Localizing engrams of olfactory memories in *Drosophila*.

subcellular organization of appetitive, aversive and extinguished memories ?

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wer keine Träume hat,

der hat auch keine Kraft zu kämpfen.

für Marina

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1 - Introduction

Learning refers to the ability to change behaviour as a result of experience. Memory refers to the persistence of such changes. The most prominent example might be that of Pavlov´s dog salivating at the sound of a bell (Pavlov, 1927). Memory, the result of learning, is expressed as a change in behavior to the same sensory input: The bell, the conditioned stimulus (CS), does not initially cause Pavlov`s dog to salivate. As the bell is repeatedly followed by meatpowder in the mouth, the unconditioned stimulus (US), the bell alone elicits the conditioned response of salivation (classical or Pavlovian conditioning). There must be relatively long-lasting changes in the interconnections or interactions among the neurons in the brain between the auditory input (sound of the bell) and the behavioral output (salivation). The memory trace, or engram, refers to those changes in the brain that are necessary and sufficient for memory storage. Until recently, the engram had remained hypothetical, because no neuronal correlate had been conclusively identified which fullfilled the criteria of being necessary and sufficient for memory formation. In mammals, it seems that memories are widely distributed within functional areas of the cortex (Lashley, 1950). Yet a majority of scientists working in that field agree that a memory trace must exist.

Much of the early work has been performed on the facilitation of the gill-withdrawal reflex in the marine snail *Aplysia*, an organism with a less complex nervous system. *Aplysia* has allowed to identify the neuronal circuitry necessary for behavioral plasticity and the biochemical machinery underlying synaptic plasticity in this organism. The close parallels between behavioral and synaptic plasticity in *Aplysia* have lead to the central dogma of memory research, that synaptic plasticity underlies behavioral plasticity (see Kandel, 2001). Yet, despite its simplicity, the nervous system of *Aplysia* has not made it possible to identify the engram. Finally, new intervention techniques in *Drosophila* made the breakthrough. They allowed to identify an engram of olfactory memory as a change at a single neuronal level within the brain. Synaptic plasticity in the output of a set of Kenyon cells in the mushroom bodies is necessary and sufficient for olfactory memory formation (Zars et al., 2000).

Zars and coworkers (2000) were able to localize the site of synaptic plasticity that is necessary and sufficient for olfactory memory formation and by that raised the question, where in the brain these memories are stored. My study contributes to this question as I was able to localize the neuronal site that is necessary for storage of olfactory memories to the same site, where *rutabaga*-dependent synaptic plasticity is sufficient for memory formation.

Moreover, I could localize the phenomenon of extinction, a special kind of forgetting, to the same structure. This result suggests a subcellular interaction between the biochemical machineries of memory trace formation on the one hand, and its modulation by extinction on the other hand.

In the second part of my thesis I compare aversive and appetitive olfactory memories using sugar and electric shock as reinforcers. This allows to localize the neuronal sites in the brain that are necessary and sufficient for formation of an appetitive olfactory memory. A comparison between electric shock and sugar memory reveals that both memories localize to the same group of cell, but require differential monoamine signaling for their formation. The results suggest the biogenic amines dopamine as specific representation of the reinforcing capacity of electric shock and octopamine in case of sugar, respectively. Concidering the anatomy of the neuronal structure the memory localizes to, a model can be suggested in which several memory traces are formed inside one and the same neuron.

1.1 *Drosophila* as a model organism to study learning & memory

Concerning the complexity of model systems, the gill-withdrawal circuit in *Aplysia* with its few neurons might mark one end of a scale. The other end is marked by those 10^8 to 10^{11} neurons that make up the mammalian brain. Right in the middle between these two extremes resides *Drosophila* with its approximately 200,000 neurons. Despite this relative small number, *Drosophila* is capable of generating complex behaviours which can be used as model systems for behavioral plasticity, such as circadian rythms (see Stanewsky, 2003), flexibility in olfactory and visually guided orientation during walking and flight (see Heisenberg, 1998; Heisenberg et al., 2001), and learning during courtship behavior (see Quinn & Greenspan, 1984; Greenspan & Ferveur, 2000).

As for associative learning behavior, the most extensively studied experimental pavlovian memory task is olfactory conditioning (Quinn et al., 1974; see Waddell &Quinn, 2001), which can be used as positively or negatively reinforced learning task depending on whether sugar (Tempel et al., 1983) or electric shock (Tully & Quinn, 1985) serves as US. Irrespective of the type of US, the training consists of two olfactory cues (CS+ and CS-), which are sequentially presented to the animals, the first accompanied by the US (CS+), the second without the US (CS-). In a subsequent test trial, the animals must choose between the two olfactory cues (CS+ vs. CS-) in a forced choice maze (see Material & Methods).

1.2 – Processing of sensory stimuli

For olfactory conditioning in *Drosophila* I use olfactory cues to serve as conditioned stimuli and either sugar or electric shock to serve as unconditioned stimuli.

Olfactory processsing is well characterised in insects. Olfactory cues are received at the antennae and the maxillary palps by receptor neurons (RN) projecting their axon to the antennal lobes (Fig. 1A, Strausfeld 1976, 1998; Stocker 1994). Here they meet two broad classes of interneurons: Antennal lobes intrinsic interneurons (LocI) poviding lateral connections between individual glomeruli (Fig. 1B, Lei et al., 2002) and two classes of projection neurons (Fig. 1C, PNs or RIs). The mayority of PNs receive dendritic input from only one glomerulum and send their axons via the inner antennocerebral tract (iACT, see Stocker, 1990) to the calyx of the mushroom bodies (MBs) and the lateral protocerebrum (LPC, see Stocker 1990; Heimbeck et al., 1997; Fiala et al., 2002; Wong et al., 2002). A second group of PNs is multiglomerular and send their axons via the medial antennocerebral tract (mACT, see Stocker, 1990) directly to the LPC, bypassing the calyx of the MBs (Jefferis et al., 2002).

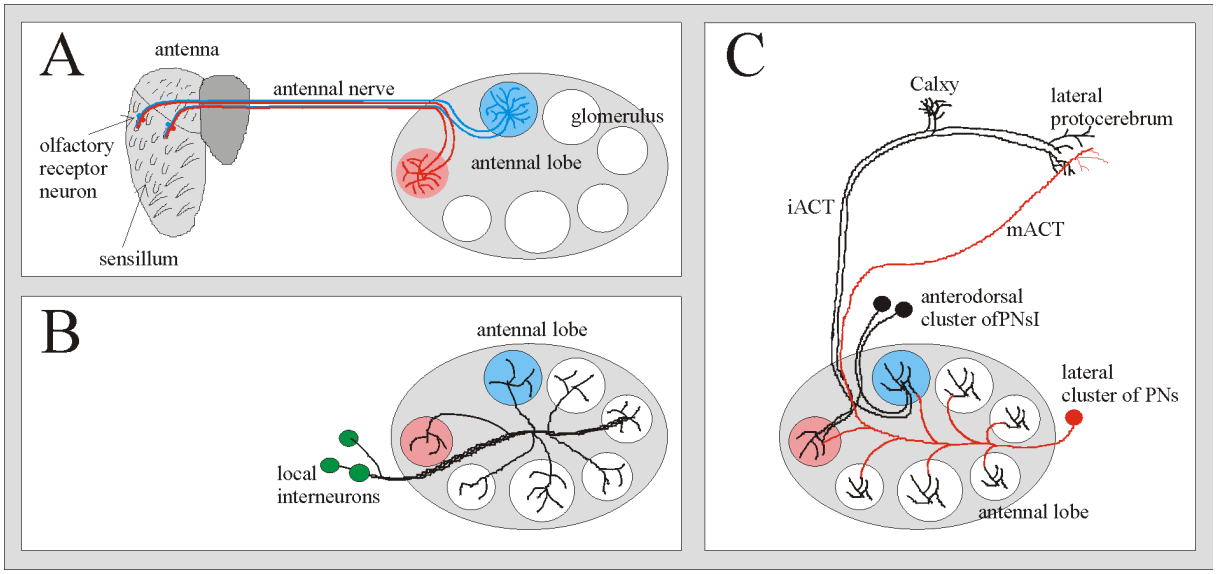

Figure 1: Elementary organisation of the olfactory pathway in *Drosophila*. The antenna houses olfactory receptor neurons that extend their axons via the antennal nerve to the antennal lobes, where they synapse in individual glomeruli **(A)**. Local interneurons of the antennal lobe provide crosstalk between the glomeruli **(B)**. The processed olfactory information is relayed to the higher order olfactory neuropiles of the mushroom bodies and the lateral protocerebrum via two anatomically distinct paths **(C)**. Relay interneurons (RI) of the anterodorsal cluster relay information from only one glomerulus via the iACT to the calyx of the mushroom bodies and the lateral protocerebrum. The RI of the lateral cluster innervate several glomeruli and extend their axon exclusively to the lateral protocerebrum via the mACT bypassing the mushroom bodies.

The intrinsic neurons of the MBs, the Kenyon cells (~2500 per MB, Technau & Heisenberg, 1982), send their axons to the anterior part of the brain where some bifurcate to form the medial and vertical lobes (Fig. 2). The fine structure of the lobes defines three sublasses of Kenyon cells in distinct subsystems, which derive from 4 neuroblast in a fixed pattern during larval and pupal development (α/β,α`/β` and γ, Crittenden et al., 1998; Lee & Luo, 1999). Efferent neurons leave the MBs via the lobes and the distal peduncle and project

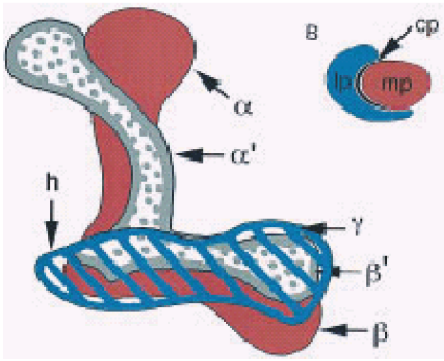

Figure 2: Lobes of the mushroom body. The medial and vertical lobes are build up by the axons of the Kenyon cells. Running their way down the peduncle **(B)** the axons sort themselfs according to the lobe system they project to. The medial projecting γ-axons are unbifurcated, whereas the axons of the α/β- and α´/β´-lobes bifurcate to send a medial and vertical projection. (This figure was taken from Crittenden et al., 1998).

to various anterior neuropil regions, contra- and ipsilateral MB lobes and the lateral protocerebrum (Ito et al., 1998; Schürmann, 1987). From there, large descending neurons relay signals through the cervical connective to the ventral ganglion, as suggested from a report in larger flies (Strausfeld et al., 1984).

The neuronal representation of an olfactory cue is spatially organised. In mammals, RNs express one type of olfactory receptor and converge onto the same glomerulus in the olfactory bulb (Mombaerts et al., 1996). In *Drosophila,* RNs express only a small fraction of olfactory receptors and the RNs expressing the same receptors converge onto a subset of glomeruli in the antennal lobes providing a spatial organisation of the first order olfactory neuropil (Vosshall et al., 2000; Bhalerao et al., 2003). A recent study by Hummel et al. (2003) revealed that axonal targeting of the RNs to the glomeruli of the antennal lobe involves the cell-surface protein Dscam to establish this type of correct connections. The spatial organisation is maintained along the olfactory pathway including the higher order olfactory centers of the MBs calyces and the lateral protocerebrum (Fiala et al., 2002; Wong et al., 2002; Wang et al., 2003).

The recently identified candidate gustatory receptors of *Drosophila* (Clyne et al., 2000; Scott et al., 2001) are expressed in specialised sensory neurons localised in gustatory sensillae. They are located on the external and internal mouthparts, tarsal segments of the legs and the anterior margin of the wings and differ in response to gustatory stimuli (see Singh,

1997; Hiroi et al., 2002). Depending on their location, they project to different regions of the suboesophageal ganglion, the tritocerebrum or the thoraco abdominal ganglion (Strausfeld 1976; Stocker and Schorderet, 1981). Sugar triggers feeding behavior in a well characterised pattern: stimulation of the tarsal sensilae leads to extension of the proboscis. Stimulating the labellar sensilae triggers contraction of the cibarial pump resulting in food-uptake (Dethier, 1976; Mourier, 1964) and stimulation of internal sensillae. The induction of these behaviors depends on two types of parameters, on the one hand on internal ones such as the nutrative status of the animal (Duve et al, 1979), and on the other hand on external parameters such as the composition of the food (von Frisch, 1935).

Electric shock is commonly used as aversive reinforcer during olfactory conditioning in *Drosophila* (Tully & Quinn, 1985). Althought it was conclusively shown that electric shock can act as a potent reinforcer (Tully & Quinn, 1985), until now no neuronal correlate for processing information about electric shock could be identified.

1.3 – Monoamine signaling and second messenger cascades

In the central nervous system (CNS) of both vertebrates and invertebrates biogenic amines are important neuroactive molecules involved in stimulus processing (see Blenau & Baumann, 2001; Müller, 2002). Physiologically they can either act as neurotransmitters, neuromodulators, or neurohormones. In insects, monoamines like dopamine (DA), tyramine and octopamine (OA) exert their effects by binding to specific membrane proteins that belong to the superfamily of G protein-coupled receptors (GPCR). A common feature of GPCR activation is the subsequent change of intracellular second messenger concentrations. Depending on which type of GPCR is activated, a change in the intracellular concentration of cAMP and/or Ca2+ is most likely to take place. As a result of GPCR activation, cAMP-concentration can either be elevated or decreased. The cellular response relies on the selective interaction between the receptor and G protein types (Gudermann et al., 1996, 1997). When the receptor binds to a Gs-type protein, the activated $G_{\alpha}s$ subunit will interact with adenylyl cyclase (AC) in the plasma membrane. This leads to an increase of cyclase activity and production of cAMP from ATP. The rise in cAMP will then activate cAMP-dependent protein kinase (protein kinase A, PKA), which can modify the properties of various substrate proteins (e.g. ligand-gated and voltage dependent ion channels, transcription factors, such as CREB, see De Cesare et al., 1999). Biogenic amine receptors are also known to inhibit adenylyl cyclase activity, mediated by interaction of the receptor with inhibitory G proteins (Gi).

Interaction of adenylyl cyclase with activated $G_{\alpha}i$ subunits most likely can compete with binding of activated $G_{\alpha}s$ subunits and thereby interferes with cyclase activation. The different intracellular messenger pathways may also be activated in parallel within the same cell when the respective receptors and coupling partners are present.

In honeybees, the monoamine OA has been shown to represent the reinforcing capacity of the sugar stimulus during conditioning of the proboscis extension reflex (Hammer & Menzel., 1998). In the monkey, midbrain dopamine neurons are selective to appetitive rather than aversive stimuli during conditioning (Mirenowicz & Schultz, 1996). For that reason, we invesigated the role of OA and DA during olfactory conditioning in *Drosophila.*

1.4 - Genetic dissection of olfactory memory

It has been proven in rats (and later also in flies), that learning and memory has a genetically determined component (Tryon, 1940; McGuire & Hirsch, 1977). This encouraged Seymour Benzer to start searching for behavioural mutants in *Drosophila* (Benzer, 1973). Using an aversive olfactory conditioning paradigm resulted in isolation of the very first learning mutants (Quinn et al., 1974). The mutants *dunce* (*dnc,* Dudai et al., 1976) and *rutagaba* (*rut,* Livingston et al., 1984) affect the cyclic AMP (cAMP) second messanger pathway. *Dunce* is lesioned in a cAMP phosphodiesterase normally degrading cAMP (Byers et al., 1981), wheras *rut* is deficient in an adenyly cyclase, synthetising cAMP (Levin et al., 1992). This cyclase is homologous to the mammalian type-1 adenyly cyclase and is responsive to both, G-protein and Ca2+/CAM dependent stimulation (Dudai et al., 1988). It was this co-activation property, that suggested the adenylyl cyclase *rut* to be a molecular detector of coincidence between the conditioned stimulus (odor) and the reinforcer (US) during pavlovian learning (Dudai et al., 1988, Abrams & Kandel, 1988).

Both the *dnc* and *rut* gene products are highly expressed in the intrinsic cells of the MBs, the Kenyon cells (Nighorn et al., 1991; Han et al., 1992; Crittenden et al., 1998). As they receive masssive olfactory input via the calyx, the Kenyon cells of the MBs have been speculated to represent the anatomical level of coincidence detection between the olfactory impulses (the CS) and the reinforcement (the US) during olfactory conditioning. Indeed, those cells have been shown to be necessary for olfactory learning by several experimental approaches (Heisenberg et al., 1985; deBelle & Heisenberg, 1994). Moreover, disrupting normal cAMP signaling in the MBs by expressing a constitutively activate $G_{\alpha}s$ subunit abolishes olfactory learning (Connolly et al., 1996). Receptors for the biogenic amines

dopamine (DAMB; dDA1) and octopamine (OAMB) have been found to be coupled via Gs proteins to adenylyl cyclase of the *rut* type and were found to be expressed at elevated concentrations in the MB lobes (Han et al., 1996; Crittenden et al., 1998; Han et al., 1998; Kim et al., 2003). All this evidence suggests the *rut*-AC to be a coincidence detector underlying the convergence of pathways from the odor and the electric shock reinforcement (Fig. 3). In fact, restoration of *rut* gene expression exclusively to the MBs is sufficient to restore normal capability for olfactory learning to *rut* mutant flies (Zars et al., 2000). In the present work, I was able to identify the unknown neuronal representation of the reinforcers sugar and electric shock. Our data suggest the biogenic amines dopamine and octopamine to be molecular representations of the reinforcing capacities of electric shock and sugar, respectively.

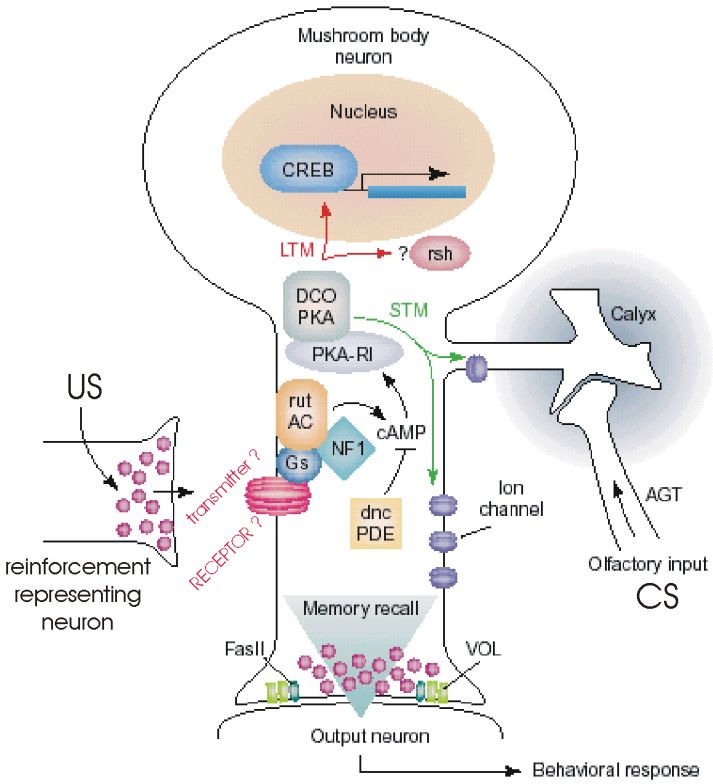

Figure 3. The cAMP cascade in learning and memory in *Drosophila*. A mushroom body (MB) neuron receives olfactory input, via interneurons of the antennocerebral tract (ACT) that synapse in the MB calyx. MBs also receive electric-shock input through yet unknown neurons. Pre-synaptic termini of the MB neuron, residing in the MB lobes, are innervated by modulatory neurons like the dorsal paired medial (DPM) neurons that might release AMN neuropeptide(s). Activation of the RUT adenylyl cyclase leads to elevation of cAMP levels in the relevant MB neurons. Longer-term stimulation of the cascade by AMN might lengthen the association and help consolidate the memory. Depending on the conditions of training and the duration of cAMP elevation, the experience results in short lived modification of synaptic connectivity (short-term memory; STM) or in longer lasting functional and structural changes (long term memory; LTM) in that neuron mediated by different mechanisms. Recall of olfactory memory requires synaptic transmission from MB neurons. DCO,

PKA catalytic subunit; PKA-R1, PKA regulatory subunit; dnc PDE, cAMP phosphodiesterase encoded by the dunce gene; Gs, stimulatory G protein; RUT, type I adenylyl cyclase; NF1, neurofibromin, rsh, radish gene product; rut, rutabaga gene product; VOL, volado gene product; FasII, fasciclinII gene product. (This figure was modified from Waddell & Quinn, 2001)

The first pure memory mutant in *Drosophila, amneasiac* (*amn*), affected a very early stage of olfactory memory leaving initial learning intact (Quinn et al., 1979). The *amn* gene encodes an apparent pre-proneuropetide neurotransmitter (Feany & Quinn, 1995; Moore et al., 1998) and is most abundant in two brain cells termed dorsal paired medial (DPM) neurons, that seem to be modulatory neurons, and that project to all the lobes of the MBs. Expressing the *amn* gene in DPM cells restores normal olfactory memory to *amn* mutant flies, and moreover blocking synaptic transmission from the DPM neurones blocks one-hour memory, but leaves immediate learning intact (Waddell et al., 2000). It is hyothesized that the AMN neuropeptide, released onto the MB lobes, could trigger a prolonged activation of the cAMP cascade, which is required for the consolidation of initial memory into more permanent memory. As the adenyly cyclase seems to be the initial event in memory formation, duration of cAMP dependent protein kinase A activation, its primary downstream target, is believed to determine whether short (STM), medium (MTM) or long-term memory (LTM) is formed (Li et al., 1996).

Expression of the PKA catalytic and regulatory subunits is elevated in the MBs (Crittenden et al., 1998; Skoulakis et al., 1993), consistent with a central role of the MBs in learning. Disrupting PKA activity globally with inducible inhibitory transgenes acutely reduces olfactory learning (Drain et al., 1991). Furthermore, flies mutated in the genes for the catalytic or regulatory subunits of PKA are deficient in learning (Skoulakis et al., 1993; Goodwin et al., 1997; Li et al., 1996). A major target for PKA phosphorylation is the transcription factor cAMP response element binding protein (CREB). Studies of flies with inducible CREB transgenes show that CREB is crucial for protein-synthesis-dependent LTM formation (Yin et al, 1994, 1995). Experiments on the *Drosophila* larval neuromuscular junction suggest that CREB-dependent transcription is crucial for new gene expression that increases synaptic efficacy (see Davis et al., 1996). This efficacy seems to be mediated by presynaptic mechanisms, including components of the neurotransmitter release machinery and cell adhesion molecules, like Volado and Fasciclin II. (Grotewiel et al., 1998; Cheng et al., 2001). Althought the relationship of these genes with memory formation is clear, its cellular mechanism of action remains unknown.

The dissection of memory into temporally distinct phases is commonly accepted and convergent with findings from vertebrate and invertebrate model systems of learning and memory (Fig. 4, Quinn and Dudai, 1976; Davis and Squire, 1984; Allweis, 1991; Squire, 1992; Folkers et al., 1993; Tully et al., 1994; Xia et al., 1997; Frankland et al., 1998; Milner, 1972; Scotville & Milner, 2000). In mammals, crosstalk between different regions of the brain is required to consolidate a memory from the short lived into more stable forms. These forms can be separated by pharmacological means and require different anatomical structures (Day & Morris, 2001). In *Drosophila*, the α-lob[illegible] necessary for a long lasting form of olfactory memory, wheras they are not necessary for the short lived form (Pascual & Preat, 2001). In this study I monitored olfactory memory for up to 3 hours trying to localise the more consolidated forms of olfactory memory.

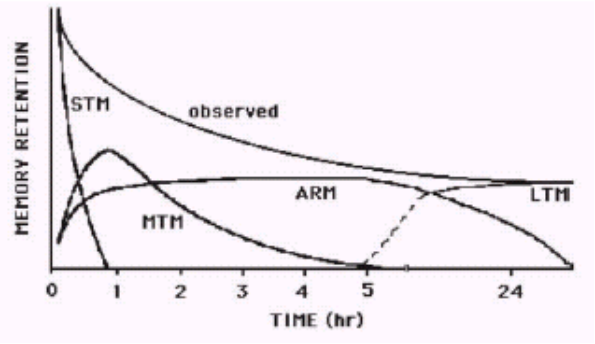

Figure 4: Behavioral model of memory formation. The decay of memory observed over time appears relatively monotone. Experimental disruptions, however, reveal several distinct memory phases underlying memory retention, including short-term (STM), middle-term (MTM), anesthesia resistant (ARM), and long-term (LTM) memory. (This figure was taken from Dubnau et al., 2003)

1.5 – Extinction of memories

The study of extinction began with Pavlov (1927), who discovered that the conditioned salivary response of his dogs to a food signaling cue diminished and finally disappeared when the cue was repeatedly presented in the absence of food. This decrease in the amplitude and frequency of a CR as a function of nonreinforced CS presentations is ubiquitous across species, and is referred to as extinction (Pavlov, 1927). Extinction is not due to forgetting of the original CS-US association, as CRs are quite resistant to loss with the simple passage of time. Rather, extinction is an active learning process that is distinct from acquisition and requires additional training to develop (Pavlov, 1927).

Some psychological theories have described extintion as an "unlearning" process dependent on a violation of the CS-US contingency established in acquisition (Rescorla and Wagner, 1972; Wagner and Rescorla, 1972). It is argued that the CS-US association mediating CR performance is weakened and ultimately lost over the course of extinction training, such that the CS loses its ability to produce a CR. An alternative hypothesis proposes that extinction is a form of new learning that counteracts the expression of the CR (Bouton,

1993; Wagner, 1981; Konorski, 1948; Pavlov, 1927). In associative terms, this process is described as the generation and strengthening of a second, inhibitory association between the CS and US representations, which acts in parallel with the excitatory association and directly opposes the tendency of the excitatory association to activate the US representation. Guided by these theoretical considerations, scientists began to search for "excitatory" and "inhibitory" responses to a CS, while the focus ranged from different brain structures or different populations of cells within a structure (e.g., glutamatergic versus GABAergic neurons) to different types of molecules within individual cells (e.g. kinases versus phosphatases; activators versus repressors of transcription) (see Myers and Davis, 2002; Bouton 1993). In this work I try to localise the cellular substrate underlying extinction of olfactory memories in *Drosophila*. The results suggest a mechanistic interaction between the molecular machineries of the existing memory trace and extinction within the same group of cells.

1.6 – Genetic tools in *Drosophila*

The most powerful advantage of *Drosophila* is its well elaborated genetics, started with Morgan in 1909. He founded forward genetics in *Drosophila* nearly one century ago. Today, a large collection of mutant alleles have been generated and established as laboratory lines. Since 2000, *Drosophila´s* genome is completely sequenced and its predicted number of about 14,000 genes (Myers et al., 2000) is accessible to "reverse genetics". Two systems are availible for transgene expression: The GAL4/UAS-system (Fig. 5) providing spatial control over transgene expression (Brand & Perrimon, 1993), and the heat-shock promoter (hsp-70 system, see Pirrota 1988) providing temporal control over ectopic transgene expression by raising the temperature. Recent progress combines these two levels of control in form of trangenic systems that provide spatio-temporal control over transgene expression by use of hormone induced transcription activators (Stebbins et al., 2001; Han et al., 2000).

The recently developed UAS-*shi*^{ts1} transgene (Kitamoto, 2001) allowed for ambient temperature dependent spatio-temporal control over synaptic transmission in identified neurons (Waddell et al., 2000; Dubnau et al., 2001; McGuire et al., 2001; Kitamoto, 2002; Schwaerzel et al., 2002). *Drosophila shibire* (*shi*) encodes the protein dynamin (Chen et al., 1991), which is involved in endocytosis and is essential for synaptic vesicle recycling and for release of vesicles (Kosaka and Ikeda, 1983). The temperature-sensitive allel *shi*^{ts1} is defective at restrictive temperatures (> 29°C) and results in rapid (~1 min) and reversible inhibition of synaptic transmission (Koenig et al., 1983). The dominant negative character could be

explained by its physiologic function in a multimeric form (Muhlberg et al., 1997; Delgado et al., 2000). Neurons overexpressing a UAS-*shi*^{ts1} allele are inhibited in neurotransmission at a restrivtive temperature (Waddell et al., 2000; Dubnau et al., 2001; McGuire et al., 2001; Kitamoto 2001, 2002; Schwaerzel et al., 2002). Its fast block of neurotransmission and the simple experimental contol of temperature makes it the most powerfull tool to study neural systems structure function correlation in *Drosophila* at the moment.

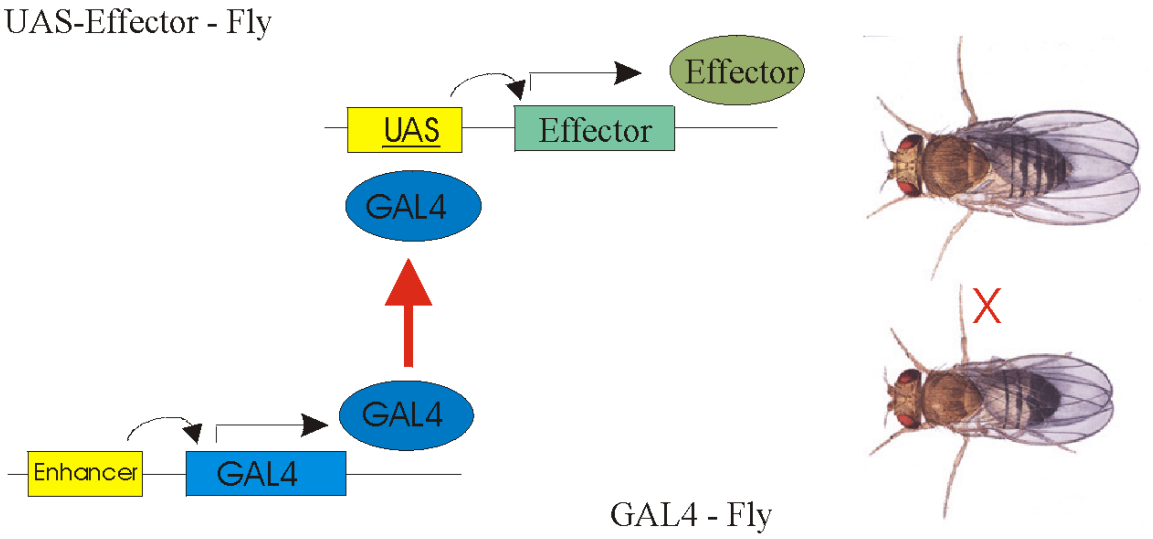

Figure 5: The GAL4/UAS-system. This system consists of two transgenic components, each inserted separate into the genomes of either the GAL4-fly or the UAS-effector fly and allows expression of any trangenic effector in a spatially restricted pattern (Brand and Perrimon, 1993). For this purpose a construct carrying the gene of the yeast transcription factor GAL4 is inserted in the *Drosophila* genome (GAL4 fly). Depending on where the construct is inserted into the genome, GAL4 expression is driven in a spatial pattern, controlled by endogenous enhancer elements in the close vicinity of the place of insertion. On a second construct the GAL4 binding sequence (UAS) and a downstream effector are encoded (UAS-Effector fly). This transgene can be expressed in the progeney of a cross between GAL4 and UAS-Effector fly. In these animals, the transgene is expressed in a GAL4-dependent manner, namely only in those cells, GAL4 is present. What makes this system powerfull is its free combination between appropriate lines by a simple cross. The GAL4-driver lines provide expression in the neurons of interest and any UAS-transgenic line provides the desired function to those neurons in the progeny of the two transgenic parental lines. For functional analysis, a large number of UAS-effectors is available.

2 - Material & Methods

2.1 – Fly care

All flies were raised on corn-meal food (Guo et al., 1996) in a 14-10 hours light-dark cycle at 25°C and 60% relative humidity. Experimental flies were fed on fresh food vials for up to 48 hours before the behavioral tests. If necessary, flies would be starved for 18 hours in empty vials equiped with moist filter paper to prevent dessication. In case of heatshock, flies were placed at 37°C for 30 minutes in empty vials. This treatment was given two times within 6 hours during the starvation procedure. To prevent direct effects of the heatshock treatment itself, flies were allowed to recover for 12 hours before testing. For behavioral experiments I used 3 to 5 day old males and females in mixed groups, either taken from homozygote lines or from progeny of crosses between homozygote parental lines. All behavioral experiments were either done in dim red light (invisible for the flies) during the training period and complete darkness during the test period at 80% relative humidity except for sugar-reactivity, which was tested for in day light and normal humidity conditions.

2.2 – Testing for perception of sensory stimuli

The test for perception of sugar was done in vertical tubes (50ml) with a 1 cm broad stripe of filter paper at the half hight of the tube, either soaked in 2 M sucrose solution or water. I scored the time starved flies spend on the filter paper (t_{filter}) during an experimental duration of 30 seconds (t_{total}), starting from the moment the fly taps onto the stripe of 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 (Fig. 6, Tully & Quinn, 1985). About 100 flies were placed into the elevator, put in register with two tubes and given one minute to choose between an electrified (12 x pulses of 130V 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 (750 ml/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}). $RI^O = \{ [N_{odor} - N_{air}] / [N_{odor} + N_{air}] \} \times 100$. The RI^O can vary between –100 (all animals avoid the odorant) and +100 (all animals choose the odorant

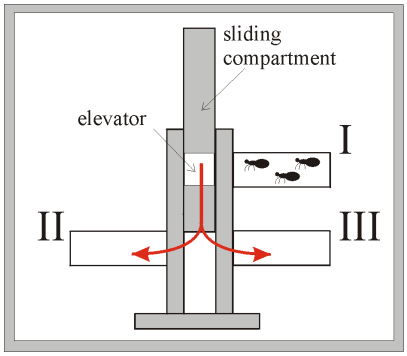

Figure 6: Principle of the T-maze device (Tully & Quinn, 1985). The apparatus consists of two horizontal tubes (II and III), which can be brought in register with an elevator mounted in a sliding device. Air is continuously pumped from the elevator at a constant flow rate of 750 ml/min. At the beginning of a test, about 100 flies are put into a starting tube (I) and from there gently tapped into the elevator, which has been brought in line with the starting tube. The test starts by pushing the sliding device down. This brings the elevator in line with the two tubes at the choice point, where the flies enter one of the two tubes. In case no stimuli are presented, the flies distribute equally between the tubes. To test for perception of electric shock this setup can be equipped with two electrifiable tubes. To test for perception of olfactory cues two test tubes can be connected to the choice point.

2.3 - Associative Experiments

I used either sugar or electric shock as reinforcers during two different pavlovian training procedures in a T-maze apparatus (Tully & Quinn, 1985). Note that the test situation was identical for both kinds of training.

For sugar learning (Fig. 7), about 100 starved flies were placed into the apparatus and during each training trial allowed to feed on 2 M sugar solution for 30 seconds. The sugar was spread onto a filter paper covering 95% of the training tubes surface. While feeding, the tube was scented with the first odor and immediately afterwards these flies were transfered to another tube for additional 30 seconds. This tube contained a filter paper soaked in water and the tube was scented with the second odor. This completed one individual training trial and, if necessary, this procedure was repeated up to four times. Flies were tested 100 seconds after training by giving them two minutes to choose between two airstreams (750 ml/min), containing either the formerly rewarded or non-rewarded odor. During a second experiment I changed the identity of rewarded and unrewarded odors, and trained a different group of flies. For each experiment I counted the number of flies choosing the rewarded (N_{reward}) and the unrewarded odor ($N_{non\ reward}$) and calculated Performance Indices as $PI_{1,2} = \{ [N_{reward} - N_{non\ reward}] / [N_{reward} + N_{non\ reward}] \} \times 100$. To rule out any non-associative effects on performance of the flies, I averaged over these two experiments with $PI = (PI_1 + PI_2) / 2$.

The test substances were diluted 36-fold in paraffin oil (Fluka) and presented in cups with various diameters to adjust concentrations: Ethylacetate in a 15 mm cup and isoamylacetate in a 16 mm cup. Under these conditions, naive flies showed no preference for one of the two substances over the other.

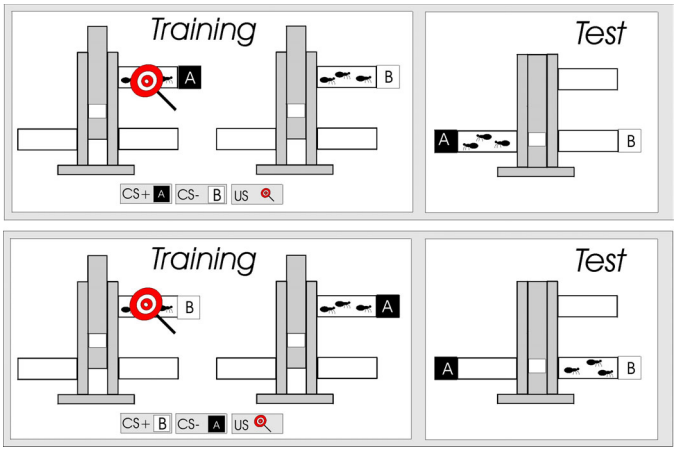

Figure 7: Sugar learning paradigm. For training about 100 previously starved flies were placed into a tube, with a surface covered with filter paper soaked in 2 M sugar solution and allowed to feed on it for 30 seconds while the tube was flushed with an olactory cue A. The flies were tapped into the elevator of the sliding compartment and this was brought in line with a second tube covered with filter paper soaked in water. The flies were allowed to enter this tube for 30 seconds while the tube was scented with odor B. After tapping the flies back into the elevator, this procedure could either be repeated, or the flies could be tested by giving them 2 minutes to choose between the two odors. The Performance Index was calculated averaging over two reciprocal experiments (upper and lower panel).

For electroshock learning (Fig. 8) about 100 flies were placed into an electrifiable tube and during one minute of training given 12 times electric shock of 130 V and 1.3 seconds duration. During that time the tube was scented with the first odor and after 45 seconds 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 seconds of air. This complets the training period. After a 100 seconds break flies were given 2 minutes to choose between two airstreams (750 ml/minute), one scented with the formerly shocked, the other one with the formerly non-shocked odor. As already described,I conducted two experiments, during which the punished odor from the first experiment became the non punished one in the second experiment. For each experiment I calculated a Performance Index as $PI_{1,2} = | [N_{non\ punished} - N_{punished}] / [N_{punished} + N_{non\ punished}] \} \times 100 |$ and averaged over these two experiments with $PI = (PI_1 + PI_2) / 2$.

I used two different sets of olfactory cues: In the case of electric shock learning (see results 3.3 & 3.4), I used benzaldehyde (Fluka) in a 5 mm cup and 3-octanol (Fluka) in a 16 mm cup as pure substances. These concentrations ellicit spontaneous avoidance behavior to a high probability. Note that during the associative experiments the PIs are positive if flies

avoid the shock associated odor in the test. In the case of comparing aversive and appetitive memories, I used starved flies and dilutions of the odorants ethylacetate and isoamylacetate (see results 3.5 - 3.8). Note that PIs are negative if flies avoid the shock-associated odor and positive if flies choose the sugar associated odor in the test. I have chosen this convention to reflect the diference in conditioned performance of the flies).

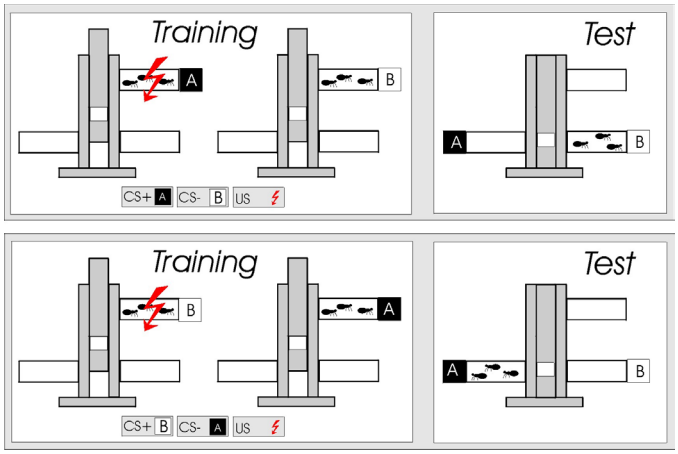

Figure 8: Electroshock learning paradigm. During training about 100 flies are placed into an electrifiable shock-tube. During exposure to the olfactory cue A, the flies receive electric shock treatment for one minute (12 x pulses of 1.3 sec duration at 130 V) followed by air and an additional minute of olfactory cue B without electroshocks. The flies are tapped into the elevator and tested for their preference between A and B for 2 minutes. From two reciprocal experiments (upper and lower panel) during which the identity of the punished odorant A or B was reversed, a Performance Index was calculated.

2.4 - The olfactory revolver device

I have developed a 4 fold T maze apparatus by modifying the apparatus originally designed by Tully & Quinn (1985). The original device (see Fig. 6) consists of a sliding compartment which can be brought in register with the training tube on the one hand or with the test tubes on the other hand. This movable “elevator“ is required to transfere the trained flies towards the position where they actually have to make their choice between the two stimuli presented in the two test tubes (see red arrows in Fig. 6). In the modified revolver, I have mounted 4 “elevators“ on a rotating disc (see inlet in Fig. 9). This design allows to train and test 4 groups of flies simultaneously.

During training with electric shock reinforcement, these flies are placed into either of 4 electrifiable training tubes. For testing the flies the elevator-disc is brought in register with the training tubes and the flies are transfered into the elevators. 4 test tubes are mounted on the the front- and back-side of the machine and the elevators is brought in line with them by rotating the elevator disc. Air flow is kept constant by a pump connected to each elevator of the sliding disc via the axis.

Experiments with sugar reinforcement were also done in this device. For this purpose flies were exposed to the CS+ in 4 training tubes equipped with filter paper soaked in 2 M sugar solution covering 95% of the surface. After 30 seconds the fies were transfered into the elevators and brought into register with 4 training tubes equipped with filter paper soaked in water. Here the flies were exposed to the CS- for 30 seconds and afterwards again transfered into the elevators. This procedure could either be repeated, or the flies could be tested, like in the case of electric shock reinforcement.

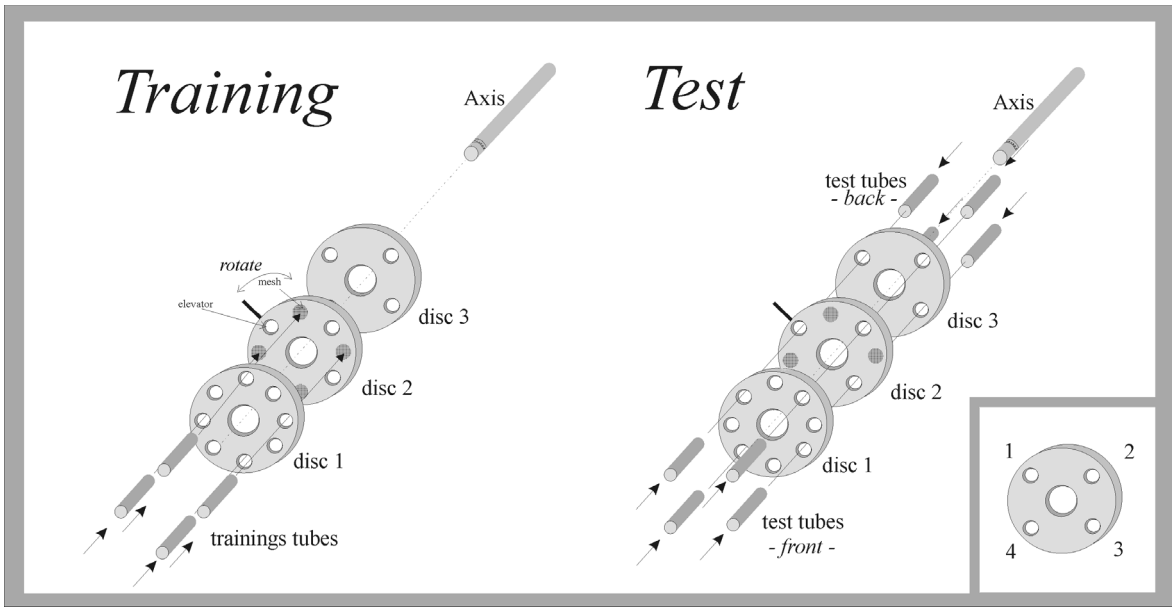

Figure 9: The olfactory revolver device. Explanation is given in the text.

2.5 – Immunohistochemistry

TAU-expression patters 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 anti-TAU antibodies (1:1000, Sigma) in PBT overnight at 4°C. A series of washes and incubation with a biotinylated anti-mouse antibodiy (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.

For whole-mount stainings, flies were anesthetized and brains were dissected in *Drosophila* ringer by stripping of the head capsule including the eyes (Rein et al., 2002). Brains were fixed overnight in 2% para-formaldehyde at 4°C. The brains were stained using different antibodies: A polyclonal rabbit anti-β-gal antibody (1:1000, Cappel, INC

Biomedicals, Seven Hills, Australia), a polyclonal rabbit anti-TAU-antibody (1:250, Sigma) and a monoclonal mouse anti-TAU-antibody (1:1000, Sigma). For neuropil staining, a monoclonal mouse antibody was used (nc82, 1: 100, Rein et al., 2002). As secondary antibodies an anti-rabbit antibody conjugated to Alexa488 (1:100, Molecular Probes, Eugene, Oregon) and a anti-mouse antibody conjugated to Cy3.18 (1:250, Jackson Immuno Research, West Grove, Pennsylvania) were used. 1 µm optical sections were acquired using a Leica CLSM / Aristoplan confocal microscope equipped with a Zeiss objective lens (Plan Neofluar 20x) with a numerical aperture of 0.8. Tissue-reconstructions were done using the AMIRA®-software.

2.6 – Genotypes

I used several Gal4-lines to drive transgene expression within the Kenyon cells of the mushroom bodies (MBs). Lines 247 (Zars et al., 2000b) , c772 (Yang et al., 1995) and D52H (Ron Davis, Baylor College Medicine, Houston Texas) drive expression in nearly all subsystems of the MB, althought to a different extent. Line GH146 drives expression in projection neurons connecting the antennal lobe with the mushroom bodies and the lateral protocerebrum (Stocker et al., 1997; Jefferis et al., 2001; Fiala et al., 2002).

I use the transgenic line TH-Gal4 carrying the yeast transcription factor GAL4 under control of regulatory regions of the tyrosine hydroxylase (*TH*) gene. TH catalyzes the first step in dopamine (DA) biosynthesis; this gene is selectively expressed in DA neurons in the CNS. The TH-GAL4 line thus provides specific experimental access to most DA neurons in the adult brain (Friggi-Grelin et al., 2003).

I used the line Canton-S (Wuerzburg) as wild-type control for the rut^{2080}-lines: The UAS-rut^{+} transgene (Zars et al., 2000a) and the 247 GAL4 enhancer (Zars et al., 2000b) were recombined onto the same 3rd chromosome and crossed into wild-type Canton-S and rut^{2080} mutant background (Schwaerzel et al., 2002). The control lines rut^{2080}; +; UAS-rut^{+} and rut^{2080}; +; 247 were handled similarly (Schwaerzel et al., 2002). Behavioral experiments were done with animals from these homozygous lines.

For temperature-dependent block of synaptic transmission, I used progeny of crosses between the homozygous parental lines UAS-shi^{ts1} (as virgin females) and GAL4-lines (as

males). The line UAS-Shi1 contains multiple inserts of UAS-*shi*^{ts1} on the 3rd chromosome, whereas UAS-Shi2 bears inserts on X and 3rd chromosomes (Kitamoto, 2001).

The enzyme *tyramine*-β-*hydroxylase (T*β*H)* is deleted in the *T*β*H*^{M18} mutant (Monastirioti, 1996). The original TβH^{M18} stock (Monastitioti, 1996) carried an additional mutation in the *white* (*w*) gene, which effects olfactory lerning and memory (Fig. 10). To circumvent this complication, I received a *T*β*H*^{M18} mutant from H. Scholz who crossed it to Canton-S and isolated recombinant TβH^{M18} lines with the w^+ allele. Non-recombinant w^+ lines were kept as controls. Due to the female-sterility of the TβH^{M18} mutation it was balanced over FM7. Homo- and hemizygous TβH^{M18} flies were tested in behavioral experiments irrespective of sex. I received heat-shock inducible Tyramine-β-Hydroxylase (TβH) flies from M. Monastirioti. In these flies, a 3 kb EcoRI fragment containing TβH-cDNA was cloned downstream of the hsp70-promoter of the pCaSpeR-hs transformation vector (Thummel et al., 1992) and transgenic flies were generated by standard procedures. A transformant carrying the insert on the 3rd chromosome was brought into TβH^{M18} mutant background by standard crosses. This line carries a wild-type *white*-cDNA within the transformation vector.

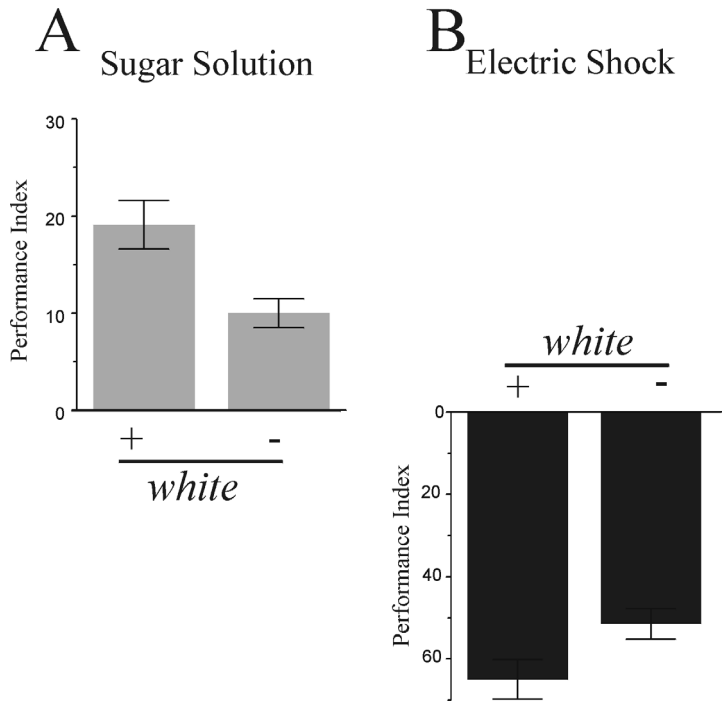

Figure 10: Effect of the *white*^{1118} mutation on olfactory learning and memory. I monitored olfactory memory directly after training with either sugar reward **(A)** or electric shock punishment **(B)**. The experiments were done with male progeny of a cross between a cantonized *white*^{1118} line (as virgin females) and a cantonized line carrying a translocation of the X-chromosome containing the white-locus (as males). The Y-linked translocation caused either red eyes (*white*$^+$) or white eyes (*white*$^-$) in half of the male progeny, respectively. Red eyed males showed significantly higher

performance of olfactory memory after training with either sugar reward or electric shock punishment (ANOVA: ps < 0,001). Interestingly, both forms of memory are reduced to the same extend (~10 units of performace). (Each data point represents the mean of six experiments plus or minus the SEM.)

I am thankful to Maria Monsterioti, Henrike Scholz, Troy Zars and Serge Birman for sharing transgenic fly stocks with me. All genotypes used in this study are summarized in table 1.

Table 1: **Genotypes of flies.**

Line	Genotype	Comment	Reference
Canton-S	wild-type	from Wuerzburg	Schwaerzel et al., 2002
white^{1118}	*white*-	cantonized	Dura et al., 1993
Dp(1;Y)*w*+	translocation of the X-chromosome (2D1 – 3D4) attached to the Y-chromosome	cantonized	Smith & Konopka, 1981
247-GAL4	*white*-, P-element containing wild-type *white*-cDNA 3rd chr.	GAL4 expression controlled by regulatory region of the *D-Mef*2 gene	Zars et al., 2000a
c772-GAL4	*white*-, P-element containing wild-type *white*-cDNA 2nd chr.	enhancer trap line, cantonized	Yang et al., 1995
D52H-GAL4	*white*-, P-element containing wild-type *white*-cDNA X chr.	GAL4 expression controlled by regulatory region of the *dunce* gene, cantonized	Ron Davis, Baylor College, Houston Texas
GH146-GAL4	*white*-, P-element containing wild-type *white*-cDNA 2nd chr.	enhancer trap line, balanced over CyO	Stocker et al., 1997
TH-GAL4	*white*-, P-element containing wild-type *white*-cDNA 3rd chr.	GAL4 expression controlled by regulatory region of the *tyrosine hydroxylase* gene	Friggi-Grelin et al., 2003
rut^{2080}	*rut*- P-element containing wild-type *rosy*-cDNA X chr.	P-element induced mutation, cantonized	Schwaerzel et al., 2002
rut^{2080}; +; UAS-rut^{+}	*rut*- P-element containing wild-type *rosy*-cDNA X chr. transgene-insertion containing wild-type *white*-cDNA 3rd chr.	P-element induced mutation, UAS-rut^{+} transgene, cantonized	Schwaerzel et al., 2002

rut^{2080}; +; 247 *rut*^{2080}; +; 247	*rut*- P-element containing wild-type *rosy*-cDNA X chr. P-element containing wild-type *white*-cDNA 3rd chr.	P-element induced mutation, GAL4 expression controlled by regulatory region of the *D-Mef*2 gene, cantonized	Schwaerzel et al., 2002
rut-rescue *rut*^{2080};+; 247, UAS-rut^{+}	*rut*- P-element containing wild-type *rosy*-cDNA X chr. P-element containing wild-type *white*-cDNA 3rd chr. transgene insertion containing wild-type *white*-cDNA 3rd chr.	P-element induced mutation, GAL4 expression controlled by regulatory region of the *D-Mef*2 gene, UAS-rut^{+} transgene, cantonized	Schwaerzel et al., 2002
UAS-Shi1	*white*-, multiple transgene-insertions containing wild-type *white*-cDNA 3rd chr.	UAS-*shi*^{ts1} transgene	Kitamoto 2001
UAS-Shi2	*white*-, multiple transgene-insertions containing wild-type *white*-cDNA 3rd chr and X chr.	UAS-*shi*^{ts1} transgene	Kitamoto 2001
TβH^{M18}	*white*+, *TβH*-	P-element based excision of the *tyramine*-β-*hydroxylase* gene	Henrike Scholz, Genetik Wuerzburg, Germany
hs-TβH	*white*-, *TβH*- transgene insertion containing wild-type *white*-cDNA 3rd chr.	P-element based excision of the *tyramine*-β-*hydroxylase* gene, heat-shock inducible transgene containing *tyramine*-β-*hydroxylase*-cDNA	Maria Monastirioti, Molecular Biology, Heraklion, Greece

3 – Results

3.1 – Validating the olfactory revolver device

To measure olfactory memory in *Drosophila,* I developed the 4 fold revolver (see Material & Methods). This device was designed to be used for olfactory conditioning with either electric shock or sugar as unconditioned stimuli (USs), allowing for 4 times faster acquisition of data compared to the old devices (Tully & Quinn, 1985; Tempel et al., 1983). Due to the 4 fold design, each choice point works individually. Whether all choice points give raise to the same results was investigated in two experiments.

First, I tested for spontaneous preference of wild-type Canton-S at any of the four choice points in the absence of olfactory cues (Fig. 11A). At all four positions flies distributed equally between the front and back tubes resulting in PIs near zero. Second, I tested olfactory conditioning in Canton-S flies using electric shock reinforcement of the olfactory cues benzaldehyde and 3-octanol. When memory was measured directly after training, memory performance was equal at all positions of the machine and not different from results measured in the old device (Fig. 11B). These results show that the four choice points give similar

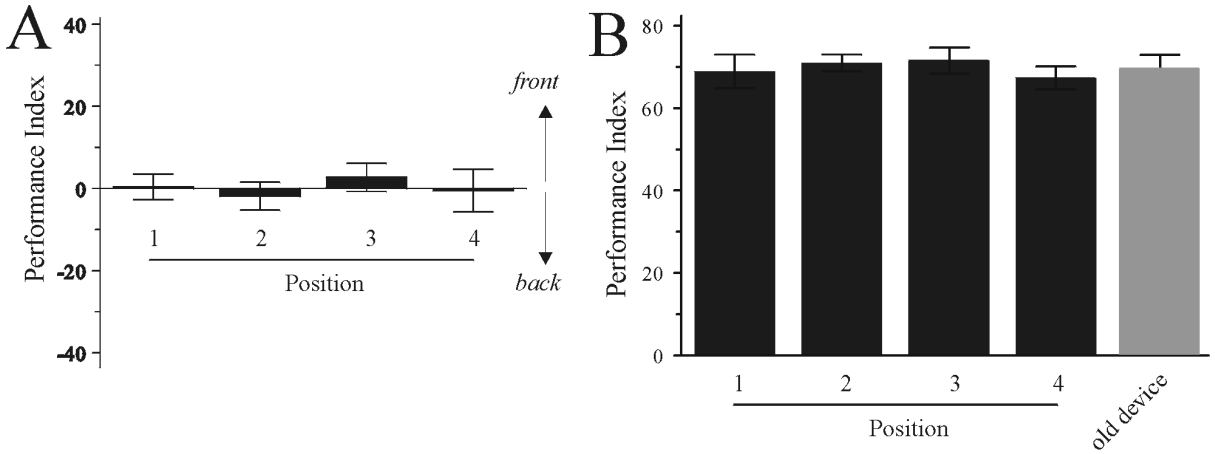

Figure 11: Testing the revolver apparatus. **(A)** Spontaneous preference of naive flies was measured in the absence of olfactory cues. Flies did not discriminate between the front- and rear-side as indicated by Perfomance Indices near zero at each of the four positions of the device (ANOVA: $p > 0.05$; $N = 4$). **(B)** Olfactory memory was measured directly after training. Performance of memory measured at the 4 choice points of the new machine was comparable to the result from the old apparatus (ANOVA: $p > 0.05$; $N = 9$).

During this study, I measured all genotypes involved in a certain experiment side-by-side in randomised order between May of 2001 and October of 2002. This long period of time enabled me to address the question whether circannual effects influence performance of olfactory memory (for an example see Farner, 1985). I pooled all data on wild-type Canton-S measured in 2001 according to the month during which the experiments were done. Post-hoc statistical analysis revealed no differences between the data acquired during different months (Fig. 11A). Moreover, the data are normally disributed (Fig. 11B) and for that reason could be analysed using parametric tests, one-way ANOVA and Duncan`s post-hoc statistical tests. (Note, that the data measured during 2002 used different dilutions of odorants and for that reason could not be included).

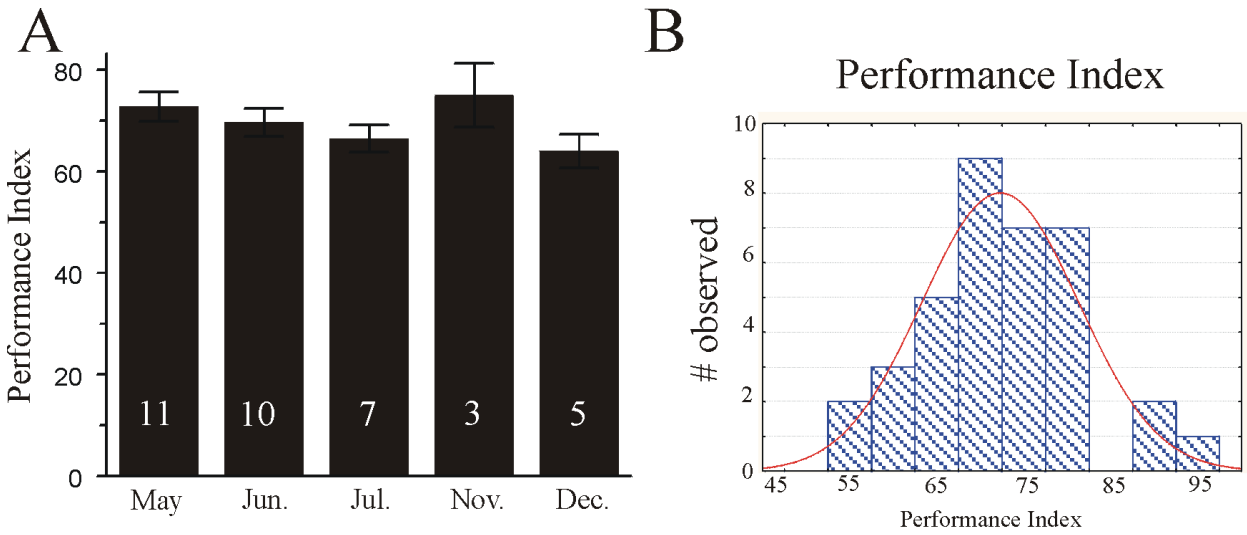

Figure 12: Long-term monitoring of olfactory memory in 2001. To reveal possible circannual effects on performance of olfactory memory we compared data acquired during different months. **(A)** Performance of electric shock memory in Canton-S flies was unaltered over a period of 8 month in 2001 (ANOVA: $p > 0.05$; N as indicated). **(B)** The 36 individual measurments followed a normal distribution (Kolmogorov-Smirnov test: $d = 0.0411141$, p = n.s.).

3.2 – Expression patterns of GAL4 lines

My attention was focused on neurons along the olfactory pathway providing CS-information, and on the other hand dopaminergic neurons, candidates for providing US-information. Correlating these structures with associative functions during pavlovian conditioning requires an anatomical analysis with respect to the identity and number of neurons that can be interfered with. Along the olfactory pathway I was interested in two types of neurons, projection neurons (PNs, Stocker et al., 1997; Heimbeck et al., 2001) connecting the antennal lobes (ALs) to the mushroom bodies (MBs) and the lateral protocerebrum (LPC), and on the other hand Kenyon cells (see Heisenberg 1998), the intrinsic neurons of the MBs.

To investigate the functional significance of these neurons for olfactory conditioning, I used cell-type specific GAL4-lines.

Kenyon Cells

I chose the GAL4-lines 247, c772 and D52H (Zars et al., 2000; Yang et al., 1995; Ron Davis, Univ. of Houston, Texas, pers. comm.) because of their Kenyon cell expression patterns (Fig. 13A-D). Althought only 247 and D52H are MB specific as seen with visualization techniques, all three lines show expression within all subsystems of the MBs. In line c772 additional expression is found in the antennal lobes and the antennal nerves. To estimate the fraction of Kenyon cells manipulatable in a particular line, the number of cells marked was counted by use of the marker gene construct UAS-nls-lacZ coding for a β-Gal protein that is transported to the nucleus (Mader, 2001; Fig. 12E-F). In females, line 247 labels 825 ± 22 (N=8) Kenyon cells per hemisphere, the number labeled in c772 is 871 ± 47 (N=6) (D52H was not counted). Since a single mushroom body consists of approximately 2,500 Kenyon cells (Technau & Heisenberg, 1982), these lines allow for manipulation of about $^1/_4$ to $^1/_3$ of those cells.

Olfactory Projection Neurons

The recently published line GH146 (Fig. 13G) drives expression of GAL4 in about 70% of olfactory projection neurons connecting the AL to the LPC and the MBs (Stocker et al., 1990; Stocker et al., 1997; Heimbeck et al., 2001). Based on their connectivity these neurons can be divided into two classes. About 90 out of the 100 neurons labeled establish dendritic arborisations in one of the 43 AL glomeruli and send their axons to the MBs and the LPR (Jefferis et al., 2001). The remaining 5–10 labeled PNs are mostly of the polyglomerular type, whose fibres bypass the calyx and extend directly to the LPR (Jefferis et al., 2001).

Dopaminergic Neurons

Biosynthesis of the catecholaminergic neurotransmitter dopamine includes hydroxylation of tyrosine, a step catalysed by the enzyme tyrosine hydroxylase (TH). The TH-GAL4 line drives espression of GAL4 under control of the 5´-upstream regulatory region of the *TH*-locus in nearly all dopamine-immunopositive cells (Friggi-Grelin et al., 2003). I visualised dopaminergic innervation of the MBs by use of a 3D-reconstruction based on whole-mount specimens, double stained with nc82-antibody as neuropil marker and anti-TAU-antibody in TH-GAL4/UAS-TAU animals (Fig. 13H-J). The MBs are innervated by the

dopaminergic system specifically in the α-lobe and at the level of the heel, which consists of fibres of the γ-system. Additionaly, I found weaker innervation at the level of the calyx.

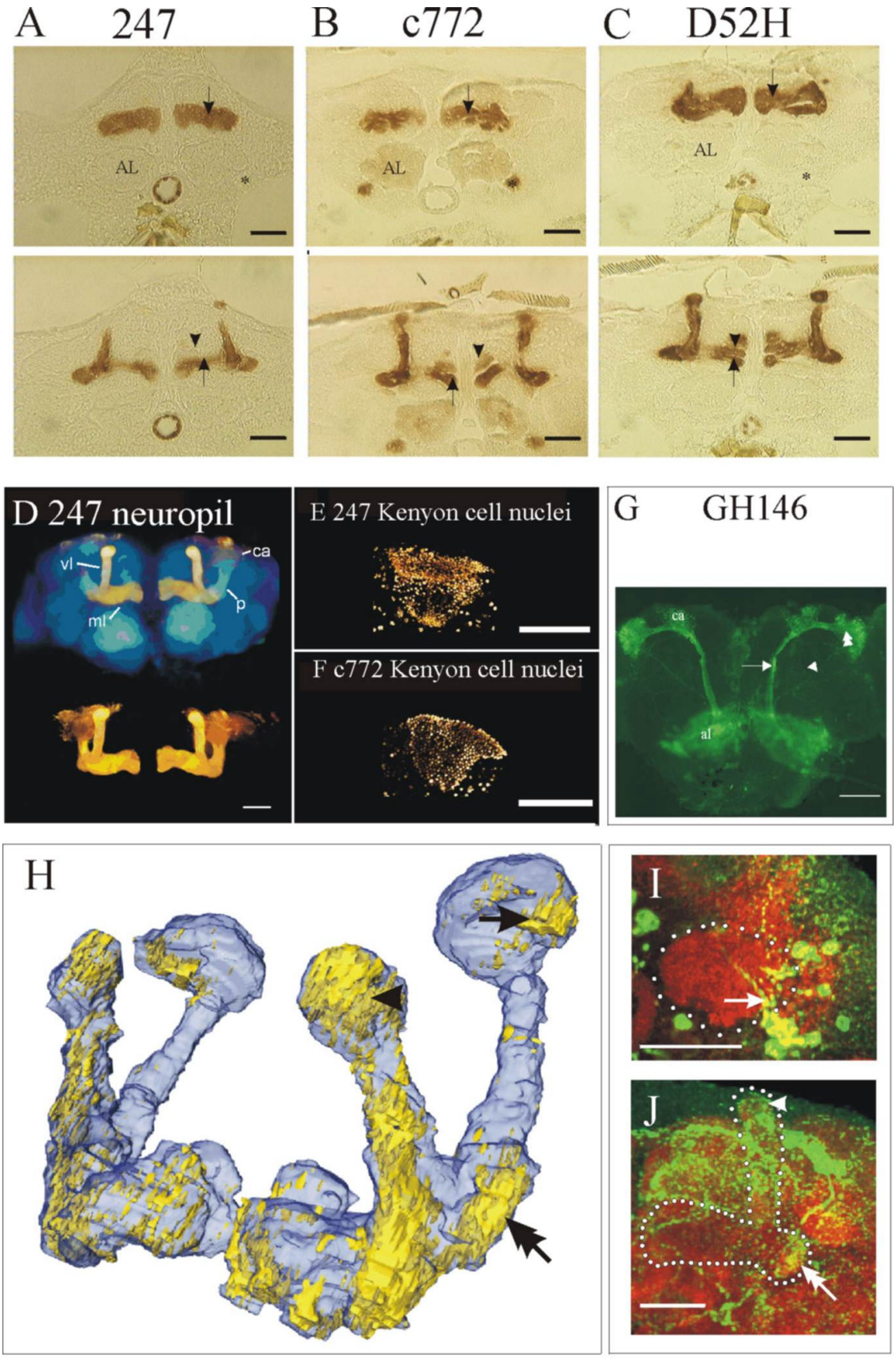

Figure 13: Immunohistochemistry of GAL4-lines (previous page). (A-C) I stained progeny of crosses between the GAL4-lines (247, c772 and D52H) and an UAS-TAU-marker line with a monoclonal anti-TAU antibody on 7 µm horizontal paraffin sections. Expression in the γ-lobes (arrow in the *upper panel*) is detected in all three lines, antennal lobes (AL) and antennal nerve (*) remain unstained, except for line c772. Expression is also detected in the medial and vertical lobes of the α/β- (arrow) and α`/β`- systems (arrowhead in the *lower panel*). **(D)** Whole-mount preparation of a 247/UAS-lacZ specimen, double stained with a monoclonal anti-β-gal-antibody (false colored in yellow) and the nc82 neuropil-marker (false colored in blue). The mushroom bodies are a paired neuropil within the brain, with a dendritic input (Calyx-ca) and axonal projections that proceed anterior in the brain via the peduncle (p), where many intrinsic cells bifurcate to form the vertical (vl) and medial (ml) lobes. The β-gal-signal is restricted to the mushroom bodies in GAL4 line 247 (*lower panel*). **(E-F)** To quantify the fraction of Kenyon cells expression of the nuclear located UAS-nls-lacZ-markergene in females of line 247 (825 ± 22, N=8) and c772 (871 ± 47 ,N=6) was used. **(G)** For visualisation of the projection neurons a whole-mount preparation of line GH146 crossed to the UAS-GFP-marker was used and GFP fluorescence was visualized. Olfactory projection neurons connect the antennal lobes (al) to the lateral horn (double arrowhead) via two tracts, the inner antennocerebral tract (ACT, arrow) forming a colateral to the calyx of the mushroom bodies (ca), and the outer ACT (arrowhead). **(H)** 3D-reconstruction of the MB-neuropil (shades of blue) and its innervation by fibres of the TH-GAL4 line (yellow). Innervation is restricted to the level of the heel (double arrow) and the α-system (arrowhead). Innervation on the level of the calyx (arrow) is weak. Reconstruction is based on whole-mount preparation of TH-GAL4/UAS-TAU specimen double stained with a polyclonal anti-TAU-antibody (false colored in green) and the nc82 neuropil-marker (false colored in red). Stacks of 10 or 30 optical sections on the level of the calyx (arrow in **I**) or the heel and α.lobes (double arrow or arrowhead in **J**). Scale bars represent 50 µm in all figures. (Figure D-F were taken from Schwaerzel et al.,2002 and Figure G was taken from Fiala et al., 2002).

3.3 – Localizing storage sites of elecric shock memory

The mushroom bodies (MBs) have been shown to be necessary for olfactory learning and memory by various experimental approaches (Heisenberg et al., 1985; deBelle & Heisenberg, 1994; Connolly et al., 1996). Even more, they are sufficient with regard to the function of the adenyly-cyclase *rutabaga (rut)*, as shown by MB-specific expression of the UAS-rut^{+} cDNA in *rut*-mutant background, suggesting that formation of olfactory memories occurs within the Kenyon cells by a cAMP-dependent mechanism of synaptic plasticity (Zars et al., 2000). This raises the question whether olfactory memories are maintained within those cells or whether synaptic plasticity in additional structures will become necessary during its consolidation. These hypotheses are tested by monitoring performance of olfactory memory at extended time points using the MB-specific GAL4 line 247 in the *rut*-rescue approach.

Wild-type Canton-S flies show a small decay of about 25% in memory over the 3 hours tested (Fig. 14). MB-rescued *rut*-mutant flies (*rut*; 247, UAS-*rut*) and wild-type flies with overexpression of the *rut*-transgene in the MBs (CS; 247, UAS-*rut*) show a performance similar to wild-type flies at all time points tested. This is in contrast to the *rut* mutant and the *rut* mutant flies with either the UAS-*rut* effector (*rut;* UAS-*rut*) or the 247 GAL4 driver (*rut;*

247) transformant alone. They show a drastically lower performance at all time points compared to MB-rescued *rut* mutant and wild-type Canton-S flies. In detail, *rut*-independent memory in the *rut*-mutant flies decayed from about 30% of the wild-type level of performance at 3 minutes to about 20% at 30 minutes and to about zero at 3 hours after training. The experiment shows that no further *rut*-dependent synaptic plasticity outside the MBs is involved in the consolidation of the memory trace during the first 3 hours after training. If one assumes any synaptic plasticity to be *rut*-dependent at this time, one must conclude that at 3 hours olfactory memory is still localised in the ~800 Kenyon cells.

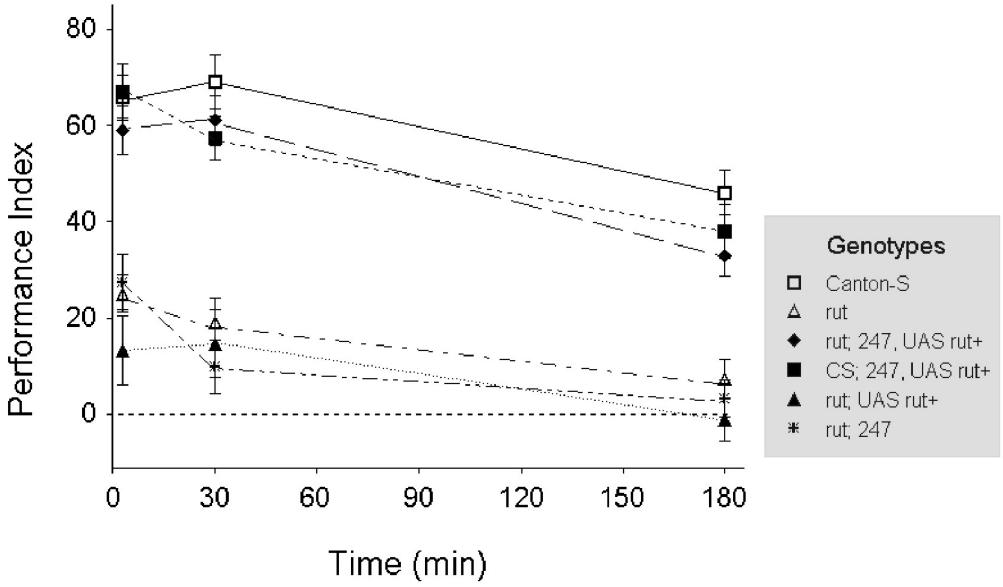

Figure 14: r*ut*-dependent memory is stored within the Kenyon cells of the MBs. Flies mutant for the *rut*-locus but with expression of the wild-type form of the gene in the mushroom bodies using the GAL4 driver 247 (*rut*; 247,UAS-*rut*+) show olfactory memory similar to wild-type Canton-S (CS) and flies overexpressing the rut-rescue construct (CS; 247,UAS-*rut*+) at 3, 30 and 180 minutes after training with electric shock (ANOVA: ps > 0.05). In contrast, *rut*-mutant flies (rut) and *rut*-control flies, expressing each of the two constructs alone (*rut*; UAS-*rut*+ and *rut*; 247) show signifficantly lower performance at all time points (ANOVA: ps < 0.005). Thus, the *rut*-mutant and *rut*-mutant controll flies show a strongly reduced performance at all time points compared to wild-type Canton-S flies (ANOVA: ps < 0.001). Each data represents the mean of at least 6 experiments plus or minus the SEMs. This figure was taken from Schwaerzel et al., 2002.

However, since *rut*-independent memory may exists, the possibility was considered that other, yet unknown molecular mechanisms of synaptic plasticity might be involved. This would open the possibility of spreading or transfering the memory trace to other regions of the brain while leaving the MBs as the only *rut*-dependent site of memory formation. To address this issue, I used MB-specific GAL4 drivers and the UAS-*shibire*^{ts1} (UAS-*shi*^{ts1}) transgene, which allows for conditional silencing of chemical synapses depending on the ambient temperature (Kitamoto, 2001). As a first step, I determined restrictive and permissive

temperature conditions within the pavlovian paradigm. I expressed temperture-sensitive UAS-*shi*^{ts1} in the Kenyon cells of the MBs using the GAL4 drivers 247 and c772 and monitored the effects on performance of 3-minute memory caused by temperature dependent inactivation of Shibire using two different lines: UAS-Shi1 with a single copy of UAS-*shi*^{ts1}, and UAS-Shi2 bearing two copies. Raising the temperature from 26°C in steps of 2 degrees lead to a decrease in memory in both GAL4 lines (Fig. 15A & B). However, to show an effect line c772 required two copies of the transgene (c772/UAS-Shi2) compared to one copy for line 247 (247/UAS-Shi1). At 32°C both GAL4-drivers (c772/UAS-Shi2 and 247/UAS-Shi1 or 247/UAS-Shi2) are maximally blocked, indicated by no further decrease in performance with further raising the temperature to 34°C. Surprisingly, performance of memory could not be blocked completely as indicated by significant performance even at the restrictive temperatures. This finding could either be attributed to only a partial block of synaptic transmission by the trangene or to relevant Kenyon cells not expressing the transgene-effector. Nevertheless, I defined 26°C as the permissive and 32°C or 34°C as the restrictive temperature for subsequent experiments.

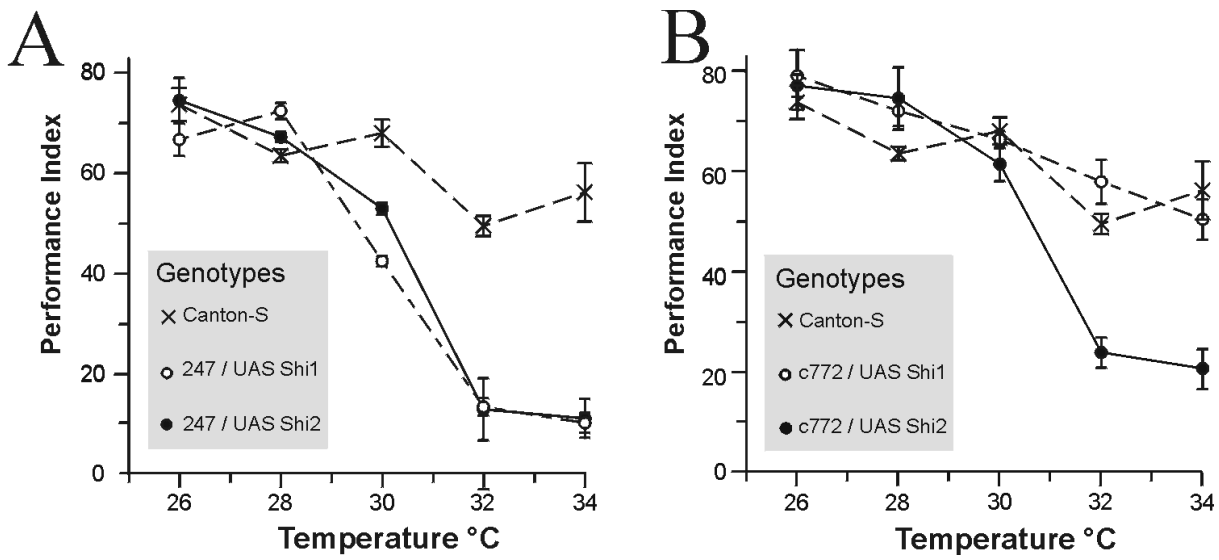

Figure 14: Temperature-dependent block of synaptic transmission. To determine permissive and restrictive temperatures we assayed performance of olfactory memory directly after training at distinct temperatures. **(A)** Flies expressing either one or two copies of the UAS-*shi*^{ts1} transgene (247/ UAS-Shi1 and 247/UAS-Shi2) show reduced memory scores compared to wild-type Canton-S flies when trained and tested at temperatures of 30°C and above (ANOVA: ps < 0.001). **(B)** Flies expressing only one copy of the transgene (c772/UAS-Shi1) show performance of memory similar to wild-type Canton-S flies at all temperatures tested (ANOVA: ps > 0.05). Adding an additional copy (c772/UAS-Shi2) resulted in signifficantly lower performance at temperatures of 32°C and above (ANOVA: ps < 0.001). Each data point represents the mean of six experiments plus or minus the SEMs. Note that data on Canton-S flies are identical in (A) and (B).

In order to investigate the functional significance of neurons along the olfactory pathway for maintaining an olfactory memory, I expressed the UAS-*shi*^{ts1} transgene in the MBs and the PNs. Experiments done on conditioning of the proboscis extension reflex in honeybees suggest an additional, perhaps redundant memory trace in the antennal lobes (Hammer & Menzel, 1998; Faber et al., 1999). In *Drosophila*, I used expression of UAS-*shi*^{ts1} in the projection neurons of the GAL4 driver GH146 to address this hypothesis for electric shock memory. Blocking synaptic transmission at the level of the MBs (GAL4 lines: 247, c772 and D52H) caused a significant reduction in performance of 3-minute memory when trained and tested at the restrictive temperature compared to genetic control flies that are heterozygous for each of the transgenes (GAL4/+ and UAS-Shi1/+ or UAS-Shi2/+), and to the same genotype at the permissive temperature (Fig. 16). Expressing the transgene in the projection neurons (GH146/UAS-Shi2), which are presynaptic to the MBs and provide them with input from the antennal lobes, also resulted in a reduction of performance at the restrictive temperature. The 247, c772, D52H and GH146 heterozygous flies were only tested at the restrictive temperature, as they already showed wild-type like performance with expression of the transgene at 26°C.

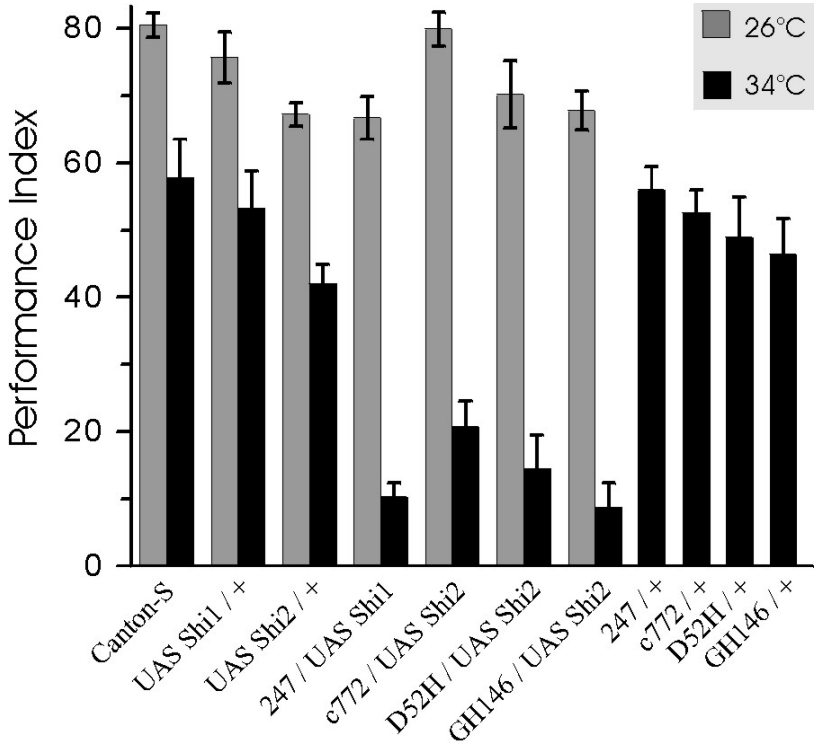

Figure 16: Olfactory memory at 3 minutes after training. All flies expressing the UAS-*shi*^{ts1} transgene in neurons along the olfactory pathway show a severe reduction in performance of olfactory memory at the restrictive temperature (black bars) compared to the permissive temperature (grey bars) and genetic controls at the retrictive tempertaure (ANOVA: ps < 0.001). Statistical analysis revealed no difference between silencing either olfactory projection neurons (GH146/UAS-Shi2) or Kenyon cells (247/UAS-Shi1, c772/UAS-Shi2 and D52H/UAS-Shi2) (ANOVA: ps > 0.05). Each data point represents the mean of six experiments plus or minus the SEMs.

As expected, blocking of MBs output effects performance of olfactory memory. The finding that blocking synaptic output from the PNs also effects performance of memory, needs sophisticated control experiments to rule out the most trivial explanation of having anosmic flies. For that reason, all genotypes showing a memory deficit and the appropiate control genotypes were tested for olfactory perception and shock reactivity to determine whether any of the above changes in olfactory memory were simply due to impairment in perceiving the task relevant stimuli. Experimental genotypes and conditions under which memory scores were normal were assumed to not negatively influence shock or odor perception. Table 2 shows that wild-type and *rut*-mutant flies were not significantly different in responding to either cue used in these experiments.

Genotype	Shock Avoidance Index	Odorant Avoidance Index
Canton S	87.3±4.2	87.3±3.8
rut^{2080}	74.9±7.1	83.3±3.8
rut^{2080}; UAS-*rut*+	78.6±6.0	94.9±1.8
rut^{2080}; 247	71.5±6.1	89.7±3.1
Canton S *	67.0±6.5	93.0±2.2
UAS *shi1*/+ *	51.7±5.0	90.3±1.3
UAS *shi2*/+ *	50.0±3.0	91.5±3.0
247/+; UAS-*shi1* /+ *	53.1±5.2	95.6±1.2
c772/+; UAS-*shi2* /+ *	58.1±5.2	91.6±2.2
GH146/+; UAS-*shi2* /+ *	48.9±3.4	89.3±1.3

Table 2: Sensory acuity tests. Neither the rescue of the *rutabaga* olfactory learning phenotype nor the silencing of Kenyon cell or projection neurons synaptic output caused behaviourally significant changes in electric shock or odorant sensitivity. Wild-type Canton-S (CS), *rutabaga* (*rut*) mutant and flies with silenced Kenyon cells or proje-ction neurons synapses (at restrictive temperature *) were tested for response to electric shock and the olfactory cues used in the learning experiments. There were no significant changes between CS and *rut*-mutant flies in either assay (ANOVA: ps > 0.05). The flies with blocked synapses from the Kenyon cells or the projection neurons were not different from wild-type Canton-S at the restrictive temperature in odor avoidance. There was a significant difference in avoi-dance of electric shock between CS-flies and flies expressing the UAS-Shi1 or UAS-Shi2 transgene, irrespective of the presence of a GAL4-driver (ANOVA: ps < 0.05). But comparing these flies, which learn normally (UAS-Shi1/+ and UAS-Shi2/+), to the rest of the memory impaired genotypes' electric shock avoidance revealed no further difference (ANOVA: ps > 0.05). Consequently, these changes in avoidance of electric shock are not significant for the olfactory learning phenotype. Each data point represents the mean of six experiments plus or minus the SEMs.

Also, flies expressing the UAS-*shi*^{ts1} transgene in the MBs or the projection neurons were investigated at the restrictive temperature (*). Those flies were not significantly different from wild-type controls in detecting the odorants used here. The odorant avoidance index is the mean of the two indices for the olfactory cues benzaldehyde and 3-octanol used in the associative experiments. Controls for electric shock avoidance showed that UAS-Shi1 and UAS-Shi2 heterozygous flies scored significantly lower than wild-type flies. However, since these flies showed normal memory formation, this is not behaviorally significant with respect

to learning. Thus, the changes in olfactory memory measured here were not simply due to changes in either of the cogent sensory modalities.

With respect to the PNs blocked flies GH146/UAS-Shi2 the esult was not unexpected. In theGAL4-line GH146 two populations of PNs are affected connecting either the antennal lobes (AL) to the calyx of the MBs and to the lateral protocerebrum (LPC), or connecting the AL directly to the LPC (Heimbeck et al., 2001). Blocking synaptic transmission in GH146 flies effects olfactory driven behavior in a concentration dependent manner, showing wild-type responses to olfactory cues at high concentration and becoming insensitive to lower concentrations (Heimbeck et al., 2001). As the olfactory cues used here were of high concentrations, GH146/UAS-Shi2 flies are still capable to show normal spontaneous olfactory driven behaviour, as do flies with the MBs output blocked. Consequently, neither the MBs themselfs nor the PNs labeled in line GH146 are necessary for this type of experience-independent olfactory behavior. On the other hand, this experiment shows that for experience-dependent olfactory behavior both, the PNs labeled in line GH146 and the MBs output are necessary. These results are in line with the hypthesis that the memory trace is indeed formed within the Kenyon cells. Blocking synaptic output from the MBs would prevent recall of the memory, whereas a block at the level of the PNs does isolate the MBs from necessary olfactory information.

In the subsequent experiments, I asked whether PNs or MBs output signaling is necessary during the distinct phases of memory acquisition and retrieval by performing training and test at either permissive or restrictive temperatures, respectively. When kept at permissive temperature throughout the experiment, performance of 30-minute memory was largely unaltered in flies expressing the UAS-shi^{ts1} transgene either in the MBs or the PNs (Fig. 17A). When trained at the permissive temperature but raised to the restrictive temperature for testing (presumably turning off output signaling), performance of memory was severely reduced in flies with either the MBs or the PNs blocked during test (Fig. 17B). Next, I inverted the temperature regime, training the flies under blocked conditions but tested them under permissive conditions (Fig. 17C). Surprisingly, performance of memory was normal in flies with UAS-shi^{ts1} expressed in the MBs (247/UAS-Shi1, c772/UAS-Shi2 and D52H/UAS-Shi2), but was still abolished in flies with expression in the projection neurons (GH146/UAS-Shi2).

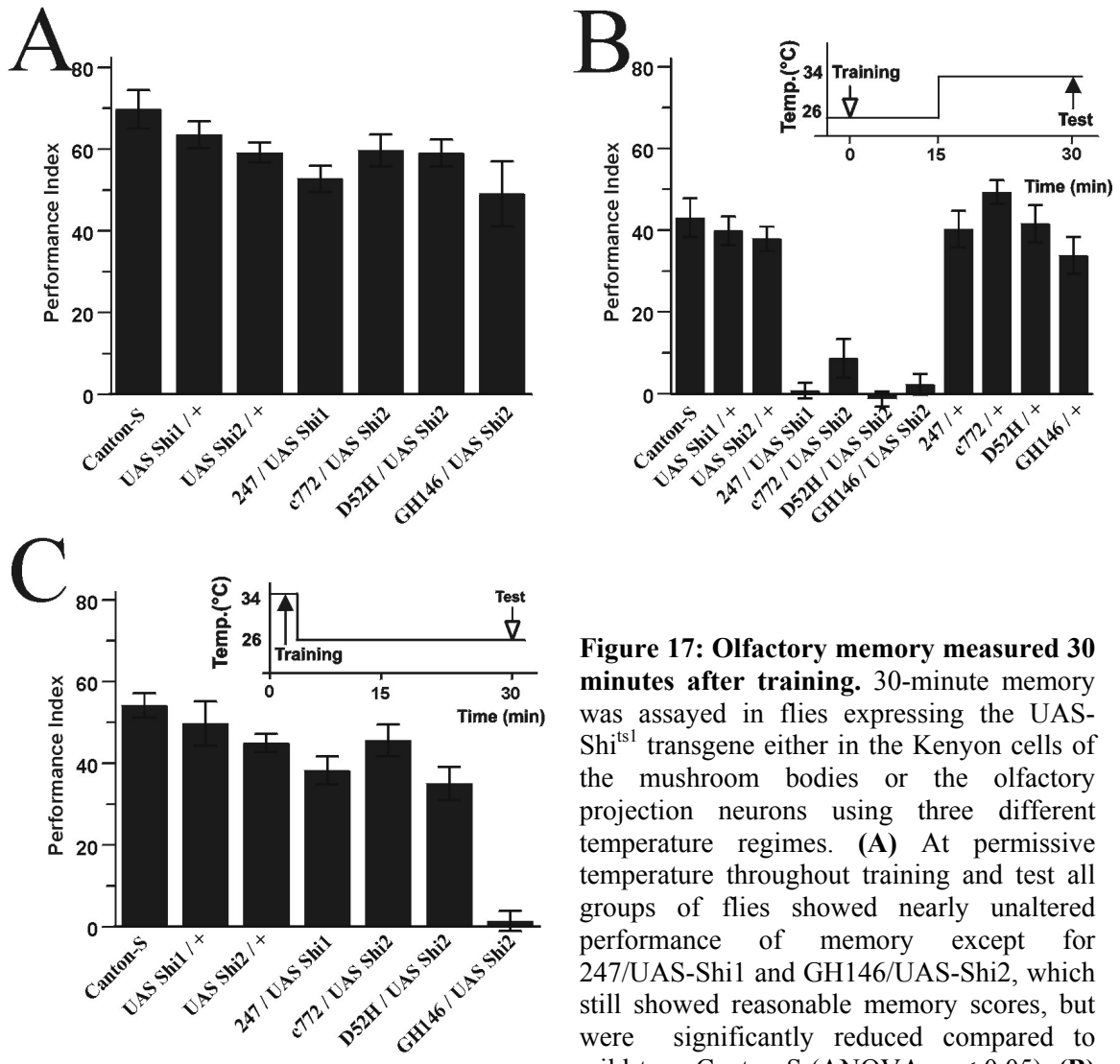

Figure 17: Olfactory memory measured 30 minutes after training. 30-minute memory was assayed in flies expressing the UAS-Shi^{ts1} transgene either in the Kenyon cells of the mushroom bodies or the olfactory projection neurons using three different temperature regimes. **(A)** At permissive temperature throughout training and test all groups of flies showed nearly unaltered performance of memory except for 247/UAS-Shi1 and GH146/UAS-Shi2, which still showed reasonable memory scores, but were significantly reduced compared to wild-type Canton-S (ANOVA: $p < 0.05$). **(B)** Raising the temperature from permissive to restrictive conditions 15 minutes before the test resulted in complete absence of memory scores in those flies expressing the UAS-s*hi*^{ts1} transgene compared to their genetic control groups (ANOVA: ps < 0.001). **(C)** Memory scores of flies expressing the UAS-shi^{ts1} transgene in the Kenyon cells of the mushroom bodies were largely unaltered compared to it´s genetic controls, when trained under restrictive conditions but tested at the lower temperature. Except for 247/UAS-Shi1 and D52H/UAS-Shi2, which still showed a reasonable, but significantly reduced memory score (ANOVA: ps < 0.05). Expression of the UAS-*shi*^{ts1} transgene in the olfactory projection neurons in GH146/UAS-Shi2 totally abolished the memory score (ANOVA: $p < 0.001$). Each data point represents the mean of six experiments, except GH146/UAS-Shi2 in **(A,** n=2) plus or minus the SEMs.

These experiments allow two conclusions: First, output signaling from the MBs is required for retrieval of olfactory memory but surprsingly not for its acquisition, indicating that synaptic plasticity can be induced upstream of the MBs synaptic output. Second, the finding that blocking output from the PNs during acquisition is preventing olfactory memory formation might indicate that synaptic plasticity within the MBs is not only a sufficient site of

synaptic plasticity for olfactory memory formation, as shown by the *rut*-rescue experiments (Fig. 14), but the only site of plasticity for this type of olfactory memory in *Drosophila*.

As time between training and test proceeds, olfactory memories consolidate and become resistant to disturbances like cooling or feeding of pharmacologic agents within 60 minutes (Tempel et al., 1983). This indicates physiological changes takeing place during the process of consolidation (Tempel et al., 1983; Tully et al., 1994, Dubnau et al., 2002). In mammals, the consolidation processes require crosstalk between different regions of the brain (Day & Morris, 2001). To test if a largely consolidated memory (Tully et al., 1994) still requires MB output, 3-hour memory was tested in line c772 expressing the UAS-Shi^{ts1} transgene (Fig 18). Unfortunately, expression of the transgene with the 247 and D52H driver lines altered memory performance even at the "permissive" temperture of 26°C in a time dependent manner, leading to a slow decay of performance (data not shown, but seen as a significant reduction already at 30 minute memory). Nevertheless, when c772/UAS-Shi2 flies were trained at the permissive temperature and tested after 3 hours having been shifted to the restrictive temperature only 15 minutes earlier, they showed no memory, in contrast to all genetic and temperature controls. As GAL4 line c772 shows obvious expression in the antennal lobes (see Fig 13B) it should be mentioned that odor perception is normal under restrictive conditions in this line expressing the UAS-Shi^{ts1} transgene (see Table 2).

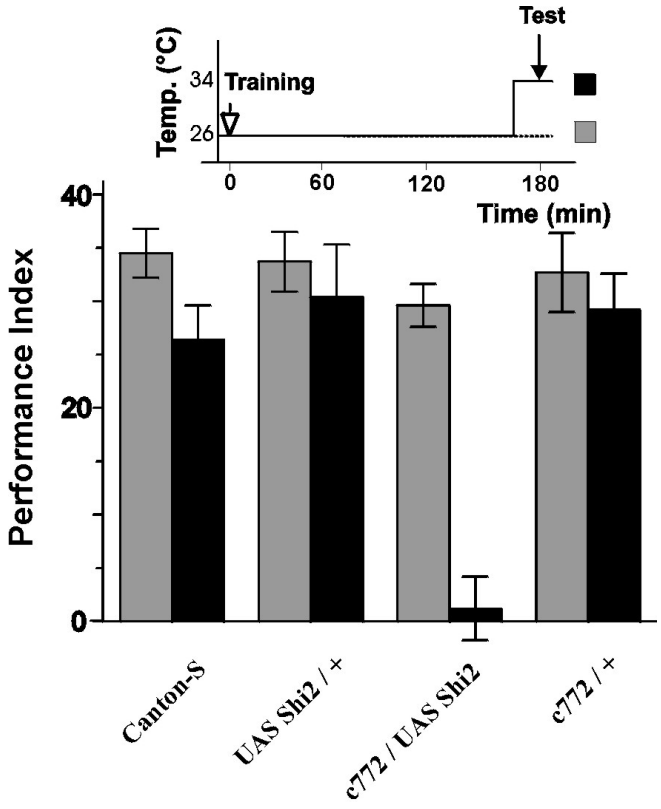

Figure 18: Olfactory memory at 3 hours. 180-minute memory of c772/UAS-Shi2 flies is completely abolished when flies are shifted to the restrictive temperature 15 minutes before test. In contrast, the same genotype at the permissive temperature and all genetic controls at either temperature did perform normally (ANOVA: ps < 0.001). Each data point represents the mean of six experiments plus or minus the SEMs.

This experiment shows that for up to 3 hours after training, synaptic output from the Kenyon cells is still necessary for flies to show memory performance. As *rut*-dependent plasticity at this synapse is also sufficient for memory perfomance after 3 hours (see Fig. 14) argues that a more consolidated form of olfactory memory is still localized within the Kenyon cells of the MBs.

3.4 –Localizing extinction of electric shock memory

Extinction occurs when a CS+, that has formed an association with a reinforcer (US) is now presented in the absence of the US. This treatment leads to a rapid decline of memory performance as the contingency between the CS+, being a predictor of the US, becomes invalid. In vertebrates, two alternative hypotheses try to explain the phenomenon of extiction, either involving a process of `re-learning` including new associations, or an erasure of the original memory trace (Bouton, 1993; Bouton et al., 1999; Myers & Davis, 2002). Whether any of these processes acts within cells or between cells is not known. With the tools developed to localize olfactory memory, it was now attempted to determine at what circuit level extinction of olfactory memories occurs in *Drosophila*.

Extinction of memory was tested by exposing the flies five times to the formerly US-associated odorant (CS+) right after training and measure performance of 30-minute memory. Since the olfactory learning paradigm used differential conditioning, that is exposure to both a punished (CS+) and an unpunished odorant (CS-), memory was extinguished by re-exposing half of the flies to the CS+ and the other half to the CS-, ruling out possible adaptation effects of re-exposure (see Dalton, 2000). In wild-type Canton-S flies, re-exposure to the CS+ induced a 40% reduction in performance compared to flies exposed to CS- (Fig. 18A). A post-hoc analysis between wild-type Canton-S flies exposed to the CS- compared to flies not re-exposed to odorants revealed no decrement in 30-minute memory (compare wild-type Canton-S in figure 14, 17A and 19A). This was not unexpected as the contingency `no punishment – CS-` did not change.

As the *rutabaga* mutation has thus far been proven to be necessary for every learning paradigm in which it was tested (see Davis, 1996) and extinction is hypothezised to be a re-learning process, it was examined whether *rut*-dependent synaptic plasticity outside the MBs was required for extinction. MB-rescued *rut*-mutant flies (*rut;* 247, UAS-*rut*+) showed

extinction and extinction control performance indistinguishable from wild-type Canton-S flies (Fig. 19A).

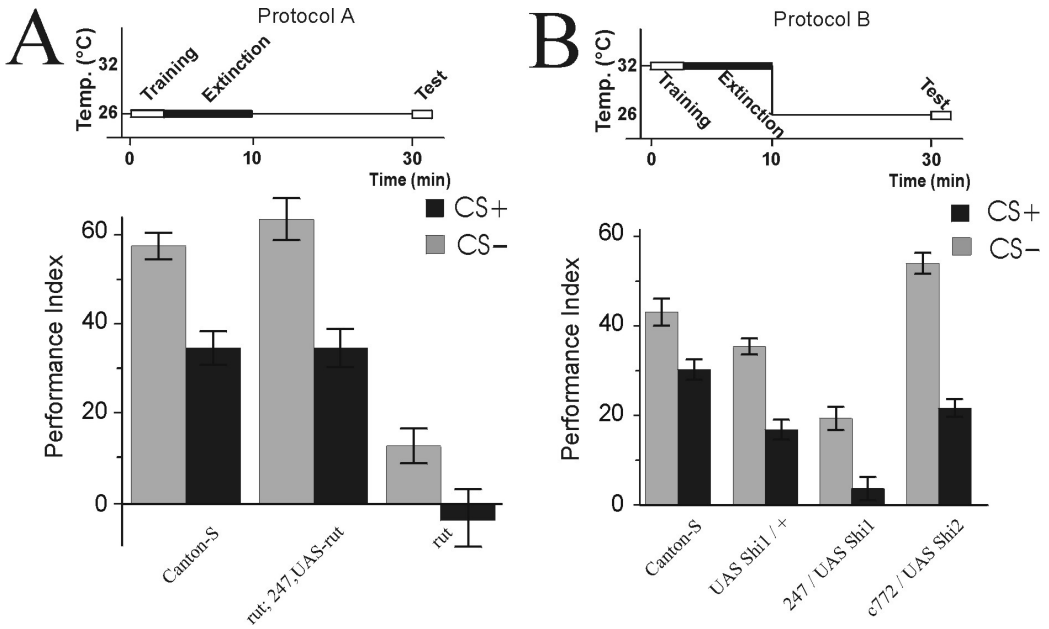

Figure 19: Extinction of olfactory memory. Flies were trained and subsequently exposed five times for 1 minute to the previously punished (CS+) or unpunished (CS-) odorant during the extinction procedure and then tested for 30 minutes memory. **(A)** Wild-type Canton-S (CS), *rut*-rescued (*rut*; 247, UAS-*rut*+) and *rut*-mutant (*rut*) flies exposed to the CS+ showed significantly lower memory scores than flies of the same genotype exposed to the CS- (ANOVA: ps < 0.05). There were no deleterious effects on performance with exposure of the CS- (compared to figure 4 and 7a for 30-minute memory scores, ANOVA: ps > 0.05). **(B)** Wild-type Canton-S, UAS-Shi1 controls (UAS-Shi1/+), 247/UAS-Shi1 and c772/UAS-Shi2 flies were trained and their memory was extinguished at the restrictive temperatures. Memory was scored at 30 minutes at the permissive temperature. Extinction occured in all genotypes, even in those groups with the mushroom body output silenced during training and extinction, 247/UAS-Shi1 and c772/UAS-Shi2 (ANOVA: ps < 0.01). Each data point represents the mean of six experiments plus or minus the SEMs. (This Figure was taken from Schwaerzel et al., 2002)

This result indicates that for extinction to occur the function of the adenyly-cyclase *rut* either is required exclusively in the set of ~800 Kenyon cells in which the memory trace is located or it is not required at all. Interestingly, even the small memory remaining in the *rut*-mutant could be extinguished. This finding favors the idea that extinction could occur independent of *rut*-function. Alternatively, one would have to argue that the *rut*-allele used here affects only memory formation but not extinction.

If extinction involves an as yet unidentified *rut*-independent mechanism of synaptic plasticity, the experiment of figure 19A does not give a hint as to its location in the brain. To restrict the possible brain regions in which it might occur, I again took advantage of the UAS-shi^{ts1} transgene. Flies expressing UAS-shi^{ts1} in the MBs with lines 247 and c772 were trained

and exposed to extinguishing stimuli at the restrictive temperature and then tested at the permissive temperature. Flies without synaptic transmission from the MBs (247/UAS-Shi1 and c772/UAS-Shi2) showed obvious extinction of their olfactory memories (Fig. 19B). Thus, extinction occurs independently of the output in Kenyon cells carrying the memory trace.

In contrast, when olfactory information was blocked at the level of projection neurons exclusively during extinction, using line GH146/UAS-Shi2, flies showed no evidence of extinction (Fig. 20). The c772/UAS-Shi2 flies within the same protocol and the GH146/UAS-Shi2 flies at the permissive temperature throughout the experiment showed obvious memory extinction.

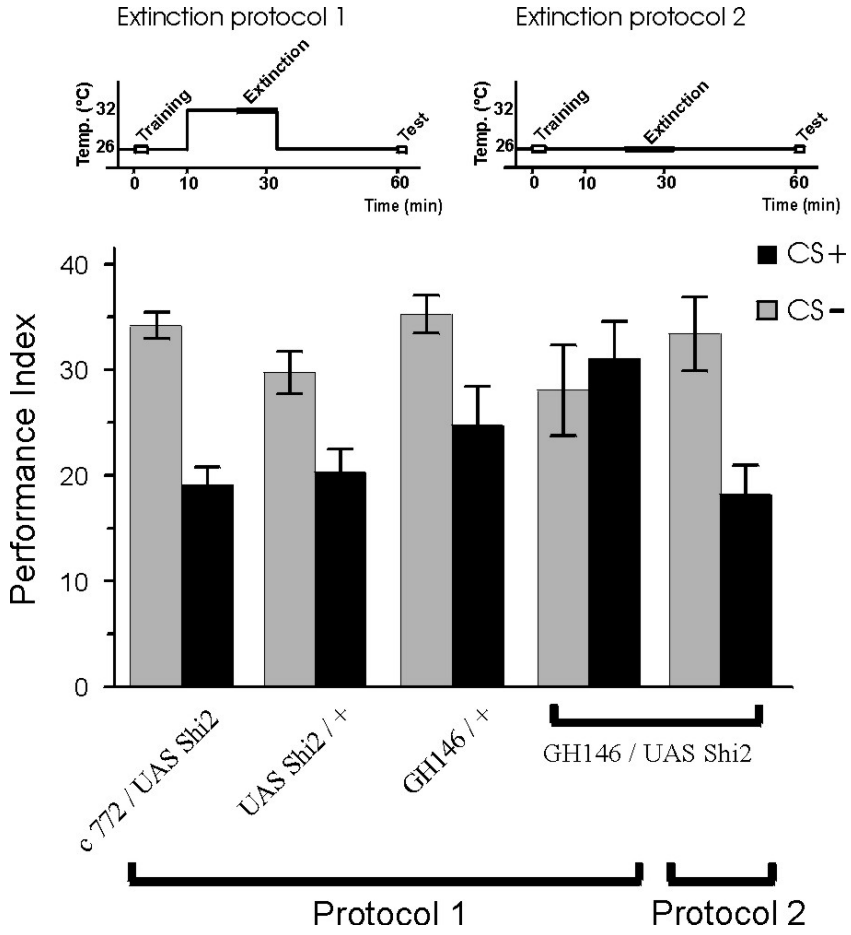

Figure 20: Extinction occurs downstream of the projection neurons. Memory was tested 60 minutes after training in flies expressing the UAS-Shi^{ts1} transgene either in the projection neurons (GH146/UAS-Shi2) or the Kenyon cells of the mushroom bodies (c772/UAS-Shi2). The extinction protocol was applied 20 minutes after training, at either the restrictive (protocol 1) or the permissive temperature (protocol 2), whereas training and test were performed at permissive temperature. When extinguished at restrictive conditions, flies expressing the transgene in the projection neurons (GH146/UAS-Shi2) showed similar high levels of memory, irrespective of whether they were extinguished with the CS+ or CS- (ANOVA: $p > 0.05$). When extinguished at the permissive temperature using the very same olfactory stimuli CS+ and CS-, the same flies showed obvious extinction of olfactory memory (ANOVA: $p < 0.01$). All other genotypes showed obvious extinction, even those flies with MB output blocked during extinction (ANOVA: ps < 0.05). Each data point represents the mean of six experiments plus or minus the SEMs.

These results can be interpreted in several ways: First, the GH146 line labels two classes of olfactory projection neurons, one innervating the MBs and the lateral protocerebrum (LPC) and the second group innervating exclusively the LPC. It remains a possibility that some unknown circuit could carry odorant information that might modulate the memory trace within the Kenyon cells or their output. Second, there might be a change in synaptic strength of a second group of Kenyon cells within the ~800 cells that are labeled in the MB GAL4 lines used here during the extinction experiments. To invoke this mechanism, one would have to imagine how this second set of cells was not recruited in the first place such that the plasticity underlying memory formation could not be initially induced. Moreover, how could odorant exposure in the absence of modulatory input from the reinforcing pathway change synaptic strength in those cells to the opposite direction of the memory encoding cells. Due to the hypotethical nature of these alternatives, the most simple explanation of the results presented here was favoured, stating that extinction is a process of intracellular memory trace suppression rather than an intercellular process.

3.5 – Comparing appetitive and aversive olfactory memories

To address the differences between positive and negative reinforced olfactory memories I compared olfactory learning in *Drosophila* using the same odors but either appetitive (sugar) or aversive (electric shock) USs. To make the comparison stringent, sugar reward learning (Tempel et al., 1983) was adapted to the 4-fold revolver machine, which is a derivative of a device originally designed for aversive electric shock learning (Tully & Quinn, 1985). As conditioned stimuli (CSs) the same two odors, namely ethylacetate (EA) and isoamylacetate (IA), were used throughout. Memory performance was tested in the same binary choice assay, and all flies were starved before the experiment. Consequently, the only difference between the two paradigms was the use of either sugar or electric shock reinforcement during the training.

Note, that here PIs are negative if flies avoid the shock-associated odor and positive if the flies chose the sugar associated odor in the test. I have chosen this convention to reflect the difference in conditioned performance of the flies. In the memory test, wild-type Canton-S flies strongly avoided an odor after having experienced it together with electric shock, PI = – 62.1 ± 4.6 (electric shock memory). They were attracted by the same odor if it had been combined once with sugar, PI = +7.8 ± 2.4 (sugar memory). As the one trial PI for sugar learning is inconveniently small, I extended the number of training cycle to two or four, yielding PIs of +17.7 ± 2.6 and 17.1 ± 2.4, respectively (Fig. 21).

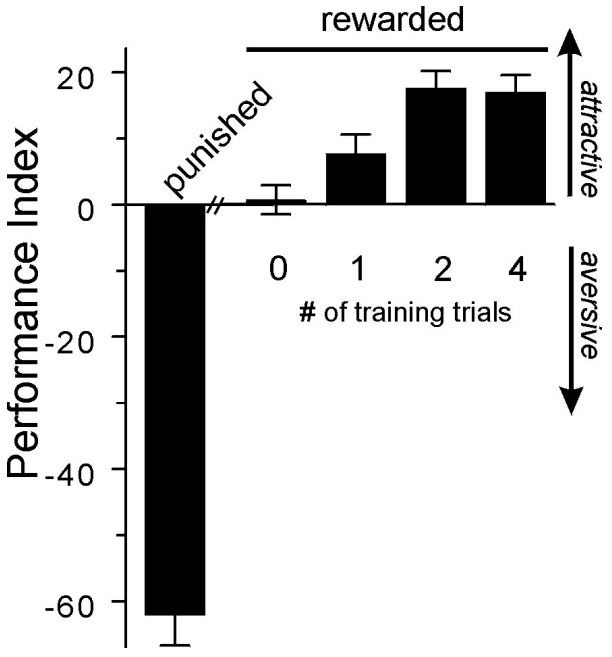

Figure 21: Comparing appetitive and aversive memory. The same olfactory cues could elicited attractive or aversive behavior, depending on the reinforcement used. Wild-type Canton-S flies acquired a strong aversive memory after a single-trial training of electric shock, recieving 12 individual pulses of 130V and 1.3 sec duration within one minute. Attractive memory needed at least two training-trials of sugar-reward to be significant (ANOVA: $p < 0.05$), and 2 additional training trials did not lead to a further increase (ANOVA: $p > 0.05$). Each data point represents the mean of six experiments plus or minus the SEMs.

3.6 – Localizing sugar reinforced olfactory memory

Sugar learning, as well as electric shock learning, has been shown to require cAMP signaling as revealed by the impairment for both types of learning in mutants affecting cAMP metabolism such as *dunce* and *rutabaga* (Tempel et al., 1983). Moreover, sugar learning, like electric shock learning, is impaired in MB mutants (Heisenberg et al., 1985). To determine whether also in sugar learning the memory trace of the odor can be localized to the MBs, I performed localization experiments similar to the ones already performed for electric shock learning using the same MB-specific GAL4 driver line 247 (see Fig. 13). r*ut*-Mutant flies were as much impaired in sugar memory (37% of wild-type per-formance; compare Fig. 22A to Fig. 21) as they were in electric shock memory (42% of wild-type performance; compare Fig. 22B to Fig 21). Mutant flies with wild-type *rut*-AC restored exclusively in the MBs (*rut*; 247, UAS-*rut*+) showed restored memory performance for both sugar and electric shock reinforcement ('rescue' groups in Fig. 22) showing that *rut*-dependent sugar and electric shock memory can be localized to the same set of about 800 Kenyon cells (see Fig. 14).

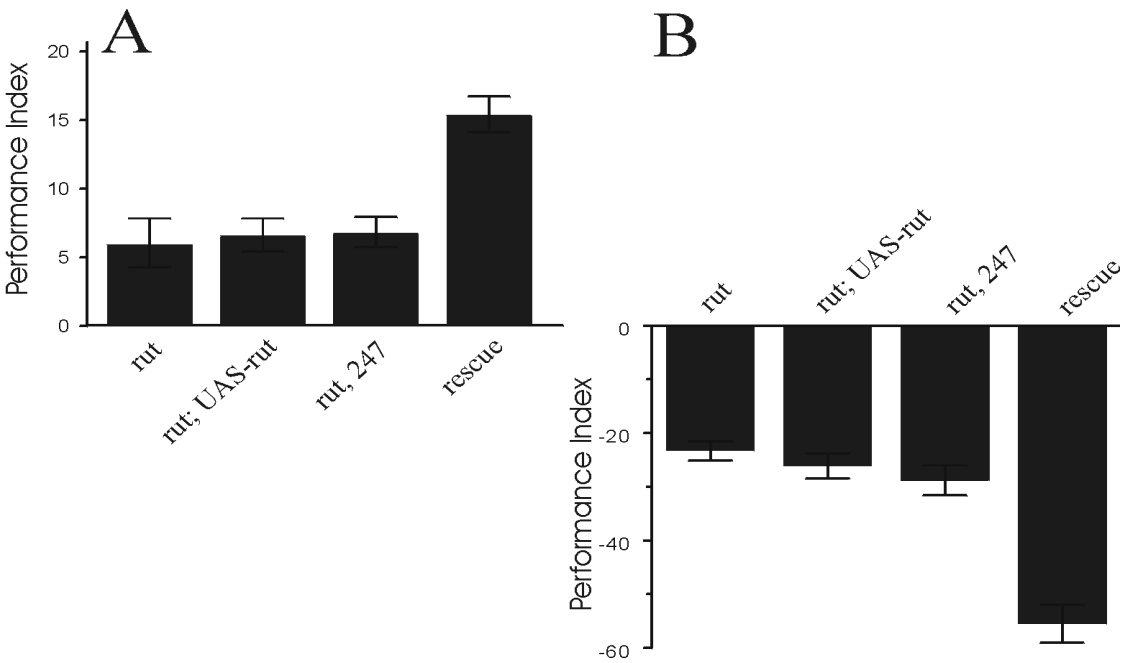

Figure 22: r*utabaga* rescue of aversive and appetitive memories. Flies mutant for the *rut*-locus (*rut* and *rut*; UAS-*ru*+ and *rut*; 247) and those with expression of the wild-type form of the gene in the mushroom bodies using the GAL4 driver 247 (rescue-group - *rut*; 247,UAS-*rut*+) are tested for olfactory memory directly after training using either sugar-reward **(A)** or electric shock punishment **(B).** In both cases *rut*-mutant performance could be rescued up to wild-type Canton-S levels (ANOVA: ps > 0.05). Each data point represents the mean of six experiments plus or minus the SEMs.

Having shown that these Kenyon cells are sufficient to restore appetitive memory, I asked whether they would also be necessary for memory formation, and therefore, used the

dominant negative UAS-*Shibire*^{ts1} allel (UAS-s*hi*^{ts1}) as effector line (Kitamoto, 2001). At the permissive temperature, both sugar and electric shock learning were intact (Fig. 23A, B grey bars), whereas at the restrictive temperature both sugar and electric shock learning were severely impaired (Fig. 23A, B filled bars). The genetic controls (247/+ and UAS-shi1/+) do not show any corresponding decrement in performance. In agreement with earlier results (see Fig. 15 & 16), memory suppression for electric shock at the restrictive temperature is not complete using the UAS-*shi*^{ts1} effector gene (Fig. 23B), whereas sugar memory is abolished completely.

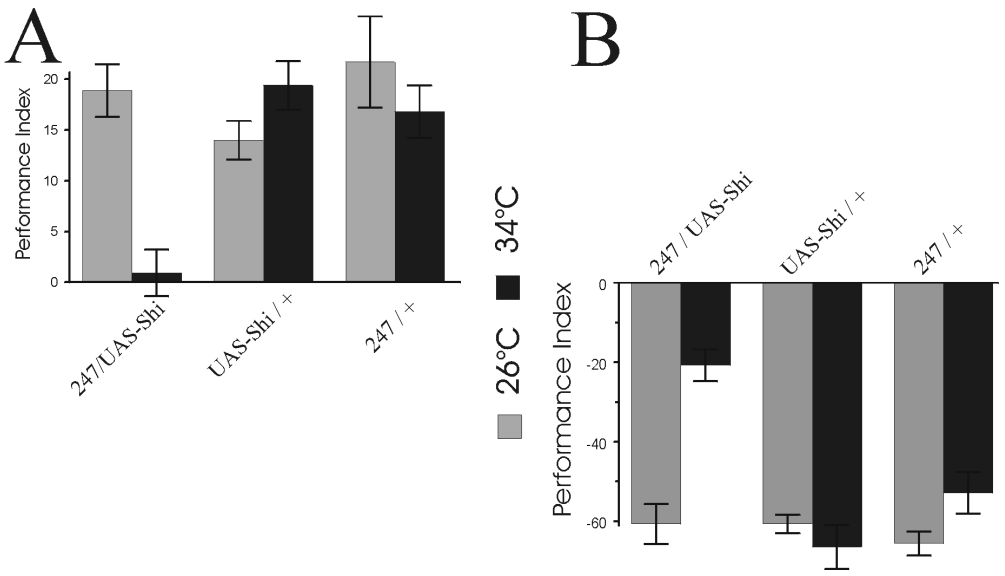

Figure 23: The mushroom bodies are necessary for sugar and elecric shock learning. (A) Restrictive temperature throughout the experiment (black bars) completely abolishes sugar memory in flies expressing the UAS-*Shi*^{ts1} transgene exclusively in the Kenyon cells of the MBs (247-Gal4 / UAS- *Shi*^{ts1}). No temperature dependent decrease is found in genetic control flies heterozygous for each of the transgenes alone (247-Gal4 / + and UAS- *Shi*^{ts1}/ +) (ANOVA: $p < 0.001$). At the permissive temperature all genotypes show normal memory (ANOVA: $p > 0.05$). **(B)** Electric shock memory is strongly reduced at the restrictive temperature in flies expressing the Shi-transgene in the Kenyon cells (247-Gal4 / UAS- *Shi*^{ts1}), compared to genetic controls at either permissive or restrictive temperature (ANOVA: $p < 0.001$). Each data point represents the mean of six experiments plus or minus the SEM.

It is a hallmark of electric shock learning that synaptic output from the Kenyon cells is only required during retrieval but not during acquisition (see Fig. 17); I tested whether this would also apply to sugar learning. I expressed UAS-*shi*^{ts1} in the MBs using the GAL4 driver 247 and measured sugar and electric shock memory in flies that were either trained at the permissive temperature but tested at restrictive temperature (Fig. 24A & C) or were trained at restrictive temperature and tested at permissive temperature (Fig. 24B & D). The outcome, that silencing of Kenyon cell output during acquisition has no effect on formation of either

form of memory, argues that both electric shock and sugar reinforcement can modulate the Kenyon cells while their output is turned off. The output from the MBs becomes necessary during retrieval of both memories, as shown by the decrement in performance of either memory, when tested at the restrictive temperature.

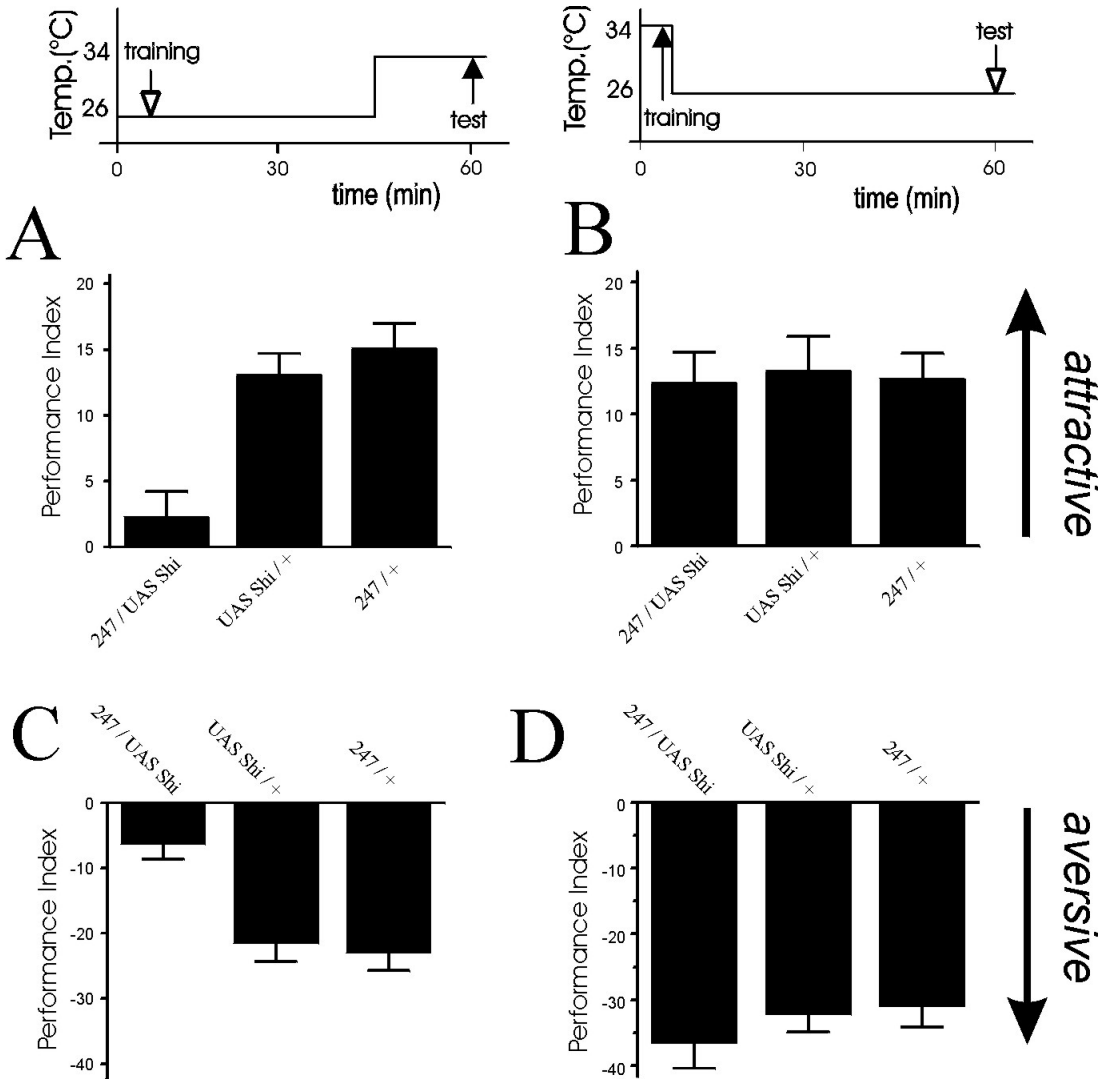

Figure 24: Mushroom body output is required for retrieval of memory. Flies expressing the UAS-*shi*^{ts1} transgene using the 247-driver (247/UAS-Shi1) were assayed for sugar and electric shock memory 60 minutes after training using two different temperature regimes.**(A, C)** Raising the temperature from permissive to restrictive conditions 15 minutes before the test resulted in a drastic decline of memory scores in 247/UAS-Shi1 flies, but not in its genetic control groups (247/+ and UAS-Shi1/+) (ANOVA: ps < 0.001). **(B, D)** Memory scores of 247/UAS-Shi flies were unaltered when trained under restrictive conditions but tested at the lower temperature. (ANOVA: ps > 0.05). Each data point represents the mean of six experiments plus or minus the SEMs.

Together these experiments show that in both, sugar and electric shock learning, the memory traces of the same odor can be localized to the same set of ~800 Kenyon cells, comprising about 30% of the MBs.

The fact that the same CS can be associated with different USs seems to be a common rule including the general accepted assumption, that these memories are stored at different locations within the brain, as suggested by results from other model organisms (for examples see Baxter & Murray, 2002; Maren,2001; Blair et al., 2001). The finding in *Drosophila* that electric shock and sugar memories about the same olfactory cue localize to the same group of Kenyon cells of the MBs obviously contradicts the above stated assumption. To further investigate the problem how appetitive and aversive memory traces of the same odor can invoke different behaviors, I focused on the modulatory systems required to represent the different USs, electric shock and sugar.

3.7 – Octopamine is required for sugar memory

In the honeybee the modulating transmitter mediating the reinforcing properties of the US in sugar conditioning of the proboscis extension reflex (PER) is octopamine (OA, Hammer & Menzel, 1998). This prompted me to investigate the role of OA in sugar and electric shock learning in *Drosophila*. I used the Tyramine-β-Hydroxylase (*T*β*H*) deficient mutant $T\beta H^{M18}$, which is blocked in the last step of the biosynthetic pathway to octopamine and shows reduced OA levels (Monatsirioti, 1996). Tested for electric shock memory, $T\beta H^{M18}$ flies perform indistinguishably from the respective wildtype control (Fig. 25A). In contrast, when tested for sugar memory (Fig. 25B) the mutant is severely impaired resulting in PIs near zero. Thus, OA is specifically necessary for sugar learning, but not for electric shock learning.

To investigate if it was indeed the TβH gene which caused this effect, I restored *T*β*H*-function using a transgene containing the wildtype *T*β*H*-cDNA downstream of the hsp70-promoter (Fig. 25C). Upon heatshock, these flies ($T\beta H^{M18}$; hsp70-*T*β*H* HS+) showed wild-type performance in sugar memory, compared to the control line in Fig. 25B. The heatshock itself has no memory-enhancing effect in mutant $T\beta H^{M18}$ flies, but I observed an intermediate phenotype in the rescue flies without heatshock ($T\beta H^{M18}$; hsp70-*T*β*H* HS-). This likely indicates background expression of the hsp70-*T*β*H* transgene with the rearing conditions of 25°C. These results support the notion that impairment in sugar learning is directly caused by loss of *T*β*H*-function.

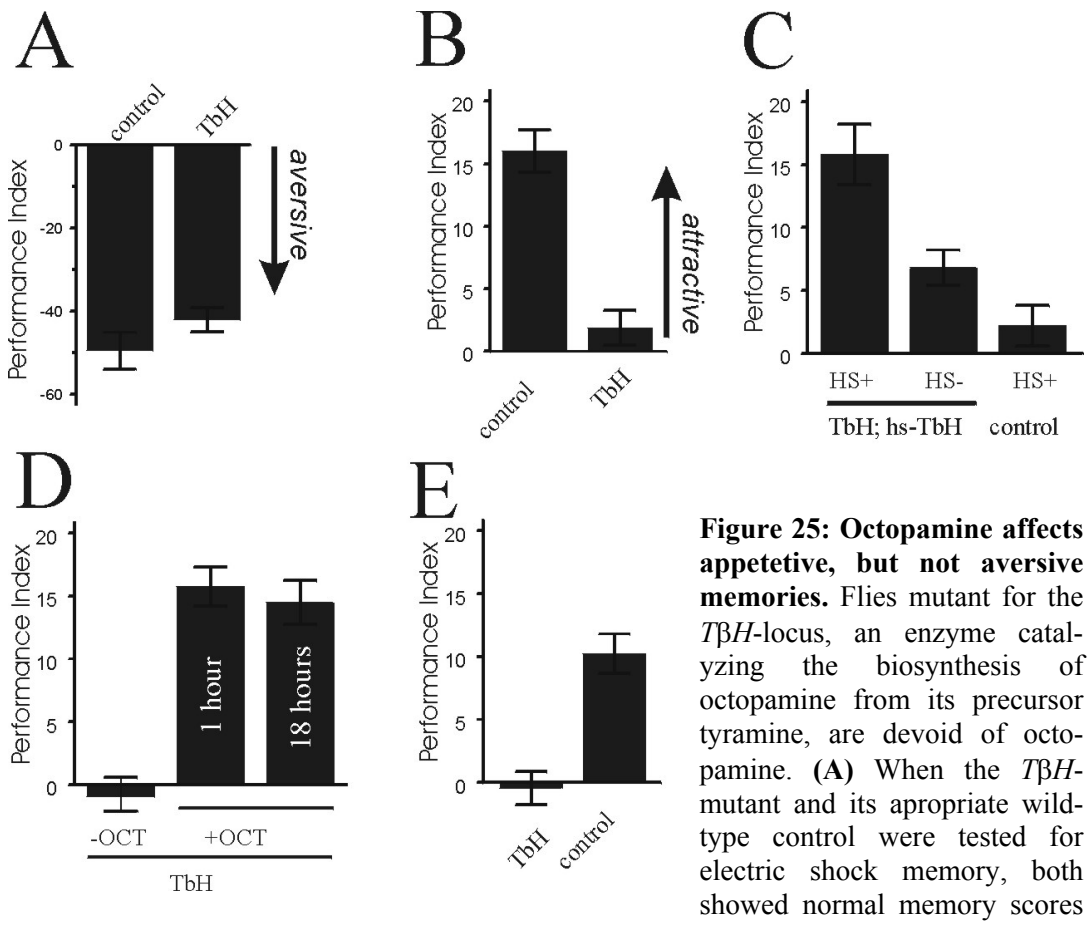

Figure 25: Octopamine affects appetetive, but not aversive memories. Flies mutant for the *T*β*H*-locus, an enzyme catalyzing the biosynthesis of octopamine from its precursor tyramine, are devoid of octopamine. **(A)** When the *T*β*H*-mutant and its apropriate wild-type control were tested for electric shock memory, both showed normal memory scores (ANOVA: $p > 0.05$). **(B)** In contrast, when tested for sugar memory *T*β*H*-mutants showed no significant memory score (ANOVA: $ps < 0.001$). **(C)**. Flies, mutant for the *T*β*H*-locus but with a heatshock-inducible *T*β*H*-construct (*TbH*; hs-TbH), showed normal performance of sugar memory upon heatshock (HS+) (ANOVA: $p < 0.05$). Neither the hs1-TbH construct alone (*TbH*; hs-TbH – HS-), nor the heatshock itself had an affect on performance, as tested in the *T*β*H*-mutant (ANOVA: $p > 0.05$). **(D)** Feeding on octopamine solution (10 mg/ml) for 1 or 18 hours before the experiment restored performance of sugar memory in *T*β*H*-mutants (ANOVA: $p < 0.001$). **(E)**. To test for a possible role of octopamine exclusively during retrieval of sugar-memory, we trained *T*β*H*-mutant flies, fed them on octopamine-solution (10 mg/ml) for one hour and afterwards tested for performance of sugar-memory. *T*β*H*-mutant flies did not show performance of memory, although the feeding itself did not abolish performance of one hour memory in control flies (ANOVA: $P < 0.001$). Each data point represents the mean of six experiments plus or minus the SEMs.

Next, I investigated whether the effect of the *T*β*H* mutation were directly attributable to a lack of OA. This is warranted, as the $T\beta H^{M18}$ mutant accumulates tyramine, the direct precursor of OA (Monatirioti, 1996). Either effect could be the cause of the learning phenotype, as tyramine is a functional neurotransmitter (Nagaya et al., 2002) and plays an as yet incompletely understood role in processing olfactory information in *Drosophila* (Kutsukake et al., 2000). To distinguish between these alternatives, $T\beta H^{M18}$ mutant flies were fed octopamine (10 mg/ml) for either 1 hour or 18 hours right before the experiment to restore octopamine within the tissue, but presumably without affecting the accumulated level of

tyramine. After feeding on octopamine, $T\beta H^{M18}$ mutant flies performed like wild-type (Fig. 25D); notably, a feeding period of 1 hour is sufficient for this effect. Thus, the defect in sugar learning in $T\beta H^{M18}$ mutant flies is likely caused directly by the lack of octopamine.

To distinguish between a role of octopamine during memory acquisition and retrieval, OA was fed to the mutant flies only after training, and memory was then tested one hour later. No rescue of memory was found in these flies, while in genetic control flies, the same octopamine feeding regime had no deleterious effect on memory (Fig. 25E). This suggests that for sugar learning, feeding OA is sufficient to restore storage, but not retrieval. According to the acquisition/retrieval problem, the same result has been found using conditioning of the proboscis extension reflex in the honeybee (Menzel et al., 1999).

3.8 – Dopamine is required for electric shock memory

Earlier experiments with flies carrying temperature sensitive alleles of the Dopa-Decarboxylase (*Ddc*) gene involved in the biosynthesis of both, dopamine and serotonine had already indicated a role of one or both of the monoamines in electric shock olfactory learning (Tempel et al., 1984). Performance in olfactory memory correlated with the concentrations of these substances in head homogenates, but this correlation could not be reproduced a few years later (Tully, 1987).

To focus on the role of dopamine in olfactory learning, I used the transgenic line *TH*-GAL4 (Friggi-Grelin et al., 2003) carrying the yeast transcription factor GAL4 under the control of a 5´ upstream regulatory region of the *Tyrosine-Hydroxylase* gene. Since Tyrosine-Hydroxylase catalyzes a metabolic step specific for dopamine biosynthesis, the gene is selectively expressed in dopaminergic neurons, as demonstrated by co-localisation of GAL4 driven reporter gene expression with anti-dopamine immunoreactivity (Friggi-Grelin et al., 2003). Hence, the *TH*-GAL4 line provides specific experimental access to most if not all dopaminergic cells.

To block the output of dopaminergic neurons, the *TH*-GAL4 driver was combined with the UAS-shi^{ts1} effector gene. Using electric shock as reinforcement, olfactory memory in *TH*-GAL4/UAS-shi^{ts1} flies was severely impaired at the restrictive temperature, while the genetic control flies (TH/+ and UAS-shi^{ts1}/+) showed normal performance (Fig. 26A, filled bars). The same experiment at the permissive temperature resulted in normal memory performance in all genotypes (Fig. 26A, grey bars). In contrast, memory performance in sugar reward learning in *TH*/UAS-shi^{ts1} flies was not negatively impaired at the restrictive temperature (Fig. 26B). These experiments show that synaptic output from DA neurons is

necessary for electric shock learning but is dispensible for sugar learning. This is the complementary result from that obtained by OA depletion above (Fig. 25) suggesting a differential role of the two monoamines in learning about sugar and electric shock.

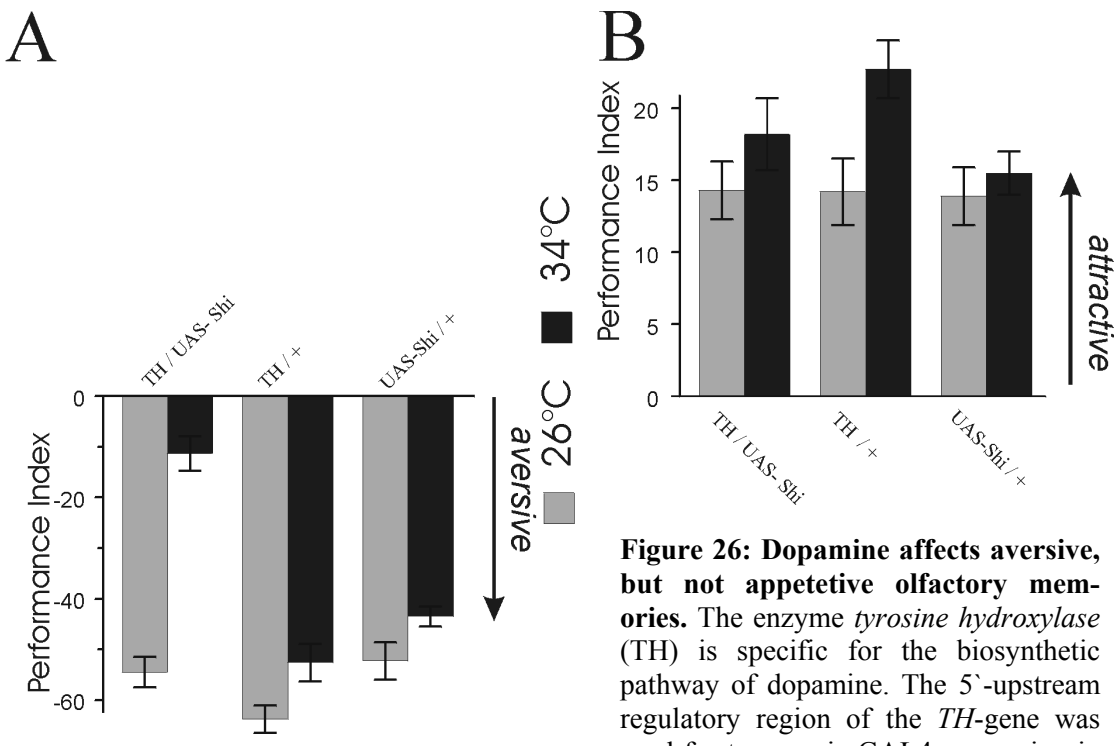

Figure 26: Dopamine affects aversive, but not appetetive olfactory memories. The enzyme *tyrosine hydroxylase* (TH) is specific for the biosynthetic pathway of dopamine. The 5`-upstream regulatory region of the *TH*-gene was used for transgenic GAL4-expression in dopamine positive cells of the TH-GAL4 line. We trained and tested TH/UAS-Shi flies and its genetic controls TH/+ and UAS-Shi/+ at these temperatures for performance of shock and sugar memory. (A) Upon block of transmission (black bars) from dopamine-positive cells performance of shock-memory was severly disturbed in TH/UAS-Shi flies, compared to it`s performance at 26°C (grey bars) and its genetic controls at both temperatures (ANOVA: $p < 0.001$). (B) In contrast, there was no temperature-dependent decrease in performance of sugar-memory in neither group (TH/UAS-Shi, TH/+, UAS-Shi/+) (ANOVA: $p > 0.05$, except for TH/+. Here performance was significantly increased at the restrictive temperature: $p < 0.05$). Each data point represents the mean of six experiments plus or minus the SEMs.

To further test this hypothesis, flies were subjected to the restrictive temperature only during either acquisition or retrieval. When trained at the restrictive and tested at the permissive temperature, *TH*-GAL4/UAS-*shi*^{ts1} flies were severly impaired as compared to *TH*-GAL4/+ control flies (Fig. 27A), suggesting that output from DA neurons is necessary for acquisition. However, retrieval is unaffected at the restrictive temperature in *TH*-GAL4/UAS-*shi*^{ts1} flies (Fig. 27B), suggesting that output from DA is dispensable for retrieval. Note that this pattern of requirement for OA and DA for acquisition but not retrieval is just the opposite of that obtained above concerning the requirement of Kenyon cell output for retrieval but not acquisition. Indeed the results show, that olfactory memory can be

acquired within the Kenyon cells of the MBs, althought their synaptic output is blocked during this process. On the other hand, this output from the MBs is necessary for retrieving the memory and modulate the behavior. The MBs have been shown to be the site of memory formation and for that reason it is required that the MBs receive input from both, the CS and the US pathways.

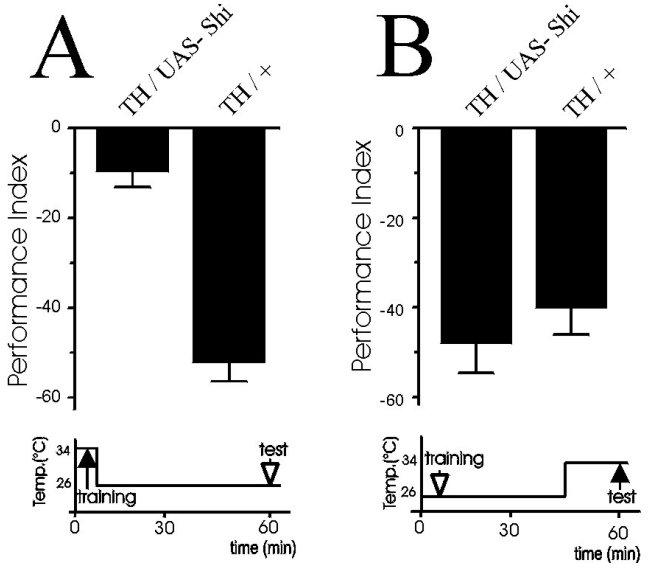

Figure 27: Dopamine-signaling is specifically needed during acquisition of memory. To distinguish between a possible role of DA transmission during acquisition or retrieval of electric shock memory, we restricted the synaptic block to either training or test phase of shock memory experiments by use of different temperature regimes. (A) Performance of one hour memory was abolished when transmission was blocked during acquisition but lowered to permissive conditions right afterwards. (ANOVA: $p < 0.001$). (B) Interestingly, perfomance was not affected when silencing of synaptic transmission was restricted to the test-phase (ANOVA: $p > 0.05$). For each experiment the mean of six experiments plus or minus the SEM are shown.

Taken together, both appetitive and aversive memories can be localized to the same set of Kenyon cells, specifically, to presynaptic modulation of their output synapses in the lobes. Furthermore, dopamine and octopamine are both involved in the acquisition rather than retrieval of these olfactory memories, the former specifically with respect to electric shock reinforcement, the latter specifically with respect to sugar reward. I like to assume that both monoamines function as modulatory transmitters directly in the association process, since both forms of olfactory memory require cAMP signaling and receptors for dopamine (DAMB, Han et al., 1996; Crittenden et al., 1998 and dDA1, Han et al., in prep.) and octopamine (OAMB, Crittenden et al., 1998; Han et al., 1998) have been found to be expressed at elevated concentrations in the MB lobes.

3.9 – Testing for perception of sensory stimuli

The *rut*-mutant genotypes showing a memory deficit and the appropriate MB-rescued *rut*-mutant flies controls were tested for olfactory detection ability, shock reactivity and sugar detection to determine whether any of the above changes in olfactory memories were due to impairment in detecting the task relevant stimuli. Table 3 shows that *rut*-mutant and *rut*-

rescued flies were not signifficantly different in responding to sugar, electric shock and olfactory cues. In case of the octopamine impaired *TβH*^{M18} mutants and the dopamine blocked *TH*-GAL4 / UAS-*shi*^{ts1} flies at the restrictive temperature, perception of olfactory cues is not impaired, as they learn with either electric shock or sugar reinforcement, respectively. Test for perception of sugar did not reveal any significant difference between the *TβH*^{M18} mutants and its wildtype control. Testing *TH*-GAL4 / UAS-*shi*^{ts1} flies either at the permissive temperature, when electric shock memory is normal or at the restrictive temperature, when memory is effected, did not reveal any significant impairment in sensing electric shock. Comparison between the sensing ability for electric shock, sugar or the olfactory cues at either the permissive or restrictive temperatures in flies with the UAS-*shi*^{ts1} transgene expressed in the MBs (247/*UAS-shi*^{ts1}) did not reveal any significant differences.

Thus, the changes in olfactory memories measured here were likely not due to changes in either of the cogent sensory modalities.

Table 3. Sensory Acuity Tests

Genotype	Shock Avoidance	Odorant Avoidance $^{1}/_{36}$	Odorant Avoidance $^{1}/_{6}$	Sugar Reactivity
rut^{2080}	68.6 ± 3.1	11.6 ± 2.1	47.0 ± 2.8	78.0 ± 6.0
rut^{2080}; UAS-rut+	76.6 ± 3.0	10.0 ± 2.0	50.5 ± 2.0	81.0 ± 5.7
rut^{2080}; 247	72.6 ± 3.0	11.6 ± 3.6	47.0 ± 2.2	74.5 ± 6.3
rut-rescue	71.7 ± 3.8	12.0 ± 2.2	53.9 ± 4.4	85.8 ± 4.0
247 / UAS-Shi (26°C)	76.2 ± 2.9	4.3 ± 2.4	50.6 ± 3.0	83.2 ± 7.9
247 / UAS-Shi (34°C)	78.2 ± 2.1	4.1 ± 3.6	43.2 ± 3.7	72.3 ± 7.9
TbH^{+} control	n.d.	n.d.	n.d.	81.8 ± 5.1
TbH^{M18}	n.d.	n.d.	n.d.	80.8 ± 5.6
TH / UAS-Shi (26°C)	79.0 ± 4.4	n.d.	n.d.	n.d.
TH / UAS-Shi (34°C)	77.0 ± 6.7	n.d.	n.d.	n.d.

Table 3: Sensory acuity tests
Neither the rescue of the *rutabaga (rut)* olfactory learning phenotypes nor the blocking of Kenyon cells nor dopamine positive cells nor the loss of *tyramine*-β-*hydroxylase (TbH)* activity caused behaviorally significant changes in electroshock, sugar or olfactory sensitivity. r*ut* mutants and flies with the Kenyon cells blocked were tested for sensitivity to the stimuli electric shock, sugar and the olfactory cues at two different concentrations, while *TbH* mutants and flies with the dopamine positive cells blocked were only tested for perception of the reinforcing stimuli sugar or electric shock, respectively. There were no significant differences between *rut*-mutant and *rut*-rescued flies in neither assay ($p > 0,05$). Blocking of the Kenyon cells at 34°C in 247/UAS-Shi flies did not affect performance in neither assay compared to non-blocked flies of the same genotype ($p > 0.05$). *TbH*-mutant flies were neither different from genetic control flies nor from all other groups tested for perception of sugar ($p > 0.05$). Acuity to electroshock and the olfactory cues were not determined (n.d.) as they showed normal performance in the shock-learning paradigma. Blocking of dopamine-positive cells at 34°C in TH/UAS-Shi flies did not affect response to electroshock compared to non-blocked flies of the same genotype ($p > 0.05$). Acuity to sugar and the olfactory cues were not determined (n.d.) as they showed normal performance in the sugar-learning paradigma. For each experiment the mean of six, in case of sugar the mean of 20 experiments was shown plus or minus the SEMs.

4 – Discussion

Zars and co-workers were able to localize an engram of aversive olfactory memory to the mushroom bodies (MBs) of *Drosophila* (Zars et al., 2000). Inspired by this finding, I first focused on the question whether it would also be possible to localize extinction, a process effecting the performance of memory.

In the first part of this study I can show that memory extinction, like memory formation and storage, localizes to the same set of neurons within the *Drosophila* mushroom bodies. This common localization suggests a model, in which memory extinction antagonizes performance of memory on a subcellular level within the same neurons.

The second part also targets memory localization, but for the first time addresses appetitive memory. I can show that memories for the same olfactory cue can be established through either sugar or electric shock reinforcement. These memories also localize to the same set of neurons within the *Drosophila* mushroom body. The further results show that two different monoamines are specificaly necessary for formation of either of these memories, dopamine in case of electric shock and octopamine in case of sugar memory, respectively. Taking the representation of the olfactory cue within the mushroom bodies into account, this suggests a model in which both memory traces may be formed at different synaptic clusters within the same cell.

4.1 – A model for formation and storage of olfactory memories

Information on the primary odor qualities of an odorant is received at the antenna, processed in the antennal lobe and signaled via projection neurons to the Kenyon cell dendrites in the calyx (Fig. 28). As proposed by Perez-Orive et al. (2002) for the locust, each Kenyon cell may respond to the coincident excitation from only a specific combination of inputs. In this way, different odorants would be represented in the MBs by the activation of different groups of Kenyon cells (Heisenberg, 2002).

For an odorant to become predictive of an electric shock, a neuronal connection between a MB output neuron and the group of Kenyon cells representing this odorant must exist. When excited, this output neuron would signal to the animal impending electric shock. We propose here that just as memory formation is the strengthening of this connection, extinction is its weakening. It has been proposed earlier that strengthening of synapses from the Kenyon cell to an output neuron occurs if the Kenyon cell is coincidentally stimulated by an odorant and a modulatory neuron signaling electric shock. The present results suggest that

extinction and thus weakening of the synapses occurs if the odorant stimulus arrives without the coincident signal from the electric shock. Although not directly comparable, the PE1 MB extrinsic cell of the honeybee has been shown to increase or decrease its firing rate when presented with CS+ or CS-odorants (Mauelshagen, 1993), supporting the contention that the output of the MB can be both up- and downregulated.

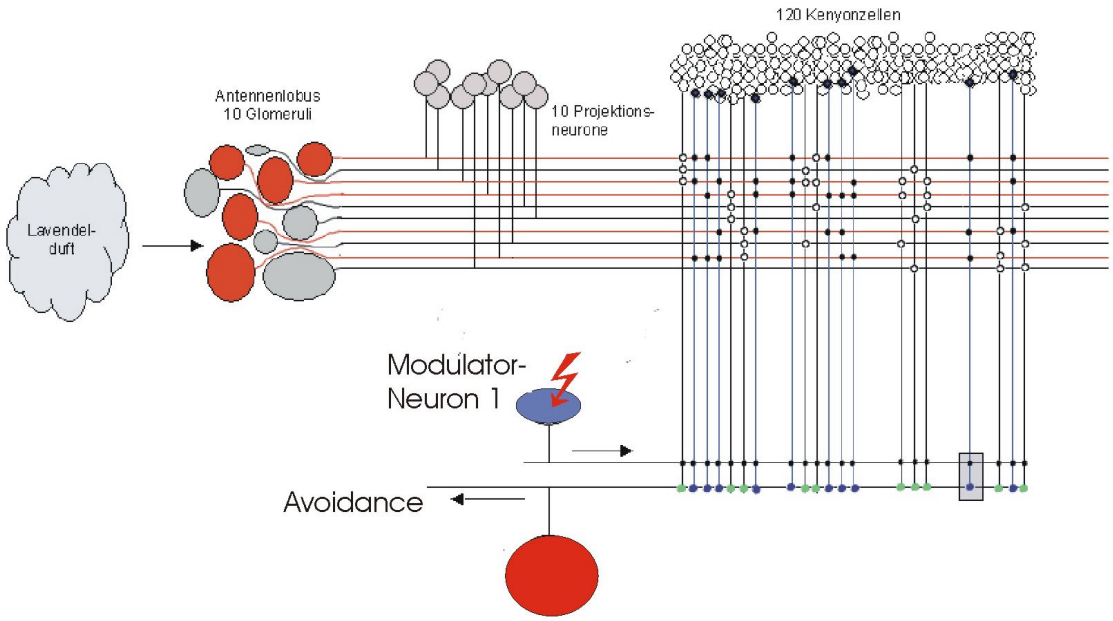

Figure 28: Hypothetical model of olfactory memory formation and storage. Perceiving a distinct olfactory cue (e.g. laven-dula) results in activation of a reproducible pattern of glomerular activity in the antennal lobe (red), relayed to the calyx of the mushroom bodies and other olfactory centers. The projection neurons (PNs) may synapse onto the Kenyon cells in a combinatory way, always 3 PNs converging onto one Kenyon cell. This divergent coding of olfactory information in the PNs (5 active glomeruli out of 10) and the Kenyon cells (10 activated out of 120) would allow for coding olfactory qualities into different ensambles of Kenyon cells activity. The hypothesised mechanism of olfactory coding in the MBs suggest an interesting mechanism for formation and storage of memories: A yet unidentified neuron could signal electric shock to all Kenyon cells, but only those which are coincidently activated via olfactory impulses could trigger the molecular machinery of memory formation and change their synapses in a plastic way (green). The architecture of the synaptic clusters formed by input- and output-neurons (grey box) will be discussed in detail. /This figure was modified after Heisenberg, 2002).

4.2 - Extinction antagonises olfactory memory at the subcellular level

Earlier studies of extinction indicate that a partial sharing of signal transduction pathways in memory acquisition and extinction is rather common. That extinction of a memory uses a subset of the signaling pathways needed for its formation has been seen in fear conditioning in the mouse and rat, and conditioned taste avoidance in the rat. Lattal and Abel (2001) showed that in the mouse, protein synthesis is needed for acquisition of spatial

learning and fear conditioning, but protein synthesis is not needed for extinction of that process. On the other hand, for fear conditioning in the rat, the NMDA receptor in the amygdala is involved in both acquisition and extinction (Lee and Kim, 1998). A similar partial use of signaling pathways in acquisition and extinction is seen in conditioned taste avoidance memory in rats (Berman and Dudai, 2001). In contrast to fear conditioning, however, protein synthesis was required in the insular cortex for both acquisition and extinction. While these studies have addressed the molecular process of extinction, they did not address the underlying circuit changes accompanying these phenomena. Consequently, the question wether extinction is an inter or intracellular process acting on memory had remained unanswered.

Two studies have explored this intra/intercellular problem. Repa et al. (2001) used electrophysiological recording in the amygdala during fear conditioning and made the intriguing observation that some neurons showed evidence of plasticity during acquisition and, even more, maintain these changes through extinction trials. This implies that modifications in other neurons should compensate for this lack of reversal in these neurons (Repa et al., 2001). A second example of an intercellular process was published by Medina et al. (2002). They used pharmacological injections into the inferior olive during extinction training of the eyelid response in the rabbit and revealed that blocking inhibitory input to the inferior olive prevents extinction. Moreover, blocking excitatory inputs to the inferior olive induced extinction even in the presence of both tone and shock (Medina et al., 2002). This result suggests regulation of the US pathway for behavioral extinction to occur. Decreasing or increasing activity of the climbing fibers either blocks or induces extinction, respectively (Medina et al., 2002). Whether there is normally an inhibitory signal connecting the tone (CS) pathway to the US pathway during tone alone presentation (mimicking a block of the excitatory input) is open. If this were true, then this would be an example of an intercellular mechanism of extinction.

For aversive olfactory learning in *Drosophila, e*xtinction appears to be an intracellular process and I now speculate on its molecular mechanism. The cAMP signaling cascade has been identified as a molecular mechanism for the associative strengthening of synapses (Byrne and Kandel, 1996; Lechner and Byrne, 1998). Coincident activation of Rut-AC by (reinforcer-induced) active heterotrimeric G protein and (CS-induced) Ca2+/CAM leads to a rise in cAMP levels and the activation of PKA. Phosphorylation products of PKA would then

lead to enhanced synaptic transmission. Now I propose that exposure to the CS+ in the absence of the reinforcer would lead to an increase in activity of some (yet unknown) antagonist that would be activated by depolarization in the absence of G protein signaling, possibly by an increase in intracellular Ca2+ concentration. This antagonist may act at the level of controlling cAMP levels, the activation state of the PKA catalytic subunit, or the phosphorylation state of PKA substrates (Figure 29).

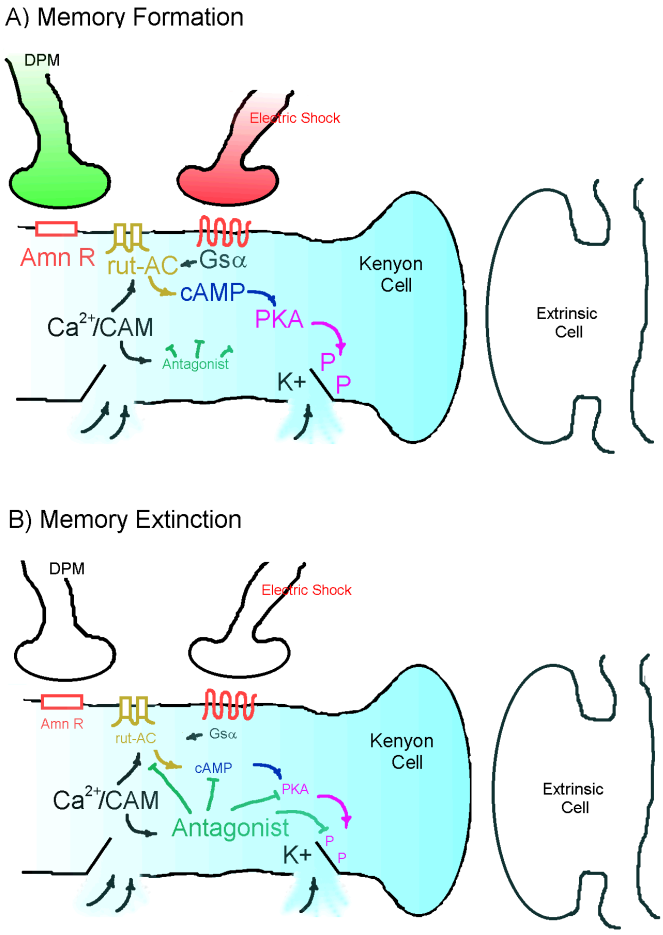

Figure 29: Model of Olfactory Memory and Extinction in Kenyon Cells. The percept of an odor is likely represented by the activation of a set of Kenyon cells. The memory template of the percept is the modification of the output synapses of this set of cells. Coincidence of, on the one hand, the electric shock signal through a heterotrimeric G protein and, on the other hand, the odorant depolarizing the Kenyon cell raising intracellular Ca2+ concentration increases cAMP levels via the Rut-AC. Protein kinase A (PKA) responds to this rise, releasing regulatory subunits and phosphorylating its products. The Amnesiac product expressed in the DPM cells transneuronally prolongs the activity of the Rut-AC and downstream signaling molecules. Exposure to the CS- alone (without electric shock) leads to a rise in intracellular Ca2+ concentration in the absence of G protein signaling. This activates an unknown antagonist of the cAMP cascade and reverses at least some of the molecular changes associated with memory formation.

Data from other systems suggest two levels in the cAMP cascade that could mediate extinction in *Drosophila* Kenyon cells. Essentially, either the level of cAMP or the activation of PKA might be the limiting factor in synaptic plasticity. In the first case, there could be an AC with properties similar to the mammalian type IX AC, which shows a reduction in cyclase activity with Ca2+/calcineurin signaling and is important for learning and memory in mice (Antoni et al., 1998; Paterson et al., 2000). If this were the case in the fly, then repeated depolarization of the Kenyon cells would decrease the levels of cAMP in the cells. There is, indeed, a gene with high homology to a type IX AC in *Drosophila*; its role in learning and memory, however, has not yet been tested (Iourgenko et al., 1997). Downstream in the cascade, a cAMP phosphodiesterase, responsive to Ca2+ signaling, could be activated by Kenyon cell depolarization.

The alternative level of regulation could be on PKA itself or its phosphorylated products. A model of synaptic plasticity in the *Aplysia* long-term sensitization system includes an activation of PKA by release and eventual degradation of its regulatory subunits that could lead to a kinase that is independent of cAMP signaling (Bergold et al., 1992; Bernier et al., 1982; Chain et al., 1999; Greenberg et al., 1987). If this model is applicable to the *Drosophila* Kenyon cells for olfactory memory, long-term activation of PKA and continued phosphorylation of its products could be a mechanism of memory storage. The odorant-induced extinction would then antagonize this process by inducing the re-binding of the PKA-regulatory subunits to the catalytic subunits, or an enzyme like Calcineurin, a Ca2+/CAM-dependent phosphatase could reverse the effects of PKA activity (Malleret et al., 2001; Zeng et al., 2001). The molecular process of extinction will probably involve a number of steps that decrease the forward signaling through cAMP and increase the reverse reactions.

4.3 – Two types of olfactory memories residing within the same neuron

Despite more than 20 years of research on olfactory learning and memory in *Drosophila,* the nature of the modulatory neurons that provide information about the unconditioned stimuli remained unknown (see Fig. 3). In paragraph 3.7 & 3.8, I show that dopamine (DA) is necessary for aversive and octopamine (OA) for appetitive learning. Moreover, as the memory traces in aversive and appetitive conditioning can be localized to the same set of about 800 Kenyon cells, the question is apparent how storage of separate memory traces for the same odor is organized within these 800 cells (Fig. 30).

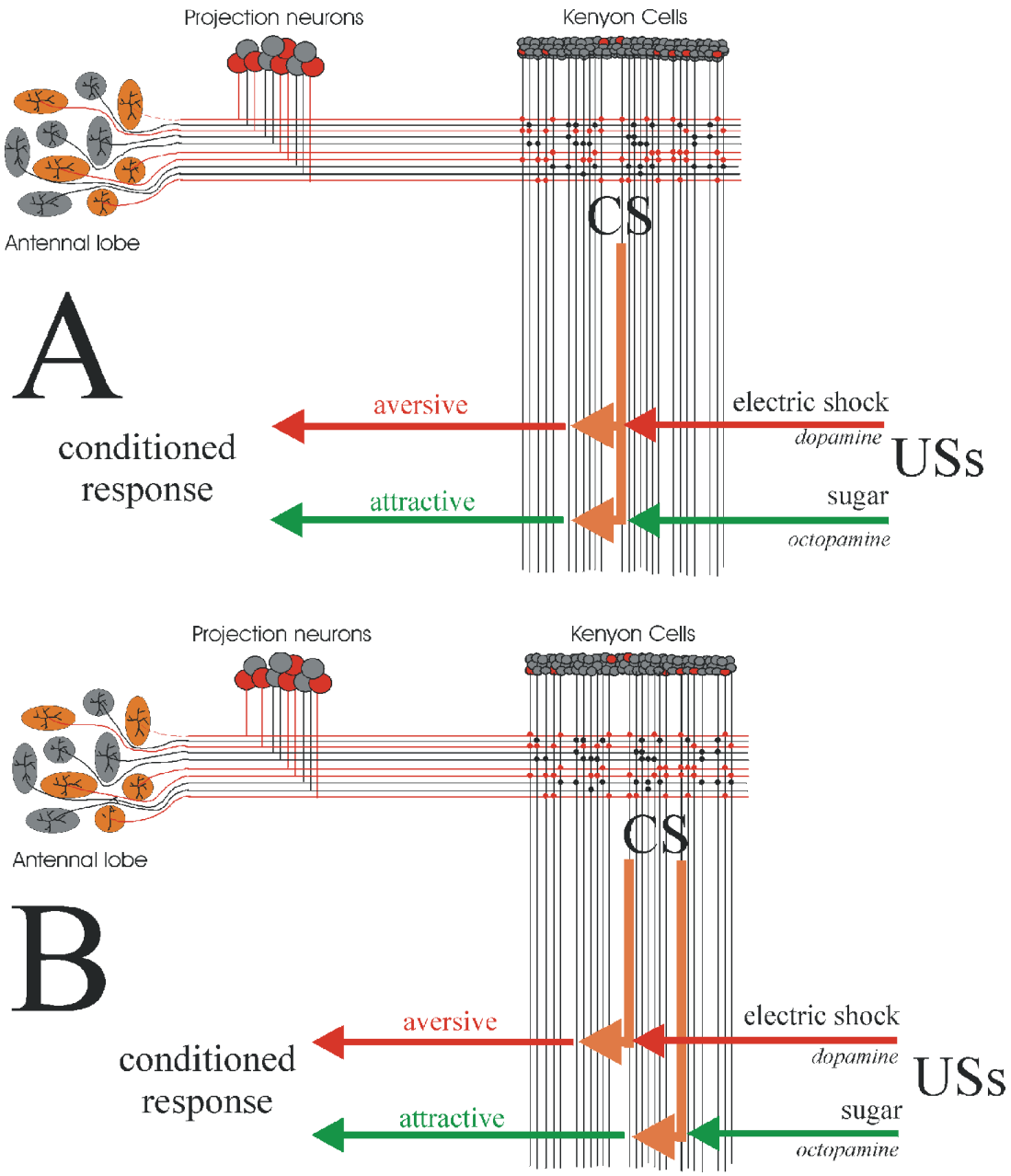

Figure 30: Alternative models of memory trace representations. Exlanation is given in text.

As shown in paragraph 3.6, appetitive and aversive memory traces reside in the same set of about 800 Kenyon cells marked in the 247-Gal4 driver line. As odors are thought to be represented in the MBs by sets of Kenyon cells (Perez-Orive, 2002; Faber & Menzel, 2001), the question arises whether each odor has but one or several representations in each MB (see Fig. 30A & B). In the former case sugar and electric shock memories of a certain odor would be formed in the very same Kenyon cells. This would imply that on a given Kenyon cell, at least two spatially separated 'synaptic clusters' exist. A MB output neuron mediating appetitive conditioned responses (CRs) and 'its' modulatory OA input neuron delivering sucrose reinforcement (Fig. 31) should have their synapses in close proximity to each other.

The same should apply to a MB output neuron mediating aversive behavior and 'its' modulatory DA input neuron delivering electric shock reinforcement. With several such US/CR neuron pairs, Kenyon cells would need several independent domains of the cAMP signaling machinery affecting only the local output synapse and being modulated only by the companion US neuron. As *Drosophila* can associate many events (USs) with odors (see above), Kenyon cells would have to accomodate many such US/CR pairs. Requirement for space at the KCs may then explain the stalk-like structure of MBs.

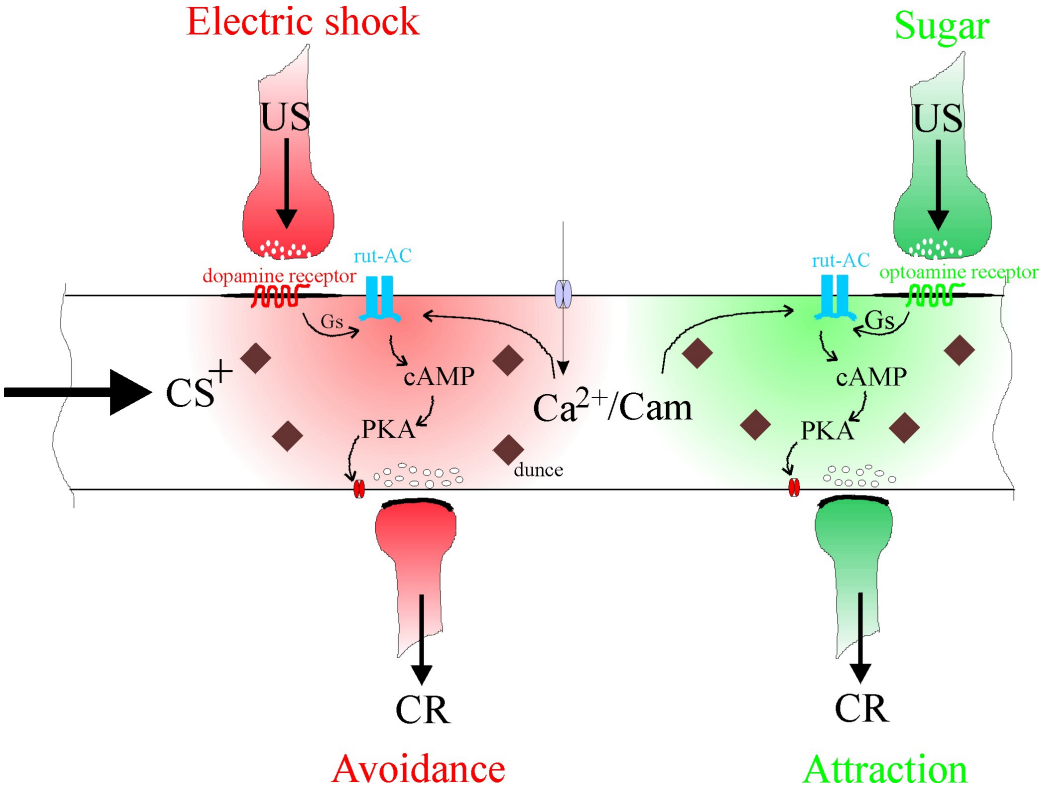

Figure 31: Model of separate memory traces within the same Kenyon cell. Olfactory cues act as conditioned stimuli (CSs) during olfactory conditioning and are likely represented by activation of unique sets of Kenyon cells at the level of the mushroom bodies (MBs). The same olfactory cue can predict several reinforcers (USs) by modification of different output synapses within the same Kenyon cells responding to the CS. Each of these modified synapses supports an individual conditioned response (CR) corresponding to the nature of the reinforcement, e.g. avoidance in case of electric shock, attraction after sugar reward. In this way, separate memory traces can be stored in an individual Kenyon cell utilising the same molecular mechanism independently at different locations along the axon. Coincident activation of the *rut*-AC, on the one hand by a Ca^{2+} increase due to the odorant (CS+) depolarizing the Kenyon cell, and on the other hand by the US signal, mediated by a membrane receptor activating a heterotrimeric G-protein, locally increases cAMP levels. Thus, the location of the increased cAMP level along the Kenyon cell axon depends on the position of the modulatory input synapse mediating the US. In case of electric shock reinforcement a dopaminergic neuron, in case of sugar reward learning an octopaminergic neuron locally activates the cAMP pathway which via PKA and phosphorylation of target proteins nearby, modulates transmission of local synapses to a MB output neuron.

The existence of two memory traces is consistent with the earlier observation that electric shock and sugar memories have different kinetics of consolidation and decay (Tempel

et al., 1983). It can be assumed that both monoamines, dopamine and octopamine, function as modulatory transmitters directly in the association process, since receptors for dopamine (DAMB; dDA1) and octopamine (OAMB) have been found to be coupled via Gs protein to adenylyl cyclase of the *rut* type and were found to be expressed at elevated concentrations in the MB lobes (Han et al., 1996; Crittenden et al., 1998; Han et al., 1998; Kim et al., 2003).

In the cellular model of memory formation, cAMP signaling modifies synapses presynaptically (Kandel, 2001). Hence, in case of *Drospohila* olfactory learning, the synapses carrying the memory trace must be the ones connecting Kenyon cells to MB output neurons. These are located in the MB lobes (including the rostral peduncle and spur, Yasuyama et al., 2002; Schürmann, 1987). Considering that the components of the cAMP signaling machinery need to physically interact with one another, one would expect them to be also located in the lobes. Indeed, *rut*-AC as well as the OA and the two DA1 receptors above are found at higher concentrations in the lobes than in the calyx and caudal peduncle (Crittenden et al., 1998; Kim et al., 2003). Consequently, the MB input neurons carrying the US for sugar and electric shock should also connect to the lobes. Their anatomical identification, however, is pending.

The TH-Gal4 line defines a set of neurons nearly identical to the fly's set of DA neurons (Friggi-Grelin et al., 2003). I like to interpret the finding that interference with acquisition in olfactory learning by blocking synaptic output from these cells indicates that indeed neurons are bocked which maintain dopaminergic synapses onto Kenyon cells in the MB lobes. The neurons labeled in the TH-Gal4 line were documented using suitable reporter genes (Fig. 13H-J). Some of them are found to innervate the MB lobe system. The neurons innervating the spur and rostral peduncle are obvious candidates for mediating the US in electric shock short term memory since this is thought to be independent of the α/β-subsystem (Zars et al., 2000). The prominent innervation of the α-lobe may be involved in long term rather than short term olfactory memory (Pascual & Preat, 2001) or may serve other types of odor learning. Unfortunately, immunocytochemistry of OA in my hands is not yet satisfactory and no OA neurons innervating the lobes have yet been reported in flies. In bees, OA immunoreactivity has been reported in the γ-lobes and the calyx (Kreisl et al., 1994).

These data predict that *oamb,* the only known gene coding for an octopamine receptor (OAMB) in *Drosophila,* should be essential for sugar reward learning. Likewise, one of the dopamine receptors of the DA1 type is predicted to be involved in electric shock learning. As the DAMB receptor gene has been tested and does not seem to be involved (K-A Han and RL

Davis, personal communication), the newly identified dDA1 gene is at present the most promising candidate.

At first sight, the finding that OA is involved in sugar reward learning agrees well with PER conditioning in the honey bee where OA is the modulatory transmitter as well (Hammer & Menzel., 1998; Menzel et al., 1999). A closer inspection, however, reveals substantial differences which can not easily be reconciled with each other. The honeybee VUMmx1 neuron appears to be octopaminergic and has been shown to carry the reinforcing properties of the US in PER conditioning; it innervates the calyces, antennal lobes and lateral protocerebrum, but not the MB lobes (Hammer & Menzel., 1998). A neuron with this arborisation in *Drosophila* would not be suited to provide the US for sugar learning unless one would postulate a fast molecular long distance signal in the cAMP pathway from the calyx to the lobes or a separation between the site of OA release and OA effect. At present it is still too early to conclude that memories in honeybee PER conditioning and conditioned osmotaxis of flies are differently organized at the circuit level.

Nevertheless, octopamine as transmitter in sugar reward learning may be evolutionarily conserved between honeybee and *Drosophila.* Unfortunately, the role of the monoamines in aversive conditioning has not yet been tested in honeybee. In the monkey, midbrain dopaminergic neurons have been described that carry the reinforcing properties of a US in appetitive but not aversive conditioning. It will be interesting to see whether the dissociation between the modulatory systems for sugar and electric shock learning also applies for additional aversive and appetitive stimuli in *Drosophila*.

Taken together, this study takes two further steps in the search for the engram. First, it is shown that extinction is localized to the same set of cells as is acquisition, and second, that aversive and appetitive memories also are localized to this same set of cells. Together with the differential requirment of DA and OA for aversive and appetitive memories, these findings suggest that the organization of both acquisition versus extinction and of appetitive versus aversive memory formation is achieved on the sub-cellular level. This view is in marked contrast to the pessimism expressed by Lashley that is might not be possible to localize an engram; whether advanced methods of intervention might eventually reveal that memory organization in mammals is also well localized and in this respect is similar to flies will be entertaining to observe.

5 – Summary

Zars and co-workers were able to localize an engram of aversive olfactory memory to the mushroom bodies of *Drosophila* (Zars et al., 2000). In this thesis, I followed up on this finding in two ways.

Inspired by Zars et al. (2000), I first focused on the whether it would also be possible to localize memory extinction.While memory extinction is well established behaviorally, little is known about the underlying circuitry and molecular mechanisms. In extension to the findings by Zars et al (2000), I show that aversive olfactory memories remain localized to a subset of mushroom body Kenyon cells for up to 3 hours. Extinction localizes to the same set of Kenyon cells. This common localization suggests a model in which unreinforced presentations of a previously learned odorant intracellularly antagonizes the signaling cascades underlying memory formation.

The second part also targets memory localization, but addresses appetitive memory. I show that memories for the same olfactory cue can be established through either sugar or electric shock reinforcement. Importantly, these memories localize to the same set of neurons within the mushroom body. Thus, the question becomes apparent how the same signal can be associated with different events. It is shown that two different monoamines are specificaly necessary for formation of either of these memories, dopamine in case of electric shock and octopamine in case of sugar memory, respectively. Taking the representation of the olfactory cue within the mushroom bodies into account, the data suggest that the two memory traces are located in the same Kenyon cells, but in separate subcellular domains, one modulated by dopamine, the other by octopamine.

Taken together, this study takes two further steps in the search for the engram. (1) The result that in *Drosophila* olfactory learning several memories are organized within the same set of Kenyon cells is in contrast to the pessimism expressed by Lashley that is might not be possible to localize an engram. (2) Beyond localization, a possibible mechanism how several engrams about the same stimulus can be localized within the same neurons might be suggested by the models of subcellular organisation, as postulated in case of appetitive and aversive memory on the one hand and acquisition and extinction of aversive memory on the other hand.

6 – Zusammenfassung

Troy Zars und seine Mitarbeiter konnten für das olfaktorische Elektoschockgedächtnis von *Drosophila* zum ersten mal die Spur eines Duftgedächtnisses in den Pilzkörpern (PK) lokalisieren. Darauf aufbauend stelle ich nun in dieser Arbeit zwei Fragen:

1. Wäre es möglich auch den Prozess der Auslöschung dieses Gedächtnissen zu lokalisieren? Obwohl die Verhaltensphysiologie der Gedächtnisauslöschung sehr gut charakterisiert sind weiss man sehr wenig über die daran beteiligten molekularen Signalmechanismen und Strukturen. In Anlehnung an die Arbeit von Zars et al. (2000) kann ich zeigen, dass sowohl die Speicherung wie auch die Auslöschung dieses Gedächt-nisses in den gleichen Kenyonzellen der PK geschieht. Diese gemeinsame zelluläre Lokalisierung legt ein Model nahe, in dem die wiederholte Präsentation des mit Elektro-schock assoziierten Duftes während der Auslöschung, intrazellulär auf die gleichen Signalwege wirkt die auch für die Bildung des Duftgedächtnisses notwendig sind.
2. Wäre es möglich auch die Spur eines attraktive Duftgedächtnisses zu lokalisieren? Ich kann zeigen, dass Gedächtnisse über den gleichen Duft sowohl attraktiv als auch repulsiv sein können, je nachdem ob Zucker oder Elektroshock während der pavlovschen Konditionierung benutzt wird. Beide Gedächtnisse sind im gleichen Satz von Kenyonzellen lokalisiert. Dies wirft die Frage auf, wie das gleiche Duftsignal mit zwei verschiedenen Ereignissen (Zucker und Elektroschock) assoziiert werden kann. Es zeigt sich, dass zwei unterschiedliche Monoamine jeweils spezifisch für das Anlegen eines der beiden Gedächtnisse verantwortlich sind; Dopamin für das Electroschockgedächtnis und Octopamin für das Zuckergedächtnis. Berücksichtigt man wie Duftreize in den PK kodiert sind, ergibt sich ein Model bei dem zwar beide Spuren in einer Zelle lokalisiert sind, diese jedoch durch die Nutzung unterschiedlicher subzellulärer Bereiche voneinander getrennt werden. Jeweils einer dieser Bereiche wäre durch Dopamin moduliert, der andere durch Octopamin.

Das Fazit dieser Studie ist, dass zwei wichtige Punkte bei der Lokalisierung von Gedächtnis-spuren aufgezeigt wurden. (1) Die Tatsache, dass beim Duftlernen von *Drosophila* mehrere Spuren verschiedener Duftgedächtnisse lokalisiert worden sind widerlegt die von Lashley aufgestellte Behauptung, dass Gedächtnisse nicht lokalisierbar sind. (2) Verschiedene Spuren können für den gleichen Duft in den gleichen Zellen angelegt werden, sofern man eine subzelluläre Organisation annimmt, wie sie sowohl für Zucker- und Elektroschockgedächtnis, als auch Gedächtnisbildung und Auslöschen vorgeschlagen werden

7 - References

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List of publications

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

Martin Schwaerzel, Maria Monastirioti, Henrike Scholz, Florence Friggi-Grelin, Serge Birman, and Martin Heisenberg. Dopamine and octopamine establish separate memory traces in the same set of neurons. *Submitted to Nature.*

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

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

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

Würzburg, den 17.02.2003

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