Sugar reward learning in *Drosophila*

Neuronal circuits in *Drosophila* associative olfactory learning

Dissertation zur Erlangung des

Naturwissenschaftlichen Doktorgrades

der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

Andreas Stephan Thum

aus Nördlingen

Würzburg, 2006

Eingereicht am:

Mitglieder der Promotionskommission:

Vorsitzender:

Gutachter:

Gutachter:

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

Table of content

1. Introduction

For centuries, researchers in the functional brain sciences have been mapping properties of behaviour to areas of the brain. Nevertheless, a simple, generally accepted model of the brain is not yet established. Without a firm idea of how the brain works as a whole, assigning functions to certain parts of the brain remains pending. This general problem is not restricted to mammals, but in other animals such as insects it might be less severe, as these have much smaller brains and distinctly less complex behaviour. Especially the fly *Drosophila melanogaster* has proven most useful for analyzing brain functions. Among the many experimental advantages it is the amenability to genetic analysis and the existing set of genetic tools that make this species a preferred model of functional brain research (Duffy, 2002; McGuire et al., 2005; Bier, 2005).

1.1 *Drosophila melanogaster*: a model organism to study learning and memory

Drosophila was introduced into the laboratory by Castle at Harvard in 1901 and soon picked up by Lutz, Loeb, Morgan, and others (Kohler et al., 1994). The major wild types currently in use date from the 1920s (Ashburner 1989). The flies are conveniently small, inexpensive, clean, harmless (except for occasional allergies), and easy to cultivate. Their generation time is only about 10 days at 25°C, and the life cycle includes easily identifiable phases (Ashburner 1989). Furthermore, it displays a rich behavioural repertoire, including positive phototaxis, negative geotaxis, and courtship. The flies are also able to link certain cues of their environment and establish a memory for such associations (Quinn et al., 1974; Tully and Quinn 1985; Hall et al., 1986, Dudai et al., 1988, Corfas and Dudai 1989; Liu et al., 1999). But it is the amenability of *Drosophila* to genetic analysis that has made the real difference. The small number of chromosomes, the convenient chromosomal cytology, the

availability of wild types and mutants, the short generation time and ease of breeding – all these initiated a systematic analysis of *Drosophila* genetics (Dudai 2002). With time, the accumulation of knowledge, the large number of genetically mapped mutations and the rich repertoire of experimental methods, have all reinforced the experimental advantages of *Drosophila*, and made it a popular model organism to study the contributions of genes to behaviour. For example genes which are involved in learning and memory investigated in an efficient, reproducible memory assay (Tully and Quinn 1985).

The next parts of the introduction will focus on the advantages of *Drosophila* which are relevant for my research. Namely the established behavioural odor learning paradigm, the huge existing set of genetic tools to intervene with behaviour, the described neuronal circuit relevant for CS and US processing and coincidence detection, and the identified genes and their suggested molecular mechanisms in learning and memory.

1.2 Classical conditioning: The beginnings and a paradigm for the fly

1.2.1 Pavlovian conditioning

About one century ago the Russian physiologist Ivan Petrovich Pavlov (1849-1936) carried out series of learning experiments. He trained dogs by paring two stimuli, a gustatory stimulus (food) - the unconditioned stimulus (US) - and an auditory one (bell) or visual stimuli - the conditioned stimulus (CS). The US elicits the unconditioned response (UR) the dogs salivate. After the pairing the CS comes to evoke a conditioned response (CR), which is similar to the unconditioned response (UR) elicited by the US. This Pavlovian conditioning (Pavlov 1906) is still a cornerstone of learning research and therefore today called classical conditioning. In the past three decades, it has accumulated renewed momentum, due to the impressive developments in behavioural, cognitive, system, and cellular neuroscience (Holland et al., 1993; Pearce and Bouton 2001; for *Drosophila* reviewed in Davis 2005;

Heisenberg 2003). Based on the learning experiments of Pavlov a new field of research emerged with more complex tasks, e.g. second-order conditioning (Rescola et al., 1980), or experiments showing that the ability of a stimulus to enter into association and control behaviour is altered by the history of the subject with this or other stimuli either before, during, or after training. For example sensory pre-conditioning (Brogden et al., 1939; Kimmel et al., 1977), conditioned inhibition (Pavlov 1927; Zimmer-Hart and Rescola 1974), learned irrelevance (Baker 1976), latent inhibition (Lubow and Moore 1959), pre-conditioning to the US (Miller et al., 1993), and US devaluation (Rescorla et al., 1973; Holland and Straub 1979). Introducing the fruitfly *Drosophila melanogaster* into the study of learning and memory (Quinn et al., 1974; Dudai et al., 1976) made it possible to exploit the integrative approach combining learning psychology and genetic intervention. This capability is still paramount. The fly can be used, in principle, to determine how many genes influence a given type of learning and how these genes interact (Davis 1993).

1.2.2 The paradigm

In 1974 Quinn and Benzer showed that *Drosophila* can learn to discriminate between different odors. Their sophisticated set of experiments excluded effects like pseudo-conditioning, excitatory states, odor preferences, sensitization, habituation, and subjective bias, which have been complications of preceding conditioning experiments in flies (Frings et al., 1941; Murphy et al., 1967; Yeatman and Hirsch 1971). To train flies, they are exposed to an odor A (the CS+) paired with electric shock (the US) as an aversive reinforcer, followed by the unpaired presentation of a second odor B alone (the CS-). This is the so called training phase, which precedes a test phase where the animals can choose between the two olfactory cues (CS+ and CS-) in a forced choice maze (see Material and Methods). In general this protocol for olfactory conditioning of *Drosophila* is still used today.

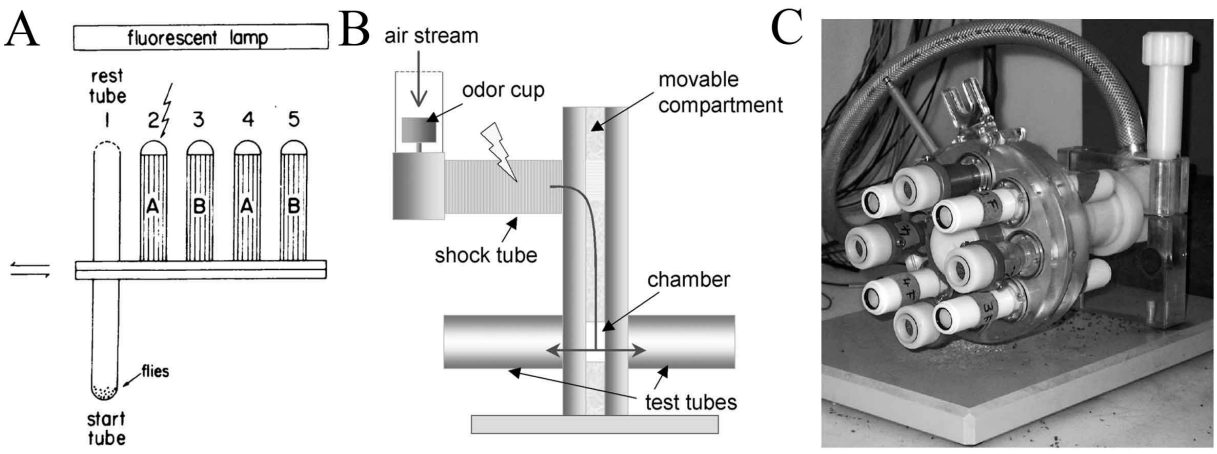


Figure 1: Basic olfactory paradigms:

A) Apparatus used for training and testing flies. Two plastic blocks holding tubes slide on a dovetail joint, so that the start tube can be shifted into register with tubes 1 through 5. Tube 1, the rest tube, is perforated for ventilation. Tubes 2 through 5 contain grids with odorants A or B. Tubes 2 and 3 are used for training. The lightning bolt indicates voltage on the grid. Tubes 4 and 5 are for testing. (Dudai et al., 1976) **B)** Improved conditioning apparatus consists of a shock tube with 95% of its inner surface covered with an electrifiable printed circuit grid, two collection tubes at a T-maze choice point for testing relative odor preferences, a sliding center compartment used to transfer flies from the training tube to the choice pint and odor tubes that house odor cups containing different odors. The odor tubes slip onto the distal ends of either the training tube or the collection tubes. Vacuum lines, with air speeds controlled by Teflon needle valves, are connected to the upper port during training and to the lower port during the test trial. (Tully and Quinn 1985) **C)** The actually used machine is a modified (Schwärzel et al. 2001) T-maze. Four independent T-mazes placed on a rotating disc, allow training and testing of four independent groups of flies simultaneously (taken from Masek 2005).

Emerging from an apparatus designed for behavioural countercurrent distribution (Figure 1A), the paradigm was improved to a purely classical one (Tully and Quinn 1985) excluding the original operant features (Quinn et al., 1974; Dudai et al., 1976). Today even automated versions (Pascual and Preat 2001) or modifications (Figure 1C) with increased throughput (Schwärzel 2003) exist. Usually electro shock is used as a reinforcer for the classical learning paradigm in *Drosophila* (reviewed in Davis 2004), but also other noxious stimuli can serve as aversive reinforcers (Quinn et al., 1974; Tully and Quinn, 1985) and even sugar as a positive US (Tempel 1983; Schwärzel et al., 2003).

1.3 Genetic intervention

Initially, neurogenetic analysis was used in *Drosophila* to assess the contribution of genes to behaviour (Hirsch 1959). With the use of single-gene mutations the field advanced into a new phase (Benzer 1967); mutants were generated at random, usually by feeding the flies with mutagenic chemicals (Ashburner 1989). The putative mutants were then screened for phenotypes, e.g. lethality, developmental problems, abnormal performance in discrete behavioural tasks. In that way a number of single gene mutants were identified in *Drosophila* affecting learning and memory rather specifically (reviewed in Davis 2004). Nowadays, mutations are induced by using virus-like transposable genetic elements, so called P-elements that mutate the fly genome by jumping into its chromosomes (O'Kane and Gehring 1987). Also other enhanced methods are in use (Yin 1995; Goodwin 1997), for instance the GAL4-UAS system (Brand and Perrimon 1993).

1.3.1 The GAL4-UAS system

Drosophila is one of the most genetically tractable metazoans. Beside its sequenced genome (Adams et al., 2000) the enormous expansion of genetic tools really defines the fly as a model organism. One particularly elegant example of tool development was the creation of the GAL4/UAS system for targeted gene expression that allows the selective activation of any cloned gene in a wide variety of tissue- and cell-specific patterns (Brand and Perrimon 1993). GAL4 encodes a protein that activates transcription in the yeast *Saccharomyces cerevisiae* induced by galactose (Laughon et al., 1984; Laughon and Gesteland 1984; Masumoto et al., 1980). It directly binds to defined site of an Upstream Activating Sequence (UAS), analogous to an enhancer element described in multicellular eukaryotes (Giniger et al., 1985). The tool uses a promoter (or enhancer) that directs the expression of the transcription activator GAL4

in a particular pattern (Phelps and Brand 1998). GAL4 in turn directs transcription of the GAL4-responsive UAS target gene in an identical pattern (Figure 2).

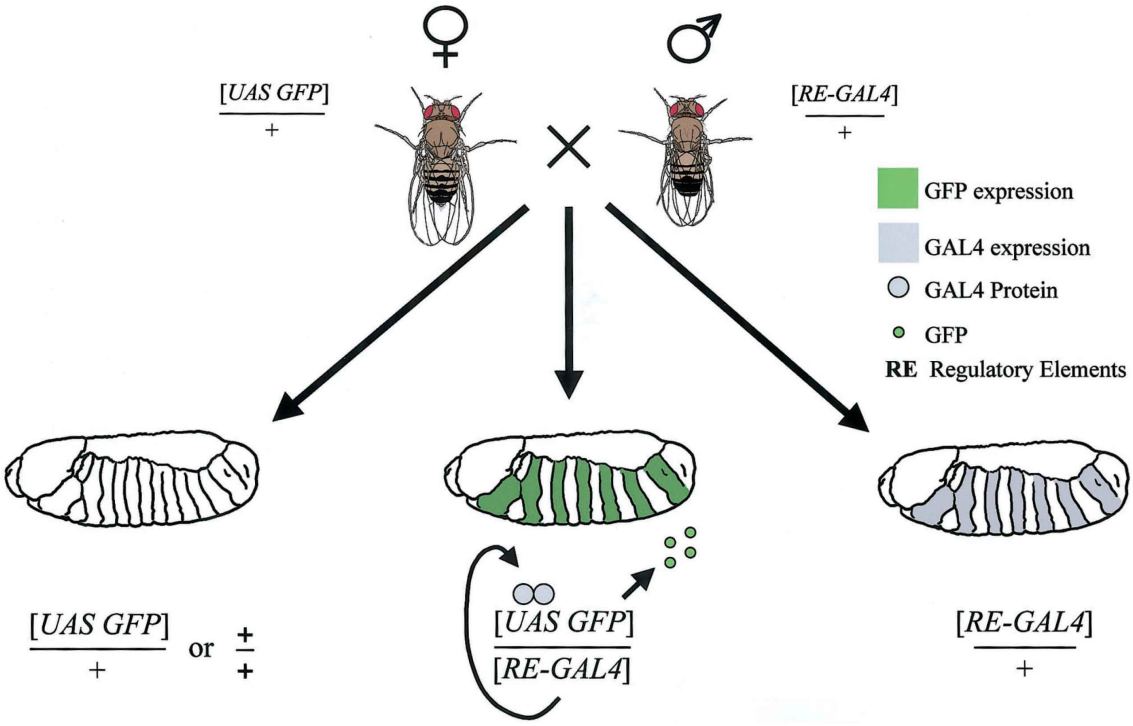


Figure 2: The bipartite UAS/GAL4 system in *Drosophila*: When females carrying a UAS responder (UAS-GFP) are mated to males carrying a GAL4 driver progeny containing both elements of the system are produced. The presence of GAL4 in an alternating segmental pattern in the depicted embryos then drives expression of the UAS responder gene in a corresponding pattern (taken from Duffy 2002).

This bipartite approach using two separate parental lines, the effector line and the driver line, has two major advantages. First, the transcriptional inactivity of the parental responder line means that transgenic responder lines can be generated for gene products that are toxic. Second, one can target the expression of any effector gene in a variety of spatial and temporal fashions by mating it with distinct GAL4 drivers (Brand and Perrimon 1993). Despite the tremendous profit for the *Drosophila* research, application of the GAL4/UAS system revealed three major problems one has to be aware of. First, the activity of GAL4 is temperature dependent. Simply by altering the temperature, a wide range of expression levels of any responder can be achieved, thereby increasing the flexibility of the system (Duffy 2002). Second, although the majority of GAL4 drivers do not appear to have deleterious phenotypic

effects on their own, instances of a GAL4 driver disrupting aspects of normal development have been reported, for example GMR-GAL4 (Freeman et al., 1996; Rister pers. communication). For such reasons it is important to ensure that the process of interest is not affected by the presence of GAL4 on its own. Third, differences in mRNA and protein stability, cellular localization, and sensitivity and timing of detection between a reporter (e.g. UAS-GFP) and the effector gene may lead to differences in the pattern of expression and affect the interpretation of results (Duffy 2002). Additionally it was shown that also the insertion site of the P-element has an effect on the expression pattern of the effector gene (Ito et al., 2003, Jenett pers. communication). Thus, an accurate understanding of the phenotypic consequences of targeting effector gene expression depends on a concomitant analysis of the expression pattern of the effector when combined with a GAL4 driver.

Although the GAL4/UAS system provided a previously unprecedented degree of temporal and spatial regulation, the lack of absolute temporal and spatial specificity of many drivers can hinder analyses in the tissue of interest. A common problem is lethality prior to the developmental stage of interest. Because of that, technically refined improvements to the inducibility of the GAL4/UAS system have been developed. Currently at least nine different approaches have been developed that provide an additional level of spatial and or temporal control to GAL4 activation. See Table 1 for a brief description of these modifications (reviewed in Duffy 2002; McGuire et al., 2003; Pascual et al., 2005; Zeidler et al., 2004)

Table 1: Modifications of the GAL4/UAS system

Modification	Application	Publication
Laser microbeam induced expression of heat-shock GAL4 in single cells	Refining the GAL4/UAS system to determined single cells in the embryo	Halfon et al., 1997
Injecting of caged, inactive GAL4-VP16 into embryos and activation by a lightbeam	Refining the GAL4/UAS system to determined single cells in the embryo	Cambridge et al., 1997
Use of hormone responsive GAL4 chimeras; GAL4-estrogen receptor chimera, GAL4-progesterone receptor chimera	Temporal control of the GAL4/UAS system in a hormone-dependent manner	Han et al., 2000 Osterwalder et al., 2001 Roman et al., 2001
Combination of a inducible tetracycline responsive transactivator (rtTA-M2-alt) With the GAL4 system	Temporal control of the GAL4/UAS system in a tetracycline-dependent manner	Positive system: Stebbins et al., 2001 Negative system: Stebbins and Yin 2001
FLP promoted recombination in cis between two FRT sites flanking a stop cassette; introduced into the GAL line or UAS line	Increased resolution of GAL4/UAS inducibility	Ito et al., 1997; Nellen et al., 1996 Pignoni and Zipursky 1997 Zecca et al., 1996
MARCM system: combination of tubGAL80, GAL4/UAS, and FLP/FRT	Mosaic analysis with a repressible cell marker	Lee and Luo 1999
Using a fusion protein containing the GAL4 binding domain and the repression domain of the insulator suppressor of hairy wing under heat-shock promoter control	Conditional repression of genes located downstream of a UAS sequence	Pascual et al., 2005
Generation of temperature-sensitive GAL4 and GAL80 based on conditionally active inteins	Temporal control of the GAL4/UAS system in a temperature-dependent manner	Zeidler et al., 2004
TARGET system: Three component system using tubGAL80^{ts}	Temporal control of the GAL4/UAS system in a temperature-dependent manner	McGuire et al., 2003

1.3.2 The TARGET system

To examine adult behaviour, it is necessary to induce the respective effector gene specifically in the adult animal in order to avoid noxious side effects, as the enhancer/promoter dependent GAL4 expression drives the effector gene expression often

during the whole development. Therefore different modifications to the GAL4/UAS system were invented to get temporal control (Table 1). Unfortunately introducing temporal control into the system often means to create new driver lines or effector lines; most often makes half of the existing stocks useless (Stebbins et al., 2001; Han et al., 2000; Osterwalder et al., 2001; Roman et al., 2001). A particularly elegant modification to circumvent effector gene expression during development allowing the use of the full set of existing driver and effector lines was the creation of the TARGET system (**t**emporal **a**nd **r**egional **g**ene expression **t**argeting; McGuire et al., 2003). Usually a P element carrying the GAL4 coding region drives the expression of GAL4 protein in a specific tissue on the basis of proximity of the P element to a tissue-specific enhancer. The GAL4 protein then binds to its cognate UAS binding site and activates transcription of the downstream effector gene, as in the conventional way. In the TARGET system, a temperature sensitive GAL80 protein ($GAL80^{ts}$), expressed ubiquitously from the tubulin 1 promoter, represses the transcriptional activity of GAL4 at 19°C and thus prevents the expression of the UAS-effector transgene, but becomes inactive at 30°C, allowing GAL4 to drive the expression of the UAS-effector transgene (McGuire et al., 2003). The invention of the TARGET system allows an adult specific onset of genetic intervention with neurotransmission in *Drosophila* using well described effector genes.

1.3.3 Effector genes to intervene with neurotransmission

Genetic modification in *Drosophila melanogaster* has been used to elucidate functions of neural circuits in behaviour (Sokolowski 2001). The genes of choice ("effector genes") can conveniently and reproducibly be expressed in defined subsets of cells using the GAL4/UAS system (Brand and Perrimon 1993). Effectors that, for instance, block neurotransmitter release or induce cell death have been used to impair neural function (Brand and Dormand 1995; Roman et al., 2004) and by that contributed in revealing the behavioural significance of neural

circuits. In my work I compared the action of five effector genes, *shibire^{ts1}* (Kitamoto 2001; Kitamoto 2002), *Tetanus toxin light chain (TNT)* (Sweeney 1995; Martin 2002) *reaper* (White and Steller 1995; Bergmann et al., 2003; Hay et al., 2004) *Diphtheria toxin A-chain* (*DTA*) (Bellen et al., 1992; Han et al., 2000) and inwardly rectifying potassium channel (*Kir2.1*; Baines et al., 2001; Nitabach et al., 2002), showing differences in their efficiency depending on the target cells and the timing of their onset during development.

Misexpression of *shibire^{ts1}* (*shi^{ts1}*), a temperature sensitive dominant negative dynamin, blocks normal endocytosis for synaptic vesicle recycling, thereby causing an impairment of synaptic transmission (Kitamoto 2001; Kitamoto 2002). Due to the temperature sensitivity of *shi^{ts1}*, synaptic output can be blocked by shifting up the temperature. This temperature-induced block of synaptic transmission is reversible within 1 min by shifting back to the permissive temperature (Koenig and Ikeda 1989; Kitamoto 2002).

Another way of inhibiting neurotransmitter release is expressing *Tetanus toxin light chain* (*TNT*), a protease specifically cleaving neuronal-Synaptobrevin (n-Syb; Sweeney et al., 1995; Martin et al., 2002). N-Syb is essential for neurotransmitter release as it regulates Ca^{2+}-dependent fast synaptic vesicle fusion (Kidokoro et al., 2003). Despite the successful application of these two effector genes that mainly block chemical synapses (Kitamoto 2002; Martin et al., 2002), electrical synapses, for example, are supposed to remain unaffected (White and Paul 1999; Phelan and Starich 2001).

Therefore, genetic ablation is an alternative intervention method as it can act regardless of synapse type (Sweeney et al., 2000). Ectopic *reaper* (*rpr*) expression induces apoptosis by activating the caspase proteolytic cascade that finally leads to DNA fragmentation and chromatin condensation (White and Steller 1995; Bergmann et al., 2003; Hay et al., 2004).

Diphtheria toxin A (DTA), in turn, is an inhibitor of protein synthesis by ribosylating elongation factor-2 (Wilson and Collier 1992). The toxicity of DTA is extreme; one molecule per cell is thought to be sufficient to cause cell death (Yamaizumi et al., 1978). Therefore, I

used the attenuated mutant I of DTA (DTI) for cell ablation (Bellen et al., 1992; Han et al., 2000).

Another alternative method which can block neuronal activity regardless of the synapse type is electrical silencing (Baines et al., 2001, Nitabach et al., 2002). In *Drosophila*, the expression of the human inwardly rectifying potassium channel, Kir2.1, hyperpolarizes neurons, thereby efficiently blocking action potential generation (Baines et al., 2001). In mammalian neurons, temporally controlled Kir2.1 expression can block neuronal excitability (Johns et al., 1999).

1.3.4 RNAi silencing

RNA interference is the technique of destructing targeted endogenous messenger RNAs at the post-transcriptional level served by small homologue dsRNAs (Birchler et al., 2003). Recent combination of the GAL4/UAS system with RNAi technology is emerging as a powerful tool for analysis of loss-of-function phenotypes. Currently, a variety of approaches have been adopted and proven successful for the directed expression of constructs that form double-stranded RNA (dsRNA) molecules (Enerly et al., 2003; Giordano et al., 2002; Kalidas and Smith 2002; Nagel et al., 2002; Piccin et al., 2001; Reichhart et al., 2003; Schmid et al., 2002). Such dsRNAs are capable of mediating gene-specific RNAi (Enerly et al., 2003; Giordano et al., 2002; Kalidas and Smith 2002; Nagel et al., 2002). Difficulties in cloning and inconsistent silencing complicate the method and led to different strategies for UAS RNAi vectors. A vector that is able to generate dsRNA molecules by simultaneous transcription of sense-antisense strands was published by Giordano and coworkers (2002) Two identical but oppositely oriented regulatory regions, each composed of a five-copy array of the UAS activating sequence coupled with an inversely oriented SV40 polyadenylation site flank a cloning polylinker on both sides. Another vector was designed to produce intron-spliced hairpin RNA (Lee and Carthew 2003). It contains inverted repeats separated by a functional

intron under control of the upstream activating sequence (UAS) such that mRNA produced by the transgene is predicted to form loopless hairpin RNA following splicing. Alternatively, UAS effector constructs that lack an intron and instead directly form a hairpin have also been utilized to mediate gene-specific RNAi. Inclusion of an intron in the construction of a UAS hairpin responder may aid in formation and transport of the dsRNA molecule (Kalidas and Smith, 2002; Reichhart et al., 2003), at least it greatly enhances the stability of inverted-repeat sequences in bacteria, facilitating the cloning procedure (Lee and Carthew 2003). However, the absence of direct comparisons between these different approaches makes it difficult to ascertain their relative efficiencies, but with each method successful gene knockdown effects have been reported.

Biochemical studies performed to investigate RNAi have complemented the genetic studies and provided valuable information about the mechanism. The trigger for RNAi in all cases studied to date is a dsRNA molecule, which is cleaved to siRNAs (Elbashir et al., 2001a; Elbashir et al., 2001b; Hammond et al., 2005). The specificity of RNAi is achieved by the nucleotide complementation between the target mRNA and the dsRNA. A DNA template of about 500– 700 bp as multiple copies or as inverted repeats is usually sufficient to trigger RNAi. The enzyme Dicer cleaves the dsRNA into 21–23 nt small RNAs. *Drosophila* has two dicers: dcr-1 and dcr-2. The dcr-1 gene plays a role in miRNA biogenesis, whereas dcr-2 is involved in siRNA production (Lee et al., 2004), although some overlap in function occurs. The role of Dicer is not merely confined to cleavage of the template to produce dsRNA but it also plays a role in the delivery of siRNA molecules to the RISC complex. RISC is a multiprotein complex endonuclease, reported to be in the range of 200–500 kDa, which ultimately brings about the cleavage of the target mRNA. The assembly of the activated RISC complex takes place in a stepwise manner that requires ATP (Pham et al., 2004). The formation of the active RISC complex is preceded by the arrangement of RLC (RISC Loading Complex), also known previously as complex A. The site of target mRNA degradation is

generally believed to be the cytoplasm. A recent study in mammalian cells proposes specific cytoplasmic bodies to be the site of target mRNA degradation by the activated RISC complex (Sen and Blau 2005).

1.4 The neuronal network

As described above during classical conditioning two chemical stimuli (odor as CS and sugar as US) are associated. What are the bases of chemosensation in *Drosophila melanogaster*? What is the neuronal network mediating the CS and US? Where does the coincidence detection take place? In the next part I will try to sum up the current answers of *Drosophila* research regarding these questions. In the second paragraph I will focus on sugar as a reinforcer because electroshock –although punishment is more often used as a US - is poorly understood (Yu et al., 2004; Riemensperger et al., 2005). In addition I mainly used sugar for classical conditioning. The third paragraph will focus on the MB as the major site of olfactory memory formation in the fly brain. As nearly nothing is known how the mushroom bodies and higher brain centres elicit a conditioned motor output, I will restrict my Introduction to the above asked questions.

1.4.1 The CS: the olfactory system of *Drosophila*

The fly has two pairs of olfactory organs, the antennae and the maxillary palps (Figure 3 and 4B). Each antenna contains about 1200 olfactory receptor neurons (ORNs), whereas each maxillary palp contains about 120 ORNs (Stocker 1994, Hildebrand and Shepherd 1997). ORNs are compartmentalized into sensory hairs called sensilla, which can be subdivided into three major morphological types: basiconic, coeloconic and trichiod. Each sensillum contains the dendrites of up to four ORNs (Figure 3). ORNs send axons to the antennal lobe (AL),

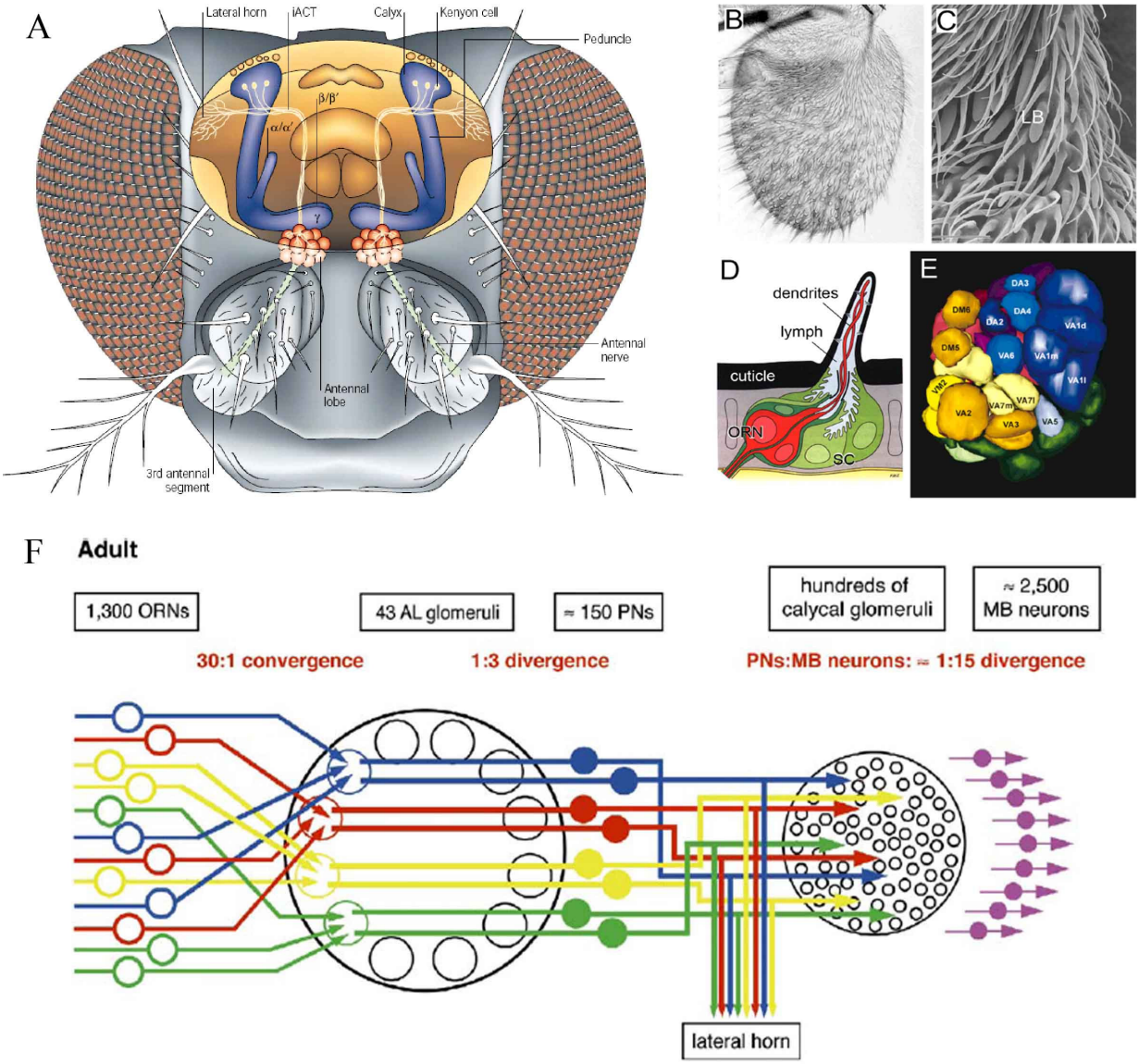


Figure 3: Organization of the olfactory system in *Drosophila*. **A)** Odor information is carried from the third antennal segments and maxillary palps (not shown) to the antennal lobe, where receptor fibres are sorted according to their chemospecificities in about 43 glomeruli. These represent the primary odor qualities, which are reported to two major target areas in the brain, the dorsolateral protocerebrum (lateral horn) and the calyx of the mushroom body. The inner antennocerebral tract (iACT) connects individual glomeruli to both areas. α/α', β/β' and γ mark the three mushroom body subsystems (Heisenberg 2003). **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) and projection neurons (PNs) (Taken from Masek 2006). **F)** Wiring diagram of the adult olfactory system (Ramaekers et al., 2005).

whose functional organization is remarkably similar to that of the olfactory bulb in vertebrates (Hildebrand and Shepherd 1997). In the AL, ORNs synapse onto about 150 second order

neurons called projection neurons (PNs, Figure 3, Stocker 1994). Additionally gabaergic local interneurons provide a means for inhibitory information transfer in the AL itself between well defined substructures called glomeruli (Stocker et al., 1990). The AL can be subdivided into 43 glomeruli (Laissue et al., 1999, Figure 3E). Individual ORNs send axons to only one or a few glomeruli, so on average, 30 ORNs project their axons to an individual glomerulus. ORNs that express the same olfactory receptor protein project to the same glomerulus (Jefferis et al., 2001, Wong et al., 2002, Marin et al., 2002). A large family of odorant receptor (Or) genes exists, all G protein coupled receptors (GPCRs). 60 genes encode for 62 odorant receptors proteins via alternative splicing (Clyne et al., 1999, Gao and Chess 1999, Vosshall et al., 1999).A deletion mutant lacking Or22a but still having an 'empty' ORN ab3A present on the antenna, provided a useful system for Or function. By ectopic expression of a Or gene in the mutant background via the GAL4/UAS system a large number of odorants can be rapidly screened for receptor activation (Dobritsa et al., 2003, Hallem et al., 2004). Nearly all of the antennal odorant receptors have now been characterized using this approach, and by comparing the odor response spectra conferred by individual odorant receptors with the odor response spectra of wild-type ORNs, many of these receptors have been mapped to ORNs from which they are derived (Hallem et al., 2004). So single odorants via binding to odor receptors and activation of ORNs, elicit complex spatial activity patterns in which most glomeruli respond to multiple stimuli and all stimuli elicit unique spatial patterns of activity across the population of glomeruli. This suggests that, as with the receptors themselves, detailed information about the molecular structure of the odor is combinatorially encoded in the pattern of activity across glomeruli (Ache and Young 2005). Because there are 43 glomeruli and 150 PNs, each glomerulus is sampled on average by 3-4 PNs (Ramaekers et al., 2005, Figure 3F). The axons of PNs project to the mushroom body (MB) and lateral horn of the brain.

1.4.2 The US: the gustatory system of *Drosophila*

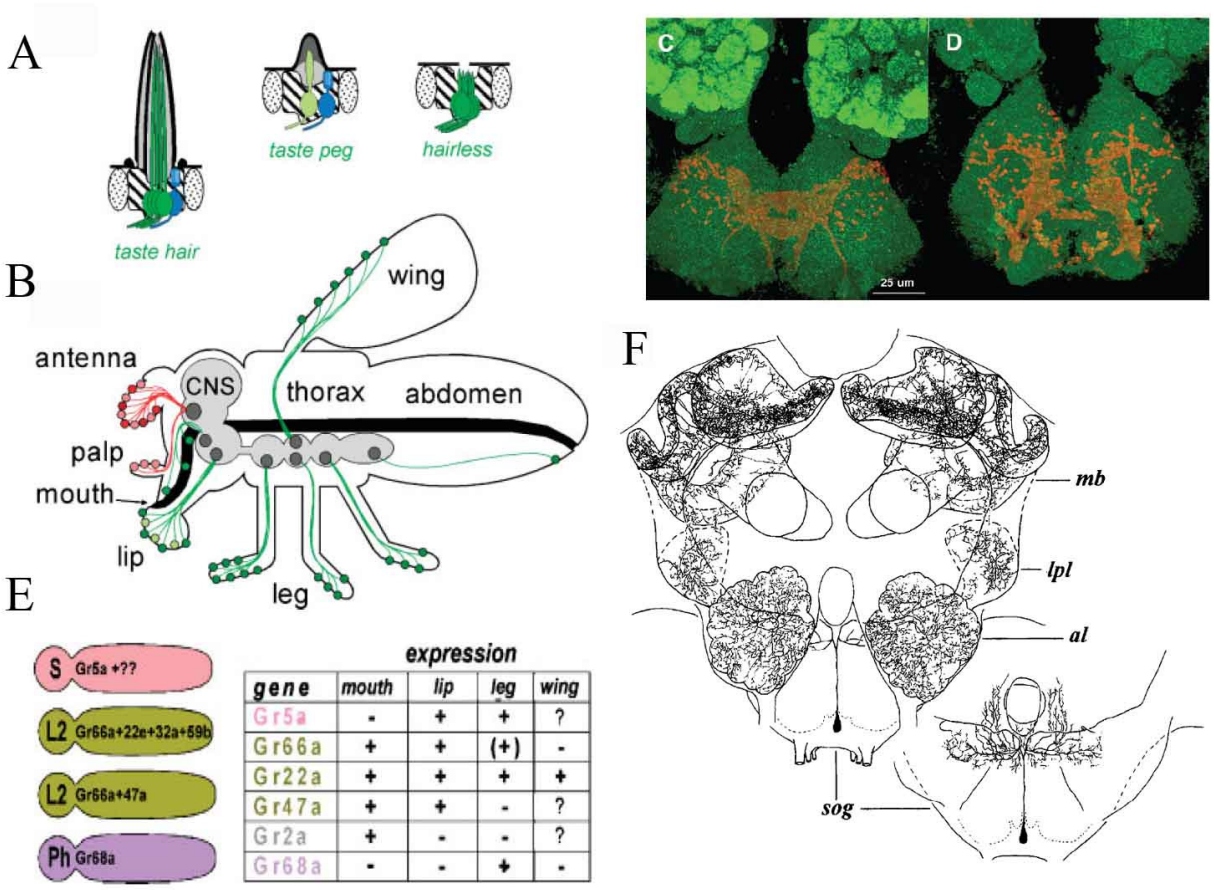


Figure 4: A) Gustatory receptor neurons (GRN in shades of green) occur in three different categories of sensilla. In taste hairs and pegs, gustatory neurons are accompanied by a mechanosensory neuron (blue). Hairless sensilla are internal and contain varying numbers of neurons. **B)**: A schematic outline of the anatomy of a fly indicates the location of chemosensory sensilla. Shades of red or green correspond to the structural olfactory or gustatory categories. The central nervous system is in grey and the digestive tract in black. GRNs converge to different brain centers (dark grey) that are associated with the different body parts of the fly (de Bryne 2006). **C)** Axonal targets of *Gr66a*-expressing neurons in the tritocerebrum/SOG as visualized using *n-synaptobrevin-GFP* as a reporter. **D)** Axonal targets of Gr5a expressing neurons. The qualitative difference in projection patterns between brains seen in (C) and (D) is striking (Thorne et al., 2005). **E)** GRNs express several receptors but probably mediate a particular taste quality (colors). S, sugar cell (pink); L2, bitter cell (olive); Ph, putative pheromone-sensitive cell (purple). The question marks in the S neuron indicate the presence of unidentified receptors for other sugars. The table shows expression of a few selected Gr genes in various taste organs (de Bryne 2006). **F)** Schematic drawing of the dendritic arborizations of the VUMmx1 neuron in the honeybee (Hammer and Menzel 1998).

The gustatory system of the fly is complex and, unlike that of mammals, not restricted to a single taste organ (Stocker 1994). Most of our knowledge about the functional properties of gustatory receptor neurons (GRNs) comes from research on taste hairs, which house the majority of GRNs and are found abundantly on different parts of the body. In *Drosophila*, a

total of approx. 660 GRNs are found in hairs on the legs, the wings, the labellum, and even the ovipositor (Figure 4B, Stocker 1994). Taste hairs are innervated by a single, unbranched dendrite of two or four GRNs combined with a single mechanosensory neuron (Dethier et al., 1976). In contrast, labellar taste pegs house only one chemosensory and one mechanosensory neuron (Falk 1976). Gustatory receptor neurons (GRN) of *Drosophila* occur in three different types of sensilla: taste hairs, taste pegs and hairless sensilla (Figure 4A, Stocker 1994, Tanimura and Shimada 1981, Rodrigues and Siddigi 1981). There are about 35 taste pegs, each containing a single GRN (Shanbhag et al., 2001). Hairless sensilla are found in three groups inside the oral cavity and house 24 GRNs that contact the food before it is pumped into the oesophagus (Stocker 1994). The four GRNs have traditionally been named after their best stimuli in the blowfly: S (sucrose), W (water), L1 (low salt) and L2 (high salt). It is convenient to think of these as the insect equivalent of our 'taste qualities' (Figure 4E). S neurons respond to at least five different types of sugars: pyranose, fructose, trehalose, and glycerol (Koseki et al., 2004, Tanimura et al., 1982, Tanimura et al., 1981, Wieczorek and Wolff 1989). The second neuron has been dubbed the water (W) neuron and was thought to rather respond to low osmolarity (Dethier et al., 1976). The L1 neuron responds to cations (Dethier et al., 1976). Recent evidence shows the L2 neuron responses to typical 'bitter' compounds such as quinine and caffeine but also to the artificial sweetener saccharin (Liscia and Solari 2000, Liscia et al., 2004).

The gustatory sensilla express a second family of G-protein coupled receptors, the gustatory chemosensory receptor (Gr) genes (Clyne et al., 2000, Dunipace et al., 2001, Scott et al., 2001). This family consists of 60 genes that encode 68 proteins via alternative splicing. Using in-situ hybridization and reporter gene constructs, 11 genes have been shown to be expressed in subsets of either adult taste neurons, adult olfactory neurons, or larval taste and olfactory neurons (Dunipace et al., 2001, Scott et al., 2001). Thus the Gr gene family may encompass both odorant and taste receptors. Functional evidence has been obtained by

analysis of a mutation in the Gr5a gene, together with heterologous expression experiments, that expressed in a subset of cells in the labellum; it encodes a taste receptor for the sugar trehalose (Dahanukar et al., 2001, Chyb et al., 2003). Importantly, electrophysiological recordings showed that loss of Gr5a did not abolish the GRNs response to other sugars, indicating that these S neurons express more than one sugar receptor protein (Thorne et al., 2004, Wang et al., 2004). Inoshita and Tanimura (2006) identified recently water gustatory receptor neurons using behavioural proboscis extension reflex and electrophysiological recordings. The water receptor is expressed in a single gustatory receptor neuron in each sensillum on the labellum and project to a specific region in the subesophageal ganglion, thus revealing the water taste sensory map in *Drosophila*. Genetic ablation of Gr66a-expressing cells abolished responses to a number of bitter tastants, suggesting that Gr66a is expressed in bitter tastant receptor cells (presumably L2 neurons). The Gr66a expressing neurons appear to be of two types, distinguished by having a different set of Gr genes co-expressed with Gr66a (Thorne et al., 2004, Wang et al., 2004). This suggests that there may be subclasses of bitter taste neurons with different responses. In addition to expression in taste organs, at least three Gr genes, Gr10a/b, Gr21a and Gr63a, are expressed in *Drosophila* antennae (Scott et al., 2001), suggesting a role in olfaction. In insects salt reception is thought to function via amiloride-sensitive DEG/ENaC sodium channels (Lindemann 1996). Two genes, Pickpocket11 and Pickpocket19, are expressed in taste sensilla, and disruption of these genes results in a diminished behavioural response to salt but not to sucrose (Liu et al., 2003).

Anatomical and cobalt filling studies have shown that GRNs from different peripheral tissues project to different regions of the suboesophageal ganglion (SOG) and tritocerebrum but lack a glomerular organization like that in the antennal lobe (Edgecomb and Murdock 1992, Kent and Hildebrand 1987). The two GRN populations defined by either Gr5a (sugar-sensitive) or Gr66a (bitter-sensitive) expression project to nonoverlapping regions of the SOG (Figure 4C and 4D, Thorne et al., 2004, Wang et al., 2004). Projections from different

peripheral tissues are also segregated in the brain, even when the neurons express the same receptor (Wang et al., 2004). Therefore, in contrast to the olfactory system, the spatial information of the gustatory input is directly represented in the neuronal activation pattern of the SOG.

In contrast to the olfactory system, in adult *Drosophila* much less is known about the neurons of the gustatory pathway connecting the SOG to higher brain centers. In larva Melcher and Pankratz (2005) published a candidate gustatory interneuron modulating feeding behaviour. These neurons collect the information from external gustatory expressing neurons at the SOG, as well as by internal pharyngeal chemosensory organs and project axons to the pharyngeal muscles, to the central neuroendocrine organ, and to higher brain centers (Melcher and Pankratz 2005). In the honeybee Hammer (1993) identified a single octopaminergic neuron, the VUMmx1 neuron (Figure 4F), that mediates the reinforcing function of the unconditioned stimulus (sugar as US) in the conditioning of the proboscis extension response (PER). The VUMmx1 neuron belongs to a group of 15 ventral unpaired median neurons of the suboesophageal ganglion, and its soma is located in the maxillary neuromere. The dendrites of VUMmx1 arborize symmetrically in the brain and converge with the olfactory pathway at three sites: the primary olfactory neuropil, the antennal lobe (AL); the secondary olfactory integration area, the lip region of the mushroom bodies (MB); and the output region of the brain, the lateral horn (LH). VUMmx1 responds to sucrose solution both at the antenna and the proboscis with long-lasting spike activity and to various visual, olfactory, and mechanosensory stimuli with low-frequency spike activity (Menzel 2001). Nevertheless also in *Drosophila* behavioral data (Schwärzel et al., 2003) suggest that octopamine mediates the reinforcement via sugar in appetitive classical conditioning. And additional anatomical data (Sinakevitch and Strausfeld 2006) preserve the possibility of at least one VUMmx1 similar octopaminergic neuron.

1.4.3 The mushroom bodies: structure and function in olfactory learning and memory

The MBs of *Drosophila* are bilaterally symmetric structures consisting of approximately 2500 intrinsic neurons, also known as Kenyon cells, per brain hemisphere (Figure 3A and Figure 5). The cell bodies of these neurons are located in the dorsal posterior aspect of the brain. Just anterior and ventral to the cell bodies, the MB neurons give rise to a dendritic field known as the calyx that receives input from the PNs. The axons of the neurons project to the anterior portion of the brain via a dense structure known as the peduncle, where they turn and give rise to the lobes of the MB (Crittenden et al., 1998; Lee et al., 1999). Several studies have demonstrated that the MB lobes can be subdivided immunohistochemically or on the basis of the expression pattern of different genes or reporter constructs (Yang et al., 1995, Crittenden et al., 1998, Strausfeld et al., 2003). The following substructures are defined: α/β, α'/β', and γ lobes, more recently β'' and the glutamatergic core neurons αc/βc (Strausfeld et al., 2003). The MB derives from four neuroblasts (Ito et al., 1997) and each neuroblast sequentially produces three types of neurons (Lee et al., 1999). The earliest born neurons, from larval hatching through the mid-third instar larval stage, project to the γ lobe. Neurons born between the mid-third instar larval stage and puparium formation project into α'/β' lobes. Finally, neurons born after puparium formation project into α/β lobes (Lee et al., 1999). The calyx of the MB has been shown to contain three major types of processes. The first type includes the dendrites of the intrinsic neurons of the MB. The second type includes the extrinsic PNs from the antennal lobe that form large synaptic boutons on the intrinsic MB neurons and are immunoreactive against choline acetyltransferase and vesicular acetylcholine transporter. The PNs form divergent synapses upon many MB cell dendrites. The third type includes GABA

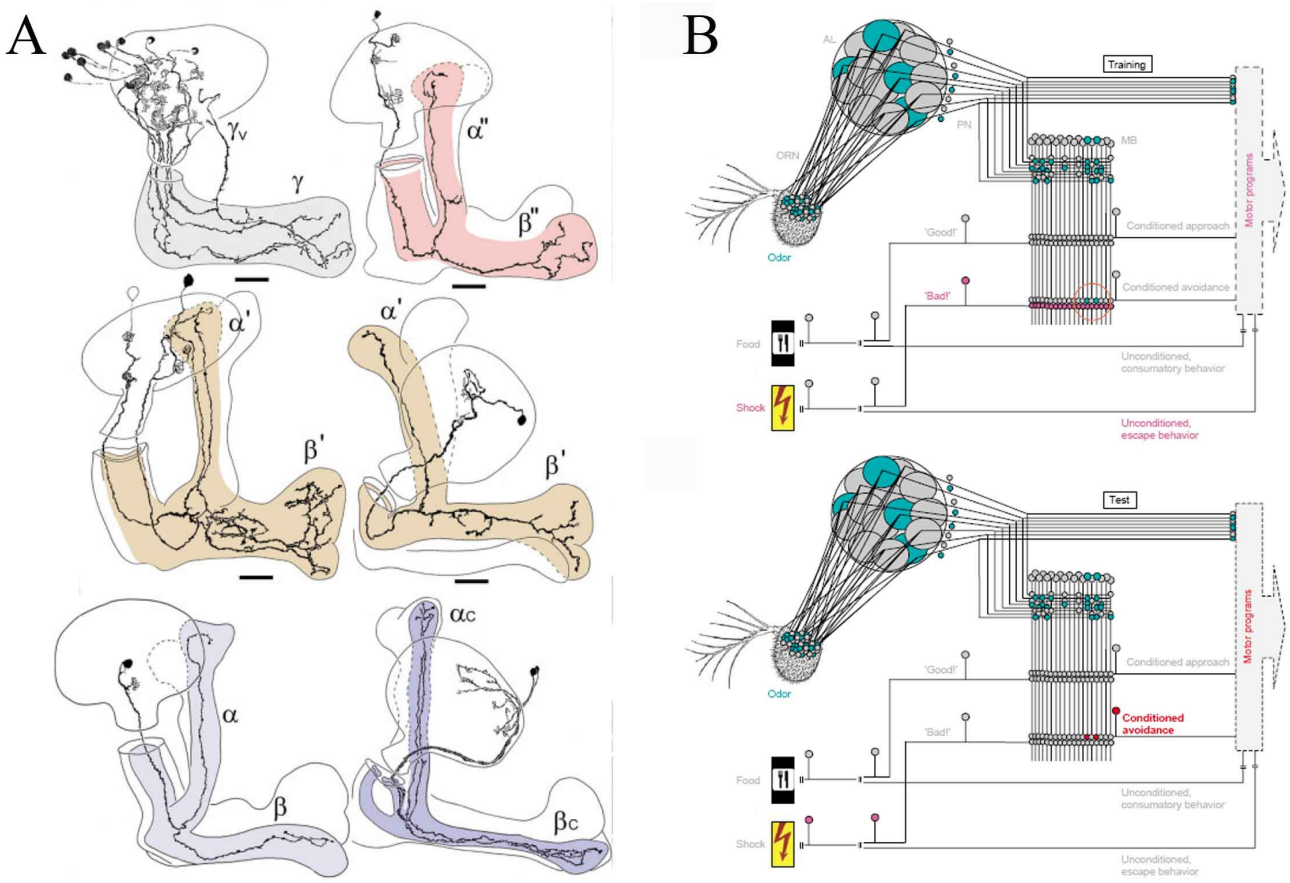


Figure 5: A) Dendritic morphologies of Kenyon cells: Examples of whole cell reconstructions and their assignments to lobes. **Top left:** γ lobe, showing one neuron with a vertical tributary. **Top right:** β'' lobe. **Middle panels:** Two examples of Kenyon cells supplying α'-β'. **Bottom left:** Single neuron supplying α-β. **Bottom right:** Two Kenyon cells supplying αc-βc (taken from Strausfeld et al., 2003). **B)** A minimal model for *Drosophila* olfactory learning. A highly simplified diagram shows the olfactory pathway. Olfactory receptor neurons (ORN) project to the antennal lobe (AL), leading to a specific combinatorial activity pattern. From there, uniglomerular projection neurons (PN) relay to the lateral horn and to premotor centers (box labeled 'Motor output'), as well as to the mushroom body (MB) calyx. Output from the mushroom bodies then projects to a variety of target regions including premotor areas. In the model, we assume that a Kenyon cell needs input from at least three projection neurons to fire. In the mushroom bodies, the activation pattern of the sensory and the projection neurons is therefore transformed into an activation pattern of the mushroom body — intrinsic Kenyon cells. A memory trace for the association between odor and reinforcement is proposed to be localized within the Kenyon cells: during training, when the activation of a pattern of Kenyon cells representing an odor occurs simultaneously with a modulatory reinforcement signal (labeled 'Good!' and 'Bad!'; potentially octopaminergic and dopaminergic neurons concerning reward and punishment, respectively), output from these activated Kenyon cells onto mushroom body output neurons is suggested to be strengthened. This strengthened output is thought to mediate conditioned behaviour towards the odor when encountered during test, during which no reinforcer is present. Activated cells or synapses and motor programs are represented by filled symbols and bold lettering, respectively (taken from Gerber et al., 2004).

immunoreactive neurons that synapse upon the MB dendrites and occasionally upon the PN boutons (Yasuyama et al., 2002). Functionally, several experiments showed that the MB

house an olfactory memory trace for electroshock associated learning by fulfilling five criteria (reviewed in Gerber et al., 2004). First, it was indirectly observed that the MB harbours the potential for neuronal plasticity (Davis 1996, Abrams et al., 1998). Second, rescue experiments using the TARGET system showed that the synaptic plasticity in the MBs is sufficient for olfactory associative learning (McGuire et al., 2003, Zars et al., 2000, Mao et al., 2004). Third, structural MB mutants (Heisenberg et al., 1985), chemical ablation of the MBs (de Belle and Heisenberg, 1994), and transgenic suppression of synaptic plasticity in this neuropile (Connolly et al., 1996), proved the necessity of synaptic plasticity in the MB. Using *shi*^{ts} to block chemical output of synapses dependent on the temperature, it was published that fourth, blocking MB output during test (McGuire et al., 2001, Dubnau et al., 2001, Schwärzel et al., 2001), and fifth, blocking input to the MB during training (Schwärzel PhD thesis 2003) prevents flies from expressing any memory. Due to these five criteria, it has been proposed that a memory trace for the association between odor and shock is localized within the Kenyon cells: when the activation of a pattern of Kenyon cells representing an odor occurs simultaneously with a modulatory reinforcement signal, output from these activated Kenyon cells onto mushroom body output neurons is suggested to be strengthened (Heisenberg 2003). This strengthened output is thought to mediate conditioned behaviour towards the odor when it is encountered during testing. If sugar is used as a reinforcer in the appetitive version of the paradigm, Schwärzel (2003) showed that the *rutabaga*-dependent synaptic plasticity in the MB is sufficient for memory formation. And also output from the MB neurons is necessary only during retrieval but not during memory acquisition.

1.5 Molecular mechanisms of learning and memory

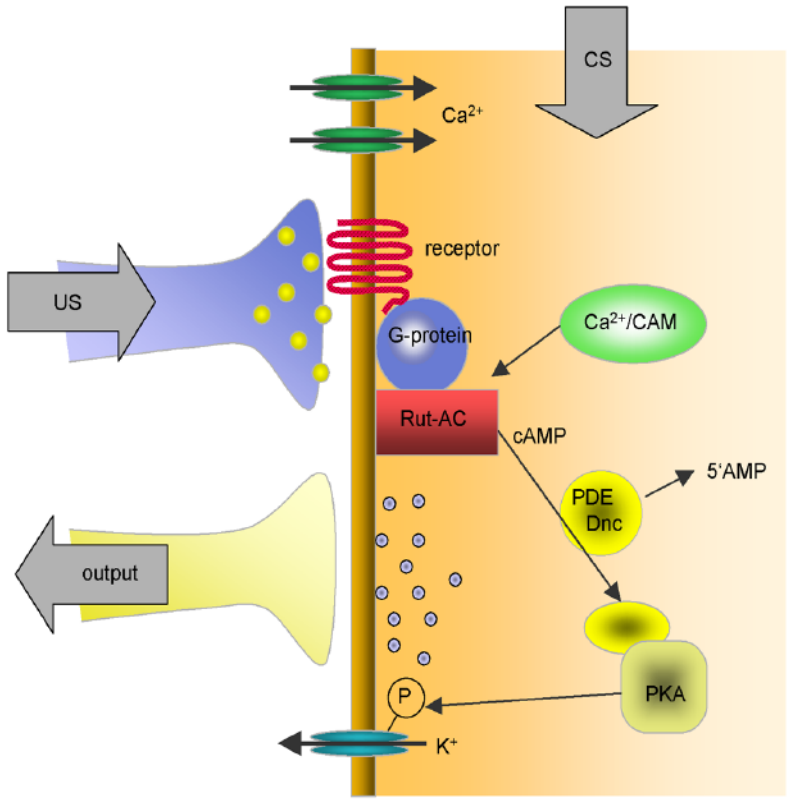


Figure 6: The molecular model of olfactory learning. Presynaptic modulation of transmission at Kenyon cell synapse to output neuron is thought to underlie olfactory short and middle term memory in *Drosophila*. Simultaneous arrival of CS and the US activates adenylyl cyclase A (Rut-AC), via calcium/ calmodulin increase (CS) and activation of G-protein coupled receptors (US). Rut-AC activation leads to an increase of cAMP. Elevated level of cAMP activate protain kinase A (PKA), which regulates target proteins at the synapse. Phosphodiaesterase (PDE) downregulates cAMP levels by degrading cAMP to 5'AMP (modified from Masek 2005).

Drosophila neurogenetics has provided independent and remarkable evidence for the role of the cyclic adenosine monophosphate (cAMP) signal transduction cascade in learning and memory. It has also been useful in dissecting phases of acquisition and consolidation of memory (reviewed in Heisenberg 2003, McGuire et al., 2005, Margulies et al., 2005). Two mutants, *rutabaga (rut)* and *dunce (dnc)* affect the cyclic AMP (cAMP) second messenger pathway (Livingstone et al., 1984, Dudai et al., 1976). *Dnc* is lesioned in a cAMP phosphodiesterase usually degrading cAMP (Figure 6, Byers et al., 1981). By performing immunohistochemistry on *Drosophila* brains with an anti-Dnc antibody, Nighorn (1991) demonstrated preferential expression of the Dnc protein in the MBs. The *rut* mutant is deficient in an adenylyl cyclase, the enzyme synthesising cAMP (Levin et al., 1992). This cyclase is homologous to the mammalian type-I adenylyl cyclase and is responsive to both, G-protein and Ca^{2+}/CaM dependent stimulation (Dudai 1988). Moreover, disrupting normal cAMP signalling in the MBs by expressing a constitutively active $G\alpha_s$ subunit abolishes

olfactory learning (Connolly et al., 1996). Receptors for the biogenic amines dopamine (DAMB, dDA1, DDR2-like) and octopamine (OAMB, Dmoa2, DmOct beta1R, DmOct beta2R and DmOct beta3R) have been found to be coupled direct via G proteins or indirect to adenylyl cyclase of the rut type and were found to be expressed at elevated concentrations in the MB lobes (Han et al., 1996; Kim et al., 2003; Hearn et al., 2002; Han et al., 1998; Lee et al., 2003; Balfanz et al., 2005; Maqueria et al., 2005). This evidence and the co-activation property suggests the adenylyl cyclase *rut* to be a molecular detector of coincidence between the conditioned stimulus (odor) and the reinforcer (US) during pavlovian learning (Dudai 1988, Abrams and Kandel 1988) resulting in an increased cAMP level of the intrinsic Kenyon cell. A third component of the cAMP signaling pathway has also been implicated in *Drosophila* olfactory learning and memory. The cAMP-dependent protein kinase (PKA) is a major mediator of signaling through the cAMP pathway (Taylor et al., 1990). Skoulakis and colleagues focused on mutants in the catalytic domain of PKA (Skoulakis et al., 1993), decreased PKA activity to 20% of normal activity demonstrated a significant impairment of memory performance (Skoulakis et al., 1993). PKA, as the primary downstream effector of cAMP modulation, has many targets that may act in concert to bring about the cellular and circuit-level changes required for learning and memory. At the minimum, PKA has been shown to modulate the Ca^{2+}-dependent K^{+} channel in *Drosophila* (Zhou et al., 2002), as well as to enhance spontaneous transmitter release via increases in Ca^{2+} influx (Yoshihara et al., 2000). One of the major phosphorylation targets of PKA is the transcription factor CREB. Phosphorylation of CREB by PKA leads to transcriptional activation by the phosphorylated CREB protein. This activated peptide has been implicated in a host of important interactions (Lonze and Ginty 2002). Original data where an isoform of the gene specifically blocks the formation of the protein synthesis-dependent component of long-term memory (Yin et al., 1994) was recently questioned, leaving the role of CREB activator protein in the formation of LTM unclear at the present time (Perazzona et al., 2004). An additional link to the cAMP

pathway has been identified with the *amnesiac* mutant that is specifically defective in short term memory. Analysis of the locus demonstrated that it encodes three putative neuropeptides, one of which has homology to the pituitary adenylyl cyclase activating peptide (PACAP; Feany and Quinn, 1995). This immediately suggested a potential mechanistic link between *amn* and *dnc* via modulation of cAMP levels by neuropeptide signaling through a G protein-coupled receptor that signals through the *rut* adenylyl cyclase (Kandel and Abel, 1995). Waddell and colleagues (2000) demonstrated that the *amnesiac*-encoded polypeptide is expressed in two neurons of the *Drosophila* brain, which they termed the dorsal paired medial (DPM) neurons. Mutations in the neurofibromatosis 1 gene produce a dominant disorder in humans characterized by nervous system tumors and learning defects. The gene product encoded at the NF-1 locus has been shown to be a Ras GTPase activating protein (Xu et al., 1990). Surprisingly, NF1 was shown in *Drosophila* to be required for the activation of the *rut* adenylyl cyclase by PACAP peptides, suggesting a dual role for this protein in regulating the Ras and cAMP pathways (Guo et al., 1997). Behavioral testing of mutants deficient for NF1 demonstrated a defect in short-term olfactory learning and memory that could be rescued by acute expression of a heat shock-NF1 transgene or by the expression of a constitutively active catalytic subunit of PKA, providing strong support that the role of NF1 in learning and memory is mediated, at least in part, by a defect in cAMP signal transduction (Guo et al., 2000).

Recent data suggest also a contribution of cell adhesion molecules and membrane receptors in learning and memory. Two cell adhesion molecules, an α-integrin subunit deleted in the *Volado* mutant and a second cell adhesion molecule, encoded by the *fasciclin II* gene, demonstrated that mutants disrupt STM (Grotewiel et al., 1998, Cheng et al., 2001). *Notch* receptors have been shown to have a diverse array of functions in both invertebrates and vertebrates ranging from cell type specification via lateral inhibition to regulation of neurite

outgrowth. Two recent reports have tied *Notch* to long-term memory in *Drosophila* (Presente et al., 2004 and Ge et al., 2004).

Additionally a dozen of mutants which are not yet analysed in detail complete the current state of learning and memory research in *Drosophila* (*aPKM*; Sacktor et al., 1993, Drier et al., 2002), *Leonardo* (Skoulakis and Davis 1996), Nalyot (deZazzo et al., 2000), *radish* (Folkers et al., 1993, Chiang 2004), *staufen* and *pumilio* (Dubnau 2003, Martin 1997), *nebula* (Chang et al., 2003), *cramer* (Comas et al., 2004), *synapsins* (Godenschwege et al., 2004), *S6KII* (Putz et al., 2004), *latheo* (Boynton and Tully 1992), *linotte* (Dura et al., 1993, Simon et al., 1998, Bolwig et al., 1995)).

1.6 Motivation to investigate sugar reward learning in *Drosophila*

Genetic intervention in the fly *Drosophila melanogaster* has provided strong evidence that the mushroom bodies of the insect brain act as the seat of a memory trace for aversive and appetitive olfactory learning (reviewed in Heisenberg, 2003). However, as most of the learning experiments of the fly use electroshock as reinforcer, sugar reward learning is hitherto fragmentarily investigated. Additionally several lines of evidence from honeybee and moth have suggested another site, the antennal lobe to house neuronal plasticity underlying appetitive olfactory memory (reviewed in Menzel, 2001; Daly et al., 2004). Therefore, exploiting the extensive set of genetic tools available to intervene with behaviour in *Drosophila*, I reinvestigated appetitve learning with respect to the underlying substrate.

In the first part, I modified a genetic tool, the TARGET system (McGuire et al., 2003), which allows the temporally controlled expression of a given effector gene in a defined set of cells, to intervene with neurotransmission. Comparing effector gene expression at different developmental timepoints which either blocks neurotransmission or ablates cells will help

answering following question: What is the appropriate effector gene for evaluating the function of neural circuits?

In the second part, by restoring Rutabaga adenlylate cyclase (rut-AC) activity specifically in projection neurons or mushroom bodies of *Drosophila*, I investigated if appetitive olfactory memory in the fly is differently organized compared to electroshock learning as suggested by several studies from other insects.

In the third part of the thesis I tried to understand how the reinforcing signals for sugar reward are internally represented. In the bee Hammer (1993) described a single octopaminergic neuron – called VUMmx1 – that mediates the sugar stimulus in associative olfactory reward learning. Is there a similar VUM neuron in *Drosophila*? What are the neurons mediating the sugar reinforcement in the appetitive olfactory learning experiments of the fly?

2. Material and Methods

2.1 Fly care

All flies were raised on corn-meal food (Guo et al., 1996) in a 14-10 hour light-dark cycle at 25°C and 60% humidity. Experimental flies were fed on fresh food vials for up to 48 hours before the behavioural tests. For the learning experiments, I used 2-4 day old males and females in mixed groups, either taken from homozygous lines or from progeny of crosses between homozygote parental lines.

2.2 Genotypes of used flies

Table 2: Used fly strains

Line	Genotype	Comment	Reference
Canton S	wild-type	from Würzburg stock collection	Schwärzel 2002
white^{1118}	white$^-$ mutant	cantonized	Dura 1993
mb 247 GAL4	*white*$^-$, P-element containing Wild-type *white* cDNA 3rd chromosome	GAL4 expression controlled by regulatory region of the *D-Mef2* gene in MB	Zars 2000
GH146 GAL4	*white*$^-$, P-element containing Wild-type *white* cDNA 2nd chromosome	Enhancer trap line, balanced over CyO, expression in PNs	Stocker 1997
NP 225	*white*$^-$, P-element containing Wild-type *white* cDNA 2nd chromosome	Enhancer trap line, balanced over CyO, expression in PNs	Ito 2003
act GAL4	*white*$^-$, P-element containing Wild-type *white* cDNA 2nd chromosome	Enhancer trap line, balanced over CyO, expression in all cells	Ito 1997
elav GAL4	*white*$^-$, P-element containing Wild-type *white* cDNA X chromosome	Enhancer trap line, expression in predominantly in neurons	Lin and Goodman 1994
D42 GAL4	*white*$^-$, P-element containing Wild-type *white* cDNA 3rd chromosome	Enhancer trap line, expression in predominantly in motoneurons	Yeh 1995

NP 7088	*white*$^-$, P-element containing Wild-type *white* cDNA 2nd chromosome	Enhancer trap line, balanced over CyO, expression in putative octopaminergic neurons	Hayashi 2002
1.3 TβH GAL4	*white*$^-$, P-element containing Wild-type *white* cDNA 3rd chromosome	Enhancer trap line, expression in putative octopaminergic neurons	Hampel unpublished
UAS-shi^{ts}	*white*$^-$, P-element containing Wild-type *white* cDNA 3rd chromosome	*shibire^{ts1}* (*shi^{ts1}*) a temperature sensitive dominant negative dynamin	Kitamoto 2001
UAS-rpr	*white*$^-$, P-element containing Wild-type *white* cDNA X chromosome	*reaper* (*rpr*) induces apoptosis by activating the caspase proteolytic cascade	Aplin and Kaufman 1997
UAS-TNTE	*white*$^-$, P-element containing Wild-type *white* cDNA 2nd chromosome	*Tetanus toxin light chain* (*TNT*) aprotease specifically cleaving neuronal-Synaptobrevin (n-Syb)	Sweeney 1995
UAS-EGFP- kir2.1	*white*$^-$, P-element containing Wild-type *white* cDNA 2nd chromosome	human inwardly rectifying potassium channel	Baines 2001
UAS-DTI	*white*$^-$, P-element containing Wild-type *white* cDNA 2nd chromosome	Diphtheria toxin inhibitor of protein synthesis	Han 2000
UAS-Gα_s*	*white*$^+$, P-element containing Wild-type *white* cDNA 2nd chromosome	constitutively active Gα_s-protein	Connolly 1996
UAS- mCD8:GFP	*white*$^-$, P-element containing Wild-type *white* cDNA 2nd chromosome	green fluorescent protein balanced over CyO	Lee and Luo 1999
UAS-n-syb-GFP	*white*$^-$, P-element containing Wild-type *white* cDNA 3rd chromosome	presynaptic green fluorescent protein	Ito 1998
rut^{2080}	*rut*$^-$ P-element containing Wild-type rosy-cDNA X chromosome	P-element induced mutation, cantonized	Schwärzel 2002
rut^{2080};+;UAS- rut+	*rut*$^-$ P-element containing Wild-type rosy-cDNA X chromosome Transgene-insertion containing wild-type *rut*-cDNA	P-element induced mutation, cantonized UAS-rut$^+$ transgene, cantonized	Schwärzel 2002

TβH^{M18}	*white*$^{+}$, *TβH*$^{-}$	P-element based excision of the *tyramine-β-hydroxylase* gene	Scholz unpublished
TβH; UAS-TβH	*white*$^{+}$, *TβH* Transgene-insertion containing wild-type *TβH*-cDNA X chromosome^{-}	P-element based excision of the *tyramine-β-hydroxylase* gene balanced over FM7A	Scholz unpublished
tubGAL80^{ts}	*white*$^{-}$, P-element containing Wild-type *white* cDNA 3rd chromosome	Temperature sensitive GAL4 inhibitor expressed in all cells via tub1 promoter	McGuire 2003

2.3 Behavioural Assays

2.3.1 Adult Paralysis and Recovery

For all experiments, 50 virgin females were crossed to 20 males, and were kept to adulthood at 18°C. Groups of either ten or twenty adult F1 flies were transferred two days after eclosion to fresh food vials using an aspirator and were left there to recover overnight at 18°C. Flies were not CO_2-anaesthetized to avoid potential negative effects of anaesthesia. Next day, these vials were placed in a 30°C incubator. To count the paralysed flies, vials were gently agitated, and the numbers of immobilised flies were measured at the given time points. In experiments using *Act5C /UAS-TNT; +/Tub-GAL80^{ts}*, genders were separated before the experiment. Because both *elav* and *UAS-rpr* are on the X chromosome, only the female progeny was examined. To assess the reversibility of the effector actions, fly vials in a 30°C incubator were shifted back to 18°C.

2.3.2 Temperature shift during development

Crosses were cultured at 18°C. Twenty third-instar (wandering) larvae were collected with a brush and transferred to a new food vial. To induce effector gene activity, food vials containing collected animals were transferred to 30°C for 24 h at three different developmental time points: third instar larva (directly after stage selection), early pupa (pupal

stage P1 to P2), or late pupa (pupal stage P10 to P12). After induction at 30°C, vials were placed again at 18°C and the number of eclosed adults was scored. Each measurement was repeated at least six times.

2.3.3 Testing for perception of sensory stimuli

The test for perception of sugar was done in vertical tubes (50ml) totally covered with filter paper with a 1 cm broad stripe of fluid- either 2M sucrose solution or water- at the half height of the tube. I scored the time starved flies spend on the wet stripe (t_{filter}) during an experimental duration of 60 seconds (t_{total}). Starting from the moment the fly taps onto the filter paper. I calculated a quantitative Reactivity Index (RI) as $RI^s = (t_{filter} / t_{total}) \times 100$. The RI^s can vary between 0 (no time spent on the filter paper) and 100 (total experimental time spent on the filter paper).

Flies were tested for perception of electric shock in a T-maze assay (Tully and Quinn 1985; Schwärzel et al., 2002). About 100 flies were placed into the elevator, put in register with two tubes and given one minute to choose between an electrified (12 pulses of 90 V DC and 1.3 sec duration at 5 sec intervals) and a non electrified tube, both equipped with copper wire. From each experiment I counted the number of flies choosing the electrified tube (N_{shock}) or the non electrified tube ($N_{non\ shock}$) and calculated a Response Index as $RI^E = [(N_{shock} - N_{non\ shock}) / (N_{shock} + N_{non\ shock})] \times 100$. The RI^E can vary between -100 (all animals choose the electrified tube) and +100 (all animals avoid the electrified tube).

To test for perception of olfactory cues about 100 flies were placed into the elevator, the tubes were put in register and the flies were given 2 minutes to choose between two airstreams (750ml/min), one scented with the test odor the other one unscented. A Reactivity Index (RI) was calculated from the number of flies choosing either the scented airstream

(N_{odor}) or the unscented one (N_{air}). RI^{O}= [(N_{odor} – N_{air}) / (N_{odor} + Nair)] x 100. The RIO can vary between -100 (all animals avoid the odorant) and +100 (all animals choose the odorant).

2.3.4 Olfactory associative learning

Six different odorants were used in the learning experiments 4-MCH and 3-OCT each ten times diluted in odor caps with diameters of 5 and 4 mm. These are traditionally used odorants for *Drosophila* learning experiments (Tempel et al., 1983). They are chemically very different from each other. IAA and AM each 36 times diluted in were used in odor cups with diameters of 15 and 16 mm because they seem 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 one of the key part of an overripe mango smell (Zhu et al., 2003). Additionally BA and EA were used both 100 times diluted in 5mm odor cups. BA was reported to be distinct from other odorants in the way how the memory is processed (Keene et al., 2004). And due to fast evaporation EA has to be exchanged every five minutes, increasing the duration of one learning trial by 20%, therefore use of this odor combination should be avoided in future experiments.

All learning experiments were either done in dim red light (invisible for the flies) during the training period or complete darkness during the test period except for sugar-reactivity, which was tested for in day light and normal humidity conditions.

Standard Pavlovian training procedures reinforced by electric shocks in a modified T-maze apparatus were applied (Tully and Quinn 1985; Schwärzel et al.,, 2002). Briefly, a group of ~100 flies were trained by receiving the first odor for 1 min in the presence of 12 pulses of electric shocks (90 V DC). After 1 min of fresh air, the tube was scented for an additional minute with the second odor but without electric shock, followed by another period of 45 sec of air. The memory test started ~100 sec after the training trial by shuttling the flies

into a choice point between the previously punished odor and control odor. They were given 2 min to choose one of them. To measure associative learning, a reciprocal experimental design was employed: two groups of flies receive either odor A with shock and B without or odor B with shock and A without. For both groups, the preference between odor A and B is measured after training. The learning index is then calculated by taking the mean preference of the two reciprocally trained groups.

Standard Pavlovian training procedures reinforced by sugar in a modified T-maze apparatus were applied (Tully and Quinn 1985; Schwärzel et al.,, 2002). Flies would be starved in groups of up to 250 animals for 18 hours at 25°C or for 42 hours at 18°C in empty vials equipped with moist filter paper to prevent desiccation. Briefly, a group of ~100 flies were trained by receiving the first odor for 1 min without sugar. After 1 min of fresh air, the tube was scented for an additional minute with the second odor in presence of sugar, followed by another period of 1 min of air. 1.5 ml of 2M sucrose solution was applied on a 7 cm x 4.5 cm piece of filter paper the day before and dried over night at room temperature. This training procedure was repeated directly afterwards. The memory test started ~160 sec after the training trial by shuttling the flies into a choice point between the previously punished odor and control odor. They were given 2 min to choose one of them. To measure associative learning, a reciprocal experimental design was employed: two groups of flies receive either odor A with sugar and B without or odor B with sugar and A without. For both groups, the preference between odor A and B is measured after training. The sequence of the odors was altered for the 3-minutes memory assay during the training in the middle of the experiment. Due to technical reasons it is hardly possible to reward the second odor, if one measures memory for longer retention intervals. In detail, I have to take the flies out of the machine after the measurement and put them into new vials. Here I cannot exclude soiling the new vials in which the animals will stay for at least one hour with sugar crystals which detach from the dried filter paper. As satisfied flies show no learning (data not shown; pers.

communication M. Schwärzel) this would make measurements of long term memory impossible. The learning index is then calculated by taking the mean preference of the two reciprocally trained groups as already described for aversive olfactory learning.

2.4 Immunohistochemistry

Microdissection of whole mount preparations was performed in the Ringer solution to remove cuticle and connective tissues. The brains were fixed in phosphate buffered saline containing 0.3% Triton X-100 (Sigma; PBT) containing 4% formaldehyde for 2 h at room temperature and subsequently rinsed with PBT three times. Blocking of samples was performed in 3 % normal goat serum (Sigma) in PBT for 1 h. The brains were incubated with the primary antibodies in the blocking solution at 4°C overnight. Rabbit anti-GFP (1:1000; Molecular Probes), or mouse monoclonal anti-Synapsin (3C11) for labelling presynapses (1:10; Godenschwege et al., 2004) were used as a primary antibody. Samples were washed four times for 10 min with PBT. The brains were incubated with secondary antibodies in the blocking solution at 4°C overnight. Alexa Fluor 488- or Cy3-conjugated goat anti-rabbit or anti-mouse, respectively, was used to detect the primary antibody. After four 10-min rinses with PBT, brains were mounted in Vectashield (Vector Labs). Confocal image stacks were taken with a Leica SP1. A stack of images was collected at 1 µm steps with a 40x objective. Images of the confocal stacks were projected and analysed with the software Image-J (NIH, USA) and organised with Photoshop (Adobe, USA) and AMIRA software.In case of Figures 11 and 16 the protocol was modified as described in the standardisation protocol of the Virtual brain project (http://genetics.biozentrum.uni-wuerzburg.de/home/VirtualBrain).

The dDA1-expression patterns were examined on paraffin sections, blocked for two hours with normal horse serum (1:50) in PBS plus 0.1 % Triton X-100 (PBT) and incubated with monoclonal mouse anti-dDA1 antibodies (1:1000; Kim et al., 2003) in PBT overnight at

4°C. A series of washes and incubation with a biotinylated anti-mouse antibody (1:200) for one hour at room temperature followed (see Buchner et al., 1988). Signal was detected using the Vectastain ABC elite kit (Vector Laboratories, Burlingame, California) following manufacturer's instructions.

3. Results

3.1 Differential potencies of effector genes in developing and adult *Drosophila*

To evaluate the potency of effector genes in living adult *Drosophila*, I expressed shi^{ts1}, *TNT*, *rpr*, or *DTI* with different GAL4 drivers. For all the effector genes except for shi^{ts1}, I used $Tub\text{-}GAL80^{ts}$ to prevent effector gene expression during development, because most GAL4 driver lines have expression earlier in the life cycle (Hayashi et al., 2002) which may lead to developmental defects persisting to adulthood. I determined the paralysis resulting from the respective gene products by counting the fraction of motile flies after mechanical agitation I used three driver lines expressing *GAL4*: (1) ubiquitously (*Act5C*), (2) pan-neuronally (*elav*), or (3) predominantly to motor neurons (*D42*). The neuronal effects can be evaluated by comparing the mutant phenotypes caused by the first two drivers.

I induced effector gene expression (or the dominant negative form of the Shi^{ts1} protein) by raising the temperature to 30°C after rearing the flies at 18°C. In Table 3 the results of this treatment for each effector/GAL4 combination are summarized. Experimental flies carrying combinations of *TNT*, shi^{ts1}, or *Kir2.1*, with any of the tested GAL4 lines showed paralysis. The paralytic effect was quickest in shi^{ts1} and slowest in *Kir2.1* if *elav* or *D42* was used (Table 3). In contrast, flies expressing *rpr* or *DTI* caused no or slow paralysis with any of the GAL4 drivers. All the control flies without *GAL4* or effector genes were measured in parallel with the experimental flies. They were still fully motile 1 day after the temperature shift (Table 4).

3.1.1 Induced paralysis by blocking chemical synapses

In combination with TNT, all GAL4 drivers were able to induce paralysis after inactivation of *Tub-GAL80^{ts}*, although the onset of the mutant phenotype took more than an hour (Fig. 7A). The three GAL4 lines had different kinetics of paralysis with their half-life at

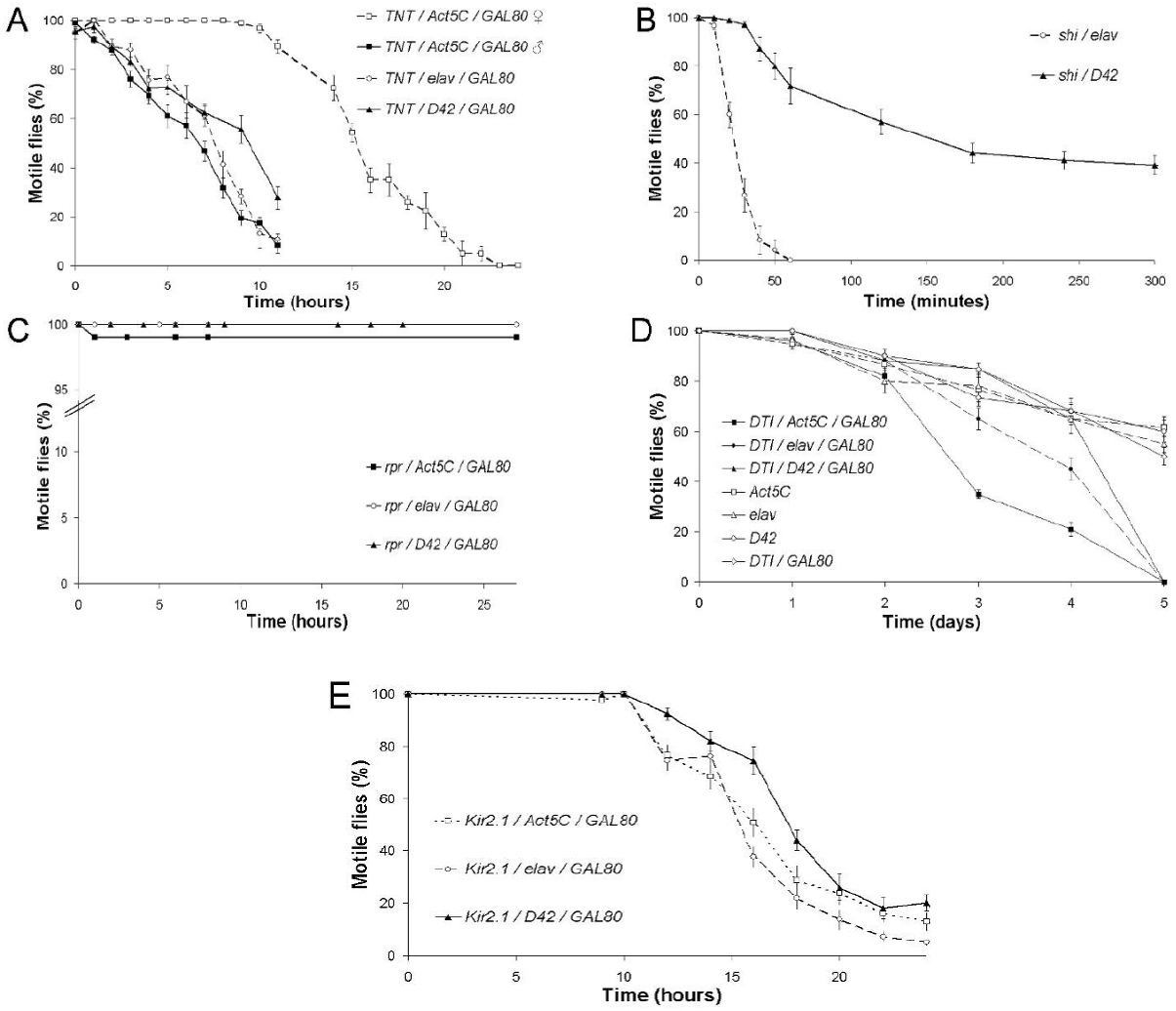


Figure 7: Temperature-dependent paralysis with three driver lines targeting *GAL4* expression to all cells (*Act5C*), all neurons (*elav*), or predominantly motor neurons (*D42*) for three different effector genes. Fractions of motile flies as a function of time after temperature shift to 30°C are indicated with mean ± s.e.m (n = 2-17). Controls either with all driver lines alone or effector genes together with *Tub-GAL80^{ts}* (except *UAS-shi^{ts1}*) alone are tested in parallel. (A) *UAS-TNT* together with *Tub-GAL80^{ts}* in combination with *D42* (filled triangle), *elav* (open circle), and *Act5C* (females: open squares; males: filled squares). (B) *UAS-shi^{ts}1* in combination with *D42* (filled triangle), *elav* (open circle). (C) *UAS-rpr* together with *Tub-GAL80^{ts}* in combination with *D42* (filled triangle), *elav* (open circle), and *Act5C* (filled squares). (D) *UAS-DTI* together with *TubGAL80^{ts}* in combination with *Act5C* (filled square), *elav* (filled circle), and *D42* (filled triangle). As controls, *UAS-DTI* together with *Tub-GAL80^{ts}* (open diamond) and the three driver lines, *Act5C* (open square), *elav* (open circle), and *D42* (open triangle) are tested alone as a heterozygote. (E) *UAS-EGFP-Kir2.1* together with *TubGAL80^{ts}* in combination with *D42* (filled triangle), *elav* (open circle), and *Act5C* (open squares).

30°C varying between 6.5 and 15 hours (Table 3). Since D42 caused the paralysis similar to the other GAL4 lines, induction of TNT in motor neurons appears to be sufficient to cause paralysis. I also found a particularly pronounced difference between genders with *Act5C*. For unknown reasons, TNT-induced paralysis of females occurred for the first time after nine hours (Table 3). A comparable delay was not seen in males of the respective genotype.

In contrast to the kinetics of TNT action, Shi^{ts1}, as reported (Kitamoto, 2001), caused quick paralysis at the restrictive temperature. At the permissive temperature (18°C) with *elav* or *D42*, flies were able to survive to adulthood. Within several minutes after shifting the temperature to 37°C, all experimental flies were paralysed (data not shown). This quick paralysis was not complete if flies were shifted to 30°C (Fig. 7B). Especially with *D42*, shifting the temperature to 30°C failed to cause total paralysis even after several hours probably due to an incomplete block of neurotransmission. Also in *elav/+;UAS-*shi^{ts1}*/+* flies, the half-life of motility was 22 min (Table 3 and Fig. 7B). Given the similar TNT-induced paralysis by *D42* and *elav*, the blocking effect of Shi^{ts1} appears to be sensitive to the combination of the amount of the expressed protein (strength of the GAL4 driver) and the restrictive temperature.

Interestingly, shi^{ts1}-expressing flies with *Act5C* failed to survive to adulthood even at 18°C, while with *elav* and *D42* shi^{ts1} did not cause any detectable detrimental effects during development. As no third instar larvae of this genotype were found, the lethal period must lie early in development (Table 5). Moreover, since the shi^{ts1} mutant is viable at 18°C (Koenig et al., 1983) and animals with neuronal expression of shi^{ts1} with *elav* survived until adulthood (Table 5), high levels of non-neuronal expression of the Shi^{ts1} protein even at the 'permissive' temperature seem to be detrimental. Taken together, the effect of shi^{ts1} on survival may vary with cell type, expression level, and temperature.

3.1.2 Genetic ablation and electrical silencing in adult flies

TNT and Shi^{ts1} are supposed to block chemical synapses, but not electrical synapses (White and Paul, 1999; Phelan and Starich, 2001). As it may, for some applications, be desirable to completely block neuronal signalling irrespective of the synapse type, I attempted genetic cell ablation as well as electrical silencing. Surprisingly, none of the driver lines in combination with *rpr* or *DTI* quickly induced paralysis upon induction of effector gene transcription (Fig. 7C and 7D). Flies were motile even one day after inactivation of $GAL80^{ts}$ (Fig. 7C). Experimental animals were indistinguishable from control flies either with the driver or *UAS-rpr* alone (Fig. 7C and Table 4). Even after 5 days of permanent induction with *Act5C* or *D42*, the *rpr*-expressing flies were still indistinguishable from controls in terms of their motility (data not shown). This may be due to the insufficient amount of Rpr protein as Rpr is active only at high dosage (White et al., 1996).

Table 3: Summary of adult paralysis: time after the onset of the expression to immobilize 50% of the adult flies.

	Act5C	*elav*	*D42*
TNT;GAL80	15 h (♀) 6.5 h (♂)	7.5 h	9 h
shi^{ts1}	- *	22 min	2.5 h
rpr; GAL80	> 5 d**	> 5 d** (♀)	> 5 d**
DTI;GAL80	2.5 d	3.5 d	4.5 d
Kir2.1; GAL80	16 hours	15 hours	17.5 hours

*: No adult fly is available because of lethality in early development at 18°C.
**: No significant difference from control flies in the measured period (5 days).

The effect of DTI on motility was also measured for five days after the induction of effector gene expression. No fast induced paralysis was achieved with DTI, as most of the

experimental flies were motile 2 days after inactivation of $GAL80^{ts}$ as well as control flies (Fig. 7D). In contrast to *rpr*, continuous induction of *DTI* expression for five days abolished motility of the experimental flies with all GAL4 drivers (Fig. 7D). Keeping the flies of the control genotype at 30°C for five days already reduced the survival to about 60% (Fig. 7D). Taken together, adult-induced *rpr* or *DTI* is not applicable for behavioural analysis as the onset of the deleterious effect, if any.

Table 4: Effecct of induced adult paralysis on control flies

Genotype	Percent of motile flies 27 hours after temperature shift to 30 °C
Act5C / +	100 ± 0.0
elav / +	100 ± 0.0
D42 / +	100 ± 0.0
TNT / + / *GAL80*	99.0 ± 0.9
shi^{ts1} / +	96.0 ± 2.2
rpr / + / *GAL80*	98.3 ± 1.1
DTI / + / *GAL80*	95.0 ± 3.1
Kir2.1 / + / GAL80	100 ± 0.0

The numbers represent the mean ± SEM (in %) based on ten to twelve independent experiments

Adult-induced Kir2.1 could block motility of the experimental flies (Fig. 7E). One day after the temperature shift, with all three tested drivers more than 80 % of the flies were immotile (Fig. 7E). The kinetics of paralysis was similar with different drivers (Fig. 7E), while the effect generally took longer than blocking chemical neurotransmission (Table 3).

3.1.3 Effector gene action during development

As *rpr* and *DTI* expression in the inducible GAL4 / UAS system did not cause quick adult-induced paralysis, I tested their efficiency during development. I shifted the temperature to 30°C for 24 hours at three different developmental periods (see details in Materials and Methods): third instar larva, early pupa, or late pupa (Table 5). Survival of individuals to

adulthood was used as a measure of killing efficiency. For further comparison, I simultaneously examined *TNT*, *shi*^{ts1}, and *Kir2.1* under the same experimental conditions. Induction of DTI for 24 hours effectively killed the animals at all three developmental stages (Table 5). These results suggest that more protein synthesis might be required during

Table 5: Adult eclosion rate after temporary effector gene expression in development

	Percent of eclosed adult flies		
	Time point of temperature shift		
Genotype	3rd instar larvae	early pupae	late pupae
Experimental			
TNT / *Act5C* / *GAL80*	0.0 ± 0.0*	0.0 ± 0.0*	2.0 ± 1.3*
TNT / *elav* / *GAL80*	0.0 ± 0.0*	1.3 ± 1.3*	0.1 ± 0.1*
shi^{ts1} / *Act5C*	N.D. (lethal before 3rd instar larvae)		
shi^{ts1} / *elav*	11.7 ± 3.1*	1.7 ± 1.7*	0.0 ± 0.0*
shi^{ts1} / *D42*	85.0 ± 5.6	93.3 ± 2.1	90.0 ± 2.6
rpr / *Act5C* / *GAL80*	22.2 ± 3.6*	31.3 ± 5.2*	88.8 ± 3.0
rpr / *elav* / *GAL80*	2.5 ± 1.6*	23.8 ± 3.8*	92.5 ± 1.6
DTI / *Act5C* / *GAL80*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
DTI / *elav* / *GAL80*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
DTI / *D42* / *GAL80*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Kir2.1 / *Act5C* / *GAL80*	0.0 ± 0.0*	2.5 ± 2.5*	0.0 ± 0.0*
Kir2.1 / *elav* / *GAL80*	24.0 ± 3.7*	21.7 ± 3.1*	2.5 ± 1.6*
Kir2.1 / *D42* / *GAL80*	2.9 ± 1.8*	2.5 ± 1.6*	22.5 ± 6.5*
Control			
Act5C / +	88.5 ± 2.0	91.7 ± 1.9	92.1 ± 1.8
elav / +	88.9 ± 2.0	92.4 ± 1.8	91.3 ± 2.0
D42 / +	87.0 ± 2.6	97.8 ± 1.5	96.0 ± 1.6
TNT / + / *GAL80*	88.8 ± 2.6	91.2 ± 3.0	92.0 ± 3.1
shi^{ts1}/ +	91.4 ± 1.4	96.7 ± 2.1	98.3 ± 1.7
rpr / + / *GAL80*	94.0 ± 2.2	90.0 ± 3.8	77.5 ± 5.3
DTI / + / *GAL80*	96.7 ± 2.1	96.7 ± 2.1	96.7 ± 2.1
Kir2.1 / + / GAL80	84.0 ± 3.1	85.0 ± 4.3	97.5 ± 1.6

Numbers represent the mean ± SEM (in %) based on six to ten individual experiments; *$P < 0.01$ (ANOVA, followed by Duncan's *post-hoc* test); each experimental group is compared to the respective controls with either GAL4 or UAS-effector alone.

development for neuronal function or viability, as the same 24-hour temperature shift in the adult caused no visible effect.

Induction of *rpr* either in early pupae or larvae also had significant effects on viability (Table 5; $P < 0.01$). In contrast, the effect of *rpr* was indistinguishable from the controls when induced in late pupae (Table 5, $P > 0.05$). The potency of *rpr* induction in the third instar larva was the strongest of the three tested time points (Table 5; $P < 0.01$, one-way ANOVA followed by Duncan's *post-hoc* test). Despite the more restricted expression pattern, *elav* was more effective than *Act5C* in combination with *UAS-rpr* when induced in the third instar larva (Table 5; $P < 0.01$, *t*-test). These results, together with those obtained with DTI, suggest that developing cells might be more sensitive to genetic cell ablation than adult ones.

TNT expression with the *Act5C* and *elav* drivers killed nearly all animals in any of the three induction periods (Table 3). Likewise, transient Kir2.1 expression efficiently interfered with development in all three induction periods, although in some cases it could not kill all animals (Table 5). Keeping the flies with neuronal shi^{ts1} expression at the restrictive temperature for one day was sufficient to kill them at all three periods, while the animals survived if it was expressed with *D42* (Table 5). *Act5C / UAS-* shi^{ts1} could not be measured because of the lethality at 18°C prior to the third-instar larva.

3.1.4 Recovery from paralysis?

To investigate whether reactivation of $GAL80^{ts}$ at the permissive temperature can revert the effects of induced effector genes, I looked for recovery from paralysis after shifting flies back to the low temperature. At the time point when about 50 % of the flies were still motile, I put the flies back to 18°C. Interestingly, with TNT or Kir2.1 all flies still motile at this time got paralysed despite the temperature shift and no animal recovered from paralysis within one day. The effects of TNT or Kir2.1 were therefore not reversible with any of the employed GAL4 drivers. The prolonged effect of TNT and Kir2.1 might be due to their stability and residual TNT and Kir2.1 synthesis.

In contrast to the irreversible effects of TNT and Kir2.1, flies carrying *UAS-shi^{ts1}* with *elav* or *D42* quickly recovered from paralysis with few exceptions. As reported by Kitamoto (2001; Kitamoto, 2002), fifteen minutes at 18°C restored locomotion after complete paralysis at 37°C. In the present experiment flies with *elav* or *D42,* however, exhibited after-effects even after extended periods at low temperature. About twenty percent of the recovered flies had a held-out wing phenotype, supposedly due to an irreversible defect in the motor neurons driving flight control muscles (data not shown). Thus, I conclude that the dominant negative effect of Shi^{ts1} protein can quickly revert in many, but obviously not all neurons.

3.2 Appetitive olfactory learning of adult *Drosophila melanogaster*

3.2.1 Improving the protocol of the appetitive learning paradigm

Schwärzel (2002) adapted the paradigm of classical olfactory conditioning for *Drosophila* to a 4 fold revolver allowing 4 times faster acquisition of data (see Introduction). This device was designed for olfactory conditioning with either electric shock or sugar as unconditioned stimuli. Although measurements using the new device allowed increased throughput and normal learning scores for aversive olfactory learning, the appetitive protocol gave rise to relatively low values (Schwärzel et al., 2003). Hence I conducted a series of experiments to improve the conditioning procedure using sugar as a positive reinforcer.

In the standard protocol, Schwärzel presented sucrose as a US for 30 seconds on wet filter paper. I presented sucrose on dried filter papers as a US for one minute. This modification increased the learning scores two- to three-fold. Second, I tested three different odor combinations which were published to be similarly avoided at certain dilutions (Masek 2005, Schwärzel 2003, Tempel et al., 1983). Isoamylacetate (IAA) and amylacetate (AM) gave the highest LI values (Figure 7A). Although the odor combination benzaldehyde (BA)/ ethylacetate (EA) gave rise to similar learning scores (Figure 7A, ANOVA: $p>0.05$), this odor

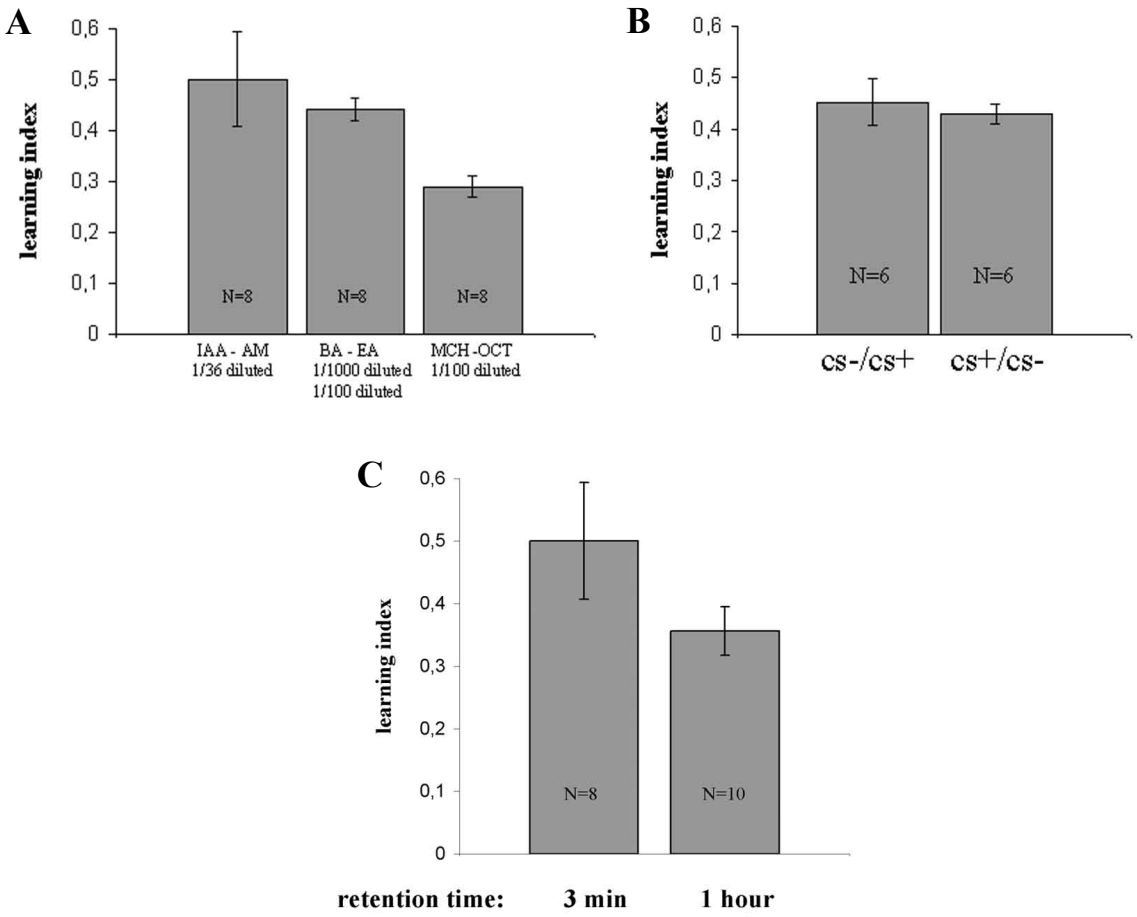


Figure 7: Improving the paradigm for appetitive olfactory learning. All experiments are done with wild-type flies raised at 25°C. **A)** Learning indices of 3-minute memory for different odors: same learning index for isoamyclacetate (IAA)/ amylacetate (AM), and benzaldehyde (BA)/ ethylacetate (EA; ANOVA: $p>0.05$; $n = 8$), but lower for 4-methylcyclohexanol (MCH)/ 3-octanol (OCT) at given dilutions (ANOVA: $p<0.05$ $n = 8$). **B)** Learning index of 3-minute memory of wildtype CS flies using BA / EA as odors. Pairing the sugar with the first or second odor makes no difference (t-test: $p>0.05$; $n = 6$). **C)** 3-minute and 1-hour memory of wildtype CS flies using IAA / AM as odors. (t-test: $p>0.05$; $n = 6$ or10)

combination proved inconvenient. As EA evaporates fast, it needs to be exchanged every 5 minutes, increasing the duration of one learning trial by 20%. 4-Methylcyclohexanol (MCH) and 3-octanol (OCT) are the most commonly used odors. Although the performance index was lower than with the two other odor combinations (ANOVA, post-hoc: Bonferroni: IAA/AM against MCH/OCT $p<0.05$ and BA/EA against MCH/OCT $p<0.01$), these odors were used in some of the experiments due to their good signal-to-noise ratio and because they have been used before (Figure 7A; Tempel et al., 1983).

In differential conditioning, non-associative learning effects can be eliminated using all four possible stimulus combinations in the training and averaging the test scores in the final memory score. For technical reasons I rewarded always the first odor (see Methods for explanation) omitting two permutations (Scherer et al., 2003). To test for non-associative effects, I compared the 3-minute memory after rewarding the first and the second odor (CS+/CS- or CS-/CS+). Figure 7B shows that the two groups of flies did not differ in their learning index (t-test: $p>0.05$). As reported before, the kinetics of forgetting in sugar reward learning is different from that in electroshock learning (Tempel et al., 1983). Reward memory "decays" more slowly. My experiments revealed a similar kinetics of the appetitive sugar memory, as there is no significant decrease in memory after one hour (Figure 1C, t-test: $p>0.05$).

3.3 A memory trace specific for appetitive olfactory

3.3.1 Comparing the localization of differently reinforced olfactory memories

Appetitive and aversive olfactory learning in *Drosophila* both use the cAMP signalling pathway - but different reinforcing systems. Moreover, for both types of learning, *rut*-dependent memories have been located in the MB (reviewed in Gerber et al., 2004). A careful consideration of the published data showed that *rut*-dependent memory in the MB is sufficient for both types of memory (Zars et al., 2000; Schwärzel et al., 2003), but the necessity of the MBs was only shown in the case of aversive olfactory learning (Connolly et al., 1996). As the necessity of a memory traces in the MBs in appetitive olfactory learning had not been shown, it was still possible that an additional memory trace upstream of the MB exists, for example in the projection neurons (PN) or antennal lobe, as suggested by the work on appetitive olfactory learning in other insects (Menzel et al., 2001; Farooqui et al., 2003, Faber et al., 2001; Daly et

al., 2004). Therefore I carried out the following experiments to see if there is a memory trace upstream of the MBs for appetitive olfactory learning.

I used the localization approach taken for electric shock and sugar learning by Zars et al. (2000) and Schwärzel et al. (2003). In addition to the MB specific GAL4 driver mb247 I used the PN-specific GAL4 driver GH146 (Heimbeck et al., 2001). *Rut* mutant flies are impaired in both types of learning (Figure 8A and 8B; Schwärzel et al., 2003). As published before, mutant flies with wild-type *rut*-AC exclusively in the MBs showed restored memory performance for both sugar (Figure 8B; ANOVA: $p>0.05$) and electroshock reinforcement

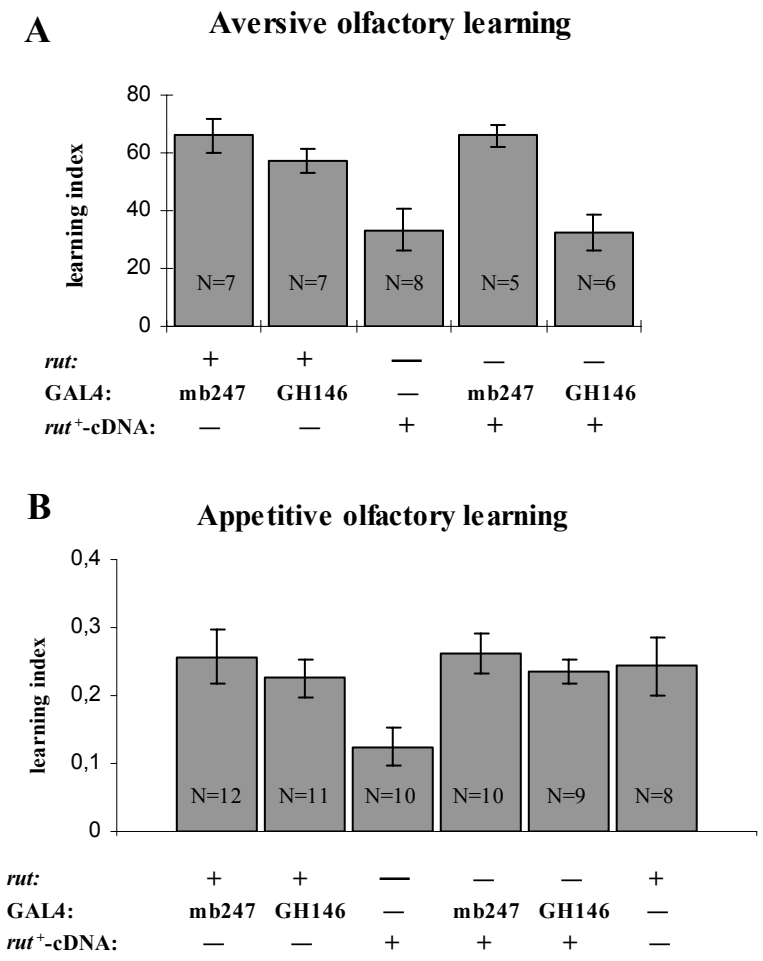


Figure 8: *Rutabaga* rescue of aversive and appetitive memories: Flies mutant for the *rut*-locus (rut, UAS-rut,) and those with expression of the wild- type form of the gene in the mushroom bodies using the GAL4 driver mb247 (rut, UAS-rut, mb247) or in the projection neurons using GH146 (rut, UAS-rut, GH146) are tested for olfactory memory directly after training using either electric shock punishment **(A)** or sugar reward **(B)**. Additionally mb247 and GH146 (and wild-type CS for appetitive olfactory learning) are tested as appropriate controls. Each data point represents the mean of 5-10 experiments plus or minus SEMs. OCT and MCH were used as odors. Aversive olfactory learning was measured by H. Tanimoto

(Figure 8A; ANOVA: $p>0.05$) at a wild-type level. These findings were in line with the results of Schwärzel et al. (2003) showing that *rut*-dependent sugar and electric shock memory can be localized to the same set of about 800 Kenyon cells (Figure 8A and 8B).

Surprisingly, with the GAL4 driver GH146 I found different results for the two types of memory. In case of electroshock learning, expression of WT *rut*-cDNA in the PNs did not rescue the memory defect (Figure 8A; ANOVA: $p<0.05$). This is in line with the accepted idea that in *Drosophila* the MBs harbour the only memory trace for aversive olfactory learning (reviewed in Gerber et al., 2004). In contrast, flies from the same cross gave rise to normal wild-type learning scores, if sugar was used as reinforcer (Figure 8B; ANOVA: $p>0.05$). This result suggests a second *rut*-dependent memory trace in the PNs specific for appetitive olfactory learning.

3.3.2 Properties of the *rut*-dependent memory trace in the Projection Neurons

To characterize the new *rut*-dependent memory trace in the PNs, I repeated the experiment with a different pair of odors. Figure 9 shows the result with the new odor combination benzaldehyde and ethylacetate. Again, *rut* mutant flies showed an about 50%

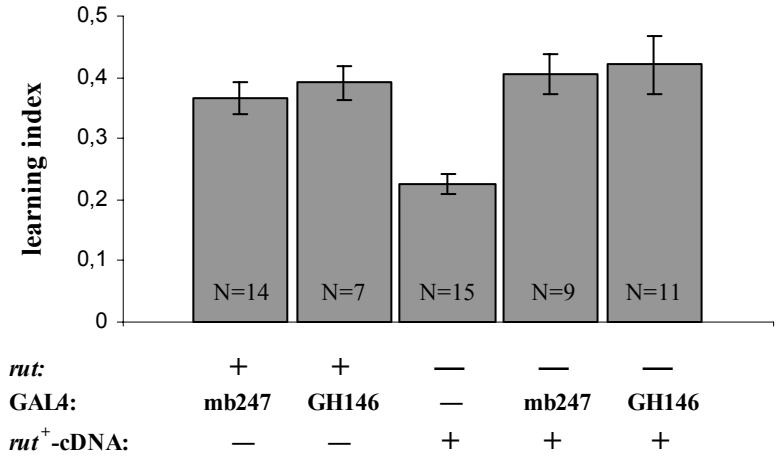


Figure 9: *Rutabaga* rescue of appetitive short-term memories with different odor combination BA and EA: Flies expressing *rut* exclusively in the MBs or PNs show wild-type learning scores, although a different odor pair was used (benzaldehyde and ethylacetate). Genotypes are the same as described in Figure 8. Each data point represents the mean of 10-14 experiments plus or minus SEMs. All flies were raised at 25°C.

reduced memory after 3 minutes (ANOVA; $p<0.01$). Expression of *rut* exclusively in the MBs was able to fully rescue this learning defect (Figure 9; ANOVA; $p>0.01$). Similarly, the

expression of *rut* in the PNs fully rescued the appetitive olfactory memory (Figure 9; ANOVA; $p<0.001$) as well as in the MBs.

In addition, I measured the stability of the new *rut* dependent memory. Figure 10 illustrates the scores for appetitive olfactory learning after increased retention times (using OCT and MCH as odors). The experiment allowed comparing the stability of the *rut*-dependent memory trace in the MB and in the PNs. After one hour the *rut* mutant showed no significant memory (Figure 10A; t-test; $p>0.05$), whereas flies expressing *rut* cDNA exclusively in the MBs (Figure10A; ANOVA; $p>0.05$) or PNs (Figure 10A; ANOVA; $p>0.05$) had learning scores not different from wild-type. Surprisingly after 3 hours the *rut* mutant showed again a memory which was significantly different from zero (Figure 10B; t-test; $p<0.001$). This suggests a second *rut* independent middle term memory component for sugar reward learning (see discussion). A statistical analysis of the 3-hours retention data

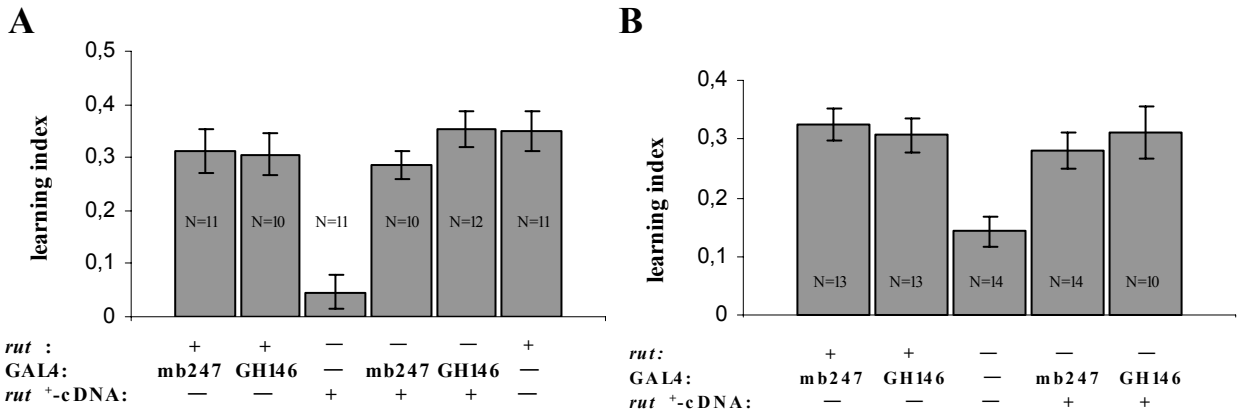


Figure 10: *Rutabaga* rescue of appetitive memories for a retention interval of one hour **(A)** or three hours **(B).** Flies expressing *rut* exclusively in the MBs or PNs show learning scores comparable to the controls. Genotypes are the same as described in Figure 8. Each data point represents the mean of 9-13 experiments plus or minus SEMs. OCT and MCH was used as odors, all flies were raised at 25°C

proved in addition no difference of the rescue in the PNs compared to that in the MBs (Figure 10B; ANOVA; $p>0.05$). Moreover, both genotypes showed equal learning scores compared to control flies (Figure 10B; ANOVA; for the MB rescue $p>0.05$; for the PN rescue $p>0.05$). In

summary, the two *rut*-dependent memories could be distinguished neither by their stability nor by their odor specificity. Further experiments are required to characterize them.

3.3.3 Additional confirmation of the *rut*-dependent memory in the projection neurons

Detailed inspection of GH146 (Heimbeck et al.,2001; Marin et al., 2002; Wong et al., 2002; Figure 11A) shows that beside its strong expression in about 100 of 150 PNs it expresses GAL4 in a small set of extrinsic MB neurons that densely innervate the MB lobes

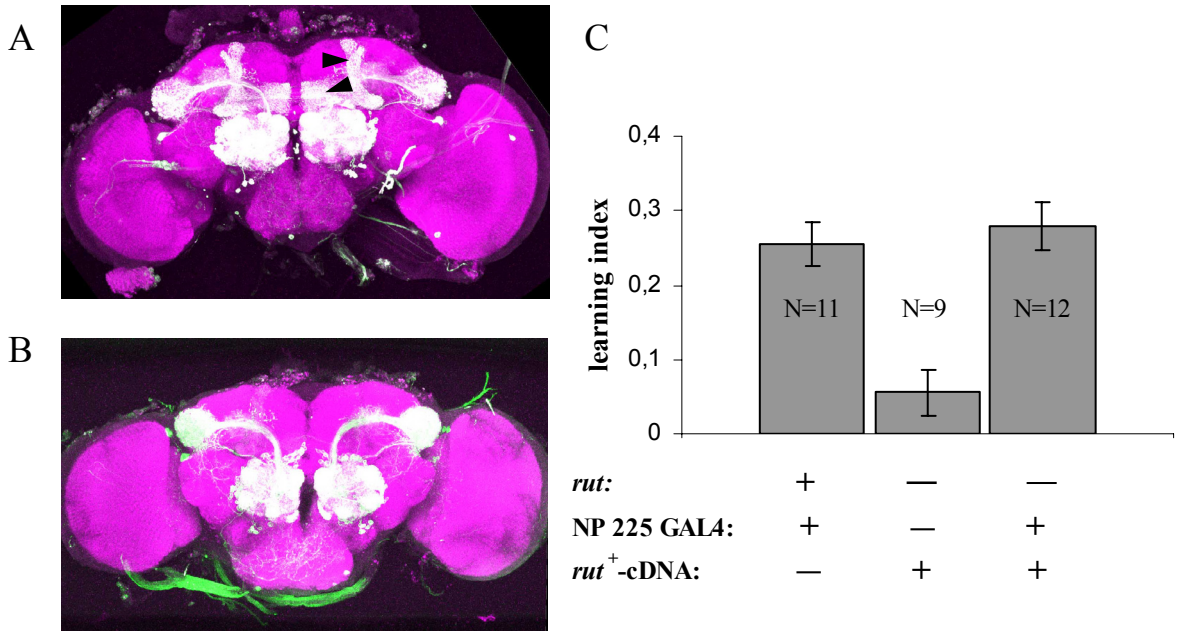


Figure 11: Projections of GH146 **(A)** and NP225 **(B)** Both drivers were crossed to UAS-CD8-GFP, brains were stained with anti-GFP (green) and the nc82 neuropil-marker (purple), scanned with a confocal microscope, and reconstructed by the AMIRA software. White and green depicts GAL4 positive patterns, showing expression in the vertical and horizontal MB lobes of GH146 (arrowheads in A). **(C)** *Rutabaga* rescue of appetitive memories for a retention interval of one hour using NP225. Each data point represents the mean of 9-11 experiments plus or minus SEMs. The projections were kindly provided by A. Jenett.

(Heimbeck et al., 2001). Although GH146 has no expression in Kenyon cells (pers. communication L. Luo) a participation of these extrinsic neurons in learning and memory cannot be excluded. For example, two MB intrinsic neurons, called DPM neurons, are required for formation of normal middle term olfactory memory (Yu et al., 2005). To

unequivocally prove that the new *rut*-dependent memory is specific for the PNs, I used another GAL4 driver line called NP225 for the memory localisation experiment. This line expresses GAL4 in a subset of about 80 PNs, but there is no detectable staining in the MBs (Ito et al., 2003; Tanaka et al., 2004; Figure 11B). Figure 11C shows one hour memory in the rescue experiments using NP225. Again the *rut* mutant has a learning score not different from zero (Figure 10C; t-test; $p>0.05$), whereas flies expressing *rut* exclusively in the PNs, under control of the PN225 driver, showed the same learning score as control flies (Figure 10C; ANOVA; $p>0.05$). This result strengthens the idea of a *rut*-dependent memory trace in the PNs specific for olfactory appetitive learning.

3.3.4 Exclusion of a developmental defect using the TARGET system

To demonstrate an acute role for *rut* in memory formation in the PNs, it is necessary to rule out a developmental brain defect. McGuire and coworkers (2003) developed the TARGET system which allows temporally and spatially restricted memory localisation experiments. I used the same method to rescue the learning defect of the *rut* mutant by expressing the *rut* cDNA specifically in the adult flies (induced 14 hours before the experiment), but not during development. As shown above the driver lines mb247 and GH146 provided rescue of the memory phenotype for appetitive olfactory learning under standard conditions (Figure 8B). First, it was tested whether *TubGAL80^{ts}* could, under permissive conditions, repress the rescue effects. Figure 12A shows the results obtained for one hour appetitive odor memory of flies that were reared and maintained at 18°C throughout the experiment. Flies of the genotype *rut*;mb247; *TubGAL80^{ts}* /UAS-*rut* had retention levels indistinguishable from those of control flies that were *rut*;UAS-*rut* alone for appetitive olfactory memory at one hour (Figure 12A; ANOVA; $p>0.05$). Flies of the genotype *rut*;GH146; *TubGAL80^{ts}* /UAS-*rut* also performed similarly to the same control flies (Figure

10A; ANOVA; p>0.05). To activate the GAL4/UAS system in the rescue experiment specifically in the adult animals, flies were reared and maintained at 18°C, but transferred (induced) 14 hours before the experiment to 30°C, a temperature restrictive for the function of *TubGAL80^{ts}* (McGuire et al., 2003). Figure 12B shows that one hour memory performance of flies carrying the GAL4 driver mb247 or GH146 in combination with the *TubGAL80^{ts}*/UAS-*rut* transgenes in the *rut* mutant background were indistinguishable from those of control flies that are wild-type at the *rutabaga* locus (ANOVA; for the mb247 rescue compared to mb247

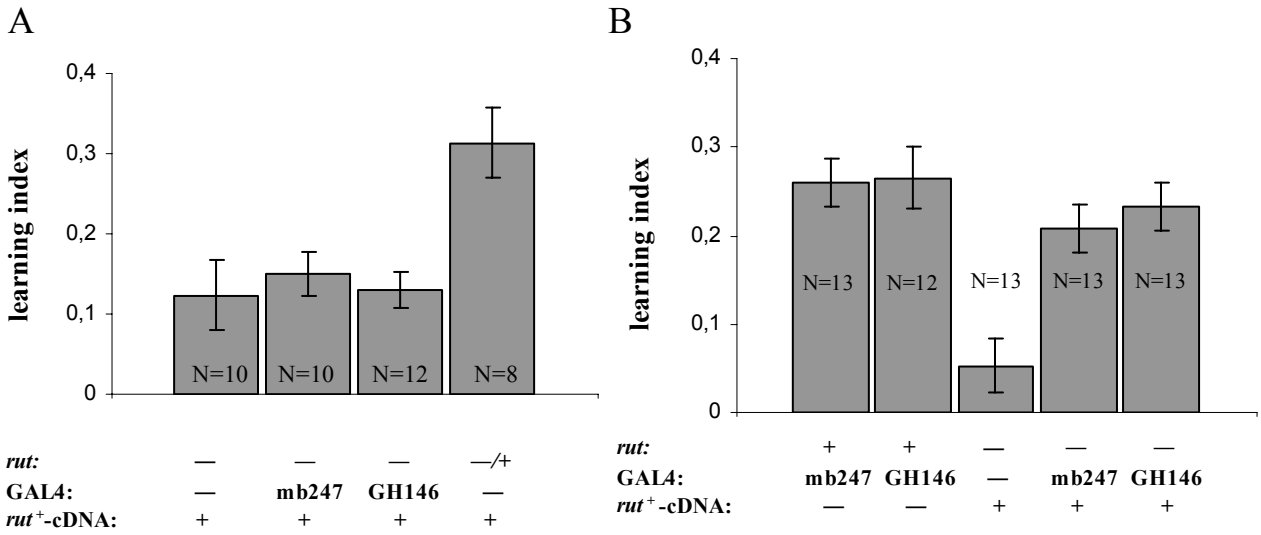


Figure 12: Temporal and spatial *rutabaga* rescue of appetitive memories via the TARGET system for a retention interval of one hour. **(A)** At 18°C under permissive conditions *TubGAL80^{ts}* is able to inhibit GAL4 transcription activating function of mb247 and GH146. **(B)** Inactivation of GAL80 by shifting the temperature to 30°C at 14 hours before the behavioural experiment fully induced the rescuing function of GAL4/UAS *rut^{+}*-cDNA. Each data point represents the mean of 8-13 experiments plus or minus SEMs. OCT and MCH were used as odors.

p>0.05; for GH146 rescue compared to GH146 p>0.05). These results showed that expression of *rut* specifically in the adult phase is sufficient to fully rescue the memory phenotype compared to flies with a wild-type *rut* locus. Therefore a defect in the development of the animal's nervous system can be excluded as the cause of the memory impairment. Figure 12A shows a further interesting result of the experiment regarding the *rut* mutant itself. Flies are shown measured after 40 hours of starvation at 18°C. Surprisingly they showed a one hour

memory significantly different from zero (t-test; p=0.019). Instead, *rut* mutant flies from the same cross, starved for 14 hours at 30°C show no learning (t-test; p>0.05). As these groups only differ in their starvation protocol, starvation itself had a pronounced effect on the memory score. When starved for more than 45 hours at 18°C *rut* mutant flies showed a completely normal performance in the memory test (data not shown).

3.3.5 Are the PNs or the MB necessary for appetitive olfactory learning?

Connolly and coworkers (1996) have shown that transgenic mushroom body expression of a dominant negative $G\alpha_s$ protein subunit that constitutively activates the adenylate cyclase

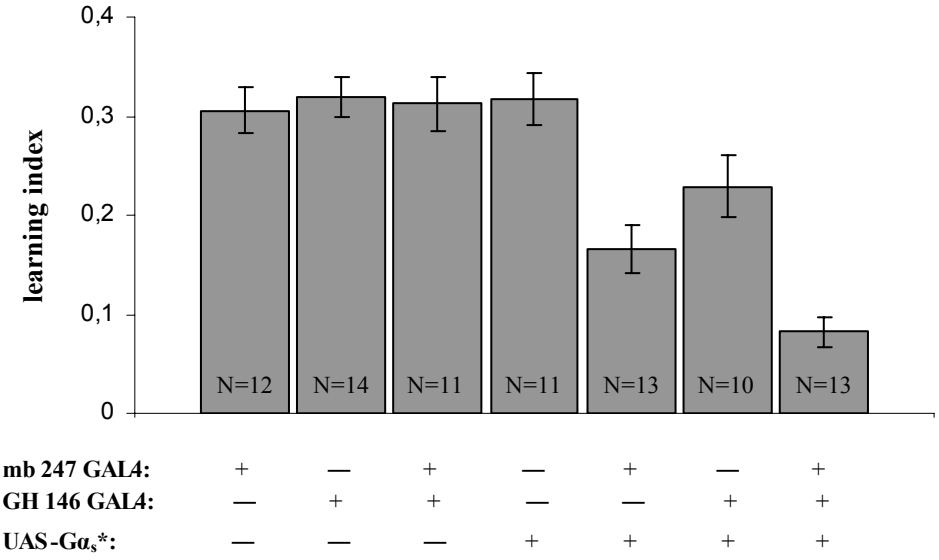


Figure 13: Impairment of appetitive olfactory memory directly after training by expression of a constitutively active $G\alpha_s$ (UAS- $G\alpha_s$*) in the MBs (mb247), the PNs (GH146), or both (mb247 and GH146). Each data point represents the mean of 10-14 experiments plus or minus SEMs. OCT and MCH were used as odors; all flies were raised at 25°C.

can completely abolish aversive associative learning reinforced by electroshock. Under the plausible assumption that a constitutively activated cyclase prevents regulation of cAMP levels and hence regulation of neuronal efficacy, this transgene seems to be a useful tool to study the necessity of neuronal plasticity of defined cells for appetitive olfactory learning. To explore this notion, I restricted disruption of cAMP signalling to the MBs, PNs, and both. I used the MB specific GAL4 driver mb247, the PN specific GAL4 driver GH146, or both in

combination with a UAS-$G\alpha_s^*$ transgene that constitutively activates adenylyl cyclases (Quan et al., 1991; Simon et al., 1991; Bourne et al., 1991). To examine appetitive associative learning in these flies, I used the standard paradigm for 3-minute memory and OCT / MCH as odors (Figure 13). Figure 13 shows for all 3 driver lines and the effector line the same learning scores. When $G\alpha_s^*$ was expressed in the MBs memory was reduced to 50 percent which was significantly different from the controls (ANOVA; $p<0.01$). Expression of $G\alpha_s^*$ in the PNs showed a decrease in learning which was on one side not different from wild-type (ANOVA; $p>0.05$), but also not different from $G\alpha_s^*$ expression in the MBs (ANOVA; $p>0.05$). The expression of $G\alpha_s^*$ in both, the PNs and MBs further decreased the memory score. Although still significantly different from zero (t-test; $p<0.001$), the decrease was significant compared to expression of $G\alpha_s^*$ in the PNs (ANOVA; $p<0.01$) but statistically not different from expression of $G\alpha_s^*$ in the MBs alone (ANOVA; $p>0.05$). As these rescue data suggest two independent, redundant memories for sugar reward learning in the PNs and the MBs, one would assume that inhibition of one memory would still give rise to full learning scores, due to the intact second one. Therefore the results shown in Figure 13 are surprising, as interference with either structure tends to decreases the memory. As $G\alpha_s$ might not only activate *rutabaga* AC but also other ACs, PKA, and channels directly and developmental defects can not be excluded it is difficult to interpret these results (see discussion).

3.4 How is the reinforcing sugar stimulus for associative olfactory learning mediated in *Drosophila*?

Given the VUMmx1 neuron in honey bee and fly, given that octopamine serves as a neurotransmitter in appetitive learning in both species, one would like to know, whether a

similar neuron also mediates the unconditioned stimulus in appetitive associative olfactory learning in *Drosophila.* Again a rescue approach to give a preliminary answer is feasible. The TβH mutant lacks the enzymatic activity to synthesize octopamine (Monastirioti et al., 1998). Restoring the activity in a given set of neurons using the GAL/UAS system in the mutant background, allows to identifiy sets of TβH-neurons sufficient to mediate the reinforcing function. In Figure 14B, as published, the TβH mutant shows a strongly reduced learning performance, that is statistically different from zero (t-test; $p<0.01$). Expression of either NP7088 GAL4 or the TβH-cDNA under UAS control in the mutant background showed the

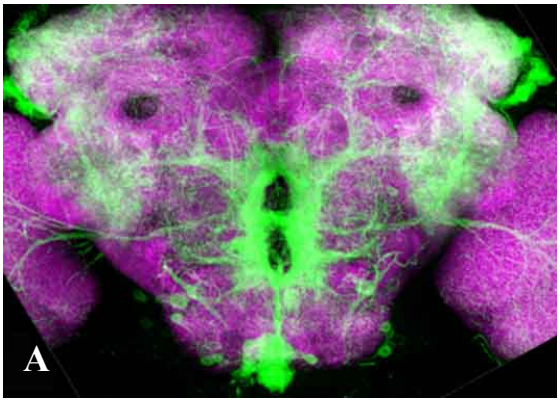
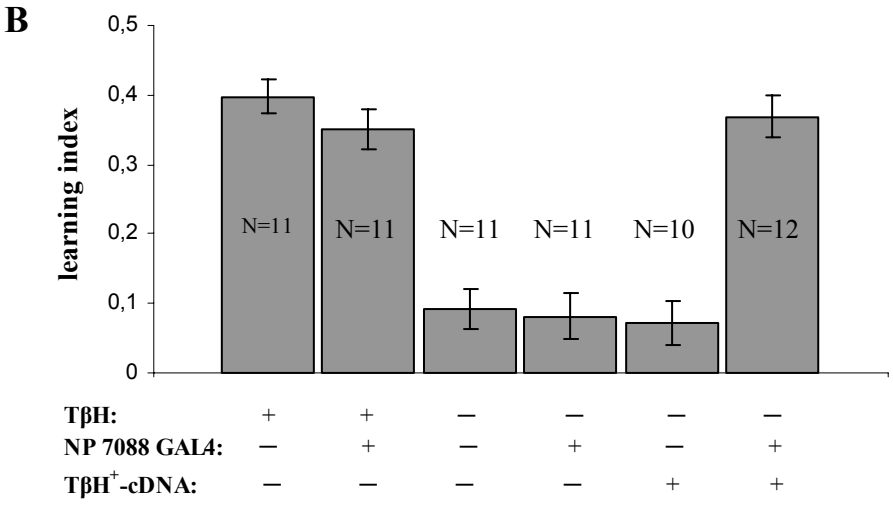


Figure 14: A) Projetion of NP7088 crossed to UAS-GFP; double staining using anti-GFP (green) and anti-synapsin (neuropil marker in purple). **B)** TßH rescue of appetitive short-term memory using IAA and AM as odors. To decrease the GAL4 expression in NP7088 flies were raised, maintained, and starved at 18°C. Each data point represents the mean of 10-12 experiments plus or minus SEMs. IAA and AM were used as odors; all flies were raised at 18°C. The expression pattern of NP7088 was kindly provided by M. Selcho.



same phenotype (ANOVA; NP7088 compared to TβH $p>0.05$; UAS-TβH cDNA compared to TβH $p>0.05$). The GAL4 driver line NP7088 originates of a collection of ~4000 GAL4 enhancer-trap lines generated by a consortium in Japan (Hayashi et al., 2002). This database has been used as a resource to search for candidate GAL4 strains with expression putatively

octopaminergic neurons including the VUM cluster. Expression of the TβH-cDNA in the mutant background driven by NP7088 fully rescued the mutant phenotype to a wild-type level (Figure 14B; ANOVA; $p>0.05$). This result showed that about 250 neurons including the VUM cluster were sufficient, if able to synthesize octopamine to rescue the TβH mutant

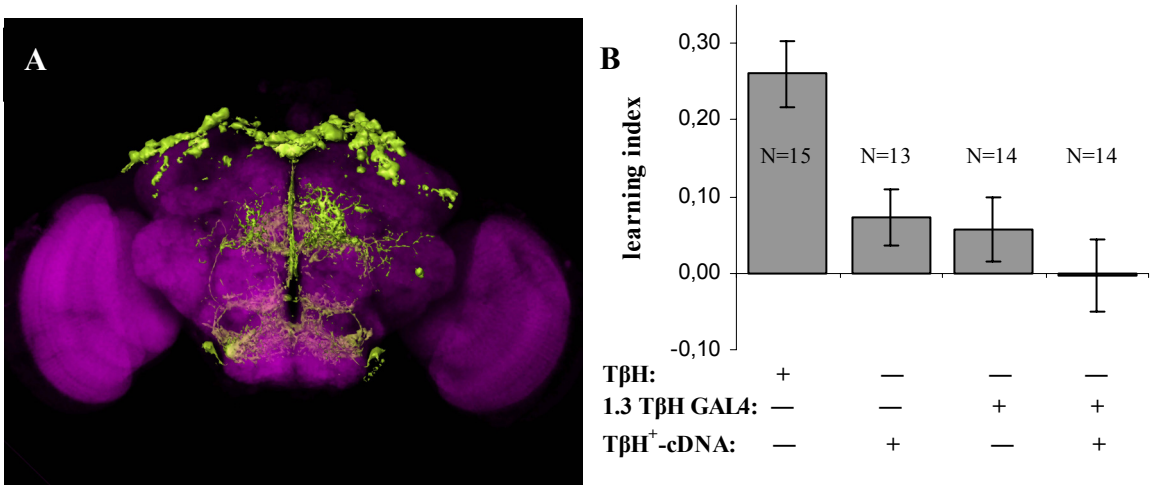


Figure 15: A) Frontal projection of 1.3TβH GAL4 crossed to UAS-GFP; double staining using anti-GFP (green) and nc82 (neuropil marker in purple). The surface of the expression pattern was rendered with AMIRA (kindly provided by A. Jenett). **B)** TβH rescue of appetitive short-term memory using IAA and AM as odors. Each data point represents the mean of 13-15 experiments plus or minus SEMs. IAA and AM were used as odors; all flies were raised at 25°C.

phenotype. To further decrease the number of cells necessary to rescue the appetitive olfactory learning, I used another GAL4 driver called 1.3TβH GAL4. This promoter GAL4 line uses a 1.3 kb upstream regulatory sequence of the TbH gene (pers. communication S. Hampel). Due to the complexity of the TβH gene locus the 1.3kb regulatory sequence limits the expression to a subset of most probably octopaminergic cells. Crossing 1.3TβH GAL4 to UAS-GFP predominantly stains innervations of the SOG, the ellipsoid body, the fan-shape body, and pars intercerebralis but not the VUM cluster (Figure 15A). Expression of TβH cDNA driven by 1.3TβH GAL4 in the mutant background seems to rescue an ethanol tolerance phenotype (pers. communication S. Hampel and A. Jenett).

As shown before, appetitive olfactory learning of control flies expressing either GAL4 or the UAS transgene in the mutant background is strongly reduced. The generally lower level of the learning performance is probably due to a different genetic background of the flies. The same results were obtained in a control experiment performed by A. Yarali. In all olfactory learning experiments described above flies were in the CS background. Whereas here flies were in the standardised genetic background for ethanol tolerance experiments (pers. communication H.Scholz); thereby causing reduced learning performance. All control flies in the TβH mutant background showed strong impairment in learning. Expressing the TβH cDNA via the driver line 1.3GAL4 TβH was not able to rescue the mutant phenotype (t-test; $p>0.05$). Taken together, the experiments with the two different GAL4 driver lines suggest a role of the VUM cluster in reinforcing sugar reward in *Drosophila*. A combined anatomical and functional analysis of GAL4 lines expressing GAL4 in different sub clusters of the 26 VUM neurons would help to further decrease the number of potential cells mediating the unconditioned stimulus in associative olfactory sugar learning in *Drosophila*.

3.5 Interference with US signalling via RNAi

Schwärzel et al., (2003) published that appetitive and aversive memory formation can be distinguished by the requirement for different catecholamines, dopamine for aversive and octopamine for appetitive conditioning. The current model for olfactory learning and memory suggests dopaminergic or octopaminergic G-protein coupled receptors activating cAMP synthesis at the Kenyon cell postsynapse. In detail, two receptors for dopamine, dDA1 and DAMB (Han et al., 1996; Kim et al., 2003) are discussed as candidates. For octopamine five receptor genes are published, namely OAMB, Dmoa2, DmOct beta1R, DmOct beta2R and DmOct beta3R (Han et al., 1998; Lee et al., 2003; Balfanz et al., 2005; Maqueria et al., 2005). In difference to the published data, most of the genes seem to be expressed rather

unspecifically (Roeder 2005) leaving the possibility to function as a presynaptic autoreceptor of dopaminergic or octopaminergic modulatory neurons. To investigate their roles in US signalling for both versions of the olfactory associative learning paradigm, I used an RNAi approach to knockdown the two most likely involved and best described GPCRs, OAMB and dDA1.

3.5.1 Knockdown of the octopamine receptor OAMB

The best described GPCR responding to octopamine is called OAMB. Although originally published to be predominantly expressed in the MB, it was later shown to be expressed in the whole fly, even in the egg-laying apparatus of females (Lee et al., 2003). Indeed, OAMB mutants show an egg-laying phenotype comparable to TβH mutants (Lee et al., 2003; Monastirioti 2003). By expressing an RNAi construct against OAMB, driven by the ubiquitous tubGAL4 driver, I tried to knock down the protein and therefore reproduce the egg-laying phenotype. The RNAi construct was cloned by T. Roeder. It consisted of about 600 nucleotides of cDNA of exon five and six, an inverse complementary genomic DNA fragment additionally including the intron between the two exons, and a small intron of the *white* gene between. The whole DNA fragment is under UAS control. As it is known that the insertion site strongly regulates the effectiveness of the RNAi construct (Natalia Funk, PhD thesis), I screened in total 26 different insertions of the UAS-RNAi-OAMB construct regarding the egg-laying phenotype. All insertions were crossed to tubGAL4/TM3. From the offspring six mated females were collected and transferred to a fresh vial with cornmeal for 5 days. After two weeks hatched adults were counted. As an internal control I measured in parallel three control genotypes treated the same way (wild-type, the TM3 balancer, and tubGAL4). Figure 17A shows the number of hatched offspring of mated tubGAL4; UAS-RNAi-OAMB females. In none of the cases the RNAi was able to fully inhibit egg-laying,

most of the insertions showed instead a weak mutant phenotype. Insertions number 25, 19, and 16 strongly decreased the number of hatched flies (Figure 16A). Using the result of this pre-screen, I expressed the insertions 19 and 25 in the MB or PN and did a standard appetitive learning experiment (Figure 16B).

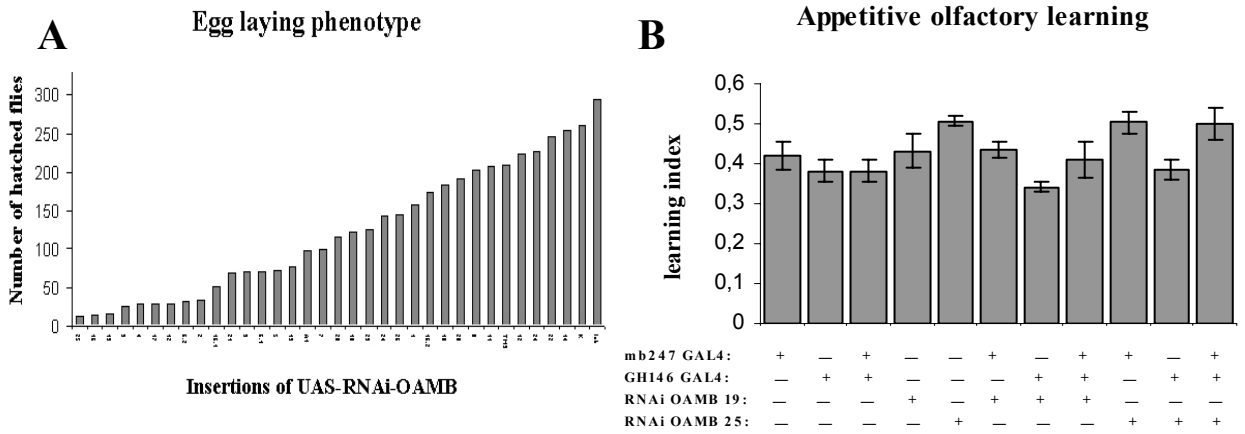


Figure 16: A) Screening of 28 different UAS-RNAi-OAMB insertions driven by tubGAL4 for an egg-laying phenotype. The different numbers represent different insertions, tub means the tubGAL4 driver alone, TM3 is a control for a third chromosomal balancer alone, K means wildtype. Values show in means of two single experiments. **B)** Appetitive olfactory learning for the two different insertions 19 and 25, either expressed in the MB or the PNs. Each data point represents the mean of 7-8 experiments plus or minus SEMs. IAA and Am were used as odors.

Surprisingly, if expressed in the MBs or PNs, none of the two insertions gave rise to significantly reduced learning performance. As I did no antibody staining against OAMB, I was not able to track the protein levels of OAMB in these cells. Therefore I can only speculate, if the lack of phenotype is due to lack of an efficient RNAi, or an unknown mechanism preserving OAMB mRNA from degradation. Additonally, an involvement of other octopamine receptors inappetitive olfactory learning would also lead to the described results.

3.5.2 Knockdown of the dopamine receptor dDA1

In *Drosophila* two G-protein coupled dopamine receptors are published, namely DAMB and dDA1 (Han et al., 1996; Kim et al., 2003). Recently it was reported that dDA1 mutants,

or DUMB mutants are impaired in normal electroshock learning (pers. communication KA. Han). Therefore I crossed 15 different insertions of a UAS-RNAi dDA1 construct to the ubiquitous GAL4 driver *Act5C* and screened the offspring for lethality, as dDA1 is published to regulate larval ecdysis (Park et al., 2004). The RNAi construct was cloned by T. Roeder. It was made of about 700 nucleotides of cDNA of the second and third exon, an inverse complementary genomic DNA fragment including the intron between the two exons, and a

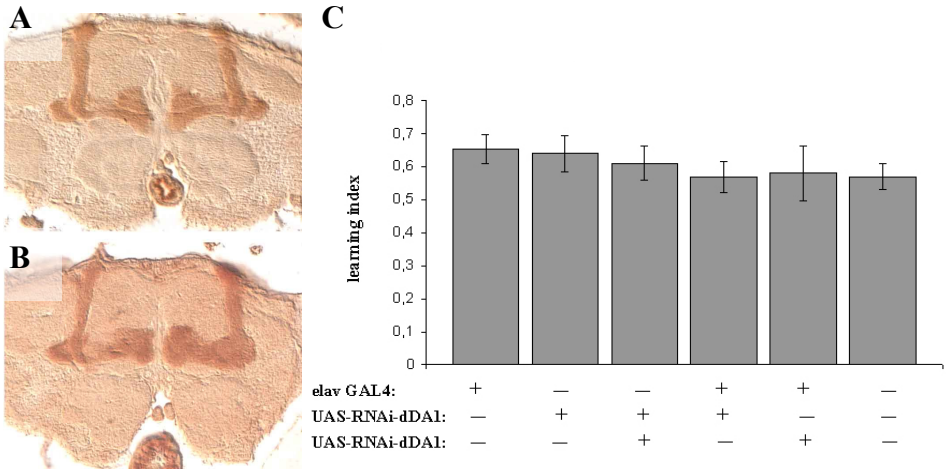


Figure 17: 7µm paraffin sections of an anti-dDA1 labelled mb247GAL4; UAS-RNAi-dDA1, UAS-RNAi-dDA1 brain **(A)** wild-type control **(B)**. **C)** Aversive olfactory learning of two different UAS-RNAi-dDA1 insertions alone or combined crossed to the pan-neural driver elavGAL4. Each data point represents the mean of 6-7 experiments plus or minus SEMs. MCH and OCT were used as odors; all flies were reaised at 25°C.

small intron of the *white* gene between. The whole construct is under UAS control. Two independent insertion lines carrying the UAS-RNAi-dDA1 construct if crossed to the GAL4 driver gave rise to no offspring (data not shown). Therefore I crossed each insertion alone or both with the pan-neuronal driver elavGAL4 and measured normal aversive olfactory memory (Figure 17C). Again, flies expressing one or two RNAi constructs in all neurons showed no significant impairment in memory for aversive olfactory learning (ANOVA; $p>0.05$). To test the efficiency of the used RNAi constructs, I monitored the protein level of dDA1 in the brain via antibody staining (Kim et al., 2003). Figure 17A and B illustrate sectioned brains of experimental (mb247; UAS-RNAi-dDA1; UAS-RNAi-dDA1) and control flies stained with

anti-dDA1. Both images showed strong staining in the vertical and horizontal lobes of the MBs. No reduction is detectable in the experimental flies (as in the behaviour, data not shown), although the RNAi constructs were driven in about one third of the Kenyon cells. This suggests that the amount of receptors in the MB Kenyon cells is not reduced and therefore cannot impair the aversive memory. Unfortunately this experiment does not allow to decide, whether dDA1 is involved in aversive olfactory memory. The results might be due to a reduced efficiency of RNAi in neurons compared to non-neuronal cells as seen in *C. elegans* (Winkelbauer et al., 2005; Syntichaki et al., 2002; Tavernarakis et al, 2000)

3.6 Testing for perception of sensory stimuli

All genotypes used for associative olfactory learning were tested for detection ability of odorants, and sugar. This determined whether any of the above changes in olfactory memories were due to impairment in detecting the task relevant stimuli. Table 6 shows the results for odor perception in the second and third column, in the forth and fifth column water and sugar perception are noted; all values are given in medians and inter-quartile range, as for several experiments the data are not normally distributed. Although there was a difference in the odor avoidance of a given genotype with respect to one odor over the year, I found no statistically significant differences in a particular experiment (Table 6). For the experiments using RNAi against dDA1 and OAMB no sensory acuity test were done as there was no difference in the learning index.

Table 6: Sensory acuity tests (values are noted in median and inter-quartile range)

Genotype	Perception of the first odor	Perception of the second odor	Water perception	Sugar perception
CantonS	0.28/0.18	0.31/0.32	0.02/0.05	0.87/0.07

mb247	0.28/0.18	0.41/0.41	0.00/0.00	0.81/0.18
GH146	0.39/0.09	0.47/0.13	0.01/0.03	0.89/0.12
rut, UAS-rut	0.27/0.19	0.38/0.07	0.02/0.05	0.94/0.05
rut, UAS-rut; mb247	0.39/0.08	0.41/0.31	0.03/0.03	0.85/0.23
rut, UAS-rut; GH146	0.29/0.23	0.36/0.20	0.02/0.07	0.90/0.13
rut, UAS-rut; mb247; tubGAL80^{ts}	0.32/0.30	0.32/0.29	0.00/0.00	0.95/0.03
mb247; tubGAL80^{ts}	0.33/0.35	0.32/0.29	0.00/0.00	0.85/0.17
rut, UAS-rut; GH146; tubGAL80^{ts}	0.30/0.27	0.42/0.11	0.00/0.03	0.77/0.15
GH146; tubGAL80^{ts}	0.33/0.27	0.43/0.27	0.00/0.08	0.91/0.02
NP225	0.28/0.28	0.47/0.21	0.00/0.00	0.87/10
rut, UAS-rut; NP225	0.25/0.07	0.50/0.25	0.00/0.03	0.87/0.12
GH146; mb247	0.28/0.25	0.34/0.12	0.03/0.07	0.90/0.12
UAS-Gα_s*	0.16/0.32	0.21/0.11	0.01/0.03	0.90/0.10
GH146, UAS-Gα_s*	0.20/0.20	0.33/0.14	0.03/0.10	0.86/0.13
mb247; UAS-Gα_s*	0.23/0.15	0.25/0.14	0.03/0.10	0.91/0.03
GH146; mb247; UAS-Gα_s*	0.19/0.07	0.35/0.27	0.00/0.03	0.89/0.10
1.3TβHGAL4	0.15/0.27	0.44/0.25	0.00/0.00	0.95/0.42
TβH, UAS-TβH	0.20/0.22	0.46/0.16	0.00/0.00	0.56/0.72
TβH, 1.3TbHGAL4	0.30/0.40	0.30/0.40	0.00/0.00	0.87/0.48
TβH, UAS-TβH; 1.3TbHGAL4	0.33/0.30	0.38/0.13	0.00/0.00	0.56/0.72
TβH	0.20/0.19	0.17/0.31	0.00/0.00	100.00/1.67
NP7088	0.25/0.23	0.33/0.21	0.01/0.03	100.00/3.33
TβH, UAS-TβH	0.13/0.18	0.25/0.17	0.00/0.00	100.00/16.67
TβH, NP7088	0.33/0.17	0.47/0.28	0.00/0.00	100.00/13.33

TβH, UAS-TβH; NP7088	0.39/0.11	0.27/0.26	0.02/0.04	100.00/36.67

4. Discussion

4.1 Induced effector gene potencies of the GAL4 / UAS system

Given the wide use of the GAL4 / UAS gene expression system for revealing the cellular basis of behaviours (reviewed in Roman, 2004), a general assessment of available effector genes is in high demand. The analysis in this study showed that the respective effector genes (*TNT*, *shi*^{ts1}, *rpr*, *DTI*, and *Kir2.1*) exerted differential interfering activities. Each one merits consideration as a tool to functionally interfere with neural circuits, but also has its shortcomings.

Adult-onset cell ablation irrespective of cell type would be an attractive tool for intervening with behavioural function. Before eclosion, DTI in combination with the TARGET system was able to kill animals. However, during adulthood it took several days of inactivation of GAL80^{ts} to impair motility. As feeding of protein synthesis inhibitors kills flies after four days (Tully et al., 1994), blockage of protein synthesis seems to be of limited use. Nevertheless, DTI might still be of use for cell ablation using driver lines with larval expression.

Similarily, *rpr*-dependent ablation turned out to be of no use in adult flies. Late pupal and adult neurons seemed to be resistant to *rpr*-dependent cell ablation. In developing cells, induced *rpr* expression was more effective, in line with the previously reported successful examples after chronic expression (McNabb et al., 1997; Busto et al., 1999; Renn et al., 1999). A restricted developmental time window of sensitivity to *rpr* expression has already been reported using ubiquitous expression by a heat shock promoter (White et al., 1996). As adult cells are also more resistant to killing by x-rays, developmental processes such as cell cycle progression may be required for triggering efficient apoptosis. As *rpr* acts with other pro-apoptotic genes, such as *head involution defective*, a combination could be more effective also

in the adult-induced neuronal interference (Zhou et al., 1997; Wing et al., 1998; Keller et al., 2002). As cell ablation would still be attractive for functional analyses of neural circuits, an effector gene causing neurodegeneration might be a better candidate for adult-onset cell ablation (Driscoll and Gerstbrein, 2003).

For the time being, *Kir2.1*, *TNT* and shi^{ts1} are the more practical effector genes to study the role of neural circuits. Although the onset of adult-induced *Kir2.1* expression was relatively slow, the temporal expression caused the expected mutant phenotype in adults and during development with all three GAL4 driver lines. As *Kir2.1* silences action potential generation regardless of the synapse type, it may serve as a useful method to trace the neural substrates of specific behaviours.

With *UAS-*shi^{ts1}, I confirmed most of the expected mutant phenotypes. In addition to its action on endocytotic processes (Kosaka and Ikeda, 1983), Dynamin has been shown to act also on the processing of other membrane vesicles including focal exocytosis (Di et al., 2003). Moreover, the involvement of Dynamin in hormone secretion at the Golgi apparatus suggests an effect of Shi^{ts1} also on the secretion of neuropeptides (Yang et al., 2001). These findings imply that Shi^{ts1} can impair neurotransmission more efficiently than the depletion of the synaptic vesicles by blocking endocytosis (Kitamoto, 2001).

So far, no case has been reported in which a chemical synapse has not been blocked by shi^{ts1}. On the other hand, its ectopic expression causes non-neuronal early developmental lethality at 18°C and its effect in certain types of neurons may be irreversible. Furthermore, the blocking activities depend on the restrictive temperature and the expression level (Kitamoto, 2002). Selecting an effector gene for behavioural analysis may always require special considerations, although this study adds to many previous examples demonstrating the usefulness of shi^{ts1} (Kitamoto, 2002).

Finally, this study confirmed the effectiveness of $GAL80^{ts}$ (McGuire et al., 2003). In the present examples the developmental expression of *TNT*, *rpr*, or *DTI* was effectively

suppressed in the presence of *Tub-GAL80*^{ts}. Without temporal control, many GAL4 driver lines have been found to be lethal with TNT (Sweeney et al., 1995; Martin et al., 2002). As driver-dependent expression during development is a problem for adult behavioural analysis using the GAL4 / UAS system, the additional temporal control by *GAL80*^{ts} is a substantial advance.

4.2 Appetitive olfactory learning

Recently, Schwärzel and coworkers (2003) "rediscovered" classical appetitive odor conditioning in *Drosophila.* Based on this work, the paradigm underwent several technical modifications leading to a 2-3 fold increase in learning scores. First, the feeding interval was twice as long as used before. As it had been shown that an increase in the number of training trials leads to raised learning scores (Schwärzel, 2003), a similar effect for prolonged training intervals was considered possible. Second, drying of the filter paper after applying the sucrose solution had a strong effect on the memory scores. As it was recently shown that water itself can be used as an aversive stimulus in learning (data not shown; Le Bourg 2005) the observed avoidance of the odor paired with the sugar solution is explicable. Sugar water itself is not only rewarding for the fly, but also has a punishing effect due to the water. Taken together these minor adjustments improved the appetitive olfactory learning experiments considerably.

4.3 A memory trace in the projection neurons specific for appetitive olfactory learning

4.3.1 Starvation is problematic

Only hungered flies approach the rewarded odor in the appetitive olfactory learning paradigm (Tempel et al., 1983). Therefore, starvation is a fundamental requirement for this kind of learning experiments. If the learning score depends on the length of starvation and if mutants are differently affected if they are hungered, are still unanswered questions as an analysis of putative starvation effects on training and memory retrieval is lacking. Thus, comparing different starvation protocols using *rut*-mutants and wild-type flies could answer the question of whether an independent memory mechanism can substitute for the *Rutabaga*-dependent one in appetitive olfactory learning under life-threatening stress conditions. For the time being a parallel measurement of internal controls for each olfactory learning experiment is the only way to avoid misinterpretations of the results. Therefore, I explicitly want to mention that only males are shown in all the localisation experiments using the *rutabaga* mutant. As the *rut* gene is on the X-chromosome (Livingstone et al., 1984), due to the crossing scheme, only hemizygous males had the required genotype. As genders were separated after the test, I also counted in parallel the females that harbour additionally to the mutated X-chromosome a wild-type one. Due to *rut* being a recessive mutant heterozygous females show normal learning scores and serve so as an internal control in all the experiments (data not shown).

4.3.2 A *rut*-dependent memory in the projection neurons

Expression of wild-type *rutabaga* in projection neurons did not rescue the defect in aversive electric shock learning. This result is in line with Connolly et al. (1998), who showed that prevention of *rut*-dependent neuronal plasticity in the MB totally abolishes aversive olfactory memory. Thereby my findings contradict critics ascribing the memory impairment to developmental defects of the MBs itself and thus suggesting a memory trace upstream of the MBs for electroshock learning.

Interestingly, *rut* mutant flies themselves showed no memory after one hour, whereas after three hours they were able to remember the rewarded odor. For aversive olfactory learning a longer-lasting form of MTM called ARM (anesthesia-resistant memory; Dudai et al., 1983; Tully and Quinn 1985) is described that was confirmed to be *rutabaga*-independent (Isabel et al., 2004). *Radish* has been shown to be a mutant lacking ARM in electroshock learning (Folkers et al., 1993). Introducing this mutant to sugar reward learning in comparison to *rutabaga* could reveal if there is a similar *rut*-independent longer lasting memory component.

In the honeybee, if an odor is presented immediately before sucrose solution, an association is formed which enables the odor to trigger the proboscis extension response (PER) in a successive test (reviewed in Menzel, 2001). Extensive studies using different approaches (Menzel et al. 1979; Erber et al. 1980; Farooqui et al., 2003; Hammer and Menzel, 1998; Faber et al., 2001) suggest memory traces at multiple and distributed sites in the brain, involving first- and second-order sensory neuropils (antennal lobe and mushroom bodies). In *Manduca sexta* Daly and coworkers (2004) reported that appetitive olfactory learning produces a restructuring of spatial and temporal components of network responses to the rewarded odor in the AL. As the learning experiments in the honeybee and *Manduca* used sugar as positive reinforcer like in my case it is suggested that olfactory memory in MBs and PNs may be conserved between insect species even at the circuit level.

By using the TARGET system, a requirement for *rut* expression during the development for normal adult learning was excluded, although in *rut* mutants developmental defects of the mushroom bodies have been observed (Balling et al., 1987; Hitier et al., 1998). The presence of the protein at the moment of the learning task in the adult fly was sufficient to rescue the impairment in memory. The same was shown for electroshock learning (McGuire et al., 2003) suggesting a general role for *rutabaga*-encoded type I adenylyl cyclase as a molecular coincidence detector independent of the reinforcer (Schwärzel et al., 2003) and cell type.

4.3.3 Are the MBs or PNs necessary for appetitive olfactory learning?

Connolly and co-workers (1998) have shown that transgenic mushroom body expression of a dominant negative $G\alpha_s$ protein subunit ($G\alpha_s^*$) that constitutively activates the adenylate cyclase cascade can completely abolish aversive associative learning. Constitutively activated cyclase prevents regulation of cAMP levels and hence regulation of neuronal efficacy (Renden and Broadie, 2003).

In this thesis transgenic expression of $G\alpha_s^*$ in MBs and PNs at the same time leads to a strong reduction in the learning performance, corroborating the results of the *rut* rescue experiments suggesting two memory traces in these cells for appetitive olfactory learning. However, as the *rut*-dependent memory traces in the MBs and PNs are supposed to be redundant, experimental manipulation of the MBs or ALs separately by $G\alpha_s^*$ should not reduce the learning performance. Contrary to this conjecture expression of $G\alpha_s^*$ in the PNs, although not significantly, tends to decrease the shown memory performance and even a loss of about 50% is seen when $G\alpha_s^*$ is expressed in the MBs. This finding seems to contradict the rescue experiments, but a detailed look at the effects of $G\alpha_s^*$ reveals several explanations. First, transgenic expression of $G\alpha_s^*$ disrupts all nine published *Drosophila* ACs (Cann and Levin, 2002; Cann et al., 2000; Iourgenko et al., 2000; Iourgenko et al., 1997; Levin et al.,

1992). It should therefore have additional effects on cAMP signalling beyond those caused specifically by the removal of *rutabaga*. Alternatively, activation of PKA-dependent phosphorylation through $G\alpha_s^*$ expression could impede modulatory changes in shared substrates or cellular systems by kinases other than PKA (Connolly et al., 1996). Another possibility is that $G\alpha_s^*$ could exert signaling effects other than through the cAMP pathway, such as through direct modulation of channels (Clapman et al., 1994). Because of all of these reported properties it is likely that $G\alpha_s^*$ also induces developmental defects in neurons. In any case, the transgenic expression of $G\alpha_s^*$ might cause defects in addition to those brought about by the absence of *rutabaga*. Therefore, I still maintain that there are two independent memory traces located in the MBs and ALs for appetitive olfactory memory. The role of $G\alpha_s^*$ expression during development could be investigated using the TARGET system (McGuire et al., 2003). A further approach would be an RNAi construct under UAS control to knock-down specifically rut AC in the MBs, the PNs, or both. A combination of the two approaches, an adult specific RNAi knock-down of *rut* in the MB, the PNs, and both should ultimately confirm the two redundant memory traces for appetitive olfactory learning in *Drosophila*.

4.4 A candidate modulatory neuron representing the appetitive US

4.4.1 Functional analysis of the VUM cluster in *Drosophila*

Despite more than 20 years of research on olfactory learning and memory in *Drosophila*, the nature of the modulatory neurons itself that provide information about the unconditioned stimuli remained unknown. So, the question of how the sugar reward (US) is represented in the fly brain is still not answered. Locally directed octopamine synthesis utilising targeted expression of wild-type TβH in the TβH mutant background suggests that some of the octopaminergic neurons labeled in the GAL4 driver line NP7088 are part of the US pathway for sugar. Of these neurons projecting to the AL or MB should be the strongest candidates.

TβH expression in a smaller set of putative octopaminergic neurons without projections to the MBs or the ALs in the driver line 1.3 TβH GAL4 had no rescuing effect (pers. communication S. Hampel). As the pars intercerebralis is labelled in 1.3 TβH GAL4 octopaminergic neurosecretion involved in olfactory appetitive learning is not likely.

4.4.2 Single-cell staining in the VUM cluster in *Drosophila*

Which neurons mediate the unconditioned stimuli in *Drosophila* olfactory learning and memory? To answer this question Mareike Selcho analyzed the GAL4 driver line NP 7088 in detail. In Figure 18 the expression pattern of the GAL4 driver line NP7088 is shown. Figure 18A shows a projection of NP7088; UAS-GFP (in green). The expression is distributed throughout the whole brain; remarkable are the dense innervations of the neuropil around the oesophagus. To identify and count the number of GAL4 positive cells, somata were stained

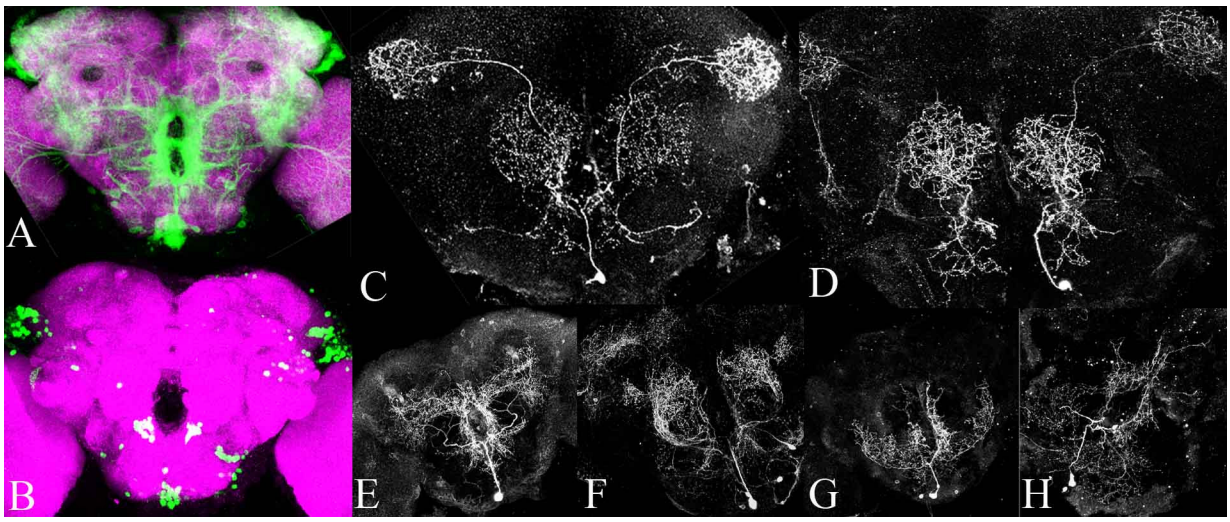


Figure 18: A) Projetion of NP7088 crossed to UAS-GFP; double staining using anti-GFP (green) and anti-synapsin (neuropil marker in purple). **B)** Projection of NP7088 crossed to UAS-nls-GFP to reveal GAL4 positive somata; double staining using anti-GFP (green) and anti-synapsin (neuropil marker in purple). **C)** Single cell staining of TDC-GAL4 using the flp-technique (Wong et al., 2002). **D-H)** Five different single cell stainings of VUM neurons in NP7088 using the flp technique (Wong et al., 2002). Data were kindly provided by M. Selcho and S. Busch.

with UAS-nls-GFP. About 250 cell bodies are stained in NP7088 including a ventral cluster near the midline, the so called VUM cluster (Figure 18B).

Mosaic clones of the VUM cluster (Wong et al., 2002; diploma thesis Selcho 2006; diploma thesis Busch 2006) illustrated the morphology of single neurons in this cluster. In Figure 18D, a neuron is described resembling in its branching pattern the VUMmx1 neuron of the honey bee. It has a cell body ventral near the midline, innervates the SEG, bifurcates near the esophagus, and sends out its axon symmetrically to the AL, MB and LH via the inner antenno-cerebral tract. The same VUMmx1 neuron is shown in Figure 18C using the driver line TDC2-GAL4. This promoter GAL4 line uses the regulatory sequence of the gene encoding a tyrosine decarboxylase (TDC, Cole et al., 2005). TDC decarboxylates tyrosine to tyramine, the precursor of OA. Therefore it is very likely that the VUMmx1 neuron in NP7088 is octopaminergic.

Taken together, the single cell staining of the VUM cluster (Selcho, 2006) using the driver line NP7088 for the first time identified a neuron similar to the honeybee VUMmx1 (Hammer 1993) innervating three olfactory brain structures, the antennal lobe, the mushroom body, and the lateral horn. As Hammer (1993) showed that this single neuron mediates the unconditioned stimulus in associative olfactory sugar learning a conserved mechanism of reward processing for the reinforcing stimulus sugar in *Drosophila* and honeybee can be suggested.

As due to technical reasons it was not possible to immuno-stain the *Drosophila* brain for octopamine, it is not known whether the used GAL4 lines are indeed labelling octopaminergic neurons (pers. communication M. Selcho). However, as the expression pattern of NP7088 is similar to TDC-GAL4 and single cell analysis of both lines revealed a similar VUMmx1-like neuron, one can suggest that a large number of octopaminergic neurons are included in NP7088. This result is strengthened by Sinakevitch et al. (2006), who showed beside overlapping clusters of cell bodies similar innervation patterns of octopaminergic neurons in the antennal lobe, mushroom body calyx, and lateral protocerebrum for *Drosophila*.

Interestingly, the fly has about twice as many octopaminergic VUM neurons as the bee (Sinakevitch et al., 2005; Sinakevitch and Strausfeld 2006; Hammer and Menzel, 1995). In detail, 26 cells in the VUM cluster of the fly compared to about 15 VUM neurons in the honeybee. The data still leave the possibility that in the fly several paired VUM neurons mediate the reinforcing function.

4.4.3 Further experiments to establish a neuronal map of the sugar US

Two series of experiments can reveal the sites were octopaminergic signalling is needed to mediate the sugar reward. First, one can analyze via RNAi knockdown if octopamine receptors in PNs and different parts of the MB are necessary for appetitive olfactory learning. Recently several G-protein coupled octopamine receptors were published in addition to OAMB (Han et al., 1998; Lee et al., 2003; Balfanz et al., 2005; Maqueria et al., 2005) making this approach laborious. Even more important, failing of the RNAi approach for OAMB leaves this method problematic in neuronal cells similar to C. elegans (Winkelbauer et al., 2005; Syntichaki et al., 2002; Tavernarakis et al, 2000). Alternatively, a rescue approach using several GAL4 drivers that stains subset of the VUM cluster could further decrease the number of candidate neurons involved in US processing. Ultimately an integrative approach combining functional and anatomical studies of the VUM cluster would answer the question, if a single VUM neuron would be found to mediate the reinforcing function of the sugar reward also in the fly. In detail, one would use the single cell mosaic analysis to block and label neurons at the same time; behavioural and subsequent immunohistochemical analysis of individual flies would directly show which neurons signal the US for appetitive olfactory learning.

4.5 A model for appetitive olfactory learning

There are distinct differences discussed above of appetitive olfactory learning with respect to aversive olfactory learning in the fly. Because of that, I would like to insert these findings into the actual not yet disproved model for appetitive olfactory learning in *Drosophila* (Heisenberg, 2003).

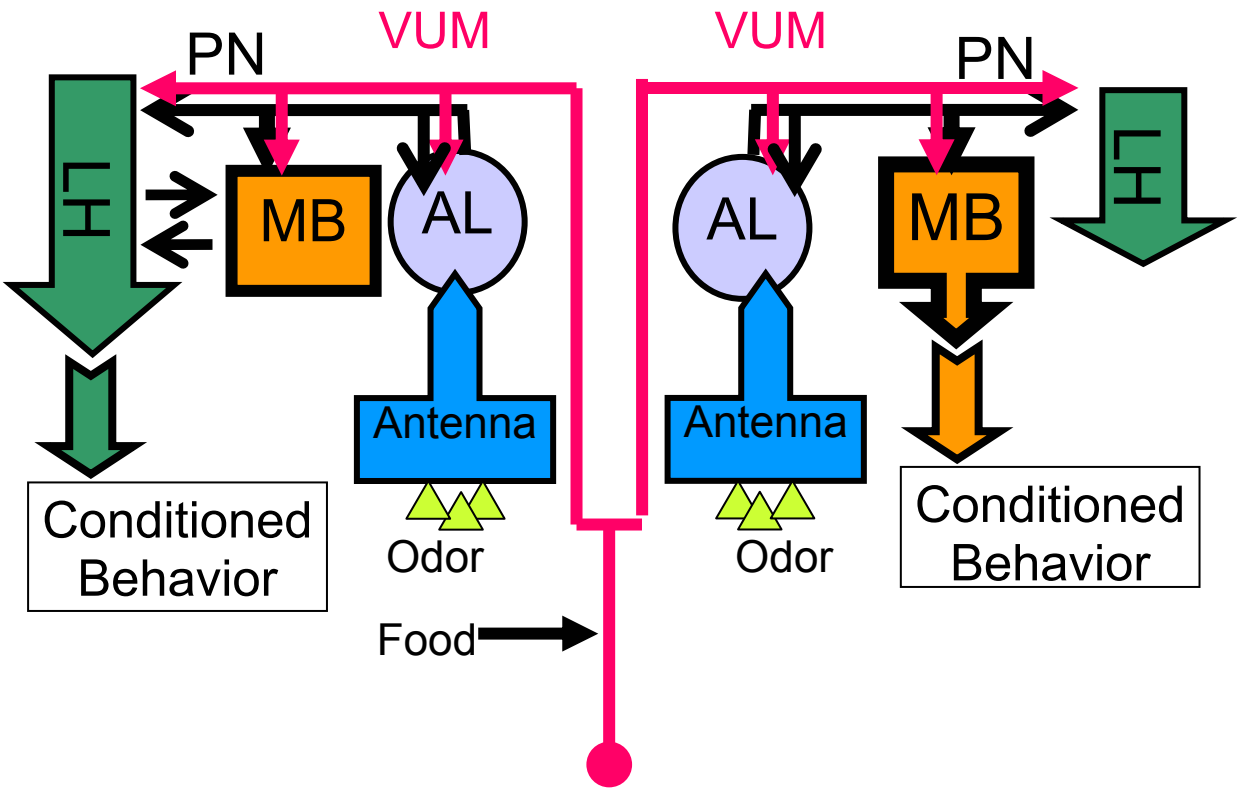


Figure 19: Two different models for appetitive olfactory learning

Appetitive reinforced learning of *Drosophila* is about half in strength compared to aversive reinforced learning, but memory decay proceeds relatively slowly after training (Figure 10; Tempel et al., 1983). Memory in wild-type flies persists for 24 hours after training with sucrose, compared to 4-6 hours after training with electric shock (Tempel et. al., 1983). This increased stability may perhaps have its origin in the redundant organisation of memory traces in different cell types. Nevertheless, as the *rut* mutant is impaired in aversive and

appetitive learning similarly, it is assumed that the cAMP cascade is in general the molecular mechanism for the associative strengthening of synapses independent of the reinforcer or the cell type. The well described olfactory pathway (reviewed in Hallem et al., 2005) and the assumption, that sugar reinforcement is mediated by neurons of the VUM cluster suggests three places of coincidence detection. The AL is innervated by octopaminergic neurons of the VUM cluster as well as by post- and presynaptic processes of the PNs (Sinakevitch and Strausfeld, 2006; Ashraf et al., 2006). In the MB calyx, beside octopaminergic neuronal endings, microglomeruli are described consisting of PN presynaptic endings and Kenyon cell postsynaptic dendrites (Yasuyama et al., 2002). Additionally several of the VUM neurons also project to the MB γ-lobe, suggesting a presynaptic modification of the cAMP signalling (Kandel, 2001). Therefore, the place of coincidence in the MB is still pending. Finally, also the LH may get input of the US and CS and therefore might work as a coincidence detector. Due to the lack of methods of specific genetic intervention and scantiness of anatomical and functional data on the LH, a prediction of the mechanistic involvement is hardly possible. Because of that, although it was not clearly shown that the PNs or MBs are necessary for appetitive olfactory learning, independent memory traces in PNs and MBs are suggested in the model.

Before conditioning an odor is represented by a certain set of PNs and a set of following MB Kenyon cells. As Schwärzel and coworkers (2003) showed that output of the MB Kenyon cells during test is necessary to elicit the conditioned response also in appetitive olfactory learning an extrinsic MB output neuron is postulated that receives synapses from all Kenyon cells. Without conditioning, these synapses would all be latent and the CR neuron would not respond to any odor. During the conditioning phase the VUMmx1 neuron or several VUM neurons independently would report the positive event – the unconditioned stimulus (US) – to all PNs and Kenyon cells. If here an odor coincides with the sugar the respective subset of PNs and Kenyon cells would be simultaneously activated by the VUM neuron(s), which will

upregulate their output synapses. As the increased firing of a conditioned set of PNs has presynapses onto the MB Kenyon cells, the respective subset of MB Kenyon cells coding for the conditioned odor would also increase their activity. Thereby, a memory trace for the association between odor and reinforcement localized within the PNs is transported to the MB Kenyon cells. The strengthened output onto MB extrinsic neurons is then thought to mediate conditioned behaviour towards the odor when encountered during test, during which no reinforcer is present. Hereby no involvement of the LH is postulated (Figure 19 right part).

Alternatively, since the LH gets both gustatory and olfactory input, extrinsic MB neurons (Ito et al., 1998) project to the LH, and GABAergic feedback neurons are suggested that project back to the MBs (Yasuyama et al., 2002; Strausfeld et al., 2003; Perez-Orive et al., 2002) another possible model can be suggested (Figure 19 left part) activating the extrinsic MB output neurons. The PNs or the MBs, establishing a memory trace for appetitive olfactory learning, provide association dependent modified olfactory information to subsequent higher brain centers in the lateral protocerebrum. A comparison of this information and differently processed olfactory signal of the protocerebrum can thereby elicit an extrinsic neuron driving the conditioned response. The comparison of the two signals may allow the distinction between a conditioned odor and increased odor intensities. One has to be aware that the comparison can take place in the Kenyon cells or further downstream, for example in the extrinsic neuron of the MB.

Taken together, the existence of similar *rut*-dependent memories in MBs and PNs allow suggesting an evolutionary conserved mechanism for appetitive olfactory learning in bee and fly. The results of this thesis also show overlapping molecular mechanisms and functional principles for aversive and appetitive olfactory learning, despite a number of significant differences. Therefore, one can speculate that the differences in appetitive and aversive olfactory learning developed during evolution from a common precursor of odorant association, due to different evolutionary pressures for the different reinforcers. Finally, the

description of a VUMmx1 neuron and the sufficiency of several cell clusters including the VUM cluster to mediate the octopaminergic sugar stimulus, are the first step in establishing a neuronal map for US processing in *Drosophila*.

5. Summary

Genetic intervention in the fly *Drosophila melanogaster* has provided strong evidence that the mushroom bodies of the insect brain act as the seat of memory traces for aversive and appetitive olfactory learning (reviewed in Heisenberg, 2003). In flies, electroshock is mainly used as negative reinforcer. Unfortunately this fact complicates a comparative consideration with other inscets as most studies use sugar as positive reinforcer.

For example, several lines of evidence from honeybee and moth have suggested another site, the antennal lobe, to house neuronal plasticity underlying appetitive olfactory memory (reviewed in Menzel, 2001; Daly et al., 2004). Because of this I focused my work mainly on appetitive olfactory learning.

In the first part of my thesis, I used a novel genetic tool, the TARGET system (McGuire et al., 2003), which allows the temporally controlled expression of a given effector gene in a defined set of cells. Comparing effector genes which either block neurotransmission or ablate cells showed important differences, revealing that selection of the appropriate effector gene is critical for evaluating the function of neural circuits.

In the second part, a new engram of olfactory memory in the *Drosophila* projection neurons is described by restoring Rutabaga adenlylate cyclase (rut-AC) activity specifically in these cells. Expression of wild-type *rutabaga* in the projection neurons fully rescued the defect in sugar reward memory, but not in aversive electric shock memory. No difference was found in the stability of the appetitive memories rescued either in projection neurons or Kenyon cells.

In the third part of the thesis I tried to understand how the reinforcing signals for sugar reward are internally represented. In the bee Hammer (1993) described a single octopaminergic neuron – called VUMmx1 – that mediates the sugar stimulus in associative olfactory reward learning. Analysis of single VUM neurons in the fly (Selcho, 2006)

identified a neuron with a similar morphology as the VUMmx1 neuron. As there is a mutant in *Drosophila* lacking the last enzymatic step in octopamine synthesis (Monastirioti et al., 1996), *Tyramine beta Hydroxylase (TβH)*, I was able to show that local *TβH* expression successfully rescued sugar reward learning. This allows to conclude that about 250 cells including the VUM cluster are sufficient for mediating the sugar reinforcement signal in the fly.

The description of a VUMmx1 similar neuron and the involvement of the VUM cluster in mediating the octopaminergic sugar stimulus are the first steps in establishing a neuronal map for US processing in *Drosophila*. Based on this work several experiments are contrivable to reach this ultimate goal in the fly.

Taken together, the described similiarities between *Drosophila* and honeybee regarding the memory organisation in MBs and PNs and the proposed internal representation of the sugar reward suggest an evolutionarily conserved mechanism for appetitive olfactory learning in insects.

6. Zusammenfassung

Arbeiten über das assoziative olfaktorische Lernen bei *Drosophila,* bei denen definierte Gruppen von Nerven genetisch verändert wurden, haben gezeigt, dass die Pilzkörper des Insektengehirns Gedächtnisspuren für aversives und appetitives Geruchslernen besitzen (Heisenberg, 2003). Hierzu wird bei der Fliege meistens Elektroschock als negativer Reiz bei der Pavlovschen Konditionierung benutzt. Leider erschwert dies einen Vergleich mit anderen Insekten, da in den meisten Studien Zucker als positiver Stimulus verwendet wird.

Interessanterweise schlagen mehrere Arbeiten bei der Biene und der Motte zusätzlich zu den Pilzkörpern einen weiteren Bereich im Insektengehirn vor, der eine Gedächtnisspur des appetitiven Geruchslernens besitzt, die Antennalloben (Menzel, 2001; Daly et al., 2004). Aus diesen Gründen habe ich mich in meiner Arbeit intensiv mit dem appetitiven Geruchslernen beschäftigt.

Im ersten Teil meiner Arbeit habe ich das TARGET System verwendet (McGuire et al., 2003), welches die zeitlich kontrollierte Expression eines beliebigen Reportergens in definierten Zellen erlaubt. Ein Vergleich verschiedener Effektoren zeigte, dass Proteine, die die Neurotransmission blocken (*Shi^{ts}; TNT, Kir2.1*), besser geeignet sind, um die Funktion neuronaler Schaltkreise in *Drosophila* zu untersuchen. Effektoren, die Zellen abtöten, entfalten lediglich während der Entwicklung ihre volle Aktivität und eignen sich daher, z.B. um das larvale Verhalten zu analysieren.

Im zweiten Teil beschreibe ich eine neue Gedächtnisspur für das Geruchslernen in den Projektionsneuronen. Die Expression des wildtypischen *rutabaga* Gens ausschließlich in diesen Zellen, rettete den Defekt im Zuckerlernen, nicht aber im Elektroschocklernen. Ferner scheinen die Gedächtnisspuren des appetitven Geruchslernens im Pilzkörper und den Projektionsneuronen gleich stabil zu sein.

Im dritten Teil dieser Arbeit wurde die Frage gestellt, wie das Belohnungssignal des Zuckers im Fliegengehirn verarbeitet wird. Hammer (1993) beschrieb in der Biene ein einzelnes octopaminerges Neuron, das VUMmx1 Neuron, welches den Zuckerreiz beim assoziativen Geruchslernen vermittelt. Eine Einzelzellanalyse des VUM clusters von *Drosophila* zeigte ein ähnliches VUMmx1 Neuron erstmals bei der Fliege (M. Selcho, Diplomarbeit). Durch die lokale Expression der *Tyramin beta Hydroxylase (TβH)*, das Oktopamin synthetisierende Enzym, im *TβH* Mutanten Hintergrund, konnte gezeigt werden, dass ca. 250 Zellen (inklusive des VUM Clusters) ausreichen, das Belohnungssignal des Zuckers zu vermitteln.

Beides, die Identifizierung eines VUMmx1 ähnlichen Neurons in der Fliege und die Eingrenzung der Neuronen, die das Belohnungssignal vermitteln, bilden die Basis für weitergehende Versuche. Diese erlauben es, neuronale Schaltkreise der US (Zucker)-Verarbeitung beim assoziativen olfaktorischen Lernen detailliert zu beschreiben.

Insgesamt legen die übereinstimmenden Gedächtnisspuren im Pilzkörper und den Projektionsneuronen von *Drosophila* und der Honigbiene nahe, dass das olfaktorische Belohnungslernen einem in der Evolution konservierten Mechanismus entstammt.

7. References

Abrams, T.W. & 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**, 128-135.

Abrams, T.W., Yovell, Y., Onyike, C.U., Cohen, J.E. & Jarrard, H.E. (1998) Analysis of sequence-dependent interactions between transient calcium and transmitter stimuli in activating adenylyl cyclase in Aplysia: possible contribution to CS--US sequence requirement during conditioning. *Learn Mem*, **4**, 496-509.

Ache, B.W. & Young, J.M. (2005) Olfaction: diverse species, conserved principles. *Neuron*, **48**, 417-430.

Adams, M.D., Celniker, S.E., et al., (2000) The genome sequence of *Drosophila melanogaster*. *Science*, **287**, 2185-2195.

Amrein, H. & Thorne, N. (2005) Gustatory perception and behavior in *Drosophila melanogaster*. *Curr Biol*, **15**, R673-684.

Aplin, A.C. & Kaufman, T.C. (1997) Homeotic transformation of legs to mouthparts by proboscipedia expression in *Drosophila* imaginal discs. *Mech Dev*, **62**, 51-60.

Ashburner, M. (1989) *Drosophila: A Lavoratory Handbook*. Cold Spring Harbor Laboratory Press, New York.

Ashraf, S.I., McLoon, A.L., Sclarsic, S.M. & Kunes, S. (2006) Synaptic protein synthesis associated with memory is regulated by the RISC pathway in *Drosophila*. *Cell*, **124**, 191-205.

Baddeley, A. (1997) *Human memory. Theory and practice*. Psychology Press, Hove.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T. & Bate, M. (2001) Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. *J Neurosci*, **21**, 1523-1531.

Baker, A.G. (1976) Learned irrelevance and learned helplessness: Rats learn that stimuli, reinforcers and responses are uncorrelated. *Journal of Experimental Psychology*, 85-133.

Balfanz, S., Strunker, T., Frings, S. & Baumann, A. (2005) A family of octopamine [corrected] receptors that specifically induce cyclic AMP production or Ca2+ release in *Drosophila* melanogaster. *J Neurochem*, **93**, 440-451.

Balling, A., Technau, G.M. & Heisenberg, M. (1987) Are the structural changes in adult *Drosophila* mushroom bodies memory traces? Studies on biochemical learning mutants. *J Neurogenet*, **4**, 65-73.

Barth, M. & Heisenberg, M. (1997) Vision affects mushroom bodies and central complex in *Drosophila melanogaster*. *Learn Mem*, **4**, 219-229.

Beck, C.D., Schroeder, B. & Davis, R.L. (2000) Learning performance of normal and mutant *Drosophila* after repeated conditioning trials with discrete stimuli. *J Neurosci*, **20**, 2944-2953.

Bellen, H.J., D'Evelyn, D., Harvey, M. & Elledge, S.J. (1992) Isolation of temperature-sensitive diphtheria toxins in yeast and their effects on *Drosophila* cells. *Development*, **114**, 787-796.

Benzer, S. (1967) Behavioural mutants of *Drosophila* isolated by countercurrent distribtion. *Proc Natl Acad Sci U S A*, **58**, 1112-1119.

Bergmann, A., Yang, A.Y. & Srivastava, M. (2003) Regulators of IAP function: coming to grips with the grim reaper. *Curr Opin Cell Biol*, **15**, 717-724.

Birchler, J.A., Pal-Bhadra, M. & Bhadra, U. (2003) *Transgene cosuppression in animals in: RNAi: A Guide to Gene Silencing*. Cold Spring Harbor Press,, Cold Spring Harbor, New York.

Bolwig, G.M., Del Vecchio, M., Hannon, G. & Tully, T. (1995) Molecular cloning of linotte in *Drosophila*: a novel gene that functions in adults during associative learning. *Neuron*, **15**, 829-842.

Bourne, H.R., Sanders, D.A. & McCormick, F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature*, **349**, 117-127.

Bower, G.H.a.H., E.R. (1981) *Theories of learning*. Prentice-Hall, Englewood Cliffs, NJ.

Boynton, S. & Tully, T. (1992) latheo, a new gene involved in associative learning and memory in *Drosophila melanogaster*, identified from P element mutagenesis. *Genetics*, **131**, 655-672.

Brand, A.H. & Dormand, E.L. (1995) The GAL4 system as a tool for unravelling the mysteries of the *Drosophila* nervous system. *Curr Opin Neurobiol*, **5**, 572-578.

Brand, A.H. & Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, **118**, 401-415.

Brogden, W.J. (1939) Sensory pre-conditioning. *Journal of Experimental Psychology*, **25**, 323-332.

Busto, M., Iyengar, B. & Campos, A.R. (1999) Genetic dissection of behavior: modulation of locomotion by light in the *Drosophila melanogaster* larva requires genetically distinct visual system functions. *J Neurosci*, **19**, 3337-3344.

Byrne, J.H. & Kandel, E.R. (1996) Presynaptic facilitation revisited: state and time dependence. *J Neurosci*, **16**, 425-435.

Cambridge, S.B., Davis, R.L. & Minden, J.S. (1997) *Drosophila* mitotic domain boundaries as cell fate boundaries. *Science*, **277**, 825-828.

Chang, K.T., Shi, Y.J. & Min, K.T. (2003) The *Drosophila* homolog of Down's syndrome critical region 1 gene regulates learning: implications for mental retardation. *Proc Natl Acad Sci U S A*, **100**, 15794-15799.

Cann, M.J., Chung, E. & Levin, L.R. (2000) A new family of adenylyl cyclase genes in the male germline of *Drosophila melanogaster*. *Dev Genes Evol*, **210**, 200-206.

Cann, M.J. & Levin, L.R. (2002) Identification of transmembrane adenylyl cyclase isoforms. *Methods Enzymol*, **345**, 150-159.

Cheng, Y., Endo, K., Wu, K., Rodan, A.R., Heberlein, U. & Davis, R.L. (2001) *Drosophila* fasciclinII is required for the formation of odor memories and for normal sensitivity to alcohol. *Cell*, **105**, 757-768.

Chiang, A.S., Blum, A., Barditch, J., Chen, Y.H., Chiu, S.L., Regulski, M., Armstrong, J.D., Tully, T. & Dubnau, J. (2004) radish encodes a phospholipase-A2 and defines a neural circuit involved in anesthesia-resistant memory. *Curr Biol*, **14**, 263-272.

Chyb, S., Dahanukar, A., Wickens, A. & Carlson, J.R. (2003) *Drosophila* Gr5a encodes a taste receptor tuned to trehalose. *Proc Natl Acad Sci U S A*, **100 Suppl 2**, 14526-14530.

Clyne, P.J., Warr, C.G. & Carlson, J.R. (2000) Candidate taste receptors in *Drosophila*. *Science*, **287**, 1830-1834.

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

Cobb, M. & Domain, I. (2000) Olfactory coding in a simple system: adaptation in *Drosophila* larvae. *Proc Biol Sci*, **267**, 2119-2125.

Cole, S.H., Carney, G.E., McClung, C.A., Willard, S.S., Taylor, B.J. & Hirsh, J. (2005) Two functional but noncomplementing *Drosophila* tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. *J Biol Chem*, **280**, 14948-14955.

Comas, D., Petit, F. & Preat, T. (2004) *Drosophila* long-term memory formation involves regulation of cathepsin activity. *Nature*, **430**, 460-463.

Connolly, J.B., Roberts, I.J., Armstrong, J.D., Kaiser, K., Forte, M., Tully, T. & O'Kane, C.J. (1996) Associative learning disrupted by impaired Gs signaling in *Drosophila* mushroom bodies. *Science*, **274**, 2104-2107.

Corfas, G. & Dudai, Y. (1989) Habituation and dishabituation of a cleaning reflex in normal and mutant *Drosophila*. *J Neurosci*, **9**, 56-62.

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

Dahanukar, A., Foster, K., van der Goes van Naters, W.M. & Carlson, J.R. (2001) A Gr receptor is required for response to the sugar trehalose in taste neurons of *Drosophila*. *Nat Neurosci*, **4**, 1182-1186.

Dahanukar, A., Hallem, E.A. & Carlson, J.R. (2005) Insect chemoreception. *Curr Opin Neurobiol*, **15**, 423-430.

Daly, K.C., Christensen, T.A., Lei, H., Smith, B.H. & Hildebrand, J.G. (2004) Learning modulates the ensemble representations for odors in primary olfactory networks. *Proc Natl Acad Sci U S A*, **101**, 10476-10481.

Davis, R.L. (1993) Mushroom bodies and *Drosophila* learning. *Neuron*, **11**, 1-14.

Davis, R.L. (1996) Physiology and biochemistry of *Drosophila* learning mutants. *Physiol Rev*, **76**, 299-317.

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

de Belle, J.S. & Heisenberg, M. (1994) Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science*, **263**, 692-695.

de Bruyne, M. & Warr, C.G. (2006) Molecular and cellular organization of insect chemosensory neurons. *Bioessays*, **28**, 23-34.

Dethier, V.G. & Goldrich-Rachman, N. (1976) Anesthetic stimulation of insect water receptors. *Proc Natl Acad Sci U S A*, **73**, 3315-3319.

DeZazzo, J., Sandstrom, D., de Belle, S., Velinzon, K., Smith, P., Grady, L., DelVecchio, M., Ramaswami, M. & Tully, T. (2000) nalyot, a mutation of the *Drosophila* myb-related Adf1 transcription factor, disrupts synapse formation and olfactory memory. *Neuron*, **27**, 145-158.

Di, Y., Li, J., Fang, J., Xu, Z., He, X., Zhang, F., Ling, J., Li, X., Xu, D., Li, L., Li, Y.Y. & Huo, K. (2003) Cloning and characterization of a novel gene which encodes a protein interacting with the mitosis-associated kinase-like protein NTKL. *J Hum Genet*, **48**, 315-321.

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

Drier, E.A., Tello, M.K., Cowan, M., Wu, P., Blace, N., Sacktor, T.C. & Yin, J.C. (2002) Memory enhancement and formation by atypical PKM activity in *Drosophila melanogaster*. *Nat Neurosci*, **5**, 316-324.

Driscoll, M. & Gerstbrein, B. (2003) Dying for a cause: invertebrate genetics takes on human neurodegeneration. *Nat Rev Genet*, **4**, 181-194.

Dubnau, J., Chiang, A.S., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Buldoc, F., Scott, R., Certa, U., Broger, C. & Tully, T. (2003) The staufen/pumilio pathway is involved in *Drosophila* long-term memory. *Curr Biol*, **13**, 286-296.

Dubnau, J., Grady, L., Kitamoto, T. & Tully, T. (2001) Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. *Nature*, **411**, 476-480.

Dubnau, J. & Tully, T. (2001) Functional anatomy: from molecule to memory. *Curr Biol*, **11**, R240-243.

Dudai, Y. (1988) Neurogenetic dissection of learning and short-term memory in *Drosophila*. *Annu Rev Neurosci*, **11**, 537-563.

Dudai, Y. (1989) *The neurobiology of memory. Concepts, findings, trends.* Oxford University Press, Oxford.

Dudai, Y. (2002) Molecular bases of long-term memories: a question of persistence. *Curr Opin Neurobiol*, **12**, 211-216.

Dudai, Y., Buxbaum, J., Corfas, G., Orgad, S., Segal, D., Sher, B., Uzzan, A. & Zvi, S. (1986) Defective cAMP metabolism and defective memory in *Drosophila*. *Acta Biochim Biophys Hung*, **21**, 177-192.

Dudai, Y., Corfas, G. & 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**, 101-109.

Dudai, Y., Jan, Y.N., Byers, D., Quinn, W.G. & Benzer, S. (1976) dunce, a mutant of *Drosophila* deficient in learning. *Proc Natl Acad Sci U S A*, **73**, 1684-1688.

Duffy, J.B. (2002) GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *Genesis*, **34**, 1-15.

Dunipace, L., Meister, S., McNealy, C. & Amrein, H. (2001) Spatially restricted expression of candidate taste receptors in the *Drosophila* gustatory system. *Curr Biol*, **11**, 822-835.

Dura, J.M., Preat, T. & Tully, T. (1993) Identification of linotte, a new gene affecting learning and memory in *Drosophila melanogaster*. *J Neurogenet*, **9**, 1-14.

Edgecomb, R.S. & Murdock, L.L. (1992) Central projections of axons from taste hairs on the labellum and tarsi of the blowfly, Phormia regina Meigen. *J Comp Neurol*, **315**, 431-444.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494-498.

Elbashir, S.M., Lendeckel, W. & Tuschl, T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*, **15**, 188-200.

Enerly, E., Larsson, J. & Lambertsson, A. (2003) Silencing the *Drosophila* ribosomal protein L14 gene using targeted RNA interference causes distinct somatic anomalies. *Gene*, **320**, 41-48.

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.

Faber, T., Joerges, J. & Menzel, R. (1999) Associative learning modifies neural representations of odors in the insect brain. *Nat Neurosci*, **2**, 74-78.

Faber, T. & Menzel, R. (2001) Visualizing mushroom body response to a conditioned odor in honeybees. *Naturwissenschaften*, **88**, 472-476.

Falk, D.R. (1976) Pyrimidine auxotrophy and the complementation map of the rudimentary locus of *Drosophila melanogaster*. *Mol Gen Genet*, **148**, 1-8.

Farooqui, T., Robinson, K., Vaessin, H. & Smith, B.H. (2003) Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. *J Neurosci*, **23**, 5370-5380.

Feany, M.B. & Quinn, W.G. (1995) A neuropeptide gene defined by the *Drosophila* memory mutant amnesiac. *Science*, **268**, 869-873.

Folkers, E., Drain, P. & Quinn, W.G. (1993) Radish, a *Drosophila* mutant deficient in consolidated memory. *Proc Natl Acad Sci U S A*, **90**, 8123-8127.

Freeman, M. (1996) Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell*, **87**, 651-660.

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J. & Birman, S. (2003) Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J Neurobiol*, **54**, 618-627.

Frings, H. (1941) An experiment on olfactory conditioning in *Drosophila melanogaster*. *Journal of Experimental Zoology*, **88**, 65-93.

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

Ge, X., Hannan, F., Xie, Z., Feng, C., Tully, T., Zhou, H. & Zhong, Y. (2004) Notch signaling in *Drosophila* long-term memory formation. *Proc Natl Acad Sci U S A*, **101**, 10172-10176.

Gerber, B., Tanimoto, H. & Heisenberg, M. (2004) An engram found? Evaluating the evidence from fruit flies. *Curr Opin Neurobiol*, **14**, 737-744.

Giniger, E., Varnum, S. & Ptashne, M. (1985) Specific DNA binding of GAL4, a positive regulatory protein of yeast. *Cell*, **40**.

Giordano, E., Rendina, R., Peluso, I. & Furia, M. (2002) RNAi triggered by symmetrically transcribed transgenes in *Drosophila melanogaster*. *Genetics*, **160**, 637-648.

Godenschwege, T.A., Reisch, D., Diegelmann, S., Eberle, K., Funk, N., Heisenberg, M., Hoppe, V., Hoppe, J., Klagges, B.R., Martin, J.R., Nikitina, E.A., Putz, G., Reifegerste, R., Reisch, N., Rister, J., Schaupp, M., Scholz, H., Schwarzel, M., Werner, U., Zars, T.D., Buchner, S. & Buchner, E. (2004) Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. *Eur J Neurosci*, **20**, 611-622.

Goodwin, S.F., Del Vecchio, M., Velinzon, K., Hogel, C., Russell, S.R., Tully, T. & Kaiser, K. (1997) Defective learning in mutants of the *Drosophila* gene for a regulatory subunit of cAMP-dependent protein kinase. *J Neurosci*, **17**, 8817-8827.

Goto, A., Blandin, S., Royet, J., Reichhart, J.M. & Levashina, E.A. (2003) Silencing of Toll pathway components by direct injection of double-stranded RNA into *Drosophila* adult flies. *Nucleic Acids Res*, **31**, 6619-6623.

Grotewiel, M.S., Beck, C.D., Wu, K.H., Zhu, X.R. & Davis, R.L. (1998) Integrin-mediated short-term memory in *Drosophila*. *Nature*, **391**, 455-460.

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

Guo, H.F., The, I., Hannan, F., Bernards, A. & Zhong, Y. (1997) Requirement of *Drosophila* NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. *Science*, **276**, 795-798.

Guo, H.F., Tong, J., Hannan, F., Luo, L. & Zhong, Y. (2000) A neurofibromatosis-1-regulated pathway is required for learning in *Drosophila*. *Nature*, **403**, 895-898.

Halfon, M.S., Kose, H., Chiba, A. & Keshishian, H. (1997) Targeted gene expression without a tissue-specific promoter: creating mosaic embryos using laser-induced single-cell heat shock. *Proc Natl Acad Sci U S A*, **94**, 6255-6260.

Hall, J.F. (1986) The conditional emotional response as a model of Pavlovian conditioning. *Pavlov J Biol Sci*, **21**, 1-11.

Hallem, E.A. & Carlson, J.R. (2004) The odor coding system of *Drosophila*. *Trends Genet*, **20**, 453-459.

Hallem, E.A., Dahanukar, A. & Carlson, J.R. (2005) Insect Odor and Taste Receptors. *Annu Rev Entomol*.

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

Hammer, M. (1993) An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature*, **366**, 59-63.

Hammer, M. (1997) The neural basis of associative reward learning in honeybees. *Trends Neurosci*, **20**, 245-252.

Hammer, M. & Menzel, R. (1995) Learning and memory in the honeybee. *J Neurosci*, **15**, 1617-1630.

Hammer, M. & Menzel, R. (1998) Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn Mem*, **5**, 146-156.

Hammond, S.M. (2005) Dicing and slicing: the core machinery of the RNA interference pathway. *FEBS Lett*, **579**, 5822-5829.

Han, D.D., Stein, D. & Stevens, L.M. (2000) Investigating the function of follicular subpopulations during *Drosophila* oogenesis through hormone-dependent enhancer-targeted cell ablation. *Development*, **127**, 573-583.

Han, K.A., Millar, N.S. & Davis, R.L. (1998) A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J Neurosci*, **18**, 3650-3658.

Han, K.A., Millar, N.S., Grotewiel, M.S. & Davis, R.L. (1996) DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron*, **16**, 1127-1135.

Han, V.K. & Carter, A.M. (2000) Spatial and temporal patterns of expression of messenger RNA for insulin-like growth factors and their binding proteins in the placenta of man and laboratory animals. *Placenta*, **21**, 289-305.

Hay, B.A., Huh, J.R. & Guo, M. (2004) The genetics of cell death: approaches, insights and opportunities in *Drosophila*. *Nat Rev Genet*, **5**, 911-922.

Hayashi, S., Ito, K., Sado, Y., Taniguchi, M., Akimoto, A., Takeuchi, H., Aigaki, T., Matsuzaki, F., Nakagoshi, H., Tanimura, T., Ueda, R., Uemura, T., Yoshihara, M. & Goto, S. (2002) GETDB, a database compiling expression patterns and molecular locations of a collection of Gal4 enhancer traps. *Genesis*, **34**, 58-61.

Hearn, M.G., Ren, Y., McBride, E.W., Reveillaud, I., Beinborn, M. & Kopin, A.S. (2002) A *Drosophila* dopamine 2-like receptor: Molecular characterization and identification of multiple alternatively spliced variants. *Proc Natl Acad Sci U S A*, **99**, 14554-14559.

Heimbeck, G., Bugnon, V., Gendre, N., Haberlin, C. & Stocker, R.F. (1999) Smell and taste perception in *Drosophila melanogaster* larva: toxin expression studies in chemosensory neurons. *J Neurosci*, **19**, 6599-6609.

Heimbeck, G., Bugnon, V., Gendre, N., Keller, A. & Stocker, R.F. (2001) A central neural circuit for experience-independent olfactory and courtship behavior in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*, **98**, 15336-15341.

Heisenberg, M. (2003) Mushroom body memoir: from maps to models. *Nat Rev Neurosci*, **4**, 266-275.

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

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

Hirsch, J. (1959) Studies in experimental behavior genetics: II. Individual differences in geotaxis as a function of chromosome vaiations in synthesized *Drosophila* populations. *Journal of Comparative and Physiological Psychology*, **52**, 304-308.

Hitier, R., Heisenberg, M. & Preat, T. (1998) Abnormal mushroom body plasticity in the *Drosophila* memory mutant amnesiac. *Neuroreport*, **9**, 2717-2719.

Holland, P.C. (1993) Cognitive aspects of classical conditioning. *Curr Opin Neurobiol*, **3**, 230-236.

Holland, P.C., Straub, J.J. (1979) Differential effects of two ways of devaluating the unconditioned stimulus after Pavlovian appetitive conditioning. *Journal of Experimental Psychology*, 65-78.

Inoshita, T. & Tanimura, T. (2006) Cellular identification of water gustatory receptor neurons and their central projection pattern in Drosophila. *Proc Natl Acad Sci U S A*, **103**, 1094-1099.

Iourgenko, V., Kliot, B., Cann, M.J. & Levin, L.R. (1997) Cloning and characterization of a *Drosophila* adenylyl cyclase homologous to mammalian type IX. *FEBS Lett*, **413**, 104-108.

Iourgenko, V. & Levin, L.R. (2000) A calcium-inhibited *Drosophila* adenylyl cyclase. *Biochim Biophys Acta*, **1495**, 125-139.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y. & Yamamoto, D. (1997) The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development*, **124**, 761-771.

Ito, K., Okada, R., Tanaka, N.K. & Awasaki, T. (2003) Cautionary observations on preparing and interpreting brain images using molecular biology-based staining techniques. *Microsc Res Tech*, **62**, 170-186.

Ito, K., Suzuki, K., Estes, P., Ramaswami, M., Yamamoto, D. & 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. & Luo, L. (2001) Target neuron prespecification in the olfactory map of Drosophila. *Nature*, **414**, 204-208.

Johns, D.C., Marx, R., Mains, R.E., O'Rourke, B. & Marban, E. (1999) Inducible genetic suppression of neuronal excitability. *J Neurosci*, **19**, 1691-1697.

Kalidas, S. & Smith, D.P. (2002) Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. *Neuron*, **33**, 177-184.

Kandel, E. & Abel, T. (1995) Neuropeptides, adenylyl cyclase, and memory storage. *Science*, **268**, 825-826.

Kandel, E.R. (2001) The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, **294**, 1030-1038.

Keene, A.C., Stratmann, M., Keller, A., Perrat, P.N., Vosshall, L.B. & Waddell, S. (2004) Diverse odor-conditioned memories require uniquely timed dorsal paired medial neuron output. *Neuron*, **44**, 521-533.

Keller, A., Sweeney, S.T., Zars, T., O'Kane, C.J. & Heisenberg, M. (2002) Targeted expression of tetanus neurotoxin interferes with behavioral responses to sensory input in Drosophila. *J Neurobiol*, **50**, 221-233.

Kent, K.S., Hoskins, S.G. & Hildebrand, J.G. (1987) A novel serotonin-immunoreactive neuron in the antennal lobe of the sphinx moth Manduca sexta persists throughout postembryonic life. *J Neurobiol*, **18**, 451-465.

Kidokoro, Y. (2003) Roles of SNARE proteins and synaptotagmin I in synaptic transmission: studies at the *Drosophila* neuromuscular synapse. *Neurosignals*, **12**, 13-30.

Kim, Y.C., Lee, H.G., Seong, C.S. & Han, K.A. (2003) Expression of a D1 dopamine receptor dDA1/DmDOP1 in the central nervous system of *Drosophila melanogaster*. *Gene Expr Patterns*, **3**, 237-245.

Kimmel, H.D. (1977) Notes from "Pavlov's Wednesdays": sensory preconditioning. *Am J Psychol*, **90**, 319-321.

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) Targeted expression of temperature-sensitive dynamin to study neural mechanisms of complex behavior in Drosophila. *J Neurogenet*, **16**, 205-228.

Koenig, J.H. & Ikeda, K. (1983) Evidence for a presynaptic blockage of transmission in a temperature-sensitive mutant of Drosophila. *J Neurobiol*, **14**, 411-419.

Koenig, J.H. & Ikeda, K. (1989) Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. *J Neurosci*, **9**, 3844-3860.

Koenig, J.H., Saito, K. & Ikeda, K. (1983) Reversible control of synaptic transmission in a single gene mutant of *Drosophila melanogaster*. *J Cell Biol*, **96**, 1517-1522.

Kohler, R.E. (1994) *Lords of the fly. Drosophila genetics and the experimental life.* The University of Chicago Press, Chicago.

Kosaka, T. & Ikeda, K. (1983) Possible temperature-dependent blockage of synaptic vesicle recycling induced by a single gene mutation in Drosophila. *J Neurobiol*, **14**, 207-225.

Kosaka, T. & Ikeda, K. (1983) Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of *Drosophila melanogaster*, shibirets1. *J Cell Biol*, **97**, 499-507.

Laissue, P.P., Reiter, C., Hiesinger, P.R., Halter, S., Fischbach, K.F. & Stocker, R.F. (1999) Three-dimensional reconstruction of the antennal lobe in *Drosophila melanogaster*. *J Comp Neurol*, **405**, 543-552.

Laughon, A., Driscoll, R., Wills, N. & Gesteland, R.F. (1984) Identification of two proteins encoded by the Saccharomyces cerevisiae GAL4 gene. *Mol Cell Biol*, **4**, 268-275.

Laughon, A. & Gesteland, R.F. (1984) Primary structure of the Saccharomyces cerevisiae GAL4 gene. *Mol Cell Biol*, **4**, 260-267.

Le Bourg, E. (2005) Humidity as an aversive stimulus in learning in *Drosophila melanogaster*. *Learn Behav*, **33**, 265-276.

Lechner, H.A. & Byrne, J.H. (1998) New perspectives on classical conditioning: a synthesis of Hebbian and non-Hebbian mechanisms. *Neuron*, **20**, 355-358.

Lee, H.G., Seong, C.S., Kim, Y.C., Davis, R.L. & Han, K.A. (2003) Octopamine receptor OAMB is required for ovulation in *Drosophila melanogaster*. *Dev Biol*, **264**, 179-190.

Lee, T., Lee, A. & Luo, L. (1999) Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development*, **126**, 4065-4076.

Lee, T. & Luo, L. (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron*, **22**, 451-461.

Lee, Y.S. & Carthew, R.W. (2003) Making a better RNAi vector for Drosophila: use of intron spacers. *Methods*, **30**, 322-329.

Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J. & Carthew, R.W. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, **117**, 69-81.

Levin, L.R., Han, P.L., Hwang, P.M., Feinstein, P.G., Davis, R.L. & Reed, R.R. (1992) The *Drosophila* learning and memory gene rutabaga encodes a Ca2+/Calmodulin-responsive adenylyl cyclase. *Cell*, **68**, 479-489.

Lin, D.M. & Goodman, C.S. (1994) Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron*, **13**, 507-523.

Lindemann, B. (1996) Taste reception. *Physiol Rev*, **76**, 718-766.

Liscia, A., Masala, C., Crnjar, R., Sollai, G. & Solari, P. (2004) Saccharin stimulates the "deterrent" cell in the blowfly: behavioral and electrophysiological evidence. *Physiol Behav*, **80**, 637-646.

Liscia, A. & Solari, P. (2000) Bitter taste recognition in the blowfly: Electrophysiological and behavioral evidence. *Physiol Behav*, **70**, 61-65.

Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., Heisenberg, M. & Liu, L. (2006) Distinct memory traces for two visual features in the *Drosophila* brain. *Nature*, **439**, 551-556.

Liu, L., Johnson, W.A. & Welsh, M.J. (2003) *Drosophila* DEG/ENaC pickpocket genes are expressed in the tracheal system, where they may be involved in liquid clearance. *Proc Natl Acad Sci U S A*, **100**, 2128-2133.

Liu, L., Leonard, A.S., Motto, D.G., Feller, M.A., Price, M.P., Johnson, W.A. & Welsh, M.J. (2003) Contribution of *Drosophila* DEG/ENaC genes to salt taste. *Neuron*, **39**, 133-146.

Liu, L., Wolf, R., Ernst, R. & Heisenberg, M. (1999) Context generalization in *Drosophila* visual learning requires the mushroom bodies. *Nature*, **400**, 753-756.

Livingstone, M.S., Sziber, P.P. & Quinn, W.G. (1984) Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a *Drosophila* learning mutant. *Cell*, **37**, 205-215.

Lonze, B.E. & Ginty, D.D. (2002) Function and regulation of CREB family transcription factors in the nervous system. *Neuron*, **35**, 605-623.

Lubow, R.E., Moore, A.U. (1959) Latent inhibition: The effect of nonreinforced pre-exposure to the conditional stimulus. *Journal of Comparative and Physiological Psychology*, 415-419.

Mao, Z., Roman, G., Zong, L. & Davis, R.L. (2004) Pharmacogenetic rescue in time and space of the rutabaga memory impairment by using Gene-Switch. *Proc Natl Acad Sci U S A*, **101**, 198-203.

Maqueira, B., Chatwin, H. & Evans, P.D. (2005) Identification and characterization of a novel family of *Drosophila* beta-adrenergic-like octopamine G-protein coupled receptors. *J Neurochem*, **94**, 547-560.

Margulies, C., Tully, T. & Dubnau, J. (2005) Deconstructing memory in Drosophila. *Curr Biol*, **15**, R700-713.

Marin, E.C., Jefferis, G.S., Komiyama, T., Zhu, H. & Luo, L. (2002) Representation of the glomerular olfactory map in the *Drosophila* brain. *Cell*, **109**, 243-255.

Martin, J.R., Keller, A. & Sweeney, S.T. (2002) Targeted expression of tetanus toxin: a new tool to study the neurobiology of behavior. *Adv Genet*, **47**, 1-47.

Martin, K.C., Casadio, A., Zhu, H., Yaping, E., Rose, J.C., Chen, M., Bailey, C.H. & Kandel, E.R. (1997) Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage. *Cell*, **91**, 927-938.

Masek, P. (2005) Odor Intensity Learning in Drosophila. *PhD thesis, Universität Würzburg*.

Matsumoto, K., Adachi, Y., Toh-e, A. & Oshima, Y. (1980) Function of positive regulatory gene gal4 in the synthesis of galactose pathway enzymes in Saccharomyces cerevisiae: evidence that the GAL81 region codes for part of the gal4 protein. *J Bacteriol*, **141**, 508-527.

Matsumoto, K., Toh-e, A. & Oshima, Y. (1981) Isolation and characterization of dominant mutations resistant to carbon catabolite repression of galactokinase synthesis in Saccharomyces cerevisiae. *Mol Cell Biol*, **1**, 83-93.

McGuire, S., Deshazer, M. & Davis, R. (2005) Thirty years of olfactory learning and memory research in *Drosophila melanogaster*. *Progress in Neurobiology*.

McGuire, S.E., Le, P.T. & Davis, R.L. (2001) The role of *Drosophila* mushroom body signaling in olfactory memory. *Science*, **293**, 1330-1333.

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

McGuire, S.E., Mao, Z. & Davis, R.L. (2004) Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. *Sci STKE*, **2004**, pl6.

McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., Riddiford, L.M. & Truman, J.W. (1997) Disruption of a behavioral sequence by targeted death of peptidergic neurons in Drosophila. *Neuron*, **19**, 813-823.

Melcher, C. & Pankratz, M.J. (2005) Candidate gustatory interneurons modulating feeding behavior in the *Drosophila* brain. *PLoS Biol*, **3**, e305.

Menzel, R. (1979) Behavioural access to short-term memory in bees. *Nature*, **281**, 368-369.

Menzel, R. (1983) Neurobiology of learning and memory: the honeybee as a model system. *Naturwissenschaften*, **70**, 504-511.

Menzel, R. (2001) Searching for the memory trace in a mini-brain, the honeybee. *Learn Mem*, **8**, 53-62.

Menzel, R. & Giurfa, M. (2001) Cognitive architecture of a mini-brain: the honeybee. *Trends Cogn Sci*, **5**, 62-71.

Menzel, R. & Muller, U. (2001) Neurobiology. Learning from a fly's memory. *Nature*, **411**, 433-434.

Mery, F. & Kawecki, T.J. (2005) A cost of long-term memory in Drosophila. *Science*, **308**, 1148.

Miller, J.S., Jagielo, J.A., and Spear, N.E. (1993) The influence of retention interval on the US preexposure effect: changes in the contextual blocking over time. *Learning and Motivation*, 376-394.

Mobbs, P.G. (1982) The brain of the honeybee Apis mellifera. I. The connections and spatial organization of the mushroom bodies. *Phil. Trans. R. Soc. Lond. B*, **298**, 309–354.

Mobbs, P.G. (1984) Neural networks in the mushroom bodies of the honeybee. *J. Insect Physiol*, **30**, 43–58.

Monastirioti, M. (1999) Biogenic amine systems in the fruit fly *Drosophila melanogaster*. *Microsc Res Tech*, **45**, 106-121.

Monastirioti, M. (2003) Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in Drosophila *melanogaster*. *Dev Biol*, **264**, 38-49.

Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K. & Budnik, V. (1995) Octopamine immunoreactivity in the fruit fly *Drosophila melanogaster*. *J Comp Neurol*, **356**, 275-287.

Monastirioti, M., Linn, C.E., Jr. & White, K. (1996) Characterization of *Drosophila* tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. *J Neurosci*, **16**, 3900-3911.

Murphy, R. (1967) Instrumental conditioning of the fruit fly, *Drosophila melanogaster*. *Animal Behaviour*, **15**, 153-161.

Nagel, J.H., Gultyaev, A.P., Oistamo, K.J., Gerdes, K. & Pleij, C.W. (2002) A pH-jump approach for investigating secondary structure refolding kinetics in RNA. *Nucleic Acids Res*, **30**, e63.

Nellen, D., Burke, R., Struhl, G. & Basler, K. (1996) Direct and long-range action of a DPP morphogen gradient. *Cell*, **85**, 357-368.

Nitabach, M.N., Llamas, D.A., Araneda, R.C., Intile, J.L., Thompson, I.J., Zhou, Y.I. & Holmes, T.C. (2001) A mechanism for combinatorial regulation of electrical activity: Potassium channel subunits capable of functioning as Src homology 3-dependent adaptors. *Proc Natl Acad Sci U S A*, **98**, 705-710.

O'Kane, C.J. & Gehring, W.J. (1987) Detection in situ of genomic regulatory elements in Drosophila. *Proc Natl Acad Sci U S A*, **84**, 9123-9127.

Osterwalder, T., Yoon, K.S., White, B.H. & Keshishian, H. (2001) A conditional tissue-specific transgene expression system using inducible GAL4. *Proc Natl Acad Sci U S A*, **98**, 12596-12601.

Park, D., Han, M., Kim, Y.C., Han, K.A. & Taghert, P.H. (2004) Ap-let neurons--a peptidergic circuit potentially controlling ecdysial behavior in Drosophila. *Dev Biol*, **269**, 95-108.

Park, S.K., Shanbhag, S.R., Wang, Q., Hasan, G., Steinbrecht, R.A. & Pikielny, C.W. (2000) Expression patterns of two putative odorant-binding proteins in the olfactory organs of *Drosophila melanogaster* have different implications for their functions. *Cell Tissue Res*, **300**, 181-192.

Pascual, A., Huang, K.L. & Preat, T. (2005) Conditional UAS-targeted repression in Drosophila. *Nucleic Acids Res*, **33**, e7.

Pascual, A. & Preat, T. (2001) Localization of long-term memory within the *Drosophila* mushroom body. *Science*, **294**, 1115-1117.

Pavlov, I.P. (1906) The scientific investigation of the physical faculties or processes in the higher animals. *Science*, **24**, 613-619.

Pavlov, I.P. (1927) *Conditioned reflexes. An investigation of the physiological activity of the cerebral cortex*. Oxford University Press, London.

Pearce, J.M. & Bouton, M.E. (2001) Theories of associative learning in animals. *Annu Rev Psychol*, **52**, 111-139.

Perazzona, B., Isabel, G., Preat, T. & Davis, R.L. (2004) The role of cAMP response element-binding protein in *Drosophila* long-term memory. *J Neurosci*, **24**, 8823-8828.

Perez-Orive, J., Mazor, O., Turner, G.C., Cassenaer, S., Wilson, R.I. & Laurent, G. (2002) Oscillations and sparsening of odor representations in the mushroom body. *Science*, **297**, 359-365.

Pham, J.W. & Sontheimer, E.J. (2004) The Making of an siRNA. *Mol Cell*, **15**, 163-164.

Phelan, P. & Starich, T.A. (2001) Innexins get into the gap. *Bioessays*, **23**, 388-396.

Phelps, C.B. & Brand, A.H. (1998) Ectopic gene expression in *Drosophila* using GAL4 system. *Methods*, **14**, 367-379.

Piccin, A., Salameh, A., Benna, C., Sandrelli, F., Mazzotta, G., Zordan, M., Rosato, E., Kyriacou, C.P. & Costa, R. (2001) Efficient and heritable functional knock-out of an adult phenotype in *Drosophila* using a GAL4-driven hairpin RNA incorporating a heterologous spacer. *Nucleic Acids Res*, **29**, E55-55.

Pignoni, F. & Zipursky, S.L. (1997) Induction of *Drosophila* eye development by decapentaplegic. *Development*, **124**, 271-278.

Presente, A., Boyles, R.S., Serway, C.N., de Belle, J.S. & Andres, A.J. (2004) Notch is required for long-term memory in Drosophila. *Proc Natl Acad Sci U S A*, **101**, 1764-1768.

Putz, G., Bertolucci, F., Raabe, T., Zars, T. & Heisenberg, M. (2004) The S6KII (rsk) gene of *Drosophila melanogaster* differentially affects an operant and a classical learning task. *J Neurosci*, **24**, 9745-9751.

Quan, F., Thomas, L. & Forte, M. (1991) *Drosophila* stimulatory G protein alpha subunit activates mammalian adenylyl cyclase but interacts poorly with mammalian receptors: implications for receptor-G protein interaction. *Proc Natl Acad Sci U S A*, **88**, 1898-1902.

Quinn, W.G. & Dudai, Y. (1976) Memory phases in Drosophila. *Nature*, **262**, 576-577.

Quinn, W.G. & Greenspan, R.J. (1984) Learning and courtship in Drosophila: two stories with mutants. *Annu Rev Neurosci*, **7**, 67-93.

Quinn, W.G., Harris, W.A. & Benzer, S. (1974) Conditioned behavior in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*, **71**, 708-712.

Quinn, W.G., Sziber, P.P. & Booker, R. (1979) The *Drosophila* memory mutant amnesiac. *Nature*, **277**, 212-214.

Ramaekers, A., Magnenat, E., Marin, E.C., Gendre, N., Jefferis, G.S., Luo, L. & Stocker, R.F. (2005) Glomerular maps without cellular redundancy at successive levels of the *Drosophila* larval olfactory circuit. *Curr Biol*, **15**, 982-992.

Renn, S.C., Armstrong, J.D., Yang, M., Wang, Z., An, X., Kaiser, K. & Taghert, P.H. (1999) Genetic analysis of the *Drosophila* ellipsoid body neuropil: organization and development of the central complex. *J Neurobiol*, **41**, 189-207.

Rescorla, R.A. (1973) Effect of US habituation following conditioning. *J Comp Physiol Psychol*, **82**, 137-143.

Rescorla, R.A. (1980) *Pavlovian second-order conditioning: studies in associative learning*. Lawrence Erlbaum Associates, Hillsdale, NJ.

Riemensperger, T., Voller, T., Stock, P., Buchner, E. & Fiala, A. (2005) Punishment prediction by dopaminergic neurons in Drosophila. *Curr Biol*, **15**, 1953-1960.

Rodrigues, V. & Siddiqi, O. (1981) A gustatory mutant of *Drosophila* defective in pyranose receptors. *Mol Gen Genet*, **181**, 406-408.

Roeder, T. (2005) Tyramine and octopamine: ruling behavior and metabolism. *Annu Rev Entomol*, **50**, 447-477.

Roman, G. (2004) The genetics of *Drosophila* transgenics. *Bioessays*, **26**, 1243-1253.

Roman, G., Endo, K., Zong, L. & Davis, R.L. (2001) P[Switch], a system for spatial and temporal control of gene expression in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*, **98**, 12602-12607.

Rybak, J. & Menzel, R. (1998) Integrative properties of the Pe1 neuron, a unique mushroom body output neuron. *Learn Mem*, **5**, 133-145.

Sacktor, T.C., Osten, P., Valsamis, H., Jiang, X., Naik, M.U. & Sublette, E. (1993) Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation. *Proc Natl Acad Sci U S A*, **90**, 8342-8346.

Scherer, S., Stocker, R.F. & Gerber, B. (2003) Olfactory learning in individually assayed *Drosophila* larvae. *Learn Mem*, **10**, 217-225.

Schmid, A., Schindelholz, B. & Zinn, K. (2002) Combinatorial RNAi: a method for evaluating the functions of gene families in Drosophila. *Trends Neurosci*, **25**, 71-74.

Schwaerzel, M., Heisenberg, M. & Zars, T. (2002) Extinction antagonizes olfactory memory at the subcellular level. *Neuron*, **35**, 951-960.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. & Heisenberg, M. (2003) Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. *J Neurosci*, **23**, 10495-10502.

Scott, K., Brady, R., Jr., Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C. & Axel, R. (2001) A chemosensory gene family encoding candidate gustatory and olfactory receptors in Drosophila. *Cell*, **104**, 661-673.

Sen, G.L. & Blau, H.M. (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol*, **7**, 633-636.

Shanbhag, S.R., Hekmat-Scafe, D., Kim, M.S., Park, S.K., Carlson, J.R., Pikielny, C., Smith, D.P. & Steinbrecht, R.A. (2001) Expression mosaic of odorant-binding proteins in *Drosophila* olfactory organs. *Microsc Res Tech*, **55**, 297-306.

Shanbhag, S.R., Park, S.K., Pikielny, C.W. & Steinbrecht, R.A. (2001) Gustatory organs of *Drosophila melanogaster*: fine structure and expression of the putative odorant-binding protein PBPRP2. *Cell Tissue Res*, **304**, 423-437.

Shanbhag, S.R., Singh, K. & Singh, R.N. (1995) Fine structure and primary sensory projections of sensilla located in the sacculus of the antenna of *Drosophila melanogaster*. *Cell Tissue Res*, **282**, 237-249.

Shields, V.D. & Hildebrand, J.G. (2001) Recent advances in insect olfaction, specifically regarding the morphology and sensory physiology of antennal sensilla of the female sphinx moth Manduca sexta. *Microsc Res Tech*, **55**, 307-329.

Shimada, I. & Tanimura, T. (1981) Stereospecificity of multiple receptor sites in a labellar sugar receptor of the fleshfly for amino acids and small peptides. *J Gen Physiol*, **77**, 23-39.

Simon, A.F., Boquet, I., Synguelakis, M. & Preat, T. (1998) The *Drosophila* putative kinase linotte (derailed) prevents central brain axons from converging on a newly described interhemispheric ring. *Mech Dev*, **76**, 45-55.

Simon, M.A., Bowtell, D.D., Dodson, G.S., Laverty, T.R. & Rubin, G.M. (1991) Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell*, **67**, 701-716.

Sinakevitch, I., Niwa, M. & Strausfeld, N.J. (2005) Octopamine-like immunoreactivity in the honey bee and cockroach: comparable organization in the brain and subesophageal ganglion. *J Comp Neurol*, **488**, 233-254.

Sinakevitch, I. & Strausfeld, N.J. (2006) Comparison of octopamine-like immunoreactivity in the brains of the fruit fly and blow fly. *J Comp Neurol*, **494**, 460-475.

Skoulakis, E.M. & Davis, R.L. (1996) Olfactory learning deficits in mutants for leonardo, a *Drosophila* gene encoding a 14-3-3 protein. *Neuron*, **17**, 931-944.

Sokolowski, M.B. (2001) Drosophila: genetics meets behaviour. *Nat Rev Genet*, **2**, 879-890.

Stebbins, M.J., Urlinger, S., Byrne, G., Bello, B., Hillen, W. & Yin, J.C. (2001) Tetracycline-inducible systems for Drosophila. *Proc Natl Acad Sci U S A*, **98**, 10775-10780.

Stebbins, M.J. & Yin, J.C. (2001) Adaptable doxycycline-regulated gene expression systems for Drosophila. *Gene*, **270**, 103-111.

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

Stensmyr, M.C., Giordano, E., Balloi, A., Angioy, A.M. & Hansson, B.S. (2003) Novel natural ligands for *Drosophila* olfactory receptor neurones. *J Exp Biol*, **206**, 715-724.

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. & de Belle, J.S. (1997) Neuroblast ablation in *Drosophila* P[GAL4] lines reveals origins of olfactory interneurons. *J Neurobiol*, **32**, 443-456.

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

Strausfeld, N.J. (2002) Organization of the honey bee mushroom body: representation of the calyx within the vertical and gamma lobes. *J Comp Neurol*, **450**, 4-33.

Strausfeld, N.J. & Hildebrand, J.G. (1999) Olfactory systems: common design, uncommon origins? *Curr Opin Neurobiol*, **9**, 634-639.

Strausfeld, N.J., Homberg, U. & Kloppenburg, P. (2000) Parallel organization in honey bee mushroom bodies by peptidergic Kenyon cells. *J Comp Neurol*, **424**, 179-195.

Strausfeld, N.J. & Li, Y. (1999) Organization of olfactory and multimodal afferent neurons supplying the calyx and pedunculus of the cockroach mushroom bodies. *J Comp Neurol*, **409**, 603-625.

Strausfeld, N.J. & Li, Y. (1999) Representation of the calyces in the medial and vertical lobes of cockroach mushroom bodies. *J Comp Neurol*, **409**, 626-646.

Strausfeld, N.J., Sinakevitch, I. & 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**, 151-169.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H. & O'Kane, C.J. (1995) Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron*, **14**, 341-351.

Syntichaki, P., Xu, K., Driscoll, M. & Tavernarakis, N. (2002) Specific aspartyl and calpain proteases are required for neurodegeneration in C. elegans. *Nature*, **419**, 939-944.

Tanaka, N.K., Awasaki, T., Shimada, T. & Ito, K. (2004) Integration of chemosensory pathways in the *Drosophila* second-order olfactory centers. *Curr Biol*, **14**, 449-457.

Tanimura, T., Isono, K., Takamura, T. & Shimada, I. (1982) Genetic dimorphism in the taste sensitivity to trehalose in *Drosophila melanogaster*. *Journal of Comparative Physiology.*, **147**, 433–437.

Tanimura, T. & Shimada, I. (1981) Multiple receptor proteins for sweet taste in *Drosophila* discriminated by papain treatment. *Journal of Comparative Physiology.*, **141**, 265–269.

Tavernarakis, N., Wang, S.L., Dorovkov, M., Ryazanov, A. & Driscoll, M. (2000) Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nat Genet*, **24**, 180-183.

Taylor, S.S., Buechler, J.A. & Yonemoto, W. (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu Rev Biochem*, **59**, 971-1005.

Tempel, B.L., Bonini, N., Dawson, D.R. & Quinn, W.G. (1983) Reward learning in normal and mutant Drosophila. *Proc Natl Acad Sci U S A*, **80**, 1482-1486.

Tempel, B.L., Livingstone, M.S. & Quinn, W.G. (1984) Mutations in the dopa decarboxylase gene affect learning in Drosophila. *Proc Natl Acad Sci U S A*, **81**, 3577-3581.

Thorne, N., Bray, S. & Amrein, H. (2005) Function and Expression of the *Drosophila* Gr Genes in the Perception of Sweet, Bitter and Pheromone Compounds. *Chem Senses*, **30**, i270-i272.

Thorne, N., Chromey, C., Bray, S. & Amrein, H. (2004) Taste perception and coding in Drosophila. *Curr Biol*, **14**, 1065-1079.

Tully, T. & Quinn, W.G. (1985) Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J Comp Physiol [A]*, **157**, 263-277.

Vosshall, L.B. (2000) Olfaction in Drosophila. *Curr Opin Neurobiol*, **10**, 498-503.

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

Waddell, S., Armstrong, J.D., Kitamoto, T., Kaiser, K. & Quinn, W.G. (2000) The amnesiac gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory. *Cell*, **103**, 805-813.

Wang, Z., Singhvi, A., Kong, P. & Scott, K. (2004) Taste representations in the *Drosophila* brain. *Cell*, **117**, 981-991.

White, K. & Steller, H. (1995) The control of apoptosis in Drosophila. *Trends Cell Biol*, **5**, 74-78.

Zhu, B.C., Henderson, G., Sauer, A.M., Yu, Y., Crowe, W. & Laine, R.A. (2003) Structure-activity of valencenoid derivatives and their repellence to the Formosan subterranean termite. *J Chem Ecol*, **29**, 2695-2701.

8. Appendix

8.1 *curriculum vitae*

Andreas Stephan Thum
Ph D Student

Lehrstuhl für Genetik und Neurobiologie
Universität Würzburg
Am Hubland - Biozentrum
97074 Würzburg
Phone: ++49 931 888 4469
Fax: ++49 931 888 4452
Email: thum@biozentrum.uni-wuerzburg.de

Surname: Thum
First name: Andreas Stephan
Date of birth: 19.04.1977
Place of birth: Nördlingen (Bayern) – Germany
Martial status: Single

Education:
1983-1987 Elementary school Wallerstein, Bayern, Germany
1988-1996 High school (Gymnasium) Nördlingen, Bayern, Germany
1996-1997 Military service

Undergraduate studies:
1997-1999 Intermediate examination in Biology, University of Würzburg, Bayern, Germany
1999-2002 Diploma in Biology: „Klonierung von Promoter-GAL4-Linien und GAL80 enhancer-trap Vektoren für *Drosophila melanogaster*“
Supervisor: Prof. M. Heisenberg

Graduate studies:
2002- PhD in Biology, University of Wuerzburg, Bayern, Germany
Toolbox *Drosophila m.*: Sugar reward learning in flies
Supervisor: Prof. M. Heisenberg

8.2 List of publications

THUM, A. S., KNAPEK, S., DIETRICH-SCHMITT, E., RISTER, J., HEISENBERG, M. and TANIMOTO, H. Differential potencies of effector genes in adult Drosophila. J Comp Neurol **in press**, 2006.

8.3 Erklärung

Erklärung gemäß §4 der Promotionsordnung für die Fakultät für Biologie der Bayerischen 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, den 15.03.2006

Andreas Stephan Thum

8.4 Danksagung

Ich möchte mich bei Prof. Martin Heisenberg bedanken, der mir die Möglichkeit gab, diese Arbeit an seinem Lehrstuhl anzufertigen. Neben der hervorragenden wissenschaftlichen Anleitung besticht dieser Lehrstuhl durch seine zwischenmenschliche Nähe. Dies ist zweifelsohne die „Schuld“ meines Doktorvaters.

Mein weiterer Dank gilt Ron, der mir fortlaufend die Grundzüge wissenschaftlicher Arbeit aufzuzeigen versuchte. Ohne seine Ideen, Ratschläge, Fliegen, fachliches Wissen, und weitere Hilfestellungen wäre diese Arbeit nicht möglich gewesen.

Henrike, Arnim, Jens, Pavel, und Eike möchte ich führ die wissenschaftliche Zusammenarbeit danken.

Des Weiteren bedanke ich mich bei Bertram für seine Unterstützung, die mir half meinen nächsten Lebensabschnitt zu planen.

Natürlich geht mein Dank auch an alle TAs. Ohne sie wäre es nicht möglich, im Lehrstuhl Genetik und Neurobiologie zu arbeiten. Speziell Susanne, da sie immer ein offenes Ohr für meine „Problemchen“ hatte.

Eva, Tilman, Mareike, Stefan, Dennis und Steffi waren – nicht nur in der Uni – bei jedem Spaß dabei. Vielleicht sollte ich hier auch dem Beamer danken?

Weiterer Dank gilt meinen nächsten Verwandten, die mich in schweren familiären Zeiten unterstützt haben. Trotz dem schmerzlichen Verlust lieber Familienmitglieder geht unser Leben weiter – nur eben anders – leider.

Ganz besonders half mir Ena. Vielen Dank dafür, dass du mich immer unterstützt hast, obwohl ich oft sehr viel von meiner Göre verlange. Danke!