool.* 14:195-206.

Erber J., Masuhr T., and Menzel R. (1980) Localization of short-term memory in the brain of the bee, *Apis mellifera*. *Physiol Entomol.* 5:343-358.

Fadool D.A. And Ache B.W. (1992) Plasma membrane inositol 1,4,5-trisphosphate-activated channels mediate signal transduction in lobster olfactory receptor neurons. *Neuron*. (5):907-18.

Feany M. B. and Quinn W. G. (1995) A neuropeptide gene defined by the *Drosophila* memory mutant amnesiac. *Science* 268:869-73.

Ferrus A. (1992) Neurogenetics of *Drosophila.* Elsevier, Vol IX, No. 1, p.11-56.

Fiala A., Spall T., Riemensperger T. and Buchner E. (2005) Representation of odor identity and intensity in central projection of *Drosophila* brain. (submitted)

Fiala A., Spall T., Diegelmann S., Eisermann B., Sachse S., Devaud J. M., Buchner E. and Galizia C. G. (2002) Genetically expressed cameleon in *Drosophila melanogaster* is used to visualize olfactory information in projection neurons. *Curr Biol* 12:1877-84.

Folkers E., Drain P. and Quinn W. G. (1993) Radish, a *Drosophila* mutant deficient in consolidated memory. *Proc Natl Acad Sci USA* 90:8123-7.

Frankland P.W., Cestari V., Filipkowski R.K., McDonald R.J. and Silva A.J. (1998) The dorsal hippocampus is essential for context discrimination but not for contextual conditioning. *Behav Neurosci* 112:863-874.

Fukushi T. (1973) Olfactory conditioning in the housefly, *Musca domestica*. *Annot. Zool. Jap*. 46 (3):135-143.

Galindo K. and Smith D.P. (2001) A large family of divergent *Drosophila* odorant-binding proteins expressed in gustatory and olfactory sensilla. *Genetics*. 159(3):1059-72.

Gao Q. and Chess A. (1999) Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. *Genomics* 60:31-39.

Gao Q., Yuan B. and Chess, A. (2000) Convergent projections of *Drosophila* olfactory neurons to specific glomeruli in the antennal lobe. *Nat. Neurosci.* 3:780-785.

Gerber B., Tanimoto H. and Heisenberg M. (2004) An engram found? Evaluating the evidence from fruit flies. *Curr Opin Neurobiol.* 14(6):737-44.

Goldman A.L., Van der Goes van Naters W., Lessing D., Warr C.G. and Carlson J.R. (2005) Coexpression of two functional odor receptors in one neuron. *Neuron* 45(5):661-666.

Gomez-Diaz C., Martin F. and Alcorta E. (2004) The cAMP transduction cascade mediates olfactory reception in *Drosophila melanogaster*. *Behav Genet.* 34(4):395-406.

Goodwin S. F., Del Vecchio M., Velinzon K., Hogel C., Russell S. R., Tully T. and Kaiser K. (1997) Defective learning in mutants of the *Drosophila* gene for a regulatory subunit of cAMP-dependent protein kinase. *J Neurosci* 17:8817-27.

Guo A., Li L., Xia S.Z., Feng C.H., Wolf R. and Heisenberg M. (1996) Conditioned visual flight orientation in *Drosophila*: dependence on age, practice, and diet. *Learn Mem*, 3:49-59.

Hallem E.A., Ho M.G. and Carlson J.R., (2004) The molecular basis of odor coding in the *Drosophila* antenna. *Cell* 117:965-979

Hammer M. and Menzel R. (1998) Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn Mem*, **5**, 146-56.

Han K.A., Millar N.S. and Davis R.L. (1998) A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J Neurosci*, **18**, 3650-8.

Han K.A., Millar N.S., Grotewiel M.S. and Davis R.L. (1996) DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron* 16:1127-35.

Han P. L., Levin L. R., Reed R. R. and Davis R. L. (1992) Preferential expression of the *Drosophila* rutabaga gene in mushroom bodies, neural centers for learning in insects. *Neuron* 9:619-27.

Hartlieb E. and Anderson P. (1999) Olfactory-released behaviours. In Hansson, B.S. (ed.), Insect Olfaction. Springer, Berlin, pp. 315-349.

Hatt H. and Ache B.W. (1994) Cyclic nucleotide- and inositol phosphate-gated ion channels in lobster olfactory receptor neurons. *Proc Natl Acad Sci USA*. 91(14):6264-8.

Heimbeck G., Bugnon V., Gendre N., Keller A. and Stocker R.F. (2001) A central neural circuit for experience-independent olfactory and courtship behavior in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 98:15336-41.

Heimbeck G., Bugnon V., Gendre N., Keller A. and Stocker R.F. (2001) A central neural circuit for experience-independent olfactory and courtship behavior in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 98(26):15336-41.

Heisenberg M. (1980) Mutants of brain structure and function: what is the significance of the mushroom bodies for behavior? *Basic Life Sci*. 16:373-390.

Heisenberg M. (1998) What do the mushroom bodies do for the insect brain? *Learn Mem* 5:1-10.

Heisenberg M. (2003) Mushroom body memoir: from maps to models. *Nature* 4:266-275.

Heisenberg M., Borst A., Wagner S. and Byers D. (1985) *Drosophila* mushroom body mutants are deficient in olfactory learning. *J Neurogenet* 2:1-30.

Hekmat-Scafe D.S., Scafe C.R., McKinney A.J. and Tanouye M.A. (2002) Genome-wide analysis of the odorant-binding protein gene family in *Drosophila melanogaster*. *Genome Res.* 12(9):1357-69.

Hekmat-Scafe D.S., Steinbrecht R.A. and Carlson J.R. (1998) Olfactory coding in a compound nose. Coexpression of odorant-binding proteins in *Drosophila*. *Ann N Y Acad Sci*. 855:311-315.

Hildebrand J.G. (1996) Olfactory control of behavior in moths: central processing of odor information and the functional significance of olfactory glomeruli. *J. Comp. Physiol. A*. 178(1):5-19.

Hildebrand J.G. and Shepherd G.M. (1997) Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu Rev Neurosci.* 20:595-631.

Inamdar M., Vijayraghavan K. and Rodrigues V. (1993) The *Drosophila* homolog of the human transcription factor TEF-1, scalloped, is essential for normal taste behavior. *J Neurogenet.* 9(2):123-39.

Isabel G., Pascual A. and Preat T. (2004) Exclusive consolidated memory phases in *Drosophila*. *Science* 304(5673): 1024-1027.

Ito K., Suzuki K., Estes P., Ramaswami M., Yamamoto D. and Strausfeld N. J. (1998) The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in *Drosophila melanogaster* Meigen. *Learn Mem.* 5:52-77.

Jefferis G.S., Marin E.C., Stocker R.F. and Luo L. (2001) Target neuron prespecification in the olfactory map of *Drosophila*. *Nature* 414:204-8.

Jefferis G.S., Marin E.C., Watts R.J. and Luo L. (2002) Development of neuronal connectivity in *Drosophila* antennal lobes and mushroom bodies. *Curr Opin Neurobiol.* 12(1):80-6.

Kay L.M. (2004) Two minds about odors. *Proc Natl Acad Sci USA*. 101(51):17569-70

Keene A.C., Stratmann M., Keller A., Perrat P.N., Vosshall L.B. and Waddell S. (2004) Diverse odor-conditioned memories require uniquely timed dorsal paired medial neuron output. *Neuron* 44(3):521-33.

Kim M.S. and Smith D.P. (2001) The invertebrate odorant-binding protein LUSH is required for normal olfactory behavior in *Drosophila*. *Chem Senses* 26:195-199.

Kim M.S., Repp A. and Smith D.P. (1998) LUSH odorant-binding protein mediates chemosensory responses to alcohols in *Drosophila melanogaster*. Genetics. 150(2):711-21.

Kim Y.C., Lee H.G., Seong C.S. and Han K.A. (2003) Expression of a D1 Dopamine Receptor dDA1/DmDOP1 in the Central Nervous System of *Drosophila melanogaster*. *Gene Expr Patterns*. 3(2):237-45.

Kitamoto T. (2001) Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J Neurobiol* 47:81-92.

Kitamoto T. (2002) Conditional disruption of synaptic transmission induces male-male courtship behavior in *Drosophila*. *Proc Natl Acad Sci USA* 99:13232-7.

Komiyama T., Carlson J.R. and Luo L. (2004) Olfactory receptor neuron axon targeting: intrinsic transcriptional control and hierarchical interactions. *Nat Neurosci.* (8):819-25.

Kosaka T. and Ikeda K. (1983). Reversible blockage of membrane retrieval and endocytosis in the garland *Cell* of the temperature-sensitive mutant of *Drosophila melanogaster*, *shibire*^{ts1}. *J Cell Biol* 97:499-507.

Kramer E. (1976) The orientation of walking honeybee in odor fields with small concentration gradients. *Physiol Entomol* 1:27-37.

Krieger J. and Breer H. (1999) Olfactory reception in invertebrates. *Science* 286(5440):720-3.

Laissue P.P., Reiter C., Hiesinger P.R., Halter S., Fischbach K.F. and Stocker R.F. (1999) Three-dimensional reconstruction of the antennal lobe in *Drosophila melanogaster*. *J Comp Neurol.* 405(4):543-52

Larsson M.C., Domingos A.I., Jones W.D., Chiappe M.E., Amrein H. and Vosshall L.B. (2004) Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* 43(5):703-14.

Laurent G. (1999) A systems perspective on early olfactory coding. *Science* 286(5440):723-8.

Laurent G., Stopfer M., Friedrich R., Rabinovich M.I., Volkovskii A. and Abarbanel H.D. (2001) Odor encoding as an active, dynamical process: experiments, computation, and theory. *Annu Rev Neurosci.* 24:263-97.

Laurent G., Wehr M. and Davidowitz H. (1996) Odour encoding by temporal sequences of firing in oscillating neural assemblies. *Nature* 384(6605):162-6.

Lee T. and Luo L. (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-61.

Levin L. R., Han P. L., Hwang P. M., Feinstein P. G., Davis R. L. and Reed R. R. (1992) The *Drosophila* learning and memory gene rutabaga encodes a $Ca2^{+}$/Calmodulin-responsive adenylyl cyclase. *Cell* 68:479-89.

Li W., Tully T. and Kalderon D. (1996) Effects of a conditional *Drosophila* PKA mutant on olfactory learning and memory. *Learn Mem* 2:320-333.

Linster C., Johnson B.A., Morse A., Yue E. and Leon M. (2002) Spontaneous versus reinforced olfactory discriminations. *J Neurosci.* 22(16):6842-5.

Linster C., Johnson B.A., Morse A., Yue E., Xu Z., Hingco E.E., Choi Y., Choi M., Messiha A. and Leon M. (2001) Perceptual correlates of neural representations evoked by odorant enantiomers. *J Neurosci* 21:9837-9843.

Liu L., Wolf R., Ernst R. and Heisenberg M. (1999) Context generalization in *Drosophila* visual learning requires the mushroom bodies. *Nature* 400(6746):753-6.

Livingstone M.S., Sziber P.P. and Quinn W.G. (1984) Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a *Drosophila* learning mutant. *Cell* 37:205-15.

Malnic B., Hirono J., Sato T. and Buck L.B. (1999) Combinatorial receptor codes for odors. *Cell*. 96(5):713-723.

Mao Z., Roman G., Zong L. and Davis R.L. (2003) Pharmacogenetic rescue in time and space of the rutabaga memory impairment by using Gene-Switch. *Proc. Natl. Acad. Sci. USA* 101(1):198-203.

Marin E.C., Jefferis G.S., Komiyama T., Zhu H. and Luo L. (2002) Representation of the glomerular olfactory map in the *Drosophila* brain. *Cell* 109(2):243-55.

Martin F., Charro M.J. and Alcorta E. (2001) Mutations affecting the cAMP transduction pathway modify olfaction in *Drosophila*. *J Comp Physiol A*. 187(5):359-70.

Martin J.R., Ernst R. and Heisenberg M. (1998) Mushroom bodies suppress locomotor activity in *Drosophila melanogaster*. *Learn Mem.* 5(1-2):179-91.

McBurney D.H. (1975) Taste and olfaction: sensory discrimination. In: Handbook of Physiology - The Nervous System III. p. 1067-1086.

McGuire S. E., Le P. T. and Davis R. L. (2001) The role of *Drosophila* mushroom body signaling in olfactory memory. *Science* 293:1330-3.

McGuire S.E., Le P.T., Osborn A.J., Matsumoto K. and Davis R.L. (2003) Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science* 302(5651):1765-1768.

McGuire T. R. and Hirsch J. (1977) Behavior-genetic analysis of *Phormia regina*: conditioning, reliable individual differences, and selection. *Proc Natl Acad Sci USA* 74:5193-7.

Meinertzhagen I.A., Govind C.K., Stewart B.A., Carter J.M. and Atwood H.L. (1998) Regulated spacing of synapses and presynaptic active zones at larval neuromuscular junctions in different genotypes of the flies *Drosophila* and *Sarcophaga*. *J Comp Neurol.* 393(4):482-92.

Milner B. (1972) Disorders of learning and memory after temporal lobe lesions in man. *Clin Neurosurg* 19:421-46.

Mizunami M., Weibrecht J.M., Strausfeld N.J. (1998) Mushroom bodies of the cockroach: their participation in place memory. *J Comp Neurol.* 402(4):520-37.

Mori K. and Shepherd G.M. (1994) Emerging principles of molecular signal processing by mitral/tufted cells in the olfactory bulb. *Semin Cell Biol.* 5(1):65-74.

Ng M., Roorda R.D., Lima S.Q., Zemelman B.V., Morcillo P. and Miesenbock G. (2002) Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly. *Neuron* 36(3):463-74.

Nighorn A., Healy M. J. and Davis R. L. (1991) The cyclic AMP phosphodiesterase encoded by the *Drosophila* dunce gene is concentrated in the mushroom body neuropil. *Neuron* 6:455-67.

Pascual A. and Preat T. (2001) Localization of long-term memory within the *Drosophila* mushroom body. *Science* 294:1115-7.

Pelosi P. (1994) Odorant-binding proteins. *Crit Rev Biochem Mol Biol.* 29(3):199-228.

Pelz C., Gerber B. and Menzel R. (1997) Odorant intensity as a determinant for olfactory conditioning in honeybees: roles in discrimination, overshadowing and memory consolidation. *J Exp Biol*. 200(Pt 4):837-47.

Perazzona B., Isabel G., Preat T. and Davis R.L. (2004) The role of cAMP response element-binding protein in *Drosophila* long-term memory. *J Neurosci*. 24(40):8823-8.

Pirrotta V. (1988) Vectors for P-mediated transformation in *Drosophila*. *Biotechnology* 10:437-56.

Pitts R.J., Fox A.N. and Zwiebel L.J., (2004) A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *Anopheles gambiae*. *Proc. Natl. Acad. Sci. USA* 10: 5058-5063.

Prasad B.C. and Reed R.R. (1999) Chemosensation: molecular mechanisms in worms and mammals. *Trends Genet.* (4):150-3

Preat T. (1998) Decreased odor avoidance after electric shock in *Drosophila* mutants biases learning and memory tests. *J. Neurosci*. 18(20):8534-8538.

Prokop A. and Technau G.M. (1994) Normal function of the mushroom body defect gene of *Drosophila* is required for the regulation of the number and proliferation of neuroblasts. *Dev Biol.* 161(2):321-37.

Quinn W. G., and Dudai Y. (1976) Memory phases in *Drosophila*. *Nature* 262:576-7.

Quinn W. G., Harris W. A. and Benzer S. (1974) Conditioned behavior in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 71:708-12.

Quinn W. G., Sziber P. P. and Booker R. (1979) The *Drosophila* memory mutant amnesiac. *Nature* 277:212-4.

Riesgo-Escovar J., Raha D. and Carlson J.R. (1995) Requirement for a phospholipase C in odor response: overlap between olfaction and vision in *Drosophila*. *Proc Natl Acad Sci USA*. 92(7):2864-8.

Rodrigues V. (1980) Olfactory behavior of *Drosophila melanogaster*. *Basic Life Sci*. 16:361-71.

Rodrigues V. and Siddiqi (1978) Genetic analysis of chemosensory pathway. *Proc Indian Acad Sci B* 87:147.

Salz H.K. and Kiger J.A. (1984) Genetic analysis of chromomere 3D4 in *Drosophila melanogaster*. *Genetics* 108:377-392.

Scoville W.B. and Milner B. (2000) Loss of recent memory after bilateral hippocampal lesions. 1957. *J Neuropsychiatr Clin Neurosci* 12:103-113.

Shanbhag S.R., Mueller B. and Steinbrecht R.A. (1999) Atlas of olfactory organs of *Drosophila melanogaster*. 1. Types, external organization, innervation and distribution of olfactory sensilla. *International Journal of Insect Morphology and Embryology* 28:377-397

Shanbhag S.R., Mueller B. and Steinbrecht R.A. (2000) Atlas of olfactory organs of *Drosophila melanogaster*. 2. Internal organization and cellular architecture of olfactory sensilla. *Arthropod Structure and Development*. 29:211-229.

Schild D. and Restrepo D. (1998) Transduction mechanisms in vertebrate olfactory receptor cells. *Physiol Rev.* 78(2):429-66.

Schürmann F.W. (1987) The architecture of the mushroom bodies and related neuropils in the insect brain. In: Arthropods brains: Its evolution, development, structure, and function. (Gupta A.P. ed.) John Wiley & Sons, New York, NY.

Schwaerzel M., Heisenberg M. and Zars T. (2002) Extinction antagonizes olfactory memory at the sub*Cell*ular level. *Neuron* 35:951-60.

Schwaerzel M., Monastirioti M., Scholz H., Friggi-Grelin F., Birman S. and Heisenberg M. (2003) Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J Neurosci*. 23(33):10495-502.

Silbering A.F., Sachse S., Eisermann B. and Galizia C.G. (2003) Odor induced activity patterns in the antennal lobe of *Drosophila melanogaster*. In Elsner and Zimmermann H.: Proceedings of the 29th Goettingen Neurobiology Conference 2003. p. 481-482.

Skoulakis E.M., Kalderon D. and Davis R.L. (1993) Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. *Neuron* 11:197-208.

Spehr M. and Leinders-Zufall T. (2005) One neuron-multiple receptors: increased complexity in olfactory coding? *Sci STKE.* (285):pe25.

Stensmyr M.C., Dekker T. and Hansson B.S. (2003) Evolution of the olfactory code in *Drosophila melanogaster* subgroup. *Proc. R. Soc. Lond. B. Biol. Sci*. 270:2333-2340.

Stocker R.F. (1994) The organization of the chemosensory system in *Drosophila melanogaster*: A review. *Cell Tissue Res*. 275:3-26.

Stocker R.F., Heimbeck G., Gendre N. and de Belle J.S. (1997) Neuroblast ablation in *Drosophila* P[GAL4] lines reveals origins of olfactory interneurons. *J Neurobiol*, 32:443-56.

Stocker R.F., Lienhard M.C., Borst A. and Fischbach K.F. (1990) Neuronal architecture of the antennal lobe in *Drosophila melanogaster*. *Cell Tissue Res.* 262:9-34.

Stoerkuhl K. F., Hovemann B. T. and Carlson J. R. (1999) Olfactory Adaptation Depends on the Trp $Ca2^{+}$ Channel in *Drosophila*. *J NeuroScience.* 19(12):4839-4846.

Strausfeld N. J., Hansen L., Li Y., Gomez R. S. and Ito K. (1998) Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learn Mem.* 5:11-37.

Strausfeld N.J. (1976) Atlas of an Insect Brain (Berlin: Springer-Verlag).

Strausfeld N.J. and Li Y. (1999) Representation of the calyces in the medial and vertical lobes of cockroach mushroom bodies. *J Comp Neurol.* 409(4):626-46.

Strausfeld N.J., Sinakevitch I. and Vilinsky I. (2003) The mushroom bodies of *Drosophila melanogaster*: an immunocytological and golgi study of Kenyon cell organization in the calyces and lobes. *Microsc Res Tech.* 62(2):151-69.

Strotmann J, Wanner I, Krieger J, Raming K and Breer H. (1992) Expression of odorant receptors in spatially restricted subsets of chemosensory neurons. *Neuroreport*. 3(12):1053-6.

Takken W. and Kline D.L. (1989) Carbon dioxide and 1-octen-3-ol as mosquito attractants. *J. Am. Mosq. Control Assoc.* 5:311-316.

Tang S. and Guo A. (2001) Choice behavior of *Drosophila* facing contradictory visual cues. *Science* 294:1543-7.

Technau G. and Heisenberg M. (1982) Neural reorganization during metamorphosis of the corpora pedunculata in *Drosophila melanogaster*. *Nature* 295:405-7.

Tempel B.L., Bonini N., Dawson D.R. and Quinn W.G. (1983) Reward learning in normal and mutant *Drosophila*. *Proc Natl Acad Sci USA* 80:1482-6.

Tryon R.C. (1940) Genetic differences in maze learning in rats. *Yk. Natl. Soc. Stud. Educ***.** 39:111-19.

Tuccini A.R., Maida P., Rovero P., Mazza M. and Pelosi P. (1996) Putative odorant binding protain in antennae and legs of *Carausius morosus*. *Insect Biochem Mol Biol 26*:19-24.

Tully T. and Quinn W.G. (1985) Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J Comp Physiol A* 157:263-77.

Tully T., Preat T., Boynton S.C. and Del Vecchio M. (1994) Genetic dissection of consolidated memory in *Drosophila*. *Cell 79*:35-47.

Uchida N. and Mainen Z.F. (2003) Speed and accuracy of olfactory discrimination in the rat. *Nat Neurosci.* 6(11):1224-9.

Vickers N.J. (2000) Mechanisms of animal navigation in odor plumes. *Biol. Bull.* 198:203-212.

Vickers N.J., Christensen T.A., Baker T.C. and Hildebrand J.G. (2001) Odour-plume dynamics influence the brain's olfactory code. *Nature 410*:466-470.

Vosshall L. B., Wong A. M., and Axel R. (2000) An olfactory sensory map in the fly brain. *Cell* 102:147-59.

Vosshall L.B., Amrein H., Morozov P.S., Rzhetsky A. and Axel R. (1999) A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* 96:725-736.

Waddell S., and Quinn W. G. (2001) What can we teach *Drosophila*? What can they teach us? *Trends Genet* 17:719-26.

Waddell S., Armstrong J. D., Kitamoto T., Kaiser K. and Quinn W. G. (2000) The amnesiac gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory. *Cell* 103:805-13.

Wang J. W., Wong A. M., Flores J., Vosshall L. B. and Axel R. (2003) Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell* 112:271-82.

Wang Y, Chiang AS, Xia S, Kitamoto T, Tully T, Zhong Y (2003) Blockade of neurotransmission in *Drosophila* mushroom bodies impairs odor attraction, but not repulsion. *Curr Biol.* 13(21):1900-4

Wang Y.L., Guo H.F., Pologruto T.A., Hannan F., Hakker I., Svoboda K. and Zhong Y. (2004) Stereotyped odor-evoked activity in the mushroom body of *Drosophila* revealed by green fluorescent protein-based $Ca2^{+}$ imaging. *J Neurosci.* 24 (29):6507-6514

Wang Z., Singhvi A., Kong P. and Scott K. (2004) Taste Representations in the *Drosophila* Brain. *Cell* 117:981-991.

West A.S. (1961) Chemical attractants for adult *Drosophila* species. *J Econ Entomol.* 54:677-681.

Wolf R., Wittig T., Liu L., Wustmann G., Eyding D. and Heisenberg M. (1998) *Drosophila* mushroom bodies are dispensable for visual, tactile, and motor learning. *Learn Mem.* 5(1-2):166-78.

Wong A. M., Wang J. W. and Axel R. (2002) Spatial representation of the glomerular map in the *Drosophila* protocerebrum. *Cell* 109:229-241.

Xia S., Liu L., Feng C. and Guo A. (1997) Drug disruption of short-term memory in *Drosophila melanogaster*. *Pharmacol Biochem Behav* 58:727-735.

Xu P., Atkinson R., Jones D.N. and Smith D.P. (2005) *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. *Neuron*. 45(2):193-200.

Yin J.C., Del Vecchio M., Zhou H. and Tully T. (1995) CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in *Drosophila*. *Cell* 81:107-15.

Yin J.C., Wallach J.S., Del Vecchio M., Wilder E.L., Zhou H., Quinn W.G. and Tully T. (1994) Induction of a dominant negative CREB transgene specifically blocks long-term memory in *Drosophila*. *Cell* 79:49-58.

Yu D., Ponomarev A. and Davis R.L. (2004) Altered representation of the spatial code for odors after olfactory classical conditioning; memory trace formation by synaptic recruitment. *Neuron* 42(3):359-61.

Zars T., Fischer M., Schulz R. and Heisenberg M. (2000) Localization of a short-term memory in *Drosophila*. *Science* 288:672-5.

Zhu J., Park K.C. and Baker T.C. (2003) Identification of odors from overripe mango that attract vinegar flies, *Drosophila melanogaster*. *J. Chem. Ecol.* 29:899-909.

8. Abbreviations

3-OCT	3-octanol
AC	adenylyl cyclase
AL	antennal lobe
AM	amylacetate
ARM	amnesia resistant memory
ATP	adenosin triphosphate
BAL	benzaldehyde
CAM	calmodulin
cAMP	cyclic adenosin monophosphate
CREB	cAMP response element binding protein
CS^+	conditioned stimulus (presented with US)
CS	conditioned stimulus
CXM	cycloheximide
DAG	diacylglycerol
DOR	drosophila odorant receptor
EA	ethyl acetate
GAL4	yeast transcription factor
Gαs	G-protein α subunit
HU	hydroxyurea
IAA	isoamylacetate
iACT	inner antennocerebral tract
IP3	inositol triphosphate
LI	learning index
LN	local interneuron
LPC	lateral protocerebrum
LTM	long term memory
mACT	medial antennocerebral tract
MB	mushroom body
MTM	middle term memory
OBP	odorant binding protein
ODE	odor degrading enzymes
OIM	odor intensity memory
OR	odorant receptor
ORN	olfactory receptor neuron
PACAP	pituitary adenylyl cyclase-activating peptide
PDE	phosphodiesterase
PI	performance index
PKA	protein kinase A
PLC	phospholipase C
PN	projection neuron
STM	short term memory
TNT	tetanus toxin
TRP	transient receptor potential
UAS	upstream activation system
US	unconditioned stimulus
WT	wild type flies

CV

Pavel Mašek

Born 18.11.1974 in Ústí nad Labem, Czech Republic

Department of Genetics and Neurobiology,
University of Wuerzburg,
Am Hubland, Wuerzburg
97074 Germany

Graduate studies:

2000 - 2005: Ph.D. in Biology, University of Wuerzburg, Wuerzburg, Germany

Undergraduate studies:

1998 - 2000: M.Sc. degree in Entomology at the University of South Bohemia, Faculty of Biological Sciences, České Budějovice, Czech Republic
Master thesis: "Ultrastructure of mayflies' cuticle: the story of larva-subimaginal ecdysis"

1994 - 1998: B.Sc. degree in Biology at the University of South Bohemia, Faculty of Biological Sciences, České Budějovice, Czech Republic
Bachelor thesis: "Morphology of the puparia of tsetse flies (Diptera, *Glossinidae*)"

Education:

1989-1993: High school (Gymnasium) Stavbařů, Ústí nad Labem, Czech Republic

1981-1989: Elementary school (ZS) Stavbařů, Ústí nad Labem, Czech Republic

Publications:

Masek P. and Heisenberg M. (2005) Odor reinforced learning in *Drosophila* with and without mushroom bodies. (Manuscript in preparation)

Masek P. and Heisenberg M. (2005) Olfactory learning independent of the cAMP-signaling pathway. (Manuscript in preparation)

Soldan T., Brunnhofer V. and Masek P. (1999) A capacity method modified for sexing of tsetse puparia: preliminary results and prospects. In: Feldmann U. (Ed) *Animal trypanosomosis: Vector and Disease Control Using Nuclear Techniques*. Proc. 2nd FAO/IAEA Seminar for Africa. Blackhuys Publishers, Leiden, pp. 129-140.

Abstracts:

Masek P. and Heisenberg M. (2004) Olfactory learning independent of the cAMP signaling pathway. The 10th European Symposium on *Drosophila* Neurobiology; Neuchatel (oral presentation)

Masek P. and Heisenberg M. (2003) Concentration learning is independent of the cAMP signaling pathway. Neurobiology of *Drosophila*; Cold Spring Harbor (poster)

Masek P. and Heisenberg M. (2002) Odor reinforced learning in *Drosophila* with and without mushroom bodies. 9th regional Drosophila meeting; Bochum (oral presentation)

Würzburg, 11.09.2005

Pavel Mašek

Erklärung gemäß §4 der Promotionsordnung für die Fakultät für Biologie der Bayrischen Julius-Maximilians-Universität Würzburg vom 15. März 1999:

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbstständig angefertigt habe und keine anderen Hilfsmittel als die angegebenen angewandt habe. Alle aus der Literatur entnommenen Stellen und Abbildungen sind als solche kenntlich gemacht.

Die Dissertation wurde weder vollständig noch teilweise an einer anderen Fakultät vorgelegt.

Würzburg, 11.09.2005

Pavel Mašek