

Dissertation

A NEW CIRCUIT FOR VISUAL MEMORY  
FORMATION:  
FROM THE OPTIC LOBES TO THE  
MUSHROOM BODIES OF *DROSOPHILA*

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## List of abbreviations

MB	Mushroom body
CS	Conditioned stimulus
CS+	Paired conditioned stimulus
CS-	Unpaired conditioned stimulus
US	Unconditioned stimulus
OPN	Olfactory projection neuron
STM	Short term memory
<i>rut</i>	<i>rutabaga</i>
cAMP	cyclic adenosine monophosphate
<i>PKA</i>	Protein kinase A
CC	Central complex
UAS	Upstream activation sequence
DBD	DNA binding domain
AD	Activation domain
ANOVA	Analysis of variance
LED	Light-emitting diode
JFRC	Janelia Farm Research Campus
SEM	Standard error of the mean
GFP	Green fluorescent protein
RFP	Red fluorescent protein
PI	Preference index
LI	Learning index
VACA	Ventral accessory calyx

## Zusammenfassung

Für alle Lebewesen ist es vorteilhaft ein bedeutsames Ereignis, wie Bestrafung oder Belohnung, mit unterschiedlichen sensorischen Reizen aus der Umwelt zu verbinden. Ein einzelnes Ereignis kann dabei zur Bildung von mehreren sensorischen Gedächtnissen führen, zum Beispiel basierend auf visuellen, olfaktorischen oder gustatorischen Reizen. Wie die grundlegenden neuronalen Mechanismen solcher verschiedener sensorischer Gedächtnisse dabei interagieren ist jedoch unklar. Zwei alternative Mechanismen sind möglich. Die verschiedenen sensorischen Eindrücke könnten über ein geteiltes Netzwerk mit dem gleichen Verstärker assoziiert werden. Andererseits könnten aber auch unterschiedliche neuronale Netzwerke benutzt werden um Gedächtnisse mit verschiedenen sensorischen Reizen zu bilden. Das neuronale Netzwerk das für das Duft-Lernen in *Drosophila* gebraucht wird wurde schon detailliert beschrieben. Wie Fliegen jedoch visuelle Reize wie Farben oder Lichtintensitäten lernen ist noch nicht bekannt. Die für visuelles Lernen meist benutzten Verhaltensapparaturen unterschieden sich zudem sehr von der Apparatur die für olfaktorisches Lernen benutzt wird besonders in Bezug auf die Aufgabenstellung und die applizierten Reize. Dies erschwert den direkten Vergleich der vorhergehenden Ergebnisse für visuelles und olfaktorisches Gedächtnis. Durch das Konstruieren einer neuen Apparatur für visuelles Lernen, die die gleiche Aufgabe von den Fliegen verlangt und in der die gleichen Belohnungs- (Zucker) und Bestrafungsreize (elektrischer Schock) appliziert werden wie im olfaktorischen Lernen, wird jedoch ein Vergleich zwischen den beiden Modalitäten möglich. Ziel meiner Doktorarbeit war es das grundlegende neuronale Netzwerk für visuelles Lernen darzulegen und dieses mit dem bereits bekannten neuronalen Netzwerk für olfaktorisches Lernen zu vergleichen. Ich fand heraus dass ein prominentes Neuropil im Fliegengehirn, der Pilzkörper, eine zentrale Rolle in der Bildung und Abfrage des visuellen Gedächtnisses spielt, wie auch beim olfaktorischen Gedächtnis. Eine spezielle Untereinheit des Pilzkörpers, die  $\gamma$ d Kenyon-Zellen, wird dabei spezifisch für das Assoziieren visueller Reize benötigt. Die gleichen Dopamin-Nervenzellen, die die Bestrafungsinformation weiterleiten, und auch die gleichen Ausgangs-Nervenzellen werden jedoch von beiden Modalitäten benutzt zur Gedächtnisbildung. Visuelle Information wird von spezifischen Projektionsneuronen bereitgestellt. Diese stellen eine direkte Verbindung zwischen der Medulla in den optischen Loben der Fliege und den  $\gamma$ d Kenyon-Zellen im Pilzkörper her. Verschiedene solcher Projektionsneurone werden dabei benötigt um unterschiedliche visuelle

Informationen wie Farbe oder Lichtintensität zu übermitteln. Aufgrund des sehr ähnlichen Netzwerkaufbaus zwischen visuellem und olfaktorischem Gedächtnis schlage ich deshalb den Pilzkörper von *Drosophila* als ein Zentrum für multi-sensorische Gedächtnisbildung vor.



## Abstract

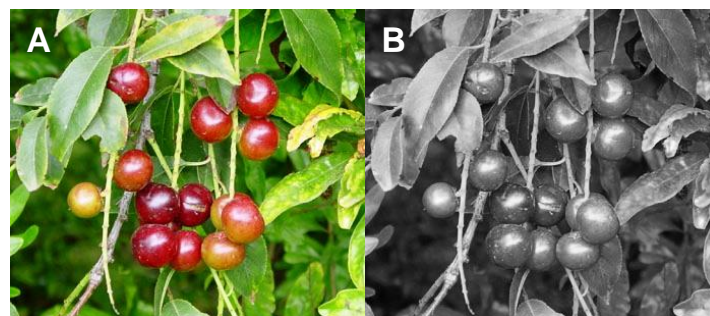
For animals it is advantageous to associate various sensory stimuli in their environment with a meaningful event, e.g., punishment or reward. A single event should thus lead to formation of sensory memories across several modalities, for example, based on olfactory, visual or gustatory cues. However, the underlying mechanisms of such memories are unclear. Two scenarios are possible: different modalities can be associated with the same reinforcement via a shared neural circuit or different neuronal circuits are employed when learning about different modalities. In *Drosophila*, the olfactory learning circuit is already well described; however, how flies learn visual stimuli like color or intensity is not known so far. Therefore it is not clear what mechanism is employed for the two different sensory memories. Also, existing setups for visual learning are very different in terms of behavioral task and stimuli application to the commonly used olfactory conditioning paradigm, which makes comparison difficult. In my PhD I aimed to identify the neural basis of the visual learning circuit and compare it to the olfactory learning circuit. Establishing a new visual learning paradigm that uses same appetitive (sucrose) and aversive (electric shock) reinforcers and requires similar task from flies as in olfactory conditioning has allowed comparison between the two modalities. The mushroom bodies (MBs) are a prominent neuropil in the fly brain and they are known for being the coincidence detector in classical olfactory conditioning. I found the MBs to be as pivotal in visual memory formation and retrieval as they are in olfactory conditioning. A specific sub-compartment of the MB, the  $\gamma$ d Kenyon cells, is specifically required for visual conditioning. However, at least aversive reinforcement and output neurons seem to be shared between visual and olfactory conditioning. Visual input to the MBs is provided by visual projection neurons that provide direct neural connection between optic lobes and the MBs and project to the  $\gamma$ d KC dendritic region. There are differential pathways mediating intensity and color information from the medulla in the optic lobes to the MB calyx. Due to this very similar circuit assembly in visual and olfactory learning, I suggest the MB as a center for multi-modal memory formation in *Drosophila*.



# 1. Introduction

## 1.1 Classical associative conditioning in *Drosophila melanogaster*

To optimally exploit resources in the environment, it is highly advantageous for animals to associate different sensory stimuli in their environment with meaningful experiences. In the early 20<sup>th</sup> century, this principle of the classical conditioning was established (Pavlov 1927). Pavlov showed that a dog can associate the ring of a bell, a normally neutral stimulus (Conditioned Stimulus = CS), with a food reward (Unconditioned Stimulus = US) when they were presented together. In such a classical conditioning experiment the animal cannot influence the exposure of CS or US. Naturally, administration of food reward elicits salivation of the dog (Unconditioned response = UR). After CS-US pairing, CS alone led to salivation (Conditioned response), thus the dog adjusted his behavior accordingly. There is also such an example in fruit flies; a ripe apple is sweet, contains nutrients and can get associated with reward. *Drosophila melanogaster* could extract different attributes (CS) of the apple, like color, shape, taste or smell to associate them with the same rewarding value (US) of the apple.



**Figure 1: Color perception adds critical information to the environment.**

**(A)** Picture containing color and brightness information. **(B)** Picture containing only brightness information. (Modified from fruitipedia.com)

The more stimuli it is able to associate, the faster and better the fly can recognize the reward in future (Figure 1, color as an example). However, the underlying mechanism of

how they learn to approach this appetitive stimulus by associating different attributes is not fully understood. The neural circuit involved in olfactory memory formation is already well described (McGuire et al. 2005); however, less is known about underlying neural mechanisms of memory formation in other sensory modalities.

*Drosophila melanogaster* is a particularly handy model organism for neural circuit dissection. Not only are the flies easy to raise and to maintain, but are also fast in their generation cycle, which makes it possible to collect many experimental flies in a short time. Furthermore their brain, consisting of 100.000 neurons, is relatively small compared to mammalian model organisms, as for example mouse (4.000.000 neurons). Identification of neural circuits in such rather simple brains is easier (Olsen & Wilson 2008) and even though they possess less neurons, they still can perform various elaborate and significant behaviors. For example, they can show aggressive behavior (Chen et al. 2002), courtship learning (Siegel & Hall 1979), different kinds of classical associative conditioning (gustatory (Masek & Scott 2010), olfactory (Quinn et al. 1974), visual (Spatz et al. 1974), operant conditioning in the heat box (Wustmann et al. 1996) and even social learning (Sarin & Dukas 2009).

A variety of visual conditioning paradigms that employ different visual stimuli like patterns or colors have been established to train fruit flies (Guo et al. 1996; Wolf & Heisenberg 1991; Menne & Spatz 1977; Spatz et al. 1974)(Table 1). However, only few details of the classical visual learning circuit are known. In my thesis I aimed to investigate the underlying circuit for visual memory formation in *Drosophila* by using a newly established visual conditioning paradigm and compare it to the well described circuit underlying olfactory conditioning.

## **1.2 Olfactory Conditioning**

### **The behavioral paradigm**

The fruit fly *Drosophila melanogaster* is capable of performing complex behavioral tasks, including classical associative learning. After association of a neutral sensory stimulus in the environment with a rewarding or punishing stimulus, the fly adapts its behavior relating to the learned stimulus. Thus, later on, it can avoid harmful

stimuli and approach rewarding stimuli. The first studies of this kind of associative learning had been done in *Drosophila* in the 1970s, where a mass of flies was trained to associate an odor with electric shock, which served as strong punishment (Quinn et al. 1974). A simple manually operated setup was built consisting of vertical tubes containing the odors and a shock grid made of copper wire. With the help of a fluorescent lamp flies were attracted to enter the training tube. However, the setup yielded rather low learning scores, probably because overcoming the naïve phototaxis response prevented some flies from walking up into the shock grid tube and receiving punishment. Later on, using the same behavioral setup, one could also reward the flies with sucrose on a filter paper while presenting them an odor (Tempel et al. 1983). In 1985 the assay got further modified and it became possible to aversively train and test flies with high throughput in the so called T-maze with purely classical training (Figure 2A)(Tully & Quinn 1985)). Application of sugar paper is also possible in this improved setup (Schwaerzel et al. 2003; Colomb et al. 2009). Since then the most widely used paradigm for olfactory learning is the T-maze also because it is possible to apply either appetitive or aversive reinforcer in the same setup where flies are performing the same behavioral task. This is a big advantage of the olfactory learning setup since one can now compare appetitive and aversive memory within one training modality. Further modifications of the olfactory assay including automatic application of odors and electric shock led to facilitation of high-throughput and multiplication of experiments (Figure 2B) (Murakami et al. 2010). Thus, these latest upgrades also make the setup highly suitable for extensive screening of libraries of various genetic drivers.

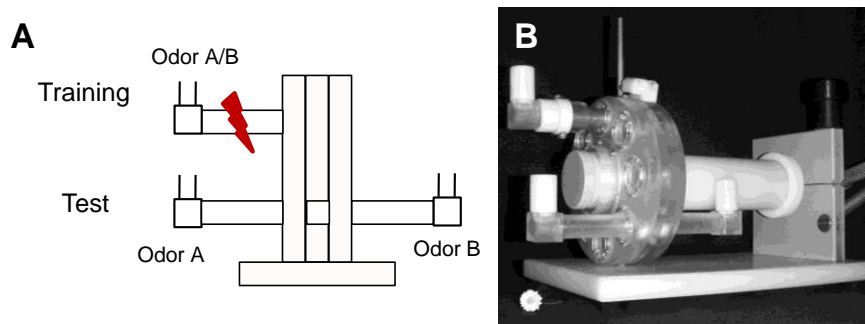
## **Conditioning procedure**

Briefly, a mass of freely walking flies gets successively trained with two odors whereas one of them is paired with reinforcement (CS+). Duration of CS presentation is one minute. The CS usually precedes the US for a few seconds (Delay conditioning). In appetitive conditioning sucrose is used as reward and presented during whole CS+ presentation. In aversive conditioning electric shock is used as punishment and flies receive 12 electric shocks of 90/100 Volt during CS+ exposure (one second shock every 5 seconds). To test the flies' memory they are released into a choice point between two horizontal tubes filled with the paired and control odor, respectively (Figure 2A). For two minutes they are allowed to choose between the two odors. Then, flies in the two tubes

are counted and approach or avoidance of the paired odor is measured to assess memory performance. Flies can go through several training trials in very short time. They can already form an association even after one training trial, meaning a single exposure of CS and US together is sufficient to induce significant learning in appetitive and aversive olfactory learning. Overall memory formation in this setup is highly reproducible and robust, but also remains flexible since various different behavioral protocols can be trained.

### Timing makes a difference in olfactory learning

Another essential task for animals is to associate non overlapping stimuli (Rescorla 1988). In nature, coincidence of CS and US is not always given, such as with food poisoning or when the nutritional value of food serves as reinforcer. Pavlov could already show that dogs are able to associate a preceding CS with a following US even when the two stimuli were separated by a gap of several minutes (Pavlov 1927). Also *Drosophila* and even their larvae are capable to form such kind of memory with olfactory stimuli (Tully & Quinn 1985; Tanimoto et al. 2004; Khurana et al. 2009; Shuai et al. 2011; Galili et al. 2011). Even when the odor precedes the US for seconds, such as in trace-conditioning, flies are still able to form an association between the two stimuli and thus



**Figure 2: Commonly used olfactory conditioning paradigm.**

**(A)** Scheme of olfactory setup. On top, single training tube that can be electrified. Two odors are presented during training consecutively via odor cups. On the bottom, flies can choose between two tubes with the two trained odors attached during test, respectively. (Modified from Tully & Quinn, 1985.) **(B)** Photograph of olfactory conditioning revolver. Four experiments can be performed in parallel. (Modified from Yarali et al., 2008).

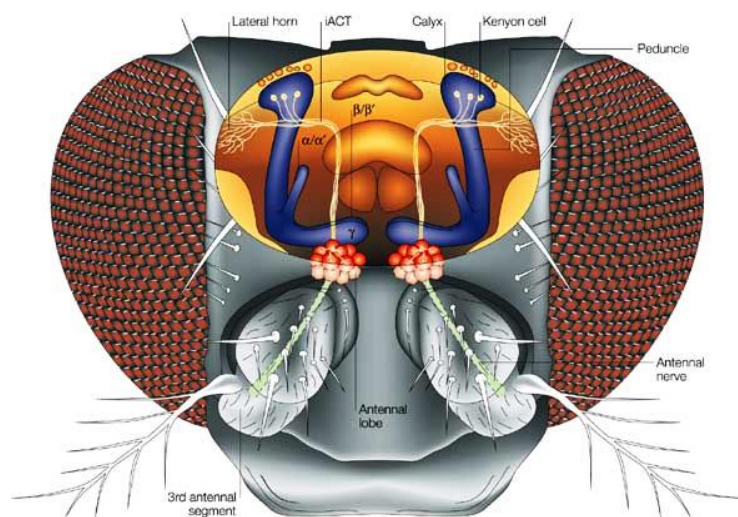
avoid the paired CS in test. However, the longer the interval (gap) between CS and US the worse flies are able to make the association. When training with stimuli in the reversed order, in the so called relief learning, where the odor follows the electric shock presentation, flies approach the paired stimuli later in test (Tanimoto et al. 2004; Yarali et al. 2008). They associate the odor with the beginning of a safety period (Sutton & Barto 1990; Chang et al. 2003) or the end of electric shock punishment (relief learning (Wagner 1981; Solomon & Corbit 1974). These two learning phenomena, trace and relief conditioning, have been rather well tested and described in *Drosophila* olfactory learning yet (Galili et al. 2011; Shuai et al. 2011; Yarali et al. 2008; Yarali & Gerber 2010). These conditioning tasks however can also be tested in mammals (Rogan et al. 2005), monkeys (Belova et al. 2007) and humans (Andreatta et al. 2010). Here, usually visual or auditory stimuli are employed.

### **The olfactory learning circuit**

By using a technically simple and flexible setup, the “T-maze” (Figure 2), it was already possible to find out many details about the underlying neural pathways for olfactory sensory processing (Figure 3), reinforcement processing and coincidence detection in appetitive and aversive classical olfactory memories in the last decades. Therefore, the most well studied learning circuit in *Drosophila* until now is by far the one for olfactory conditioning.

Olfactory information is perceived via ~1300 olfactory receptor neurons in the antennae and the maxillary palps (Stocker 1994). Each olfactory receptor neuron expresses one of ~60 specific membrane receptors (Clyne et al. 1999). The ones containing the same receptors convey odor information to ~ 50 specific glomeruli in the antennal lobe (Couto et al. 2005). Here the information can be modulated by > 1500 mostly inhibitory local interneurons (Chou et al. 2010). From the antennal lobe ~ 150 olfactory projection neurons (OPNs) project the odor information to higher brain centers like lateral horn and/or the mushroom bodies (MBs)(Wong, Wang, and Axel 2002; for review; Liang and Luo 2010)(Figure 3). The lateral horn seems to be more involved in mediating naïve behavior (Wang et al. 2003), whereas the MB is needed for associative memory (de Belle & Heisenberg 1994; Zars et al. 2000; Heisenberg et al. 1985). The MB comprises of ~2.000 MB intrinsic neurons (Aso et al. 2009) that were first described via

Golgi staining by Kenyon (Kenyon 1896), thus they are called Kenyon cells (KCs). Their dendritic region forms the calyx, closely situated below the cell bodies in the posterior region of the brain (Figure 4). Here, olfactory information is received via OPNs from the antennal lobe. The MB axons further penetrate into the anterior central brain, where they bifurcate and form a vertical and a medial lobe. The KCs can be divided into three subtypes (Crittenden et al. 1998). There are ~980  $\alpha/\beta$  neurons and ~360  $\alpha'/\beta'$  neurons that innervate the vertical and the medial lobes but also ~660  $\gamma$  lobe neurons that only innervate the medial lobe (Aso et al. 2009)(Figure 4). There is differential requirement of the lobes for different phases during memory formation. The  $\gamma$ -lobe is especially required during short term memory formation (2 minute memory; (Zars et al. 2000)) whereas the other lobes are rather involved in formation of longer lasting memories (Krashes et al. 2007; Blum et al. 2009).

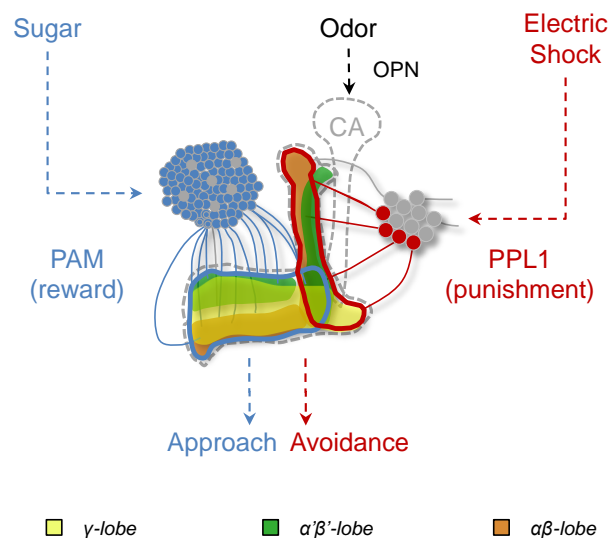


**Figure 3: Olfactory sensory circuit**

Open head of a fly. The prominent structures of the central brain are visible. Odors are perceived via olfactory receptor neurons inside the antennae that project to the antennal lobe (red). Projection neurons further mediate the olfactory information to higher brain centers, such as the MB or the Lateral Horn. MB is depicted in blue showing the dendritic region, the primary calyx, on top and the peduncle and lobes. (Modified from Heisenberg, 2003).



All lobes receive input from extrinsic MB neurons, e.g. neuromodulatory neurons like the dopamine neurons (Tanaka et al. 2008). Different clusters of dopamine neurons in the central brain were shown to convey information about appetitive and aversive reinforcement to the MB, respectively (Figure 4). In olfactory conditioning mostly sucrose is used as reward (Schwaerzel et al. 2003) and electric shock as punishment (Tully & Quinn 1985). Dopamine neurons from the PAM cluster mediate appetitive sugar information (C. Liu et al. 2012; Burke et al. 2012); whereas dopamine neurons from the PPL1 cluster mediate aversive information about electric shock to the lobes (Aso et al. 2010; Claridge-Chang et al. 2009). Hence both inputs, US and CS, reach the MB lobes and calyx, respectively. Thus, olfactory information can be modulated in the MB, which is the center for coincidence detection in olfactory classical learning, by the reinforcement information provided. Several output neurons that are projecting into the surrounding brain areas could then mediate for example approach or avoidance of the paired stimulus in olfactory classical conditioning (Ito et al. 1998; Dubnau & Tully 2001; Tanaka et al. 2008)(Figure 4).

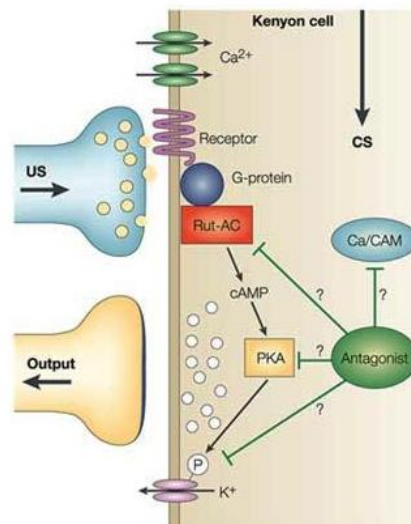


**Figure 4: The olfactory learning circuit inside the mushroom body of *Drosophila***

Odor is mediated from the antennal lobe to the MB primary calyx (CA) via OPNs. Information about appetitive and aversive reinforcement is mediated via PAM and PPL1 dopamine neurons to the MB medial and horizontal lobes, respectively. Output of the MB KCs is necessary for behavioral execution (Approach/Avoidance).

## Coincidence detection

To achieve neuronal plasticity, information about CS and US has to coincide and activate two different signaling pathways inside a single cell. Only if this activation happens at the same time, the synaptic output will get modified (Hawkins et al. 1983). The first steps in describing this signaling pathway in *Drosophila* were made possible by further analyzing mutants that had impairments specifically in learning (for example: Phosphodiesterase *dunce*; (Dudai et al. 1976; Byers et al. 1981), adenylate cyclase *rutabaga* (*rut*); (Aceves-Pina et al. 1983; Livingstone et al. 1984; Levin et al. 1992)). All these mutants have defects in genes required in the cAMP (cyclic adenosine monophosphate) signaling pathway (Figure 5). *rut* gets activated by calcium influx dependent on CS exposure as well as through a G-Protein-coupled-transmitter-receptor activated by US application. Once activated, *rut* then catalyzes the conversion of adenosine triphosphate (ATP) to cAMP. Increased cAMP levels lead to activation of the cAMP-dependent protein kinase A (PKA) that might act on downstream synapse proteins via phosphorylation (CREB, (Yin et al. 1994)) and in this way can induce neuronal plasticity.



**Figure 5: The coincidence detection mechanism inside the Kenyon cells**

The *rutabaga* protein (red) gets activated by parallel  $\text{Ca}^{2+}$ -influx caused by CS application and activation of a G-protein-receptor coupled cascade caused by US application. Thus, the catalyzed cAMP can activate PKA which acts on downstream synapse proteins to induce neuronal plasticity. (Modified from Heisenberg, 2003).

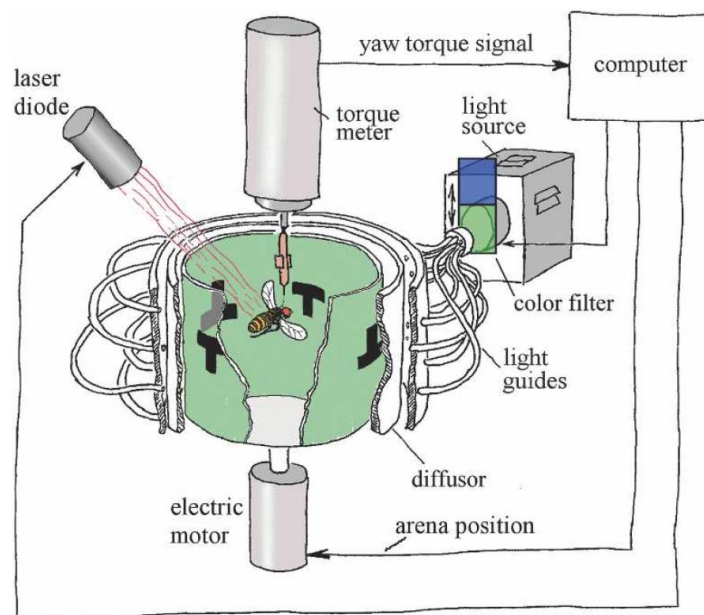
Mutation of the catalytic subunit of the PKA also leads to impairment in olfactory learning (DCO = PKA mutant; (Skoulakis et al. 1993; Lane & Kalderon 1993)). Also in other animals, cAMP was shown to be a crucial player of the learning and memory mechanism (Lechner & Byrne 1998; Kandel 2001). Interestingly, all these proteins are highly enriched inside the *Drosophila* MB (Nighorn et al. 1991; Han et al. 1992; Skoulakis et al. 1993), suggesting this neuropil to be important for memory formation. Indeed rescue experiments in the *rut* mutant background showed that restoring activity in the MB is sufficient to overcome the olfactory learning and memory impairment (Zars et al. 2000; McGuire et al. 2003; Schwaerzel et al. 2002). Since the memory impairment is not complete, other biochemical mechanisms must account for the remaining memory, such as via the Gilgamesh protein pathway (Tan et al. 2010).

### **1.3 Visual conditioning**

#### **Visual conditioning setups**

A variety of paradigms were established to classically train fruit flies with visual stimuli such as colors (see Table 1). Most of them were used the first time in the 1970s and are rarely used nowadays. In 1974, a setup was employed presenting visual stimuli (spatial discrimination learning with blue and yellow) paired with electric shock in a training alley (Spatz et al. 1974). The flies passed through illuminated funnels and reached a choice point after training. However, practicability was affected since the duration of a single run was five hours. Other paradigms employed various other color stimuli (see Table 1, always differential conditioning with two colors) paired with different aversive reinforcers, like quinine (Quinn et al. 1974) or shaking (Menne & Spatz 1977; van Swinderen et al. 2009). However, these studies showed that flies can perform color discrimination tasks and color learning, and therefore possess true color vision (Menne & Spatz 1977).

The most commonly used paradigm nowadays for visual conditioning is the flight simulator (Wolf & Heisenberg 1991). Here, a single fly is tethered on a hook inside a cylindrical flight arena (Figure 6). A torque-meter is used to measure the yaw movements of flies. In closed loop experiments, the information about yaw movement of the fly is processed and fed back onto the screen where the visual stimuli are shown in the flight arena, allowing the fly to steer through an artificial environment. For visual learning experiments flies are trained with either two different patterns (upright T vs. downright T, bars with different elevation, etc. (Wolf & Heisenberg 1991)) or color stimuli (green vs. blue (Wolf & Heisenberg 1997)), one of them paired with laser punishment (CS+). During test the flies can again choose between two patterns or two colors, and approach or avoidance of a pattern or color during flight is used as measurement for memory. In the commonly used closed loop learning protocol, the fly can actively avoid the punishment by flying towards the non-punished stimuli during the training phase. Since the fly can learn about the influence on the reinforcer of its own action, this protocol includes an operant component (Brembs & Heisenberg 2000). In contrast, during purely classical conditioning, animals are not able to influence the duration of exposure or strength of the CS or US.



**Figure 6: The flight simulator paradigm used for visual conditioning.**

A single fly is tethered on a hook inside an arena. From the walls of the arena different visual stimuli (e.g. T patterns or colors) are presented. Punishment can be applied by a laser diode. The fly's turning reaction is measured by a torque meter above and the signal is transmitted to a computer. The turning signal of the fly can be integrated with the shown visual stimulus and thus the fly can steer through an artificial environment (closed loop). (From Brembs & Wiener, 2006).

Visual Stimuli	Reinforcer	Setup	Reference
<b>Yellow/Blue</b>	Electric shock	Freely walking flies in a tunnel	(Spatz et al. 1974)
<b>Blue/Green UV/Blue</b>	Quinine	Freely walking flies in a Y-maze	(Quinn et al. 1974)
<b>Yellow/Blue</b>	Shaking	Freely walking flies in a container	(Menne & Spatz 1977)
<b>Colors</b>	Sucrose	Freely walking flies in an arena	(Heisenberg 1989)
<b>Blue/Green</b>	Laser	Tethered flying fly in an arena	(Wolf & Heisenberg 1991)
<b>Light/Dark</b>	Quinine	Freely walking flies in a Y-maze	(Le Bourg & Buecher 2002)
<b>Cyan/Purple</b>	Shaking	Freely walking flies in a container	(van Swinderen et al. 2009) - Supplementary
<b>Blue/Green</b>	Sucrose Formic acid	Freely walking flies in an arena	(Schnaitmann et al. 2010)

**Table 1: Paradigms for visual conditioning that employ a classical learning protocol.**

Various different combinations of visual stimuli (wavelength/intensities) have been trained with different appetitive and aversive reinforcers since the 1970's. Most commonly used assay is by far the flight simulator, however usually a partly operant protocol is applied.

Recently I, along with Christopher Schnaitmann, developed a new visual conditioning assay using sucrose or formic acid as appetitive or aversive chemical reinforcer, respectively (see Table 1)(Schnaitmann et al. 2010). Visual stimuli and reinforcers on a filter paper were presented to flies from beneath in a cylindrical arena. Appetitive conditioning produced rather high and very robust learning scores. However,

aversive reinforcement with formic acid led to significant but not very high memory scores.

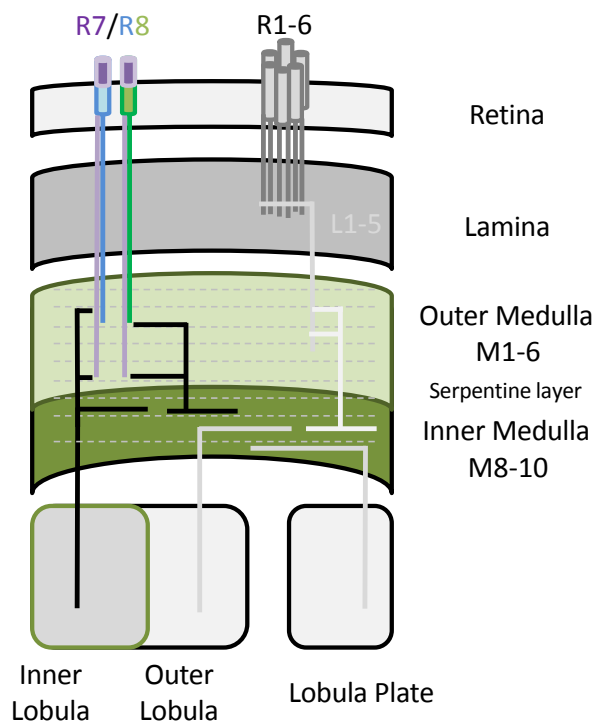
### **Does timing make a difference in visual learning?**

Relief and trace conditioning are well established in olfactory conditioning (see above). However, when studying the temporal dynamics of conditioning, a problem in using odorants can be that residual odors stick to the experimental setup and therefore distort the learning results (Galili et al. 2011). Using a different sensory modality, like visual stimuli that are also used for conditioning with mammals and humans in trace or relief learning, not only facilitates stimulus application, but also residual stimuli cannot be held to be accountable for significant learning values. Indeed there are already several studies that suggest that insects can possibly perform at least trace conditioning with visual stimuli. The first report is from the early 1930s in honeybees (Opfinger 1931). Here, color presented during the approach of a food source is learned better than the color presented during feeding. In the past 50 years, several more visual trace conditioning studies have been carried out with honeybees (Menzel 1968; Grossmann 2010) showing that visual stimuli are well suited to study this learning form. Also *Drosophila* is probably able to form a memory trace of visual stimuli, e.g. flies can remember the position of a vanished visual object and use this information later on for navigation (Neuser et al. 2008). Employing the newly established setup I first aimed to investigate if *Drosophila* is capable of mastering trace and relief conditioning with visual stimuli like color cues and if similar effects as in olfactory conditioning can be found. Such result would suggest that trace and relief conditioning is a common learning phenomenon in *Drosophila* that can be learned independent of the sensory modality used.

### **Visual processing in the optic lobe**

Visual stimuli like moving objects, colors and patterns are perceived by the photoreceptors in the ommatidia of the fly eye. Each eye contains ~800 ommatidia in the retina. Inside each single ommatidia, eight photoreceptors R1-8 are housed (Review: (Wolff & Ready 1993), Figure 7). R1-6, the outer photoreceptors, are tuned to broad

chromatic stimuli and were therefore thought not to be involved in color discrimination (Troje 1993) but only in motion vision (Review: (Hardie 1985; Borst 2009)). However, several studies claim that these broadband receptors can definitively be used for color discrimination (Fischbach 1979; Schnaitmann et al. 2013). R7 and R8 respond to more specific wavelengths and could therefore be relevant for color discrimination (Harris et al. 1976; Heisenberg & Buchner 1977; Fischbach 1979; Troje 1993; Fukushi 1994). 30% of the fly ommatidia contain the photoreceptor-pigment Rh3 in R7 (355nm, UV) and Rh5 (460nm, blue) in R8 and are called pale ommatidia. The other 70% contain Rh4 in R7 (335nm, UV) and Rh6 in R8 (530nm, green) and are called yellow ommatidia. Both types of ommatidia are stochastically distributed in the retina (Franceschini et al. 1981; Wernet & Desplan 2004). Overall, the optic lobe of *Drosophila* consists of ~60,000 cells and can be further divided into four information processing neuropiles, the lamina, the medulla, the lobula and the lobula plate (Fischbach & Dittrich 1989), review: (Morante & Desplan 2004)). All photoreceptor axons project to deeper neuropiles in the fly optic lobe. R1-6



**Figure 7: Visual sensory circuit.**

Visual stimuli are perceived via eight different photoreceptors in the retina. R1-6 project to the lamina, from where L1-5 transmit the perceived signal to the medulla layers M1-5. R7-8 directly project to the medulla layer M3/4 and M6. Inside the medulla, several extrinsic and intrinsic transmedullar neurons (Mi, Mt, Tm, Tmy) modulate the visual information and also mediate it to further neuropiles like lobula and/or lobula plate.

already harbor in the lamina where they connect with different large monopolar cells (L1-3) and one amacrine cell (amc). L1 and L2 in the lamina form the major input for the motion detection circuitry (Rister et al. 2007; Joesch et al. 2010). The following medulla is the biggest neuropil in the optic lobe containing ~40.000 cells (Hofbauer & Campos-Ortega 1990) and is comprised of layers M1-10 orthogonal to the photoreceptor projection (Figure 7)(Fischbach & Dittrich 1989). The more distal layers M1-6 receive direct and indirect input from receptor neurons; however, the inner layers M7-10 do not receive direct input from photoreceptors. The different lamina neurons L1-5 connect to the corresponding outer medulla layers M1-5. R7 and R8 directly project further down from the retina to the medulla. Here, R7 innervates Medulla layer M6, whereas R8 innervates Medulla layer M3/M4. Several transmedullar neurons (Tm) collect information from the different layers M1-10 and are connected to further downstream neuropiles like lobula, lobula plate or the protocerebrum. To be able to see different colors, color-opponent cells that process input from different photoreceptors are necessary. Tm5 and Tm9 in the medulla were suggested as such, since they receive direct input from R7/R8 and also indirect input from R1-6 in the medulla and provide output not only to the lobula but also to layer M8 in the medulla (Gao et al. 2008). Also projections from lobula and lobula plate expand into the protocerebrum of the fly. Specific visual projection neurons have been described to project from the lobula to the central brain (Otsuna & Ito 2006). A similar glomerular architecture in the protocerebrum has been suggested (Mu et al. 2012) as described in the olfactory sensory pathway with the antennal lobes (Couto et al. 2005).

### **Visual processing in the central brain**

Thus, a possible pathway for color perception in the optic lobe of the fly can already be followed in detail with the help of anatomical studies; however, description about function of distinct neuropiles and cells is missing for the most parts. Also the visual memory formation pathway is only poorly described. What was shown so far is that, in contrast to the olfactory learning circuit, the Central Complex (CC), another prominent neuropil in the fly central brain is critical for visual pattern learning in the flight simulator (Pan et al. 2009; Liu et al. 2006; Wang et al. 2008). Mutation of the *rutabaga* protein also leads to impairment of different kinds of visual pattern memory. Restoration in the CC fully rescues the memory impairment. Interestingly, different subdivisions of



the CC fan shaped body seem to represent information about different kinds of such learned patterns (Pan et al. 2009; Liu et al. 2006).

The role of the MB in visual processing and learning is contradictory. Even though the MB was shown not to be involved in classical visual learning tasks in *Drosophila* (Heisenberg et al. 1985), there are several studies showing that visual information must be processed in the *Drosophila* mushroom body to some extent (van Swinderen et al. 2009; Barth & Heisenberg 1997). Also performing different experiments that demand more complex visual memory tasks in the flight simulator assay revealed a requirement of the MBs. One example is the 'context generalization' training, where the flies learn to associate a specific pattern with punishment while the background color between training and test situation is changed from white to monochromatic lights (Liu et al. 1999). Wild-type flies can master this task; flies lacking functional MB however, cannot recognize the pattern in the changed background and are thus not able to generalize between the different backgrounds. In another flight simulator experiment it was shown that MB-blocked flies possess altered decision making behavior. When asked to decide between one of two trained visual stimuli with different saliency, like color contrast and height of a bar (Zhang et al. 2007), wild-type flies abruptly change their preference upon saliency change. MB-block flies however, show linear choice and slowly change their preference. Another task in the flight simulator is operant learning where flies learn about the influence of their own action on the reinforcer (Brembs & Heisenberg 2000). Wild-type flies, trained with mixed operant/classical conditioning, need to execute a large amount of training trials to learn the isolated operant component. Here, blocking the MB facilitates formation of the operant learning component and flies are able to establish the operant memory trace only after a few training trials (Brembs 2009). These studies clearly indicate that MBs are at least necessary to modulate visual information processing in different conditioning tasks. However, although many experiments with such different visual stimuli had been performed, especially by employing the flight simulator, it has not been understood yet how flies learn about color or intensity information of a visual stimulus.

## 1.4 Drawing a comparison between conditioning setups using different sensory modalities

To summarize, so far different neuropiles in the *Drosophila* brain were shown to be required for memory formation with different sensory modalities, such as visual and olfactory cues. However, comparing results obtained with these diverse behavioral assays is not reasonable, since these assays employ very specific and different behavioral tasks (e.g. flight orientation, binary choice by walking flies, etc.) and apply mostly different conditioning or protocol designs (Pitman et al. 2009; Neuser et al. 2008; Brembs 2008; Brembs & Wiener 2006). One-to-one comparison of paradigms used for behavioral learning experiments in visual and olfactory conditioning reveals tremendous differences concerning reinforcement, fly maintenance, amount of flies and protocol.

	Visual learning in the Flight Simulator	Olfactory learning in the T-maze	New visual learning assay
Flies	Single fly	Mass assay	Mass assay
Task	Flying, tethered	Freely walking	Freely walking
Reinforcement	Aversive: laser beam	Aversive: electric shock	Aversive: electric shock
		Appetitive : sucrose	Appetitive : sucrose
Protocol	Mixed classical/operant	Purely classical	Purely classical
High-throughput	No	Yes	Yes

**Table 2: Comparison between the flight simulator, the olfactory conditioning assay and the newly established visual paradigm.**

Points of comparison are amount of flies, task the fly has to perform, reinforcement that can be applied, training protocol and if the assays are high-throughput compatible. All in all, the new visual conditioning paradigm shares more similarities with the olfactory paradigm than with the flight simulator paradigm.

Especially the flight simulator stands out when comparing visual and olfactory paradigms and is therefore the least compatible assay in comparison with the common olfactory conditioning setup (see Table 2). However, learning paradigms should be designed to be comparable when contrasting memories of different sensory modalities (Guo & Guo 2005; Zhang et al. 2013; Scherer et al. 2003; Gerber, Scherer, et al. 2004; Hori et al. 2006; Mota et al. 2011). Only by employing similar conditioning assays will it be possible to discover common neural circuits between modalities, otherwise one cannot clearly state that found defects are due to a difference in the paradigm used or the modality used. An interesting attempt to compare visual and olfactory conditioning was done by Yadin Dudai and colleagues (Dudai & Bicker 1978). They tested flies in the visual shaking assay (Menne & Spatz 1977) and the olfactory conditioning assay (Tully & Quinn 1985): However, especially in visual classical learning they could not obtain consistent results testing different wild type strains. Their results suggest that a more salient and potent aversive reinforcer than shaking is necessary to obtain stable memory scores in visual aversive learning. In the olfactory conditioning paradigm electric shock was used as reinforcer and indeed it was possible for them to obtain stable memory scores testing all wild-type strains. Overall electric shock seems to be a more potent reinforcer than shaking the flies or present quinine or acid as punishment (Table 1) (Schnaitmann et al. 2010). Another advantage of the olfactory conditioning assay is that it is possible to train flies with appetitive and aversive reinforcers employing the same setup (Table 2). However, none of the previous used visual conditioning paradigms allows such simple application and switching of appetitive and aversive reinforcers.

Therefore, we aimed to establish a new visual paradigm that includes all the advantages of the olfactory paradigm and would allow us to compare appetitive and aversive visual learning with appetitive and aversive olfactory conditioning. Critical features such as the conditioning design, binary choice between two conditioned stimuli in the test, appetitive and aversive reinforcer and altered distribution of flies as an indicative of memory performance should be kept similar. A visual appetitive assay (Schnaitmann et al. 2010) employing same reinforcer, sucrose as reward, as in olfactory conditioning (Tempel et al. 1983; Schwaerzel et al. 2003) was already established a few years ago. Here, a group of freely walking flies receives subsequent presentation of two visual stimuli (green/blue) from beneath, where one of the colors is paired with sucrose reward, presented on filter paper. Having similar setups for different modalities allows direct comparisons of mechanisms underlying appetitive visual and olfactory memories.

To be also able to compare aversive visual and olfactory memory, we developed an associative learning assay where electric shock punishment can be paired with visual stimuli (Vogt, Schnaitmann, et al. n.d.). Constructing a 'transparent' shock grid we can present visual stimuli from beneath as in the appetitive setup. This now allows comparisons of appetitive and aversive visual memories since we can apply the same visual stimulation. With this paradigm, flies have to perform the same behavior task in appetitive and aversive visual learning and the only variable between the two protocols is the nature of the US. This is important for being successful in finding differential neuromodulator circuits underlying appetitive and aversive memories for one modality (Schwaerzel et al. 2003; Honjo & Furukubo-Tokunaga 2009; Vergoz et al. 2007; von Essen et al. 2011; Gerber, Scherer, et al. 2004). Furthermore, only now can we compare both kinds of visual and olfactory memories, because so far there were no visual learning paradigms available that could be contrasted to both, appetitive and aversive, olfactory memories in adult *Drosophila*.

## **1.5 The role of the MB in insect behavior**

### **The MB as a center for multi-modal memory formation in *Drosophila***

The MB of *Drosophila* clearly plays a pivotal role in olfactory memory formation. However, meaningful experiences like food reward or punishment should drive animals to form different associative memories of a sensory-rich environment. Thus, the induced memory should usually not be restricted to a single sensory cue in the environment like an olfactory stimulus. Not much is known about how animals associate different sensory cues like olfactory and visual stimuli with a single positive or negative experience, respectively. There are two possible alternative mechanisms how such multi-modal memories could be processed: On the one hand, distinct neuropil could host memory traces of different sensory stimuli and therefore information about same reinforcement must be processed in different regions in the brain for example as in the MB and CC of the fruit fly (Zars 2010). On the other hand, a single circuit could be shared and same reinforcement neurons could be employed for learning about different sensory stimuli, for example as inside the MBs. The latter circuit was found to be employed in mammals.

During fear conditioning in rats, auditory and visual stimuli information converges in the amygdala, where association takes place for both stimuli (Campeau & Davis 1995). In insects no such circuit has been described. Pharmacological experiments in crickets however showed that dopaminergic neurons are required for both, olfactory and visual learning (Unoki et al. 2005; Unoki et al. 2006; Mizunami et al. 2009), but circuit mechanisms underlying such multi-modal memories driven by a single appetitive or aversive stimulus remained unknown.

In other insects it was already shown that the MB indeed receives input from different sensory stimuli (Honeybee (Mobbs 1982), Ants (Ehmer & Gronenberg 2004; Gronenberg & Lopez-Riquelme 2004), Cockroach (Li & Strausfeld 1997)). Since the MBs are highly conserved structures across many insect species, having a closer look on the anatomy and the overall behavioral impact of MBs in different insect groups can probably give a hint on the general function of this prominent neuropil.

### **Sensory processing in the MB across insect species**

The MBs in insects have been first described in 1850 (Dujardin 1850). From early experiments (Faivre 1857) one could conclude that the MBs are less involved in performing simple motor action since even decapitated insects can still walk and survive. However, they were unable to perform complex movement patterns. This was confirmed by a study in *Drosophila* showing that flies have prolonged walking activity after blocking MBs (Martin et al. 1998). They do not show locomotion deficits, but seem to be unable to perform pausing. Other defects also have been found when testing flies for shock habituation but not initial shock response (Acevedo et al. 2007) or sleep rhythm (Joiner et al. 2006). Therefore, the MB was thought to provide a certain degree of performing voluntary actions. Across insect species, the MB is known to be mainly involved in modulation of olfactory information since the PNs provide most prominent input to the MB calyx (Ehmer & Gronenberg 2004; Li & Strausfeld 1997; Liang & Luo 2010). The MBs not only show strong response when applying olfactory stimuli during whole cell recordings in vivo (*Drosophila* (Turner et al. 2008)), but additionally olfactory memory is strongly impaired when ablating or blocking the output of the *Drosophila* MBs via genetic modifications (Heisenberg et al. 1985; Zars et al. 2000). Also in the honeybee, localized cooling of the MB impairs olfactory learning (Erber et al. 1980).

However, some insect species possess not only olfactory, but also multimodal afferents projecting to the calyx or the lobes of the MB (Kenyon 1896; Li & Strausfeld 1997). These inputs provide information about different sensory stimuli, like gustatory, visual, olfactory or mechano-sensory cues. Different insect groups indeed possess olfactory and also prominent visual projection neurons to the MB calyx such as ants (Ehmer & Gronenberg 2004) or honeybees (Grünewald 1999; Mobbs 1982). Even though *Drosophila* probably needs to perform less complex behaviors than e.g. honeybees, they have a similar lifestyle with similar diurnal activity rhythm and requirement in flexibility when searching for food patches or mating partners. Thus, *Drosophila* would gain an advantage from flexibility in processing visual cues related to such elemental behaviors that can be provided by a connection between MB and optic lobes. Taken together the already performed studies with visual stimuli in the flight simulator (see 1.3) with the anatomical data from other insects, it would be reasonable to search for a visual memory trace in the *Drosophila* MBs.

## **1.6 *Drosophila melanogaster* as a model organism to study the neural circuit underlying visual memory**

A big advantage in working with *Drosophila* is, of course, the enormous variety of genetic tools that are currently available. Using these highly specific tools it is possible to detect and dissect underlying neural circuits of different behaviors in the relatively small brain of a fly, even up to a level of single neuron manipulation.

### **A classical approach to study the function of a neuronal circuit**

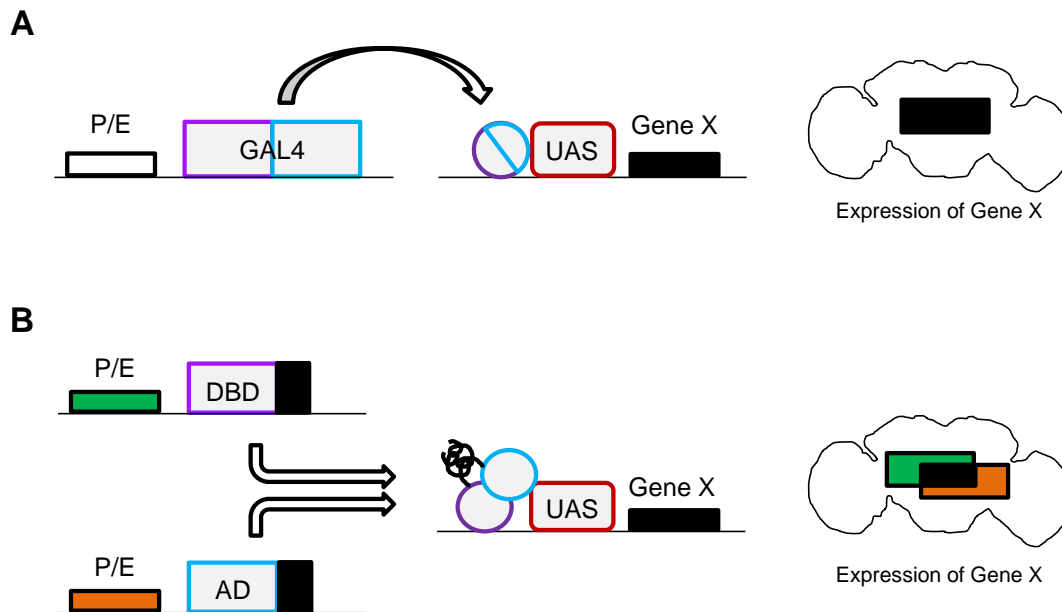
Already in the early 1970s, genetic tools were used to find specific important neuropiles and genes that are important for classical olfactory conditioning in the fly. By chemical mutagenesis, phenotypic mutants were produced bearing single gene deficits. Testing mutants revealed impairments specifically in olfactory learning for the phosphodiesterase *dunce* (Dudai et al. 1976; Byers et al. 1981) and the adenylate-cyclase *rutabaga* (*rut*) (Aceves-Pina et al. 1983). Other chemically induced mutants with

perturbed MBs were employed to detect the significance of this neuropil for different learning paradigms (mbm, mbd (Heisenberg et al. 1985)). However, in these structural mutants, every individual fly had to be dissected and checked for actual depletion since the effect was varying. Another approach, however with the same problem, was chemical ablation of specific neuropil such as the MB, by feeding hydroxyurea to larvae (de Belle & Heisenberg 1994). However, some of the mutants were still used to identify specific roles of genes.

### **Binary expression systems as genetic tools for dissecting neuronal circuits**

Introduction of binary transcription factor systems in *Drosophila* clearly enhanced the possibilities of genetic dissections. The GAL4-UAS system is probably the most widely used method (Figure 8)(Fischer et al. 1988; Brand & Perrimon 1993). Briefly, in one fly, an endogenous promotor or enhancer controls the expression of an inserted P-element including the GAL4-protein-sequence (driver line). The expressed GAL4-protein itself has no effect since it originates from yeast and is not interacting with the fly genome. In another fly, an inserted upstream activation sequence (UAS) controls the expression of the favored transgene (effector line). The UAS can only be activated by the expressed GAL4-protein and no other endogenous proteins of the fly. Only by crossing these two flies and having both p-element insertions present, is the transgene expressed under the control of the endogenous promotor or enhancer. This technique enables endless pairings of GAL4-driver line and UAS-effector line modules. Another component specific to this system is the GAL80-protein (Lee et al. 1999) which blocks the activation domain of the GAL4 protein. Thus, overlapping expression of GAL4 and GAL80 leads to non-functionality of the whole system. Employing this method one can further specify the expression pattern by expressing both, the GAL4 and the GAL80 proteins, under the control of distinct promotors. A further new method to specify expression patterns was developed by splitting the GAL4 protein, the so called Split-GAL4 system (Figure 8)(Luan et al. 2006). Split-GAL4-lines have high specificity in expression pattern, since here the DNA-binding domain (DBD) and the activation domain (AD) of the GAL4-protein are independently targeted by two different promotors. With this system, the transgene is only expressed in the intersection of the expression patterns where the functional GAL4-protein can be formed (Luan et al. 2006). Like that, higher specificity in the expression

pattern of the GAL4 protein can be achieved. It is even possible to have only a single neuron labeled in the brain of the fly. Other systems that can be combined for example to achieve double staining of different driver lines are the Q system (Potter et al. 2010) and the LexAop-system (Szuts et al. 2000; Lai & Lee 2006).



**Figure 8: Functional mechanism of the GAL4 and Split-GAL4 technique**

**(A)** A GAL4-P-element is inserted after an endogenous promoter or enhancer (in this case the P-element contains a promoter sequence) in the driver line. The expressed GAL4-protein acts on the UAS-sequence of a second insertion in the genome provided by the effector line. The UAS sequence controls the expression of an effector gene X. Thus, the expression pattern of the gene X resembles the one of the endogenous protein controlled by the promoter or enhancer that drives the GAL4-protein. **(B)** When using the Split-GAL4 technique, the DBD-domain of the GAL4 protein is expressed under a different enhancer/promoter than the AD-domain. By additionally expressing a Zink-finger protein sequence the two protein-halves can bind and form a functional GAL4-protein that activates the UAS sequence when both are present. Thus, the expression pattern of the Gene X resembles the overlap of expression of the two selected promoters/enhancers.

Different transgenic effectors can be expressed via these binary systems. Fluorescent markers, like green or red fluorescent proteins (e.g. UAS-GFP/UAS-RFP) can be used to visualize the expression pattern of the GAL4-driver lines. Combining two different binary systems (e.g., LexAop and GAL4/UAS) gives the possibility to express two halves of a split GFP protein under the control of two different promoters (Split-GFP).



Reconstituted GFP thus only gives visible fluorescence signal where the marked cells connect very close, as for example in synapses (Feinberg et al. 2008; Pech et al. 2013).

A variety of different tools also exist to manipulate neural activity. In this study I mostly employed *shibire<sup>ts1</sup>* (*shi<sup>ts1</sup>*, (Kitamoto 2001)), a temperature sensitive mutant dynamin protein, that is functional in permissive temperature (<30°C) but blocks synaptic transmission in restrictive temperature (>30°C). Activation of neurons can be achieved by employing dTrpA1 (Hamada et al. 2008), a heat-activated Transient Receptor Potential (TRP) family ion channel. The dTRPA1-channel opens at temperatures higher than 29°C. These temperature sensitive tools also have the advantage that their functionality is reversible by bringing flies back to colder temperatures and that they are not active during development.

## 1.7 Aim of the thesis

Anatomical data in other insects suggests the MB as a conserved higher brain center for memory formation and storage of different kinds of sensory stimuli and even higher cognitive functions in insects. In *Drosophila*, the MB was already shown to be involved in different sensory conditioning tasks. Olfactory conditioning, as well as gustatory conditioning (Masek & Scott 2010) and courtship conditioning (Keleman et al. 2012) are all MB dependent. In my thesis I want to investigate the role of the MB for visual classical learning in *Drosophila* and compare it to the well described role in olfactory learning. Therefore, I and my colleague developed a new classical visual learning setup that uses sucrose and electric shock as appetitive and aversive reinforcers, respectively, similar as in olfactory conditioning. Also a similar task is demanded from the flies even though different sensory stimuli are used as CS. Thus, flies are trained and tested under similar conditions as in olfactory conditioning, which will allow a direct comparison of the two modalities. Employing genetically modified flies, I will block the synaptic output of MB-specific GAL4 lines to find neurons necessary for the visual conditioning task. Specific requirements of neurons during behavioral training and test, respectively, will additionally reveal functional implications. Using even more specific Split-GAL4 lines will allow me to further dissect functions of single cell types extrinsic or intrinsic to the MB. Additionally I will describe in detail anatomical structures required for visual memory formation.

## 2. Materials & Methods

### 2.1 Flies and genetic crosses

*Drosophila melanogaster* were reared in mass culture at 25°C, at 60% relative humidity, under a 12-12-hour light-dark cycle on a standard cornmeal-based food. The Canton-Special (CS) wild type strain was used for all experiments that did not include genetically modified flies and for control crosses including WT flies. For experiments, 1-3 day old flies were collected in fresh food vials and kept overnight at 25°C and 60% relative humidity.

All used transgenes are inserted in the *w* mutant background if not otherwise stated. F<sub>1</sub> progenies of crosses between females of *UAS-shi<sup>fs</sup>* (Kitamoto 2001), *MB247-GAL80;UAS-shi<sup>fs</sup>* (Krashes et al. 2007), *UAS-mCD8::GFP* (Lee & Luo 1999) or WT-females and males of *MB247-GAL4* (Schulz et al. 1996), *+c305a-GAL4* (Krashes et al. 2007), *17D-GAL4* (Martin et al. 1998), *201y-GAL4* (Yang et al. 1995), *c205-GAL4* (Li et al. 2009), *GH146-GAL4* (Stocker et al. 1997), *VT8475-GAL4* (VDRC, Vienna, Austria), *GMR28F07-GAL4* (Janelia Gen1 line, Ashburn, Virginia, USA) or Canton-S males were used. Further, I tested *w<sup>+</sup>,rut1;UAS-rut* mutant lines with defect adenylate cyclase protein kindly provided by Li Liu (Pan et al. 2009). *rut1;UAS-rut* were crossed with *c205-GAL4* and *MB247-GAL4* to induce rescue in specific neuropiles.

To specify requirement of MB input neurons, such as dopaminergic neurons, requirement of specific KCs of the MB and MB output neurons in visual learning we utilized specific Split-GAL4 lines manufactured in Janelia Farm Research Campus. For generation of Split-GAL4 lines the two GAL4 halves (DBD and AD) were inserted in specific sites in the fly genome; *attp40* and *attp2* on second and third chromosome, respectively (Table 10, Table 11 and Table 12). F<sub>1</sub> progenies of crosses between females of *UAS-shi<sup>fs</sup>* (Kitamoto 2001), *20XUAS-IVS-Shibire[ts1]-p10* (Pfeiffer et al. 2012), *UAS-mCD8::GFP* (Lee & Luo 1999), *mb247-DsRed; mb247-splitGFP11*, *UAS-splitGFP1-10* (MB-split-GFP-line (Pech et al. 2013)), *UAS-myr-CD8::Cherry*, *13F02-LexA/Cyo; LexAop-GFP/TM2* (for double labeling of VPns and MB calyx), *UAS-Denmark::mCherry*; *UAS-Syt::GFP* (Nicolai et al. 2010), *y-w<sup>-</sup>, hsp70-flp [X]; UAS>CD2 y+>mCD8::GFP* (Wong et al. 2002), or WT-females and males of specific Split-GAL4

lines (kindly provided by Gerry Rubin, Janelia Farm, Ashburn, Virginia, USA) or Canton-S males were used. Further Split-GAL4 lines tested beyond the screening were generated by inserting 52B07-p65ADZp into attP40 and 52H01-ZpGdbd into attP2 (*MB262B*), 19B03-p65ADZp into attP40 and 39A11-ZpGdbd into attP2 (*MB607B*), 28F07-p65ADZp into attP40 and 10E05-ZpGdbd into attP2 (*425B*) and 52G04-p65ADZp into attP40 and 49F03-ZpGdbd into VK00027 (*334C*). In two effectors (*UAS-shi<sup>fs</sup>*, *20XUAS-IVS-Shibire[ts1]-p10*) used, the X chromosomes of strains were replaced with that of wild-type Canton-S (*w<sup>+</sup>*) since *white* mutation could have some effect on the synapses of the visual systems (reviewed by (Bicker 2001)).

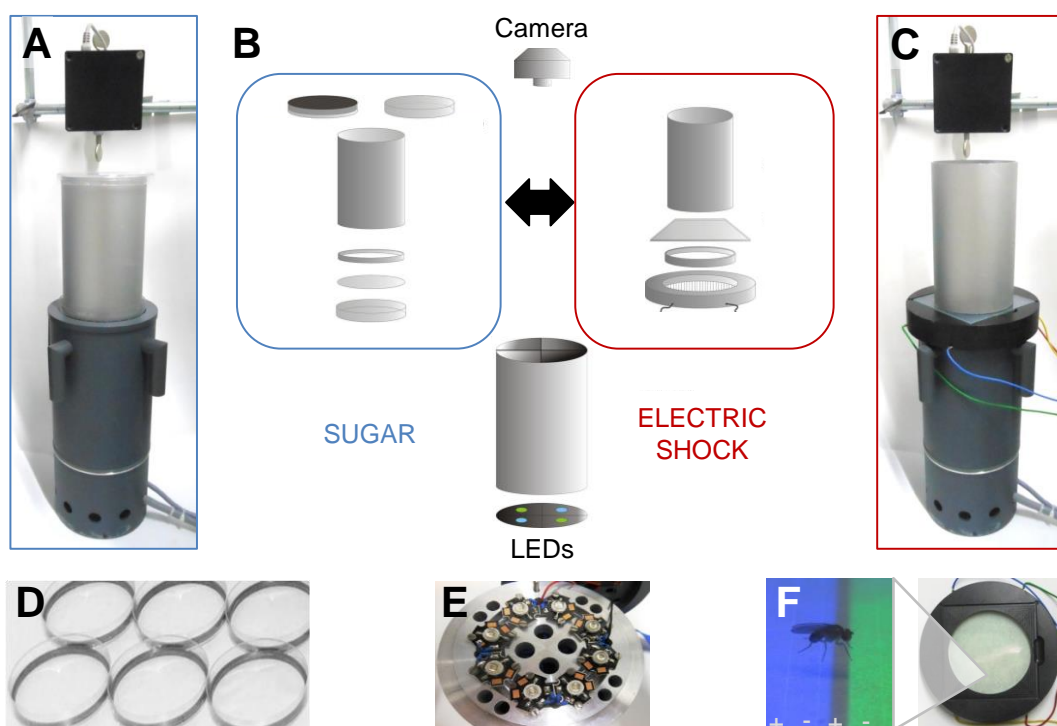
Flies with the correct genotypes were sorted under CO<sub>2</sub> anesthesia at least two days before experiments. For *rut*-rescue only males of the F<sub>1</sub>-progeny were used to perform behavioral experiments. For appetitive conditioning experiments 2-4 days old flies were starved in moistened empty vials to the mortality rate of approximately 20% (Schnaitmann et al. 2010). For aversive conditioning, starvation was not applied. For each behavioral experiment I used 30-40 mixed male and female flies. All experiments were performed in dim red light and in a custom made plastic box, containing a heating element on the bottom and a fan for air circulation.

## 2.2 Visual conditioning setups

### Visual stimulation and behavioral recording

We designed exchangeable conditioning arenas for reward and punishment application (Figure 9A-C). For both modules light-emitting diode (LED) arrays were used to apply visual stimuli (Green/Blue light) from beneath the flies (Figure 9B,E). We constructed a stimulation module using computer-controlled high-power LEDs with peak wavelengths 452 nm and 520 nm (Seoul Z-Power RGB LED) or 456 nm and 520 nm (H-HP803NB, and H-HP803PG, 3W Hexagon Power LEDs, Roithner Lasertechnik) for blue and green stimulation, respectively. LEDs were housed in a base (144 mm below the arena), which allowed homogeneous illumination of a filter paper as a screen. Using a custom made software and controlling device we were able to illuminate four quadrants of the arena independently. For separate illumination of each quadrant, the light paths of

LEDs were separated by light-tight walls in a cylinder with air ducts (Figure 9A-C). The intensities were controlled by current and measured using a luminance meter BM-9 (Topcon Technohouse Corporation) or a PR-655 SpectraScan® Spectroradiometer: 14.1 Cd/m<sup>2</sup> s (blue) and 70.7 Cd/m<sup>2</sup> s (green). Each quadrant was equipped with an Infrared-LED (850 nm), which was used for background illumination, e.g. during preference/avoidance test.



**Figure 9: Modular appetitive and aversive visual learning**

**(A-C)** Experimental setups used for appetitive and aversive visual conditioning. Scheme shows single components **(B)** of exchangeable conditioning arenas for sugar reward **(A)** and electric shock punishment **(C)** that share the same light source and video camera **(B)**. **(B)** Appetitive setup: cylindrical Fluon-coated arena closed from top with opaque lid during training or transparent lid during test. Exchangeable Petri dish on the bottom to present sugar or water soaked filter paper during training and neutral filter paper during test. Filter paper is clamped in the dish by a plastic ring. Aversive setup: the circular arena consists of a transparent electric shock grid, removable Fluon-coated plastic ring and transparent lid. The cylinder on top isolates each setup from the others and creates a similar closed visual scene as in the appetitive setup. **(D)** Petri dishes containing either dried sugar solution or water for training and neutral filter paper for test. **(E)** Visual stimulus source with one blue and one green high power LED per quadrant. **(F)** The conditioning arena with the transparent electric shock grid and a magnification with visual stimulation and a fly. Alternating stripes marked by + and – symbols indicate electric shock application.

To analyze behavioral experiments I recorded the distribution of the flies during test phase from above with a CMOS camera (Figure 9A-C)(Firefly MV, Point Grey, Richmond, Canada) controlled by custom made software (Schnaitmann et al., 2010). Four setups were run in parallel unless otherwise stated.

#### *Apparatus for appetitive conditioning*

For appetitive sugar conditioning, I used an apparatus module containing a cylinder closed on both sides with Petri dishes (Figure 9A,B,D)(Schnaitmann et al. 2010). A filter paper was clamped in the bottom dish with a plastic ring with which I was able to present sugar reward in training. This dish could be exchanged during training to either contain filter papers with dried sugar reward or water control. On top, either an opaque lid was present during training or a transparent lid during test. The transparent lid enabled video recording in test. Cylinder walls and lid were covered with Fluon (Fluon GP1, Whitford Plastics Ltd., UK), so that flies were not able to hang on them and were forced to walk on the bottom petri dish.

#### *Apparatus for aversive conditioning*

For aversive electric shock conditioning, I used an apparatus module containing an arena with a transparent shock grid (Figure 9B,C,F). The arena itself consisted of the transparent shock grid on the bottom, a plastic ring as a wall and a glass lid. The shock grid was a custom made glass plate (9x9 cm) covered with ITO (indium tin oxide), a conductive transparent substance. A grid was laser-structured onto the ITO glass in order to insulate the positive and negative electrodes (1.6 mm width with 0.1 mm of gaps). The width of the lasered lanes was chosen in a way that flies always close the electric circuit and receive punishment. Alternating current was applied to protect electrodes from a biased pigmentation. The two halves of the grid can be independently controlled. The plastic ring (wall) and the glass lid were coated with diluted Fluon (10%; Fluon GP1, Whitford Plastics Ltd., UK) to prevent flies from walking on the lid and wall. Consequently, flies were forced to stay on the shock grid on the bottom of the arena. A filter paper was clamped on the backside of the shock grid and served as a screen. The aversive setup was designed by Christopher Schnaitmann and built with the help of Stephan Prech (Electronics workshop, MPI of Neurobiology).

### 2.3 Behavioral protocols for appetitive and aversive learning

The design of conditioning (differential conditioning followed by the binary choice without reinforcement) and visual stimulation are the same in appetitive and aversive conditioning (Figure 11; (Schnaitmann et al. 2010)). Briefly, approximately 40 flies were subjected to a single conditioning experiment and introduced into the arena using an aspirator. During the training the whole arena was illuminated for one minute alternately with green and blue light, one of which was paired with reinforcement (CS+).

For appetitive conditioning, high concentration of sucrose was presented as a reward (Schnaitmann et al. 2010). The sucrose solution (2 molar (M)) was soaked in filter paper and subsequently dried. During an inter-CS-interval (ICSI) of 12 s, training petri dishes on the bottom were exchanged. The cylinder was turned and the flies were gently tapped onto the lid, then the training petri dish, now on top, was exchanged in a quick way and the whole cylinder was turned back onto the LED array.

For aversive conditioning, one second of electric shock (AC 60 V) was applied 12 times in 60 s during CS+ presentation. The two consecutive CS+ and CS- presentations are intermitted by 12 s of an interval without any illumination (Schnaitmann et al. 2010).

Such a training trial was repeated four times if not otherwise stated (Figure 13). The test started 60 s after the end of the last training session. Unlike in the training phase, only two diagonal quadrants of the arena were illuminated with the same color (unless otherwise stated) to allow flies to choose between the two visual stimuli. In the test, the distribution of the flies was video recorded for 90 s at 1 frame per second (Schnaitmann et al. 2010). No US was presented in the test period with visual stimuli. For aversive conditioning, a 1 s shock pulse (90V) was applied 5 s before the beginning of the test to arouse the flies (Figure 17A). However, when testing flies in high temperature (33°C) this additional shock was dispensable for aversive memory retrieval (Figure 17B). Two groups undergoing reciprocal CS–US contiguity (Green+/Blue– and Blue+/Green–) were trained in the same setup consecutively. A green preference index (PI) was calculated for both groups. If all flies sit on the green visual cue during test, the PI has a value of 1. A PI of 0 indicates that flies are evenly distributed on the two visual cues.

$$PI = \frac{(\# \text{flies on green visual cue}) - (\# \text{flies on blue visual cue})}{\# \text{Total flies}}$$

The difference in PI of these two groups in visual stimulus preference was then used to calculate a learning index for each video frame. Half of the trained groups received reinforcement together with the first presented visual stimulus and the other half with the second visual stimulus to cancel the effect of the order of reinforcement (Schnaitmann et al. 2010).

$$LI = \frac{(PI (\text{Green paired with reinforcer})) - (PI (\text{Blue paired with reinforcer}))}{2}$$

## 2.4 Sensory controls

Control responses to sugar or shock were performed with the same arena modules as those for appetitive and aversive conditioning. To be able to record distribution of the flies, but not disturb the choice behavior, the arenas were backlit only with infrared-LEDs. Flies were given a choice between the US presented as in the training and no US in the two halves of the arena. The choice was recorded 60 s using also the same video setup as for conditioning. A preference index was calculated by subtracting the numbers of flies on the half with a stimulus and on the control half of the arena, which was divided by the total number of flies.

$$PI = \frac{(\# \text{flies on half with US}) - (\# \text{flies on control half})}{\# \text{Total flies}}$$



## 2.5 Protocol modifications in visual learning

### ISI conditioning

To train flies with non-overlapping CS and US stimuli, meaning different inter-stimulus-intervals (ISIs), the conditioning protocol was changed. The CS duration was shortened to 15s and also shock presentation was reduced to three shocks of 1 s every 5 s (Figure 12). Due to shorter CS and US presentation, amount of training trials was increased to eight (Figure 13). Time interval between CS presentations (ICSI) was prolonged to 2 min to be able to exclude any effect of the control color on the formed memory (Figure 13). Using this protocol flies were then trained with different ISIs, negative numbers indicating that the CS+ preceded the US, positive numbers indicating that the US preceded the CS+ for the specific amount of seconds.

Neuronal output required during conditioning	Possible function in neuronal circuit
Training	US pathway
Test	site of coincidence detection or memory storage
Training & Test	CS Pathway

**Table 3: Temporal shi<sup>ts</sup>-block either during training or test can reveal several functional requirements of neurons within a neural circuit**

### Temperature manipulation

By use of a heating element and fan I was able to raise the temperature surrounding the apparatus up to steady 33°C. Like this, induction of temperature sensitive tools such as shi<sup>ts1</sup> or TrpA1 was possible. In temperature shift experiments with shi<sup>ts1</sup>, flies were transferred into moistened empty vials during temperature switch

from permissive (25/26°C) to restrictive (31/33°C) temperature or vice versa (Table 3). Behavioral test was performed 40-45 minutes after training, so that flies could acclimate to the temperature switch.

### **Aversive conditioning without context change**

Unlike the standard classical conditioning protocol, CS+ and CS- were simultaneously presented in the two halves of the arena during training and test (Figure 18). Only one half of the arena displaying CS+ was electrified. Flies were allowed to choose one of the differently cued two halves for 20 s, and such a training trial was repeated eight times with an inter-trial interval of 20 s of no stimulus. The sides of CS/US presentation were pseudo-randomized to avoid potential association with the regularity of the to-be-shocked side. During the test, the two halves were illuminated as in training, but without shock, allowing no visual context change between training and test.

### **Green intensity conditioning protocol**

To train flies with different light intensities, exactly the same electric shock application protocol was used as for aversive color conditioning (Figure 41, Figure 42). Blue and green visual cues were replaced by presenting dark and light green (1:10 ratio) as CS, thus the flies could only use the intensity difference as indicator for punishment. The intensities were controlled by current and calibrated using a luminance meter BM-9 (Topcon Technohouse Corporation) or a PR-655 SpectraScan® Spectroradiometer: 101.0 Cd/m<sup>2</sup> s (bright-green), and 10.1 Cd/m<sup>2</sup> s (dark-green).

## 2.6 Split-GAL4 MB Screening

### Setups

For the MB-Split-GAL4 screening performed in collaboration with the Janelia Farm Research Campus (HHMI, Ashburn, Virginia, USA), appetitive conditioning experiments were performed in Munich with the same control as explained above. Aversive conditioning experiments were performed at the Janelia Farm Research Campus (JFRC) in Ashburn, Virginia, USA. Therefore, the aversive conditioning setup was slightly modified and expanded (Design by Christopher Schnaitmann and Stephan Prech). 20 single shock grids could be used in parallel so that a throughput level of  $n = 80-100$  per day could be reached (see Figure 10 for 10 of the 20 arrays). Full automation of the setup was achieved by controlling all applied electric shock and visual stimuli by a single PC for all 20 setups.



**Figure 10: Modular aversive visual learning assay used during the screening in JFRC**

One half of the experimental setup for aversive visual conditioning screening project; twenty setups were used in total. The cylinders on the top rack isolate each setup from the others and create a similar closed visual scene.

## Screening lines and protocol

The Split-GAL4 lines were assembled by ~400 different lines containing either an activation domain (p65ADZp) or a DNA-binding domain (ZpGdbd), respectively, controlled by a specific promoter in JFRC. From ~2,500 different intersection combinations, 80 Split-GAL4-lines were selected for the MB-screening project by Yoshinori Aso (JFRC). The lines specifically labeled intrinsic (18 lines, Table 10), input (33 lines, Table 11) or output (29 lines, Table 12) neurons of the MB. For visual conditioning I crossed the lines with *w+;;20XUAS-IVS-Shibire[ts1]-p10* (Pfeiffer et al. 2012), to block synaptic output during training and test. Flies were tested 2 min after training in appetitive and aversive conditioning. During primary screening the experimental group was tested in parallel with the *shi/w-* control ( $n = 8$ ). Lines that showed significant phenotype or strong tendency for impairment were retested in the secondary screening together with *shi/w-* control and *+Split-GAL4* control ( $n = 8$ ) unless otherwise stated. Lines that showed consistent significant phenotype over both screening phases were additionally tested for visual memory in permissive temperature and for sensory defects in restrictive temperature unless otherwise stated. Primary and secondary screening for aversive visual conditioning could be finished within 6 weeks at the Janelia Farm Research Campus. Control tests for phenotypic lines in aversive conditioning were performed in Munich. For appetitive conditioning, primary screening, secondary screening and control tests could be finished in Munich for MB output lines. For input and KC lines most of the primary appetitive screening could be finished in Munich. However, no secondary screening and control tests could be performed due to time limitations for experiments.

## 2.7 Olfactory conditioning

Standard olfactory conditioning was applied as performed previously (Tully & Quinn 1985; Schwaerzel et al. 2002). Differential conditioning design (odors: 3-octanol/Benzaldehyd for MB-block, 3-octanol/4-methylcyclohexanol for *rut-rescue*) followed by the binary choice without reinforcement was similarly as in visual conditioning. During training, flies were exposed to 60 s of an odor paired with 12 pulses

of electric shock (CS+, 100 V/90 V) and subsequently to a control odor that also lasted 60s (CS-). Memory performance was tested 3 min after training.

## 2.8 Statistics

Statistical analyses were performed with Prism5 software (GraphPad). Groups that did not violate the assumption of normal distribution (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test) were analyzed with parametric statistics: one-sample *t*-test or one-way analysis of variance (one-way ANOVA) followed by the planned pairwise multiple comparisons (Bonferroni). Experiments with data that were significantly different from the assumptions above were analyzed with non-parametric tests, such as Mann-Whitney test or Kruskal–Wallis test followed by Dunn's multiple pair-wise comparison. The significance level of statistical tests was set to 0.05.

### Screening statistics

In the primary screening, *shi/w*- control data was pooled over all tested lines. During analysis, experimental lines *shi/Split-GAL4* were compared to pooled control data and analyzed with non-parametric statistics: Mann-Whitney test followed by the planned pairwise multiple comparisons (Benjamini Hochberg). For secondary screening, selectively tested experimental lines (*shi/Split-GAL4*) were compared to pooled *shi/w*-control data and *+/Split-GAL4* control data. They were analyzed with non-parametric statistics: Kruskal Wallis test followed by Dunn's multiple pair-wise comparisons or parametric statistics: one-way ANOVA followed by the planned pairwise multiple comparisons (Bonferroni). The significance level of statistical tests was set to 0.05.

## 2.9 Immunohistochemistry

### Antibodies

Adult fly brains were dissected, fixed and stained using standard protocols (Aso et al. 2010). Synapsin antibody (AB) staining, discs large AB staining or N-cadherin AB staining was used to visualize the neuropil. Anti-GFP AB was used to increase the intensity of the GFP signal. Dsred AB was used to increase the mCherry/DsRed signal.

Staining	Primary Antibodies	Secondary Antibodies
GFP	$\alpha$ -GFP (rabbit, 1:1000) Invitrogen	$\alpha$ -rabbit Alexa 488 (1:1000) Invitrogen
Synaptotagmin (GFP)	$\alpha$ -GFP (rat, 1:100)	$\alpha$ -rat Alexa 488 (1:250)
GRASP (GFP)	$\alpha$ -GFP (mouse, 1:100) neuromab	$\alpha$ -mouse Alexa 488 (1:200) Invitrogen
Discs large background	$\alpha$ -dskl (mouse, 1:50)	$\alpha$ -mouse Cy3 (1:250)
Synapsin background	$\alpha$ -synapsin (mouse, 1:100) (Klagges et al. 1996)	$\alpha$ -mouse Cy3 (1:250) Dianova
Synapsin background	$\alpha$ -synapsin (mouse, 1:100) (Klagges et al. 1996)	$\alpha$ -mouse Alexa 633 (1:250)
Denmark (dsred)	$\alpha$ -dsred (rabbit, 1:100)	$\alpha$ -rabbit Alexa 568 (1:250)
GRASP (dsred)	$\alpha$ -dsred (rabbit, 1:100)	$\alpha$ -rabbit Cy3 (1:200)
n-Cad background	$\alpha$ -nCad (rat, 1:100) DSHB	$\alpha$ -rat Alexa 633 (1:200) Invitrogen
DCO (PKA-C1)	$\alpha$ -DCO (rabbit, 1:2000) (Skoulakis et al. 1993)	$\alpha$ -rabbit Cy3 (1:200) Jackson Immunoresearch
R7/R8	$\alpha$ -MAb24B10 (mouse 1:50), DSHB (Zipursky et al. 1984)	$\alpha$ -mouse Cy3 (1:250) Jackson Immunoresearch

**Table 4: Antibodies used for immunostaining**

## Cell polarity

Polarity of VPNs and  $\gamma$ d KCs was analyzed by crossing VPN Split-GAL4 lines *425B*, *334C* and *MB419B* with *w-; UAS-Denmark::mCherry; UAS-Syt::GFP* (Nicolai et al. 2010), respectively. Thus, synaptotagmin-GFP staining marks the axonal regions of the neurons, whereas Denmark-mCherry staining marks the dendritic regions of the neurons.

## Connectivity between VPNs and the MB

To characterize and show connectivity between VPNs and  $\gamma$ d dendrites (ventral accessory calyx, VACA) we performed several anatomical experiments. DCO antibody labels preferentially the MB (Crittenden et al. 1998). Antibody staining in parallel with expression of *MB419B-GFP* expression shows that  $\gamma$ -lobes are also strongly labeled (Figure 38). To show connectivity, DCO antibody staining was performed in parallel with expression of GFP in VPNs (*425B-Split-GAL4*, *334C-Split-GAL4*). In a second experiment, to perform double labeling between VPNs and MB calyx I recombined *w-; UAS-myr-CD8::Cherry/CyO; LexAop-GFP/TM2* with *w-; 13F02LexA*. The resulting line was then crossed with specific Split-GAL4 lines for VPNs (*425B-Split-GAL4*, *334C-Split-GAL4*). Thirdly, to visualize connections between VPNs and the VACA I crossed *w-; mb247-DsRed; mb247-splitGFP11, UAS-splitGFP1-10* (Pech et al. 2013) with Split-GAL4 lines for VPNs (*425B-Split-GAL4*, *334C-Split-GAL4*). Reconstituted GFP will be expressed in the fly brain where the neurons come very close or even form synapses during development and in adult flies.

## Heat shock Flip-out protocol

To obtain single-cell flip-out staining, males of the *425B-Split-GAL4* were crossed with females of *y-w-, hsp70-flp [X]; UAS>CD2 y+>mCD8::GFP/CyO; TM2/TM6b* (Wong et al. 2002) to obtain  $F_1$  progeny carrying GAL4 insertion, *hsp70-flp* and *UAS>rCD2,y+>mCD8-GFP*. Crosses were raised at 25 °C. One to six days before eclosion a mild heat shock was given by placing the vial into a 32°C incubator to remove

the FLP-out cassette (rCD2, y+) in a subset of the neurons, thereby allowing the expression of *mCD8-GFP* in these neurons and *CD2* expression in the remaining neurons. The duration of the heat shock was 60–90 min. The eclosed flies were then transferred into a fresh vial and 2- to 5-day-old flies were used for dissection.

### **Double labeling with photoreceptor neurons**

To specify the medulla input region in the VPN lines we performed double labeling of *425B-Split-GAL4/UAS-GFP* and *334C-Split-GAL4/UAS-GFP* with a specific antibody for inner photoreceptors R7/R8 (mouse mAb24B10, (Zipursky et al. 1984)), respectively.

### **Analysis of confocal data**

Frontal optical sections of whole-mount brains were sampled with a confocal microscope (Olympus FV1000). Images of the confocal stacks were analyzed with the open-source software Fiji (Schindelin et al. 2012). Confocal pictures of all 80 tested Split-GAL4 lines were provided by the Janelia Farm Research Campus Flylight team. Background is stained with nc82. Split-lines are stained with 5xUAS-myr-sm::GFP inserted in VK0005. Neuronal transmitter was assigned by double staining with either glutamine (dVGluT), gamma-aminobutyric acid (GABA; GAD1) or acetylcholine (ChAT) antibodies. Specific expression patterns of the Split-GAL4 lines inside and outside the MB were assigned by Yoshinori Aso (Janelia Farm Research Campus, Table 10, Table 11 and Table 12).



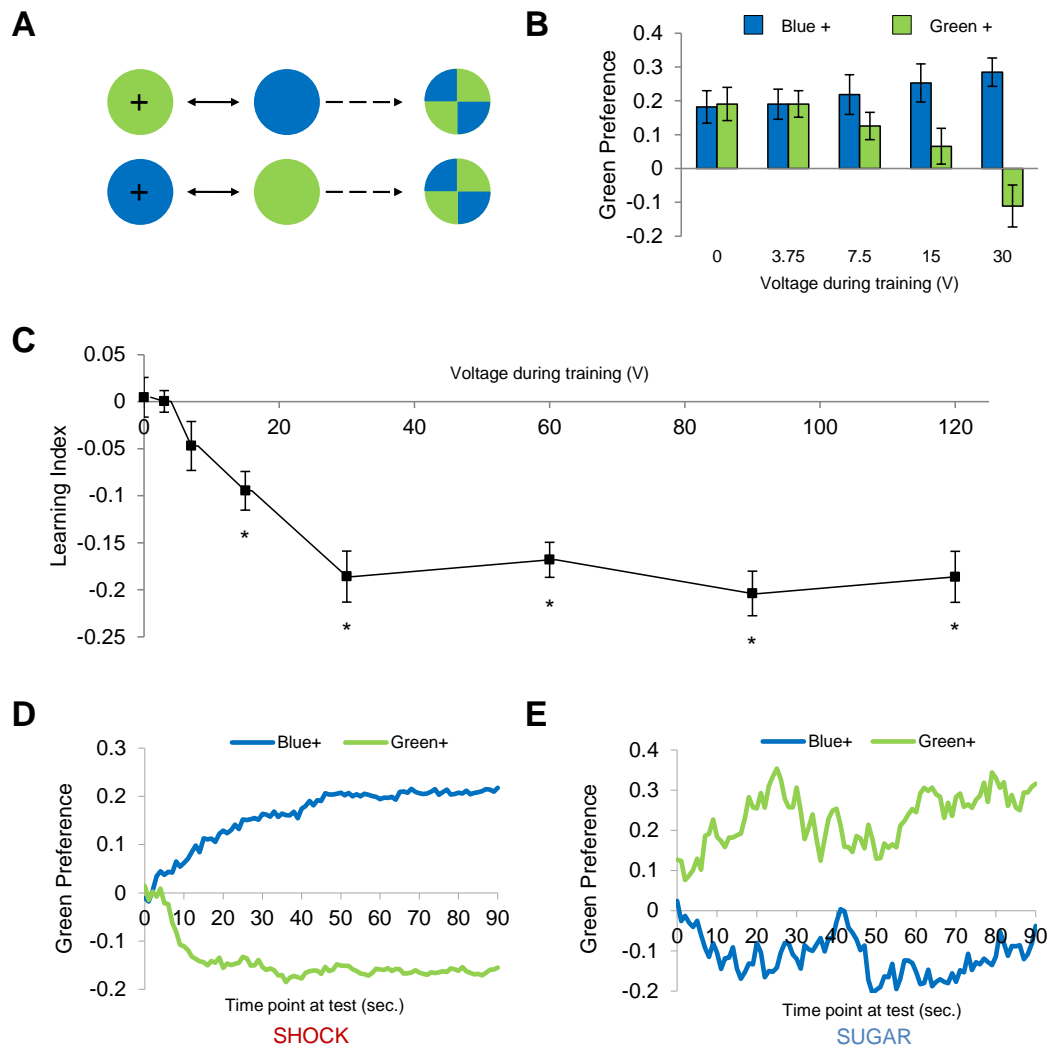
## **3. Results**

### **3.1 Visual conditioning paradigm**

By employing a newly established visual setup containing two exchangeable modules for reward and punishment, respectively, it was possible to train flies with both reinforcers under same visual stimulation and applying the same protocol. Both modules are built in a way that allows illumination from beneath the flies, either through sucrose soaked filter paper or a translucent electric shock grid. Also the same reinforcers, sugar and electric shock, are employed as in the well tested olfactory conditioning paradigm. Therefore a comparison between the two learning setups with different modalities is possible.

#### **Green preference and memory formation**

During training flies are differentially conditioned with green and blue stimuli, respectively. One of the stimuli is paired with either punishment or reward during the training phase (+, Figure 11A). In the test both stimuli are presented simultaneously to the flies and they are free to make a choice. Flies show a clear bias in towards the green stimulus in test when trained with no voltage or low voltage in both reciprocals (pooled green preference over 90s of test; Figure 11B). When training with higher voltages (15-120V) the green preference is shifted and flies show significant aversive memory (Figure 11B-C). Video recording allows analysis of the distribution of the flies on the visual stimuli at every second during test. After roughly 20-30 s of the test the flies reach a stable distribution between the two stimuli. When trained with sucrose flies approach the rewarded color in test (Figure 11D), whereas when trained with electric shock flies avoid the punished color in the test (Figure 11E (Schnaitmann et al. 2010)). All bar graphs in further figures represent the pooled data of calculated learning indices from both reciprocal green preferences during 90s of the test. Overall the memory scores for visual learning are stable and reproducible so that further experiments can be performed.

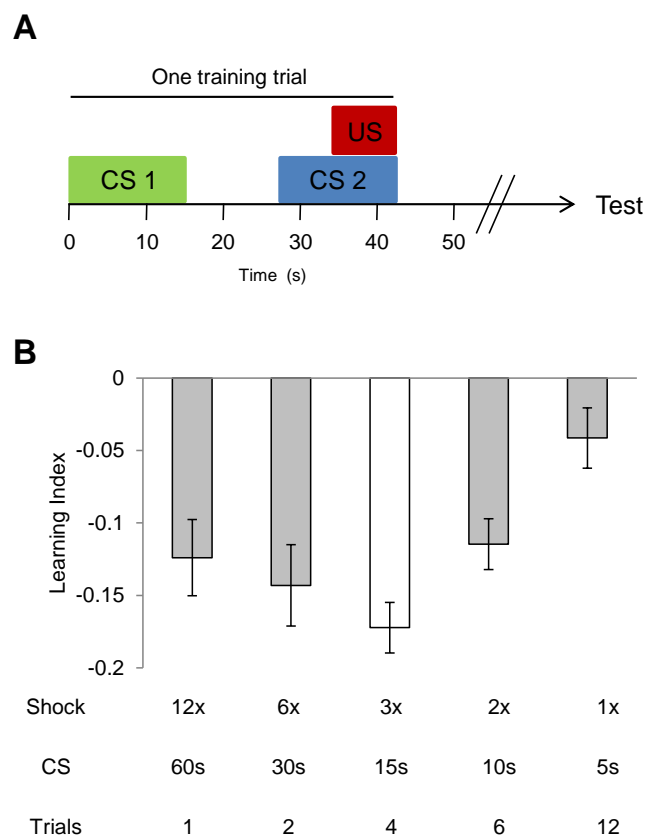


**Figure 11: Flies shift their naïve bias for visual stimuli after appetitive and aversive conditioning.**

**(A)** Differential conditioning protocol with chromatic stimuli. Two reciprocals (+ represents punished or rewarded color during training) are performed to obtain one learning index. **(B)** Pooled green preference after aversive conditioning with different voltages (0-30V) at 25 °C. Flies show naïve bias for the green stimulus after training with 0V in both reciprocals (Blue+/Green+ represent punished color during training),  $n = 15$ . **(C)** Aversive visual memory depends on shock intensity (One-way ANOVA,  $p < 0.001$ ). Flies show significant memory from 15 V (One sample t-test,  $p < 0.001$ )  $n = 15$ . No difference in performance is found among training with 30-120 V (*post-hoc* pairwise comparisons  $p > 0.05$ )  $n = 16-30$ . **(D)** Green preference of flies after aversive conditioning with 60 V at 25 °C. During every second of the test green preference is calculated independently for both reciprocals (Blue+/Green+ represent punished color during training),  $n = 16$ . **(E)** Green preference of flies after appetitive conditioning with 2M sucrose on filter paper at 25 °C. During every second of the test green preference is calculated independently for both reciprocals (Blue+/Green+ represent rewarded color during training),  $n = 6$ . Bars and error bars represent mean and SEM, respectively.

### 3.2 ISI conditioning

In ISI conditioning the time interval between the CS+ and the US (Inter-stimulus-interval) is varied. The CS+ usually precedes and at least partly overlaps with the US in the commonly applied delay conditioning protocol (Figure 12A). However, it is also possible to train the flies with the CS+ either preceding the US (negative ISI) or following the US (positive ISI) without temporal overlap. In olfactory conditioning, varying the ISI leads to different conditioning effects such as relief and trace learning (Tanimoto et al. 2004; Galili et al. 2011; Yarali et al. 2008). It is not known if flies can perform similarly when training with visual stimuli.



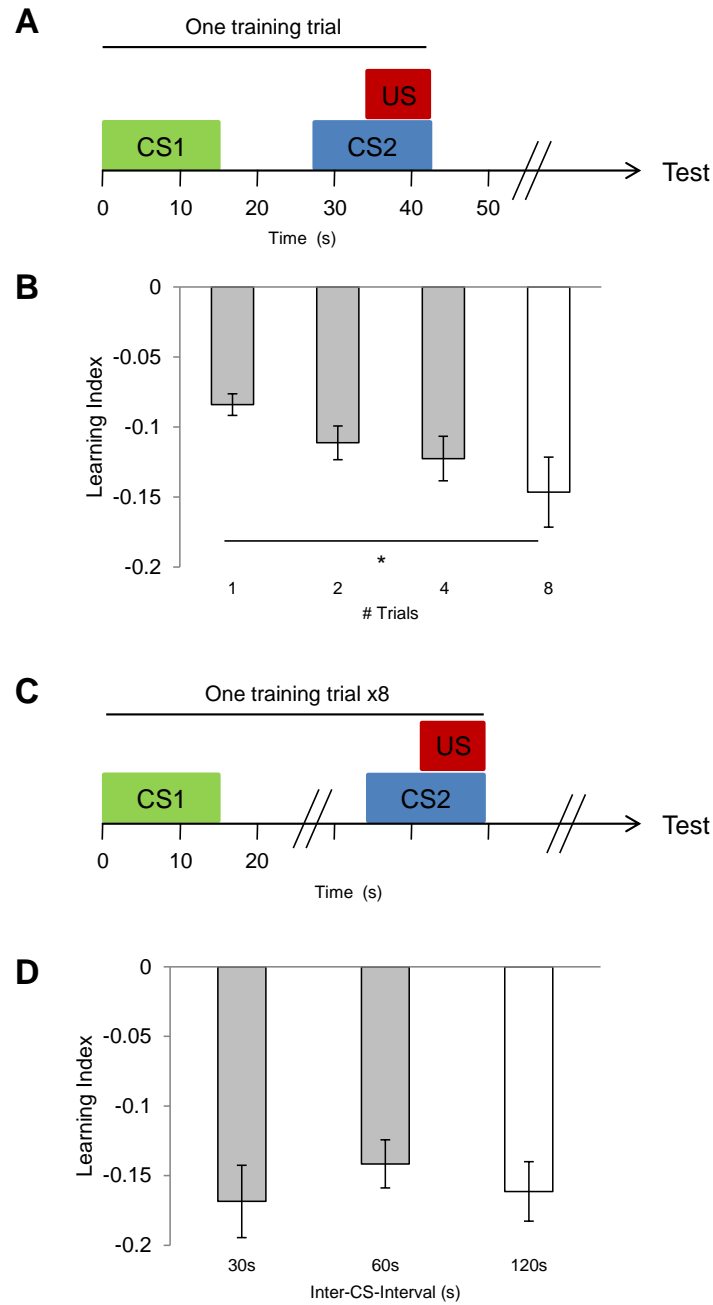
**Figure 12: Delay conditioning in relation to different protocol parameters.**

**(A)** Training protocol for normal delay conditioning of adult flies with two visual stimuli (CS = green/blue (15s)) and electric shocks (US). Inter-CS-Interval (ICSI) is always 12 s. The to-be-learned visual stimulus precedes the US application for 4 s (Inter-stimulus-interval (ISI) = -4). **(B)** To find best conditions for delay conditioning I varied amount of training trials. All experimental groups received same amount of US (12 shocks) and CS (60 s). Visual delay memory depends on variation in protocol (Kruskal-Wallis test,  $p < 0.01$ ). Flies showed significant memory with 1, 2, 4 and 6 trials (One sample t-test,  $p < 0.001$ ). Applying 12 Trials with 5 s CS and 1 shock did not reveal significant learning (One sample t-test,  $p < 0.05$ ). Significant difference in memory performance was found between 4 trials and 12 trials (*post-hoc* pairwise comparisons  $p > 0.05$ ),  $n = 15-20$ . Bars and error bars represent mean and SEM, respectively.

## Adjusting the conditioning protocol

To find the best parameters for ISI conditioning with visual stimuli, I tested different CS durations and matched shock applications (Figure 12). All tested experimental groups received the same amount of CS exposure and shock, however in different amount of trials. Worst performance was found when training flies with 5 s of CS and one electric shock per trial, even though 12 trials were performed (Figure 12B). Best memory scores were found with CS duration of 15 s and application of 3 electric shocks, presented to the flies in 4 trials. Using this protocol flies showed significant memory performance with even a single training trial (Figure 13A-B). However, comparing between training with one, two, four and eight training trials revealed that there is a tendency for improved delay conditioning with higher amount of training trials performed (Figure 13B). Thus, for all further experiments within the ISI-project I applied eight training trials with the CS duration of 15 s and three electric shocks each.

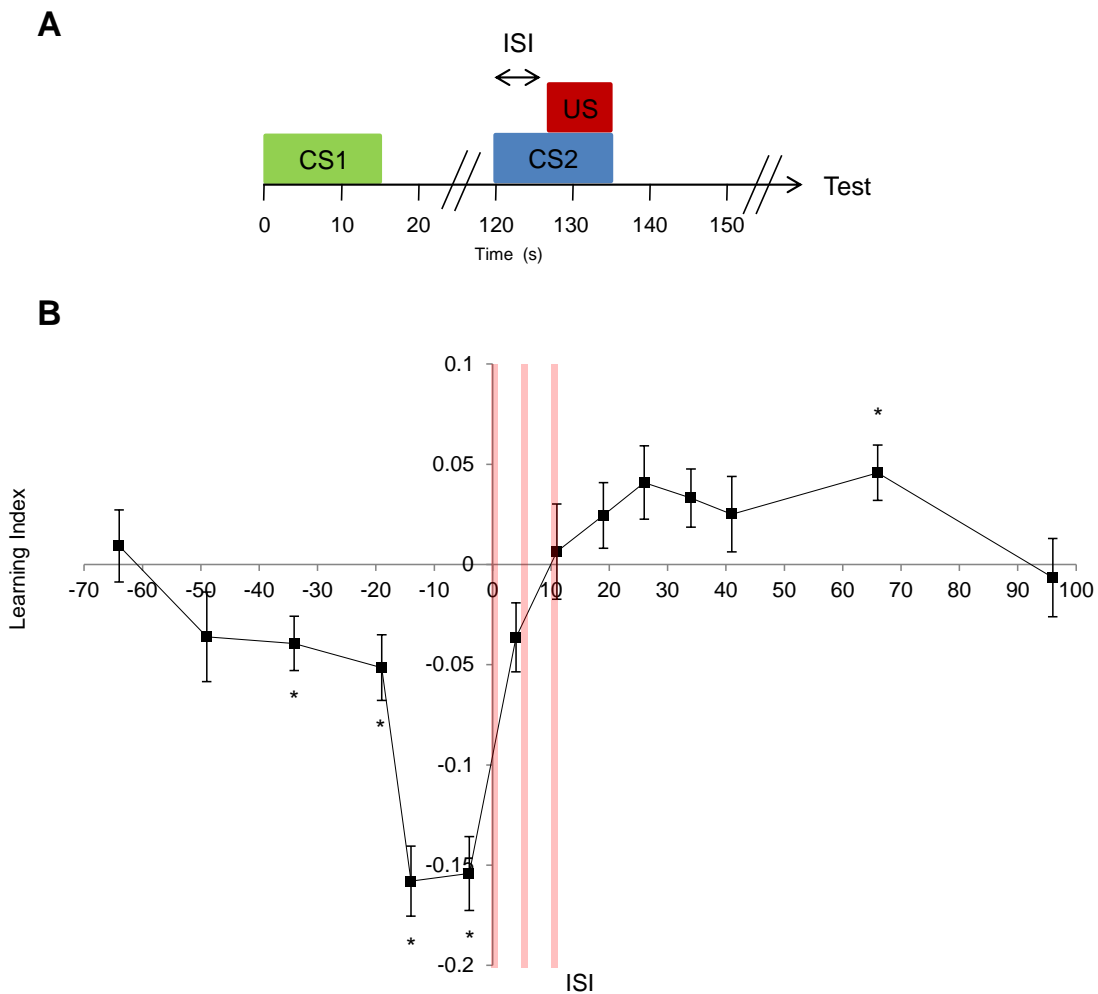
When conditioning with different ISIs the CS+ and US are presented with a temporal gap. Thus, it is critical to be able to exclude any associations of the US with a control CS-. Increasing the temporal period between presentation of US/CS+ and CS- should prevent such association. Therefore I tested if a prolongation of the inter-CS-interval (ICSI) influences the delay conditioning performance (Figure 13C-D, 30s, 60s or 120s). No significant difference was found in memory performance among all tested groups. Thus, the longest interval was chosen to be applied also in ISI conditioning experiments. Within these conditions the CS- and US are always presented with a temporal gap of 120s and no association should be formed between these stimuli.



**Figure 13: Delay conditioning in relation to amount of training trials and Inter-CS-Interval.**

**(A)** Training conditions for delay conditioning. 15s CS/ICS12s/ISI = -4. **(B)** Every amount of training trials leads to significant learning (Mann-Whitney test,  $p < 0.05$ ). Significant difference in memory performance was found between conditioning with one trial and 8 trials (Kruskal Wallis test, *post-hoc* pairwise comparisons  $p > 0.05$ ),  $n = 16$ . **(C)** Training conditions for delay conditioning. 15s CS/8 Trials/ISI = -4, Duration of ICSI was varied. **(D)** Variation in ICSI from 30 s to 120s. Delay memory does not depend on duration of Inter-CS-Interval (One-way ANOVA,  $p > 0.6$ ) All groups show significant memory performance (One sample t-test,  $p < 0.001$ ),  $n = 20$ . Bars and error bars represent mean and SEM, respectively.

## Visual trace and relief learning



**Figure 14: Timing dependent memory formation in *Drosophila*.**

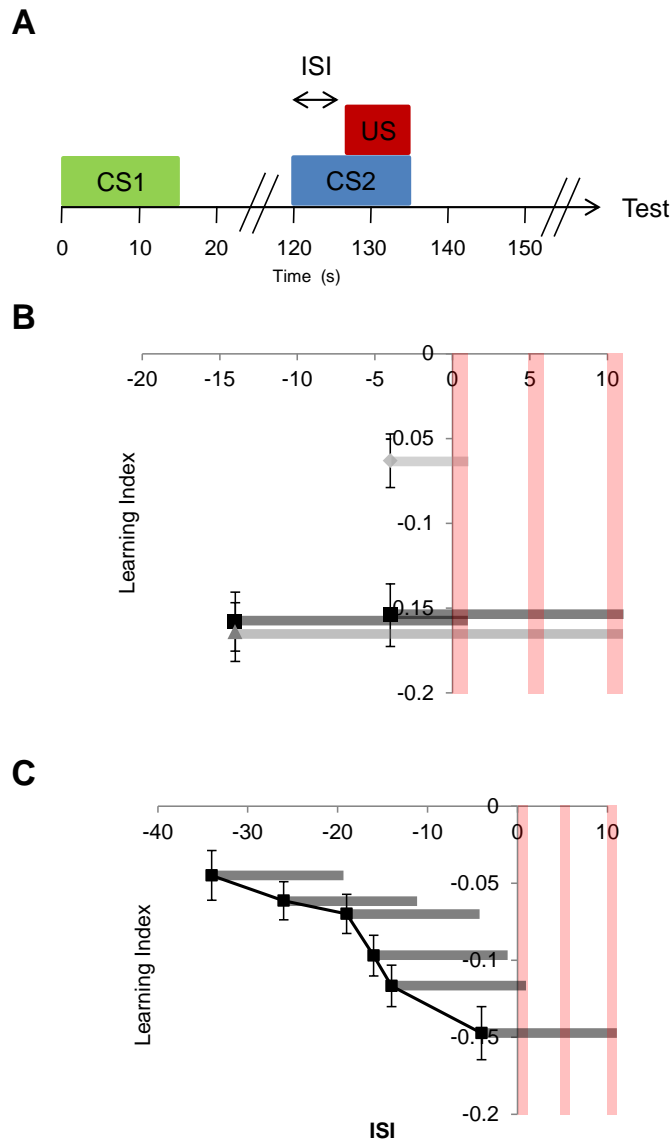
**(A)** Timeline of a single training trial. The inter stimulus interval (ISI) is the interval between onset of US and onset of CS+ in seconds. Eight training trials were applied with varying ISI values **(B)** Conditioned behavior as a function of the ISI. Red stripes indicate electric shock application. Memory performance depends on stimulus timing (One-way ANOVA,  $p < 0.001$ ). Flies show significant aversive memory in delay conditioning (One sample t-test,  $p < 0.001$ , ISI = -4, -14) and when there is a gap introduced between preceding CS+ and US presentation (One sample t-test,  $p < 0.01$ ; trace learning, ISI = -19, -34). Flies show significant appetitive memory when CS+ follows US presentation with a gap (One sample t-test,  $p < 0.01$ ; relief learning, ISI = +66). If the US preceded the CS, but still overlapped (One sample t-test,  $p > 0.05$ ; ISI = +4, +11), no significant memory was found. If the two stimuli were too far apart in time (ISI = -64, +96) flies showed no conditioned behavior (One sample t-test,  $p > 0.5$ ),  $n = 16-40$ . Marker and error bars represent mean and SEM, respectively.

Using the modified protocol I tested flies with ISIs varying from -66 (CS+ precedes US for 66 s) to +96 (US precedes CS+ for 96s)(Figure 14B). Changing the presentation timing of the paired CS led to aversive memory formation when the CS preceded the punishment with a temporal overlap (delay conditioning, Figure 14B). This was even true when the two stimuli did not overlap in their presentation (trace conditioning = aversive ISI, Figure 14B). If the US preceded the CS presentation with an overlap flies showed weak aversive or no memory. However, when the CS followed the electric shock without temporal overlap the flies approached the paired CS in the test (relief conditioning = positive ISI, Figure 14B). Thus, flies are capable to form both kinds of associative memory, relief and trace conditioning, not only with olfactory but also with visual stimuli.

### **Dissecting visual delay and trace conditioning**

Shorter presentation of CS (5s) does not lead to strong memory formation in delay conditioning even with application of three electric shocks (Figure 15B; compare to Figure 12B = 1 shock/5s CS). By increasing the CS duration to 25s I wanted to test whether prolonged presentation could improve learning. However, no significant improvement was found (Figure 15). Earlier onset and prolonged presentation of CS does not improve delay conditioning memory.

To understand the precise mechanism for trace conditioning, I tested shorter intervals of negative ISIs. Flies showed significant aversive memory up to an ISI of -34 (gap of 19s between CS+ and US, Figure 14). Therefore I tested six ISI-combinations ranging from -4 to -34 (Figure 15C; -4/-14/-16/-19/-34). The learning index linearly decreased with increasing gap between the stimuli. Overall, introducing a gap between the two stimuli leads to a decrease in learning, but the effect is not significant with rather short gap (1s). Increasing the gap leads to significant decrease in performance compared to delay conditioning (Figure 15C). Thus, it could be possible that the saliency of the stimulus decreases with increasing temporal gap between CS+ and US.



**Figure 15: Further dissection of delay and trace conditioning**

**(A)** Timeline of a single training trial. The inter stimulus interval (ISI) is the interval between onset of US and onset of CS+. Eight training trials were applied with varying ISI values. **(B)** CS duration (5s, 15s, 25s) and onset of CS was varied (ISI = -4, -14). Delay memory depends on CS duration (one-way ANOVA,  $p < 0.001$ ). All groups show significant memory performance (One sample t-test,  $p < 0.001$ ). Prolonged CS presentation (15 s, 25 s) leads to significantly better memory performance than short CS presentation (5 s) (*post-hoc* pairwise comparisons  $p > 0.05$ ),  $n = 20-36$ . **(C)** ISI value was varied across negative values for trace conditioning (ISI = -4 to -34). Trace memory depends on ISI value (Kruskal-Wallis test,  $p < 0.0001$ ). Flies were able to perform significant memory with all selected ISI values (Mann-Whitney test,  $p < 0.05$ ). Linear decrease in learning with increasing gap size (*post-hoc* pairwise comparisons  $p < 0.05$  for -34/-14, -34/-4, -26/-4),  $n = 16-24$ . Red stripes indicate electric shock application. Grey stripes indicate CS duration. Marker and error bars represent mean and SEM, respectively.

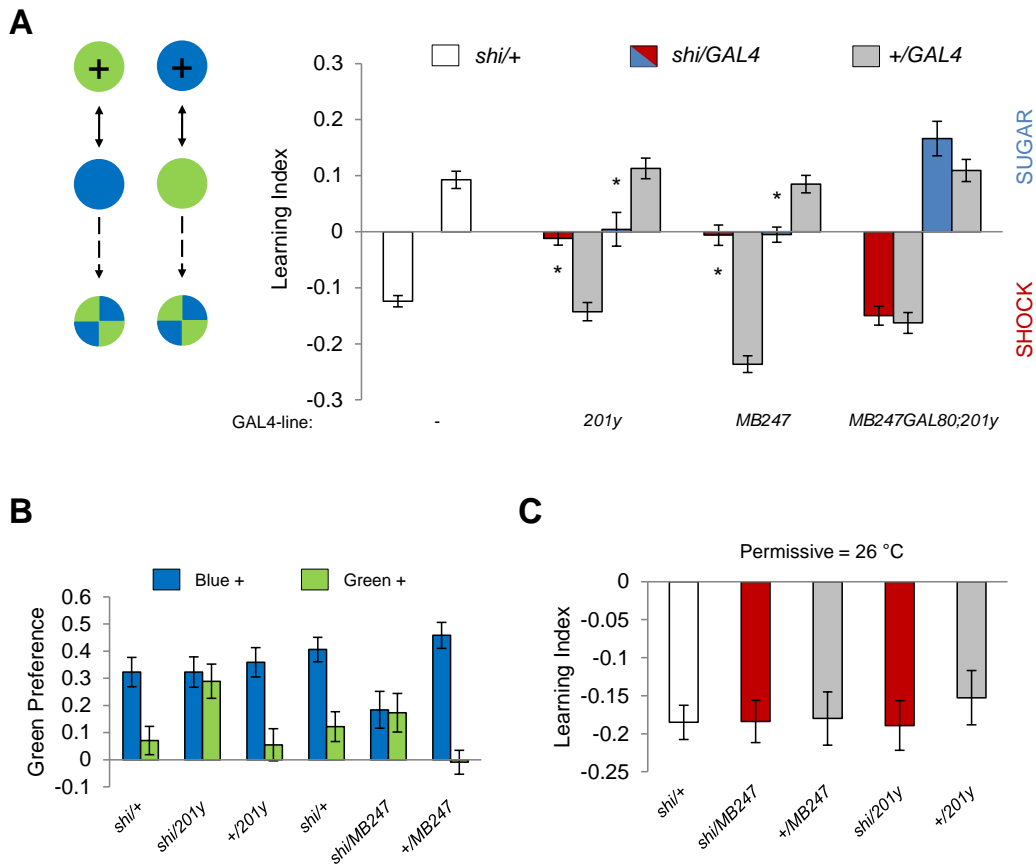


## Visual conditioning experiments with WT-flies reveal similar learning effects

The previous data sets show that flies perform trace and relief conditioning with similar effects in the presented visual conditioning paradigm and the commonly used olfactory conditioning paradigm. Thus, it should be possible to compare results obtained in both assays, although flies have overall lower memory scores in the visual task as in the olfactory task. The memory scores for visual learning are stable and reproducible, thus further experiments have been performed with genetically modified flies to dissect the underlying neural circuit for this visual learning task.

### 3.3 Requirements of Kenyon cells for visual memories

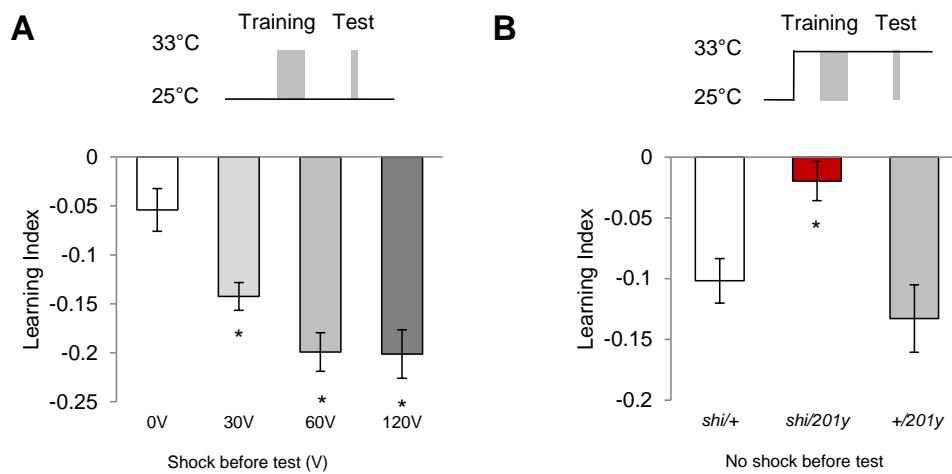
In olfactory conditioning, the MB plays a pivotal role in memory formation. Information about CS and US coincide and lead to formation of an associative memory. If also the visual information is modulated in the MBs, the output of KCs should be essential for visual appetitive and aversive memories. To block the output of KCs, two distinct GAL4 drivers labeling  $\alpha/\beta$  and  $\gamma$  neurons (*201y* (Yang et al. 1995), *MB247* (Zars et al. 2000)) were used to express *sh<sup>ts</sup>* achieving a continuous block of the neuronal output during training and test. Appetitive and aversive memories of the experimental groups were significantly impaired (Figure 16A). Discriminability of visual stimuli was intact (Figure 16B) and the learning phenotype could only be found in restrictive temperature but not permissive (Figure 16C). By blocking GAL4 transactivation of *201y* in the MB using *MB247-GAL80* it is possible to control for the influence on memory formation of the expression outside the MBs. Additional expression of *MB247-GAL80* revealed full restoration of the impaired memories (Figure 16A). Thus, we conclude that visual memories also require the output of Kenyon cells.



**Figure 16: MB is required for appetitive and aversive visual memories.**

(A) Blocking output of KCs labeled with *201y-GAL4* and *MB247-GAL4* leads to significant impairment in both appetitive and aversive memories (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ ).  $n = 10-14$ . *MB247-GAL80* restores impaired memory with *201y-GAL4* (*post-hoc* pairwise comparisons,  $p > 0.05$ ).  $n = 10-14$ . (B) Visual stimulus preference in the memory test after aversive conditioning. The blue and green bars represent the punished color during training in both reciprocals (Blue+ and Green+). Deviation from zero shows bias in visual choice, thus animals are able to discriminate visual stimuli in test. (C) Aversive learning performance is not impaired when testing flies at permissive temperature (26 °C) (one-way ANOVA,  $p > 0.05$ ),  $n = 13-14$ . Bars and error bars represent mean and SEM, respectively.

At permissive temperatures, an additional pulse of electric shock shortly before testing of at least 30V is required to perform significant visual memory display (Figure 17A). At restrictive temperatures, this additional shock pulse is not required for significant memory scores (Figure 17B), probably due to increased arousal of the flies by the elevated temperature. To keep the protocol consistent I applied the extra shock in all aversive conditioning experiments. The requirement of the MB was not dependent on increased arousal of flies prior to the test by a pulse of electric shock (Figure 17B).

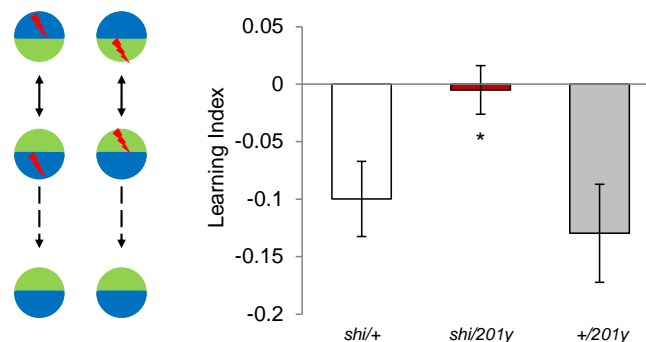


**Figure 17: Shock before test is necessary at low temperature but dispensable at high temperature.**

**(A)** A single shock (1 s) before test of at least 30 V is needed in 25 °C to obtain significant learning (0V; One sample t-test,  $p>0.05$ , 30-120V; One sample t-test,  $p<0.001$ ),  $n = 16$ . **(B)** Conditioning in 33 °C does not require shock before test. (Control groups: One sample t-test,  $p<0.01$ ).  $n = 10-11$ . Visual memories without shock before test require intact MBs (one-way ANOVA, *post-hoc* pairwise comparison,  $p<0.05$ ).  $n = 10-11$ . Bars and error bars represent mean and SEM, respectively.

### 3.4 Partly operant training protocol

The MBs have been suggested to be dispensable for some forms of basic visual learning, especially ‘flight simulator’ learning (Wolf et al. 1998). They are required for instance when the learning context is changed between training and test (Liu et al. 1999; Brembs & Hempel de Ibarra 2006). The newly established conditioning design actually also involves a change in the context of visual stimulation: the entire conditioning arena is homogeneously illuminated during training, whereas green and blue lights are simultaneously presented in the four quadrants of the arena in the test (Figure 16A). Thus, a modified conditioning design was applied in which both visual cues were simultaneously presented throughout training and test (Figure 18). This necessarily introduced an operant component in the training similar as in the standard flight simulator learning; flies can avoid to be electrified by staying away from the associated color. Using this conditioning design control flies significantly avoided the punished color; however, flies with the blocked MBs had strong impairment in visual memory (Figure 18). Thus, we conclude that the new visual learning assay requires the MBs independently of the conditioning design (classical vs. operant) and of context changes between training and test.



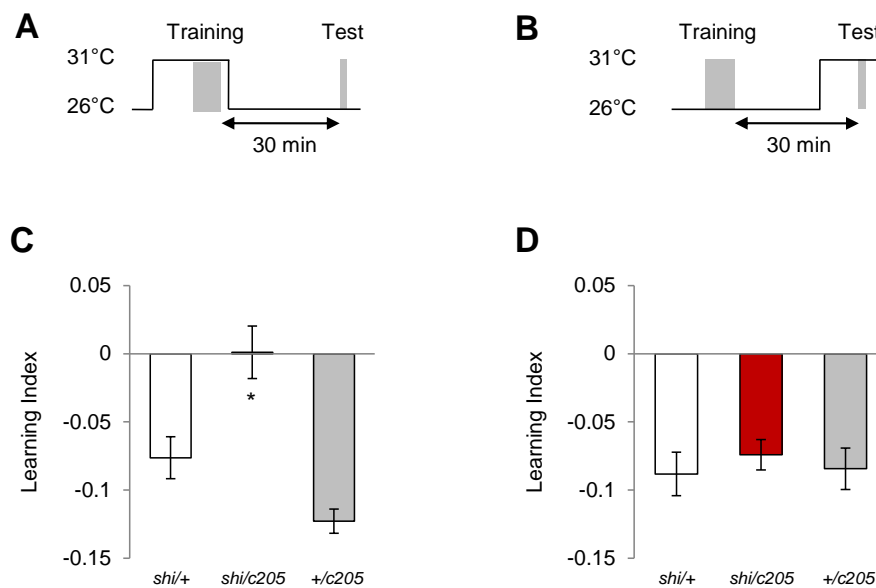
**Figure 18: MB is necessary for visual aversive conditioning with partly operant component.**

Conditioning protocol with operant component and with visual context maintained between training and test.

Visual memories with the modified protocol require intact MBs (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ ).  $n = 14-15$ . Bars and error bars represent mean and SEM, respectively.

### 3.5 Requirement of the central complex for visual aversive learning

The CC has been found to be important for some forms of basic visual learning, especially for pattern learning in the flight simulator (Liu et al. 2006; Pan et al. 2009). It was shown to be necessary and sufficient for establishing a memory trace of different patterns (elevation, angle). Blocking the output of a specific CC-GAL4-line *c205* either during training or test revealed that the CC seems to play a different role in the new visual conditioning paradigm. Here, the intact CC is required for classical visual memory acquisition, but not retrieval (Figure 19). This stands in contrast to the results in the flight simulator where blocking the output during test leads to impairment of pattern memory. In flight simulator tests, flies were not impaired in perceiving sensory stimuli such as punishment and colors (Table 8) (Brembs 2009).

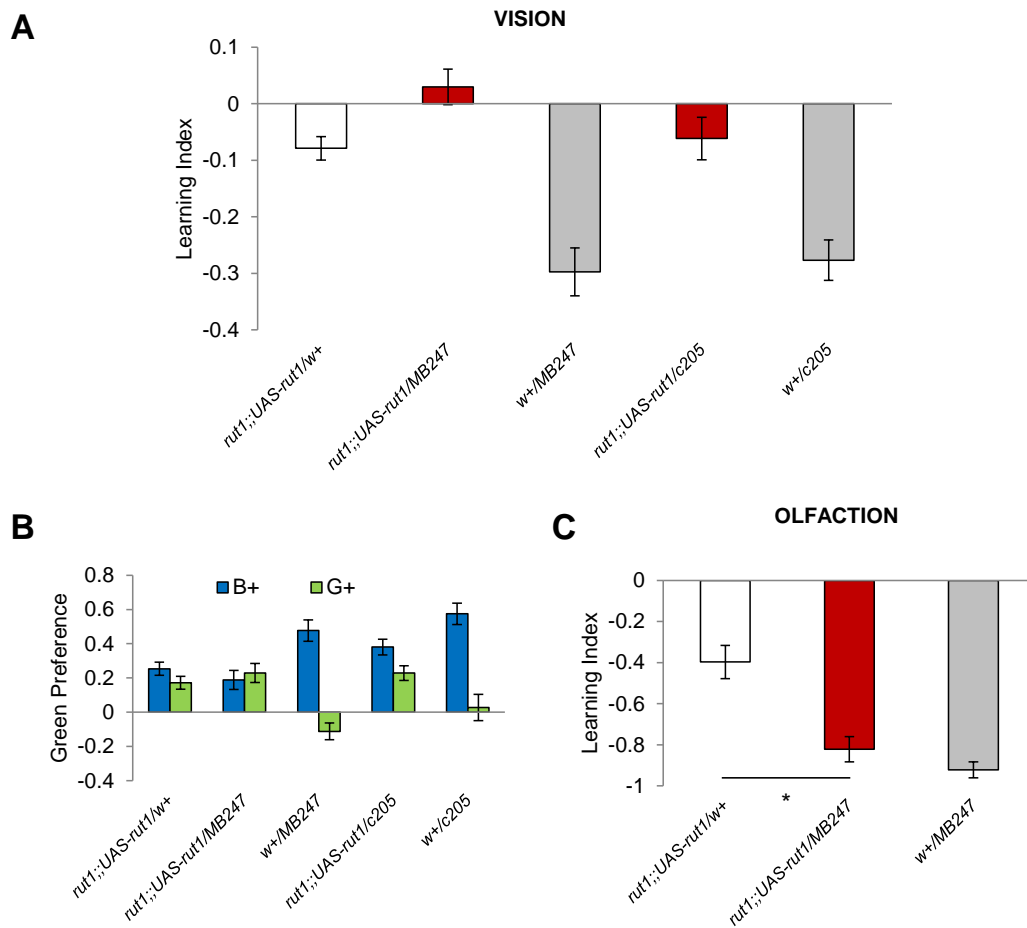


**Figure 19: Output of central complex neurons is required only during aversive visual memory acquisition.**

(A-B) Scheme of the temperature shift protocol to block the output of corresponding neurons during training (A) or test (B). (C-D) Output of neurons labeled with *c205-GAL4* is only necessary during appetitive training (one-way ANOVA, *post-hoc* pairwise comparison,  $p < 0.05$ ) but not test (one-way ANOVA,  $p > 0.05$ ).  $n = 5-7$ .

### 3.6 MB as a coincidence detector for visual classical learning

#### Requirement of *rut* in visual classical conditioning



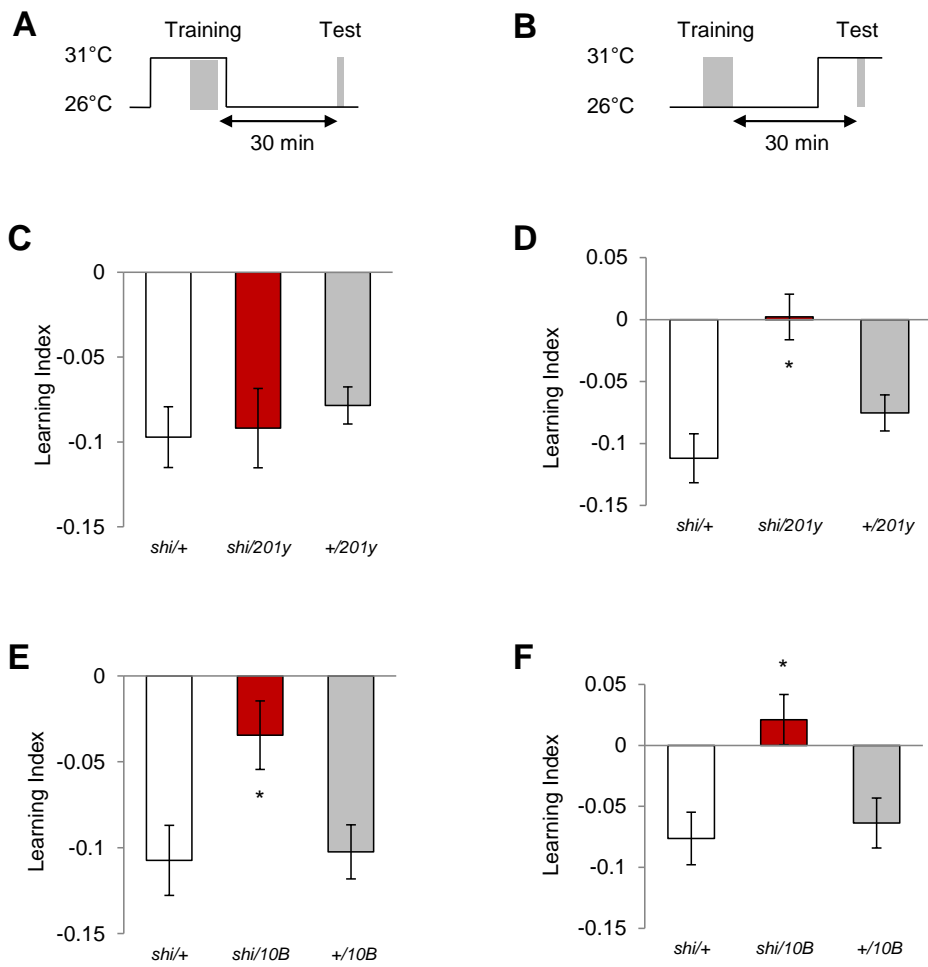
**Figure 20: *rut* mutation leads to impairment in aversive visual conditioning that cannot be rescued by restoring *rut* inside the MB or CC.**

**(A)** In visual conditioning, *rut* mutant phenotype (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.001$ ) cannot be rescued by expressing the *rut*-protein only in the CC (one-way ANOVA,  $p > 0.05$ ) or by expressing the *rut*-protein only in the MB (one-way ANOVA,  $p > 0.05$ ),  $n = 11-27$ . **(B)** Visual stimulus preference in the memory test after aversive conditioning. The blue and green bars represent the punished color during training in both reciprocals (Blue+ and Green+). Deviation from zero shows bias in visual choice, thus animals are able to discriminate visual stimuli in test. **(C)** In olfactory learning *rut* mutant phenotype can be rescued by expressing the *rut*-protein only in the MB (Kruskal-Wallis-test, *post-hoc* pairwise comparisons,  $p < 0.05$ , data acquisition by Stephan Knapek),  $n = 8$ .

The *rut* protein is described as the so called coincidence detector in olfactory learning in the MB (Zars et al. 2000). Also, in visual learning in the flight simulator this protein is critical for intact pattern memory formation and retrieval, however not in the MB but in the CC (Liu et al. 2006; Pan et al. 2009). Whether the *rut* protein is needed for learning about visual cues like color or intensity is not clear so far. Therefore, I tested *rut* mutant flies for aversive visual conditioning. The mutants showed strong impairment in visual conditioning (Figure 20A) similar to the effect found in the flight simulator paradigm, yet discriminability of visual stimuli remained intact (Figure 20B). To further specify the *rut* requirement for the new visual paradigm, I performed *rut*-rescue experiments either in the MB or the CC of *Drosophila*. In line with published results, rescue of *rut* in the MB could fully restore olfactory memory (Zars et al. 2000); however rescuing *rut* in neither the MB nor the CC could restore the visual learning ability in the new assay (Figure 20A,C). Thus, even though the MB is required for this kind of visual learning, rescuing the *rut* protein in this neuropil is not sufficient to restore visual memory.

### **MB output is required during training and test**

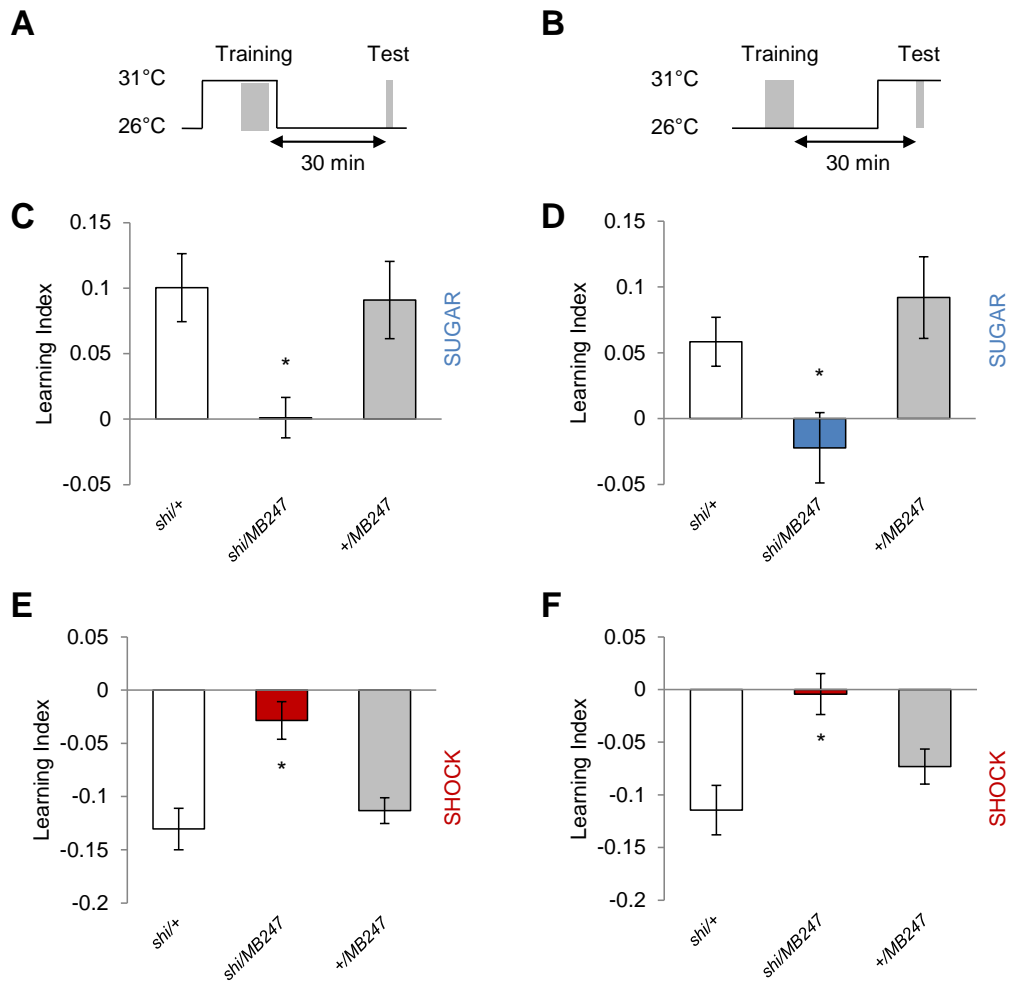
Exploration of the temporal requirement of the MB output for the formation and/or the retrieval of visual memory was performed by transiently blocking the MB output with different GAL4-Lines (Figure 21A-B, Figure 22A-B). Blocking output of neurons labeled by *201y-GAL4* ( $\alpha/\beta$ ,  $\gamma$ -neurons) revealed selective requirement for the retrieval but not the formation of aversive visual memory (Figure 21C-D). Interestingly, the transient block of a broader population of KCs using *MB247-GAL4* ( $\alpha/\beta$ ,  $\gamma$ -neurons) and *MB010B-Split-GAL4* ( $\alpha/\beta$ ,  $\alpha'/\beta'$ ,  $\gamma$ -neurons) (Bräcker et al. 2013) revealed additional requirement in memory acquisition (Figure 21E-F, Figure 22E-F). Acquisition and retrieval of appetitive memory were similarly impaired (Figure 22C-D). As in olfactory memory (Krashes et al. 2007; Dubnau et al. 2001) different KCs may mediate acquisition and retrieval of visual memories.



**Figure 21: Requirement of MB output during aversive visual memory formation and retrieval depends on expression of GAL4 driver line.**

(A-B) Scheme of the temperature shift to block the output of corresponding neurons during visual training (A) or test (B). (C-D) Output of 201y-GAL4 is dispensable during training (one-way ANOVA,  $p > 0.05$ ),  $n = 20-22$ , however necessary during aversive test (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.01$ )  $n = 12-17$ . (E-F) Output of MB010B-GAL4 is necessary in aversive training and test (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ ),  $n = 7-13$ . Bars and error bars represent mean and SEM, respectively.



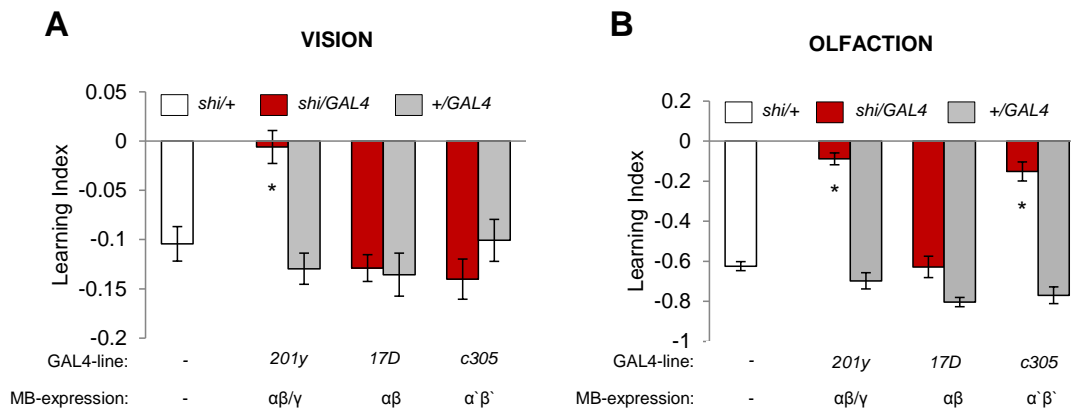


**Figure 22: MB output is needed during visual memory acquisition and retrieval.**

(A-B) Scheme of the temperature shift to block the output of corresponding neurons during training (A) or test (B). (C-D) Output of neurons labeled with *MB247-GAL4* is necessary during appetitive training and test (one-way ANOVA, *post-hoc* pairwise comparison,  $p < 0.05$ ).  $n = 10-16$ . (E-F) Similarly, output of *MB247*-labeled neurons is needed during aversive training and test (one-way ANOVA, *post-hoc* pairwise comparison,  $p < 0.05$ ).  $n = 10-28$ . Bars and error bars represent mean and SEM, respectively.

## Different Kenyon cell subsets are required for visual and olfactory memories

By comparing the requirement of different KC subsets in aversive visual and olfactory memories using *c305-GAL4*, *17D-GAL4* and *201y-GAL4* I could show that specific KC subsets are required for visual and olfactory conditioning, respectively. Blocking the  $\alpha'/\beta'$  neurons with *c305a* selectively impaired olfactory memory (Figure 23), whereas the pattern of defects were the same in blockades with *201y* ( $\alpha/\beta$ ,  $\gamma$  neurons) and *17D* ( $\alpha/\beta$  neurons). Using *17D/shi<sup>ts1</sup>* did not significantly affect either memory, while using *201y/shi<sup>ts1</sup>* strongly impaired both visual and olfactory memories (Figure 23). Hence, visual and olfactory memories surely recruit partly different KC subsets. The requirement of the  $\gamma$ -lobe neurons seems to be shared among both modalities. Given the preferential olfactory representation in the  $\alpha'/\beta'$ -neurons (Turner et al. 2008), specific contribution to olfactory learning is in order with previous publications.



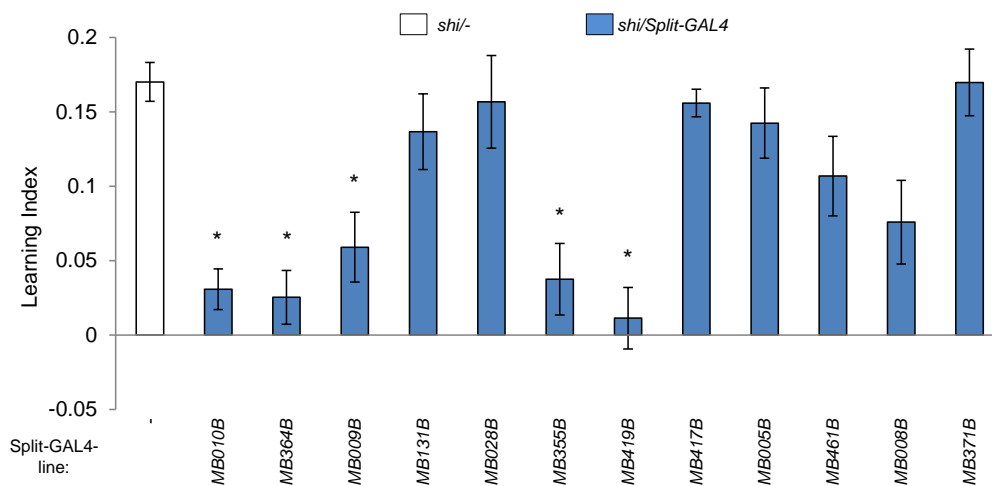
**Figure 23: Overlapping, yet distinct sets of MB-lobes are needed for aversive visual and olfactory learning.**

**(A)** Only blocking output of neurons labeled with *201y-GAL4* ( $\alpha/\beta/\gamma$ -lobes) during visual conditioning leads to memory impairment (one-way ANOVA, *post-hoc* pairwise comparison,  $p < 0.01$ ),  $n = 12-19$ . **(B)** In contrast, in aversive olfactory memory, blocking output of neurons labeled with *201y-GAL4* ( $\alpha/\beta/\gamma$ -lobes) and *c305-GAL4* ( $\alpha'/\beta'$ -lobes) leads to significant memory impairment (one-way ANOVA, *post-hoc* pairwise comparison,  $p < 0.001$ ).  $n = 10-22$ . Bars and error bars represent mean and SEM, respectively. Olfactory conditioning was performed by Kristina Dylla.

### 3.7 MB-Screening

Previous results already presume that the MB plays a pivotal role not only in appetitive and aversive olfactory conditioning but also in visual conditioning. Especially the  $\gamma$ -lobe neurons seem to be important for both kinds of conditioning. To further identify the specific function and required cell types of the MB I screened ~ 80 MB intrinsic and extrinsic Split-GAL4 lines (see Appendix; Table 10, Table 11 and Table 12) in visual appetitive and aversive conditioning. The selection covered lines for all lobes, dopamine and octopamine input lines and several described (Tanaka et al. 2008) and not yet described output lines. In parallel the same lines were tested by my colleague Toshiharu Ichinose for 2h appetitive and aversive olfactory conditioning. Thus, comparison not only to already published olfactory data but also to results of the new Split-GAL4 lines tested for olfactory conditioning is possible.

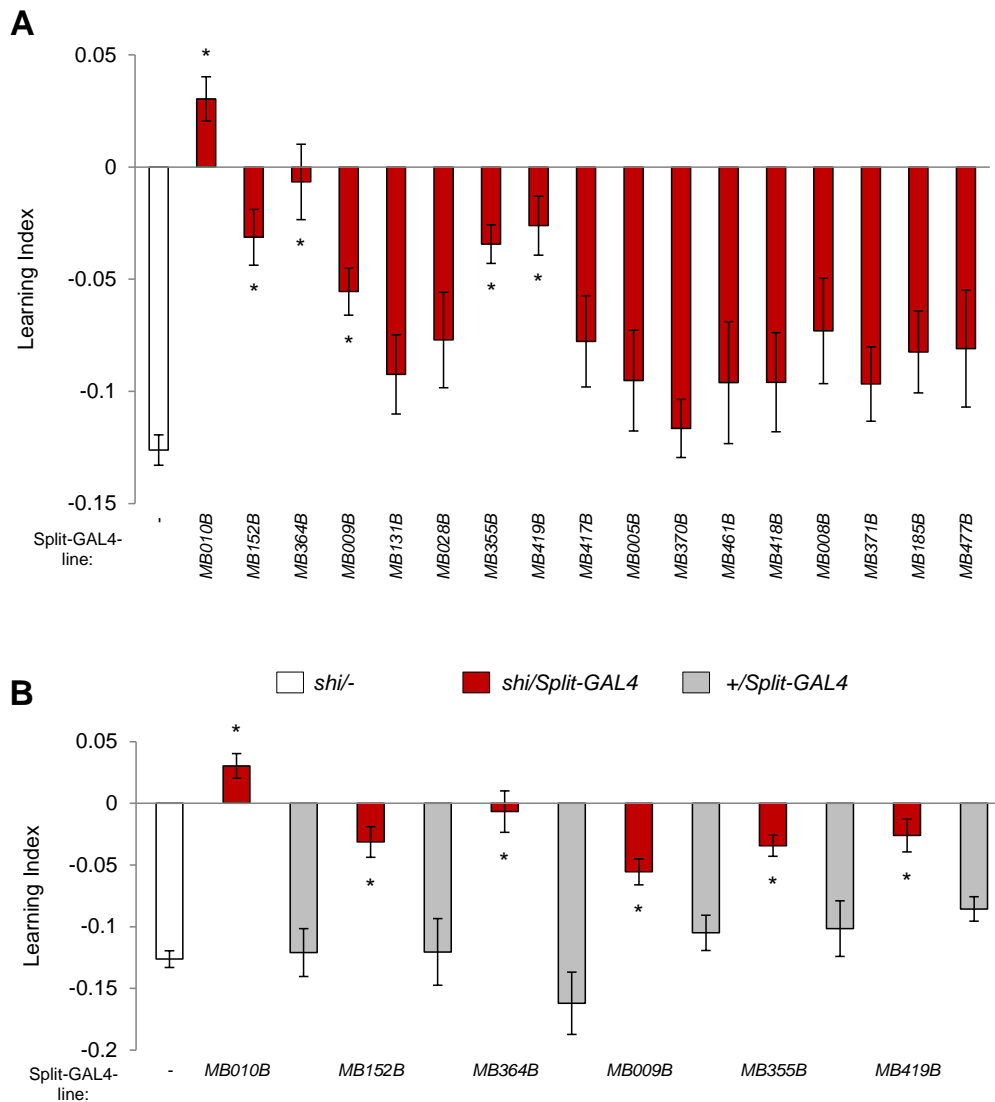
#### The $\gamma$ d lobe KCs are important for visual conditioning



**Figure 24: MB  $\gamma$ d-lobes are required for visual appetitive memory.**

Blocking output of specific MB-lobe subsets during appetitive conditioning showed that  $\gamma$ d-lobes are specifically required (Mann Whitney-test,  $p < 0.05$ ).  $n = 4-36$ . Bars and error bars represent mean and SEM, respectively.

In olfactory memories, different lobes of the MBs perform specific functions. By testing the Split-GAL4 selection for MB lobes in visual conditioning it was possible to precisely contrast the lobe requirement in visual learning (see Appendix, Table 10 for tested lines) (Bräcker et al. 2013). Blocking the output of the  $\gamma$ d neurons (MB355B, MB419B), the  $\gamma$ -lobe neurons (MB009B) as well as the entire KC population (MB010B, MB364B, MB152B) impaired both appetitive and aversive visual memories (Figure 24, Figure 25, Table 5). Blocking the lines labeling  $\alpha'/\beta'$  neurons or  $\alpha/\beta$  neurons did not



**Figure 25: MB  $\gamma$ d-lobes are required for visual aversive memory.**

**(A)** Blocking output of specific MB-lobe subsets during aversive conditioning showed that  $\gamma$ d-lobes are specifically required (Mann Whitney-test,  $p < 0.01$ ).  $n = 8-68$ . Bars and error bars represent mean and SEM, respectively. **(B)** Secondary screening of phenotypic lines with +/Split-GAL4 control (Kruskal-Wallis test, *post-hoc* pairwise comparisons,  $p < 0.05$ ),  $n = 7-119$ .

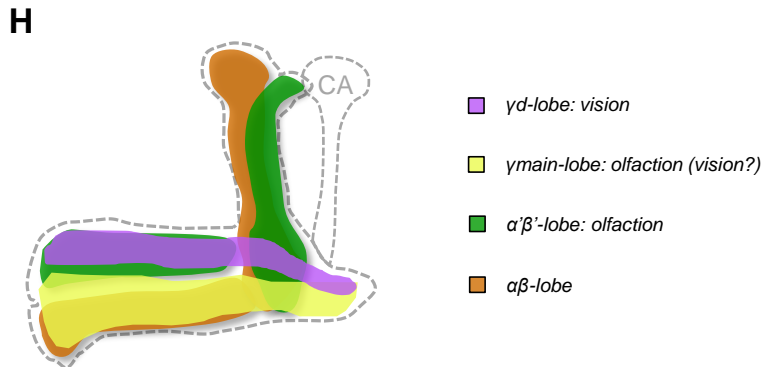
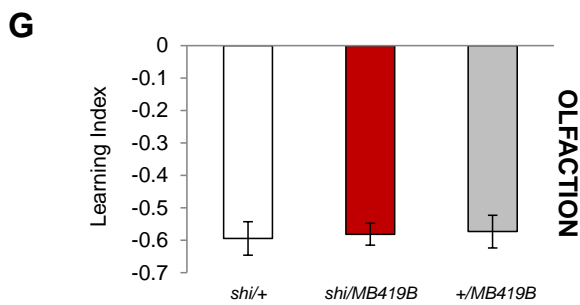
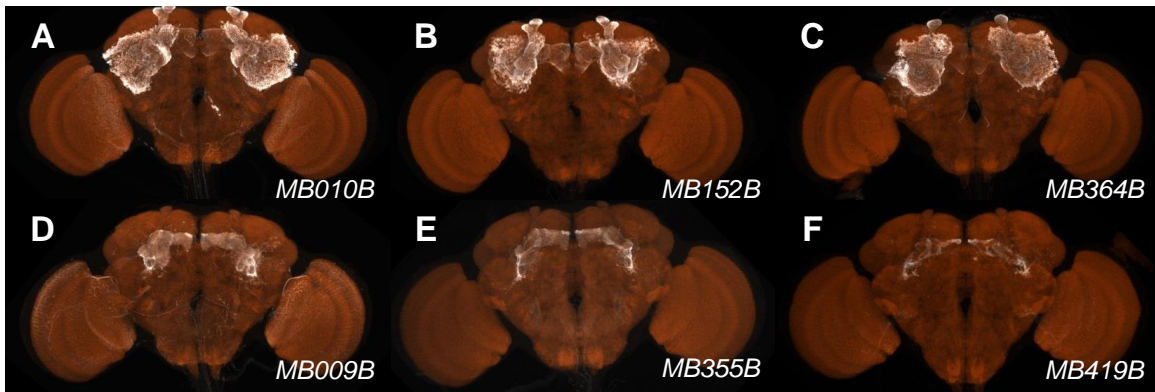
significantly reduce the performance compared to the controls (Figure 24, Figure 25). The requirements of the KC subsets for appetitive and aversive memories were strikingly similar as already found in olfactory conditioning (Kim et al. 2007; Trannoy et al. 2011; Qin et al. 2012), suggesting the commonality of the circuit mechanism between visual and olfactory memories.

	KC $\gamma$ d	Kc $\gamma$ main	KC $\alpha$ '/ $\beta$ 'a	KC $\alpha$ '/ $\beta$ 'm	KC $\alpha$ '/ $\beta$ 'p	KC $\alpha$ '/ $\beta$ 'p	KC $\alpha$ '/ $\beta$ 's	KC $\alpha$ '/ $\beta$ 'c(o)	KC $\alpha$ '/ $\beta$ 'c(i)
<i>MB010B</i>	Grey	Grey	Black	Black	Black	Black	Black	Black	Black
<i>MB152B</i>	Grey	Grey	Black	Black	Black	Light Grey	Dark Grey	Light Grey	Light Grey
<i>MB364B</i>	Black	Black	White	White	White	Dark Grey	Black	Black	Light Grey
<i>MB009B</i>	Grey	Grey	White	White	White	White	White	White	White
<i>MB355B</i>	Dark Grey	Light Grey	White	White	White	White	White	White	White
<i>MB419B</i>	Black	White	White	White	White	White	White	White	White

**Table 5: Expression of MB intrinsic lines that were tested during screening and showed impaired appetitive and aversive learning**

*MB152B* was only tested for aversive conditioning. On top is the expression pattern inside the MB depicted. Grey intensity reflects expression strength in different MB subsets (e.g. darker grey = stronger expression).

I tested two other lines that label the  $\gamma$ d-lobe but did not show any impairment in visual learning (see Table 10, Appendix). *MB131B* preferentially labels  $\gamma_{\text{main}}$ -lobe and only stochastically the  $\gamma$ d-lobe, thus probably some flies possess intact  $\gamma$ d-lobes. *MB028B* indeed labels the  $\gamma$ d-lobe; however, the intensity of the expression is very low compared to the lines that showed phenotype. It seems as if only strong neural block leads to phenotype. An additional role for the  $\gamma_{\text{main}}$ -lobe seems to be possible; however, only testing a specific line for this neuropil can reveal the requirement. To see if there is a general requirement for short-term-memory formation and retrieval, the output of the specific  $\gamma$ d Split-GAL4-line *MB419B* was blocked during training and test for 2 min. aversive olfactory memory (Figure 26, experiment performed by Toshiharu Ichinose). Blocking the neural output with  $\text{sh}^{\text{TS}}$  did not impair olfactory memory. Thus, the  $\gamma$ d-lobe of the MB could be specifically required for non-olfactory stimuli processing or even only for processing of visual stimuli.

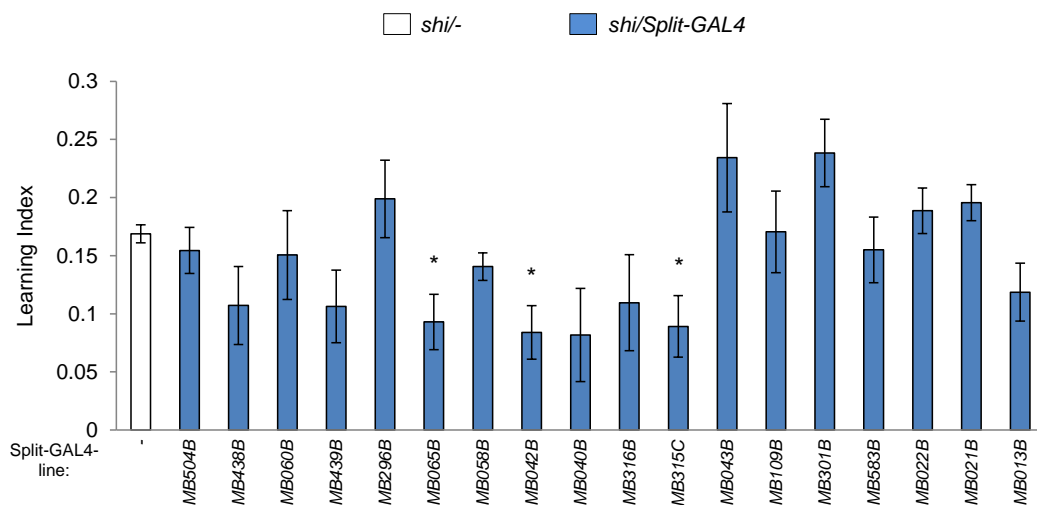


**Figure 26: MB  $\gamma$ -lobes are required for visual but not aversive olfactory memory.**

**(A)** *MB010B-Split-GAL4* labels all lobes of the MB. **(B-C)** *MB152B-Split-GAL4* and *MB364B-Split-GAL4* label  $\alpha/\beta$  and  $\gamma$ -lobes of the MB. **(D)** *MB009B-Split-GAL4* labels  $\gamma$ -lobes of the MB. **(E-F)** *MB335B-Split-GAL4* and *MB419B-Split-GAL4* label  $\gamma$ -lobes of the MB. Pictures from Flylight, JFRC. **(G)** Blocking output of neurons labeled by *MB419B-Split-GAL4* did not lead to impairment in aversive olfactory conditioning (one-way ANOVA,  $p > 0.05$ ),  $n = 8-10$ . Data acquisition by Toshiharu Ichinose. Bars and error bars represent mean and SEM, respectively. **(H)** Circuit model of olfactory and visual short-term memories in the MB-lobes. Visual and olfactory information is probably conveyed to distinct sets of KCs. (CA = Calyx).

## Differential requirement of dopamine neurons for appetitive and aversive learning

Requirement and sufficiency of different dopamine neurons for appetitive and aversive visual memories are strikingly similar to those in olfactory memories. The blockade of dopamine neurons of the PPL1 cluster with *TH-GAL4* (data not shown) or *MB504B-Split-GAL4* (data not shown, Figure 28) selectively reduced aversive visual memory similar to what was found in olfactory learning (Claridge-Chang et al. 2009; Aso et al. 2012). The blockade with *DDC-GAL4* or *R58E02-GAL4* (data not shown) that label different dopamine neurons of the PAM-cluster substantially impaired appetitive memory similar to what was found in olfactory learning (C. Liu et al. 2012). Employing more specific lines during the screening I could narrow down the requirement of neurons for appetitive and aversive visual learning.



**Figure 27: Specific dopamine neurons of the PAM Cluster are required for appetitive visual conditioning.**

Blocking output of specific dopamine neuron subsets that project to the MBs during appetitive conditioning in primary screening led to potential impairment in various different split-GAL4 lines (Mann Whitney-test,  $p < 0.05$ ).  $n = 7-105$ . Bars and error bars represent mean and SEM, respectively.

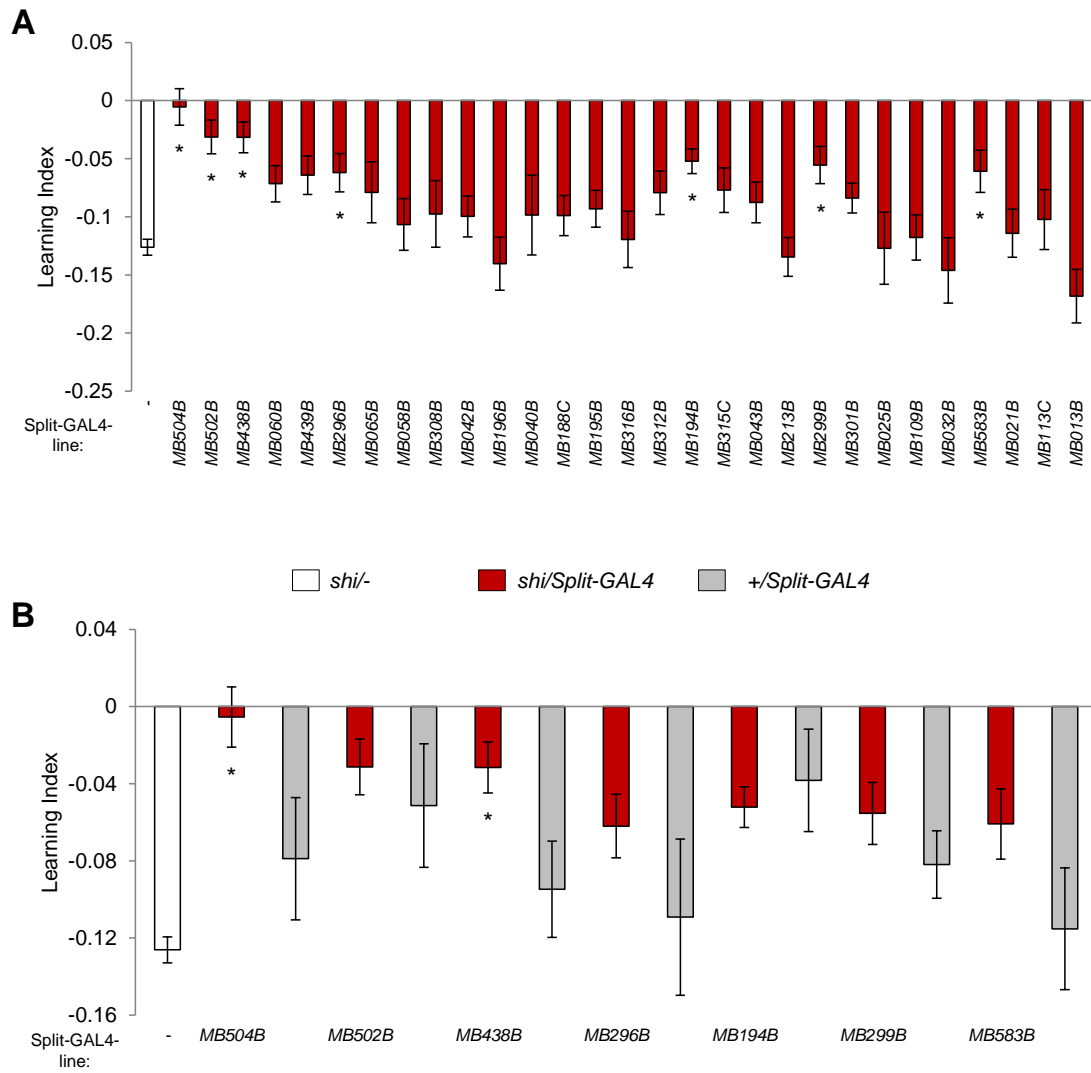
### *Appetitive MB-input*

Only selected MB-input lines were tested in appetitive conditioning during primary screening. No secondary screening could be performed due to time constraints within the screening project. Interestingly the lines with strongest impairment indeed label cells of the dopamine PAM-cluster (Figure 27). Consistent impairment or tendency for impairment was found in lines labeling  $\gamma$ 5-output (Table 6: MB040B, MB042B, MB315C) and  $\gamma$ 4-output (Table 6: MB040B, MB042B, MB316B). One line labeling neurons of the PPL1 cluster showed tendency for impairment (MB065B); however, no consistent expression of a specific cell type could be found here (see Appendix; Table 11). Thus, strongest candidates for sugar information output are dopamine PAM-cluster neurons that project to  $\gamma$ 4 and  $\gamma$ 5 compartments of the MB.

### *Aversive MB-input*

All Split-GAL4 lines labeling potential afferent connections to the MB were tested during primary and secondary screening. The Split-GAL4 line *MB504B* which had already been shown to be impaired in visual aversive conditioning could be confirmed as phenotypic also within the screening (Figure 28). Another line, *MB438B* that also specifically labels neurons from the PPL1 cluster, was also found to be impaired (Figure 28). Both lines strongly label the MP1 neurons, which project to the  $\gamma$ 1 lobe compartment of the MB (Table 6). These neurons are also labelled by *MB502B-Split-GAL4*, however during secondary screening the WT-control showed similar impairment as the experimental group during primary screening (Figure 28B).





**Figure 28: Specific dopamine neurons of the PPL1 Cluster are required for aversive visual conditioning.**

(A) Blocking output of specific dopamine neuron subsets that project to the MBs during aversive conditioning in primary screening led to impairment in various different Split-GAL4 lines (Mann Whitney-test,  $p < 0.005$ ),  $n = 8-119$ . (B) In secondary screening when additionally testing  $+/Split-GAL4$  control two phenotypes could be confirmed. Blocking output of  $MB504B-Split-GAL4$  and  $MB438B-Split-GAL4$  led to memory impairment (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ ),  $n = 7-119$ . Bars and error bars represent mean and SEM, respectively.

	MP1	MV1	V1	PPL1 $\alpha$ 3	PPL1 $\alpha$ '3	PPL1 $\gamma$ 1	PAMY12- $\gamma$ 4	PAMY3	PAMY4	PAMY5 ( $\beta$ '2a)	PAM $\beta$ 1(MVP1)	PAM $\beta$ 1p ped	PAM $\alpha$ 1(MVP1)	PAM $\beta$ 2sp(M3)	PAM $\beta$ 2cp	PAM $\beta$ '1	PAM $\beta$ '2a	PAM $\beta$ '2m	PAM $\beta$ '2p	
MB 504B	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey
MB 438B	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey	Dark Grey
MB 042B	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey
MB 040B	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey
MB 316B	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey
MB315 C	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey	Light Grey

**Table 6: Expression of dopamine lines that were tested during screening and showed impaired appetitive and aversive learning, respectively**

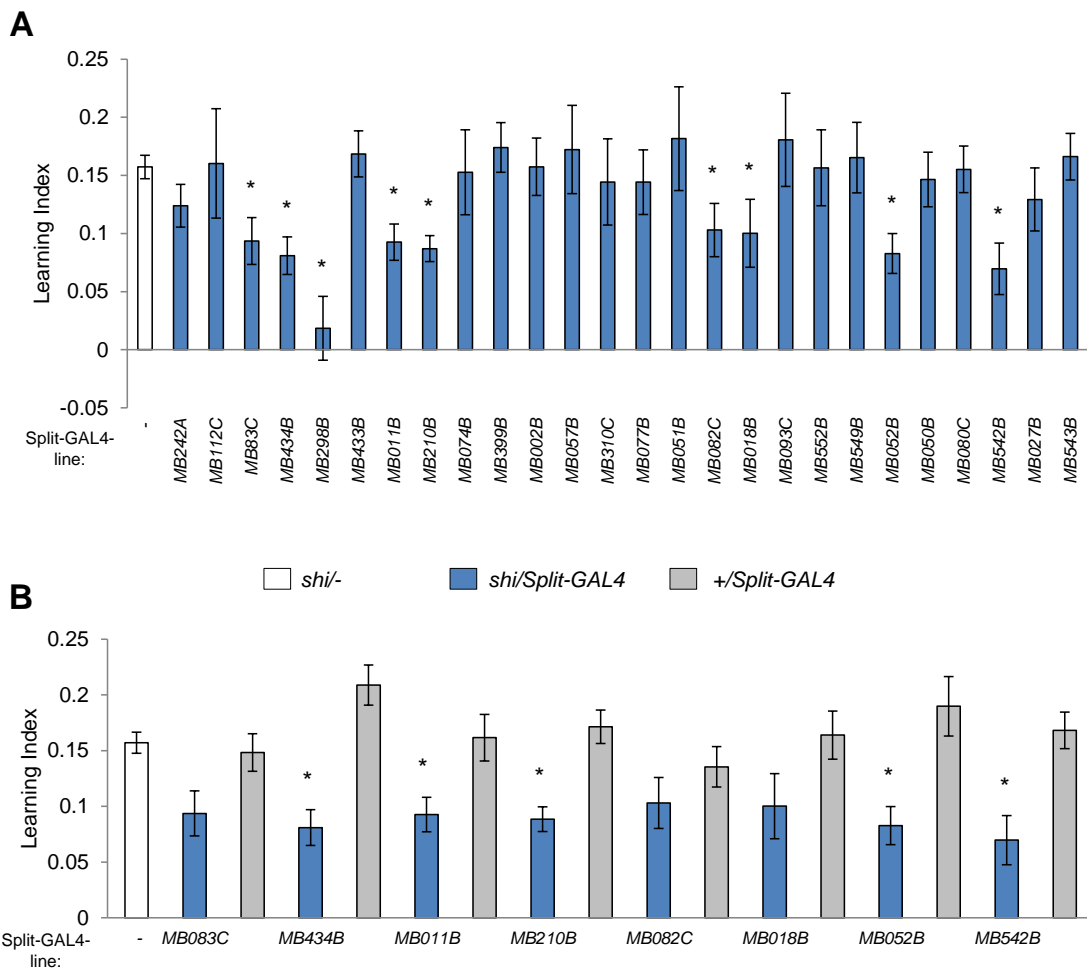
On top is the expression pattern depicted (red label = impaired in aversive conditioning; blue label = impaired in appetitive conditioning). Grey intensity reflects expression strength in different dopamine neuron subsets (e.g. darker grey = stronger expression).

### Differential requirement of potential MB-output neurons for appetitive and aversive conditioning

So far, no MB-output neurons have been described as necessary for visual short term memory. Thus, with the screening it was possible for the first time to find specific requirement of potential MB-output neurons that could be involved in memory formation or retrieval. One of the MB-output Split-GAL4 lines showed impairment for appetitive and aversive conditioning, *MB298B* (Figure 29A, Figure 30A). This line was however excluded after primary screening since the flies seemed not to be able to differentiate between the trained visual stimuli. Indeed this line shows strong expression in the optic lobes in addition to the MB-output neurons (data not shown).

## MB-output neurons required for appetitive conditioning

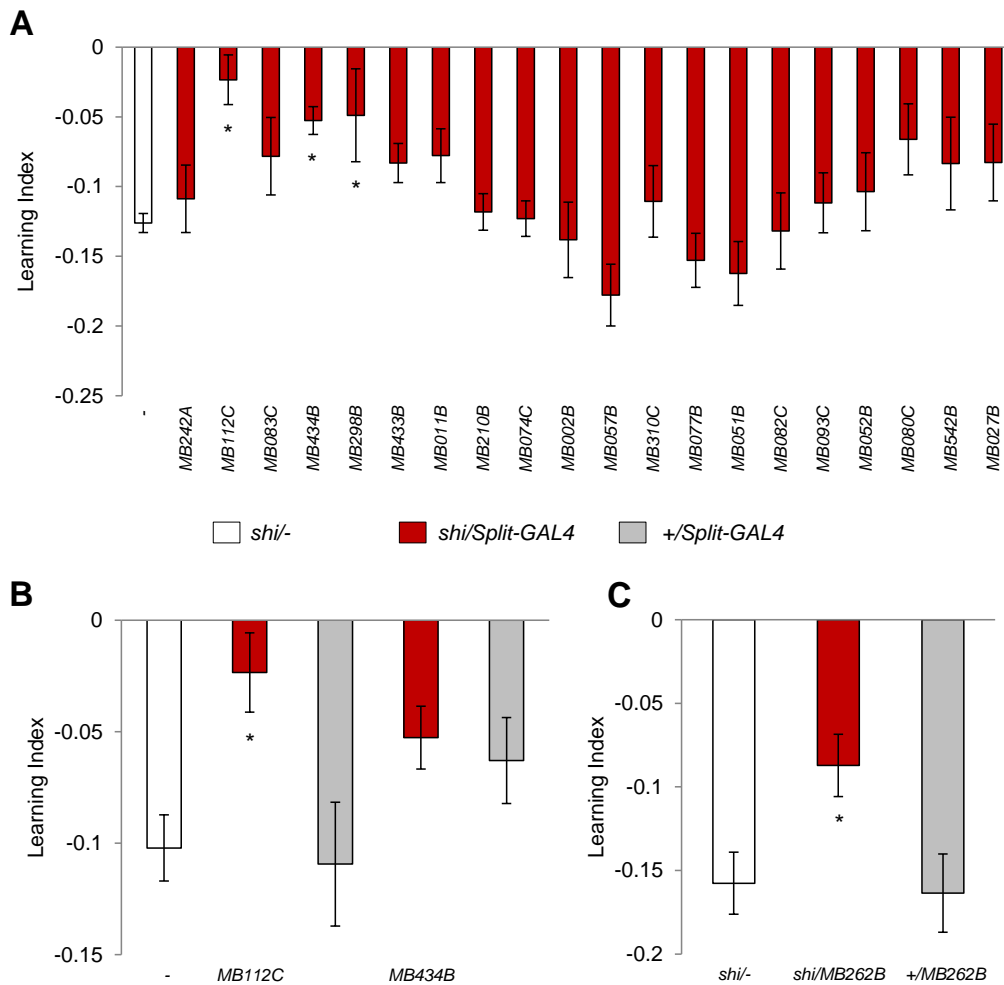
Since no previous results existed to allow selection of output neurons, all available MB-output Split-GAL4-lines were tested during primary and secondary screening in visual appetitive conditioning.



**Figure 29: Several potential MB-output neurons are required for appetitive visual memory formation.**

**(A)** Blocking output of specific MB-output neurons during appetitive conditioning in primary screening led to impairment in various different Split-GAL4 lines (Mann-Whitney-test,  $p < 0.05$ ).  $n = 7-40$ . **(B)** In secondary screening when additionally testing  $+/\text{Split-GAL4}$  control five of the previously phenotypic lines could be confirmed (Kruskal-Wallis test, *post-hoc* pairwise comparisons,  $p < 0.05$ )  $n = 8-73$ . Bars and error bars represent mean and SEM, respectively.

In line with the results for the input neurons, output neurons receiving information from the  $\gamma 4$  and  $\gamma 5$  lobe compartment of the MB are also required (Figure 29B, Table 7, GluT-positive neurons: *MB434B*, *MB011B*, *MB210B*). Additionally, blocking output from a specific region of the vertical lobes also impairs appetitive memory (Figure 29B, Table 7, V2 $\alpha$ 2, ChAT-positive neurons = *MB052B*, *MB542B*). Thus, information about sugar reward could modulate visual information in the  $\gamma 4/5$  compartment of the medial lobe and from here provide behavioral output through connected neurons.



**Figure 30: A specific MB-output neuron is required for aversive visual memory formation.**

(A) Blocking output of potential MB-output neurons during aversive conditioning in primary screening led to impairment in various different Split-GAL4 lines (Mann-Whitney-test,  $p < 0.005$ ).  $n = 8-119$ . (B) In secondary screening when experimental lines were tested together with all controls one phenotype could be confirmed.

Blocking output of *MB112C-Split-GAL4* led to memory impairment (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ )  $n = 8-25$ . (C) An additional line *MB262B-Split-GAL4* that labels a similar neuron showed impaired aversive visual memory (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ ),  $n = 9-13$ . Bars and error bars represent mean and SEM, respectively.

### *MB-output neurons required for aversive conditioning*

All MB-output Split-GAL4 lines were tested during primary and secondary screening in visual aversive conditioning. Also here in line with the results for input neurons, a single inhibitory output neuron was found to be required receiving input from the  $\gamma$ 1 lobe compartment (Figure 30B, Table 7, MVP2, GABA-positive neuron: MB112C). The requirement could additionally be shown by testing a different Split-GAL4 line labeling the same neuron (Figure 30C, MB262B). The MVP2 neuron was already described anatomically but not functionally (Tanaka et al. 2008).

### **Summary Screening**

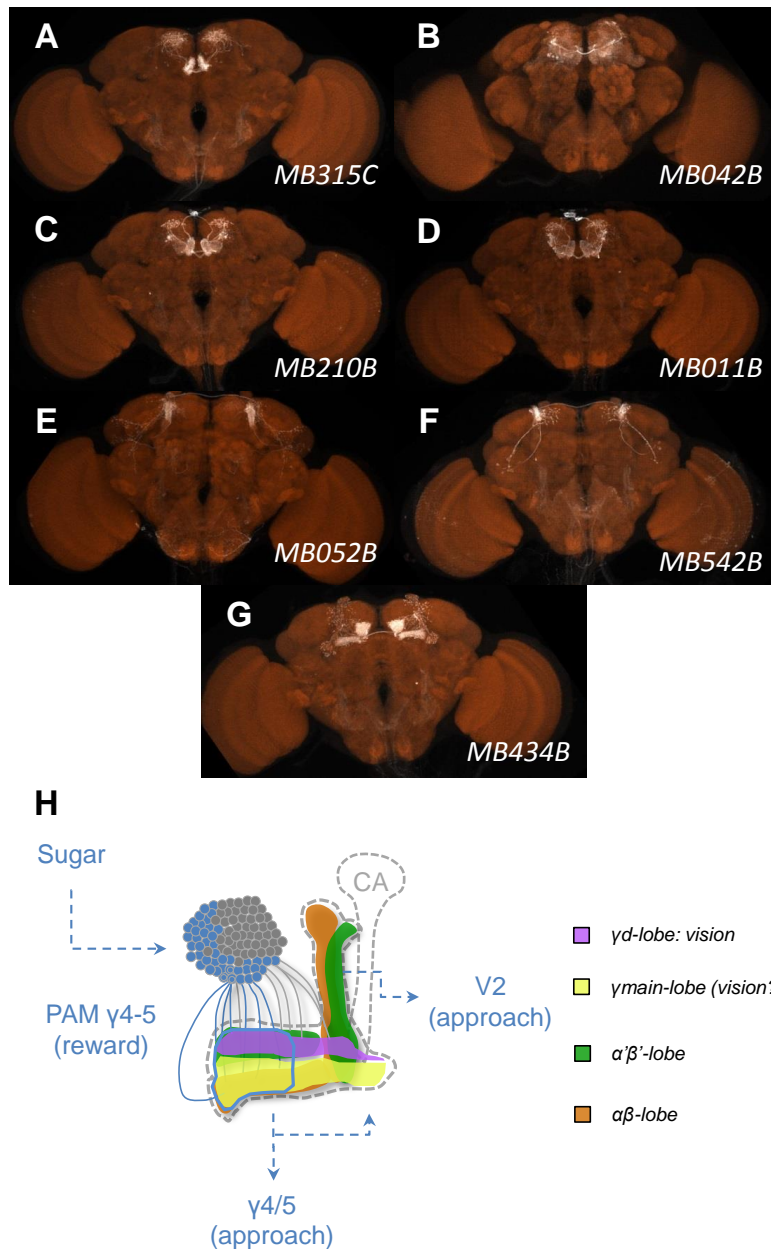
Different sub-compartments of the MB  $\gamma$ -lobe were found to be required for different visual memory tasks. The  $\gamma$ 4 and  $\gamma$ 5 sub-compartment seem to be specifically required for appetitive short term memory processing (Figure 31), whereas the  $\gamma$ 1 sub-compartment is specifically required for aversive short term memory processing (Figure 32).

However, it seems to be possible that the two different pathways could also interact with each other via feedback loops provided by MB-output neurons (Figure 33). The  $\gamma$ 4 output neuron, required in appetitive conditioning, projects back to the  $\gamma$ 1/2 sub-compartment (Figure 31G). Here, it could modulate visual aversive memory (Figure 33A). The MVP2 output neuron, required in aversive conditioning, projects back to the  $\alpha/\beta$ -lobes (Figure 32C). Here, it could modulate appetitive memory output (Figure 33B).

	outputy1(MVP2)	outputy3β'1	outputy4->y12	Outputβ1(MV2)	outputy5β'2a(M4-6)	outputβ2 β'2a(M4-6)	Outputβ'2mp(M4-6;	outputβ'1	outputα1	outputy2α'1	outputα'2(V4)	outputα3(V3)	outputα2(V2asc)	outputα2(V2α2p)	outputα'3p(V2α'3p)	outputα'3m(V2α'3m)	outputα'3a(V2α'3a)	outputα'1(V2α'1)
MB 112C	Black																	
MB 434B			Black	Black														
MB 011B					Black	Grey												
MB 210B					Black	Grey												
MB 052B													Black	Grey	Grey	Grey	Grey	Grey
MB542 B													Black					

**Table 7: Expression of potential MB-output lines that were tested during the screening and showed impaired appetitive and aversive learning, respectively**

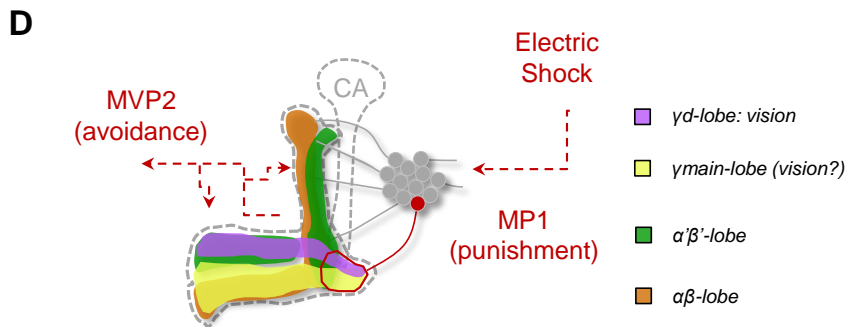
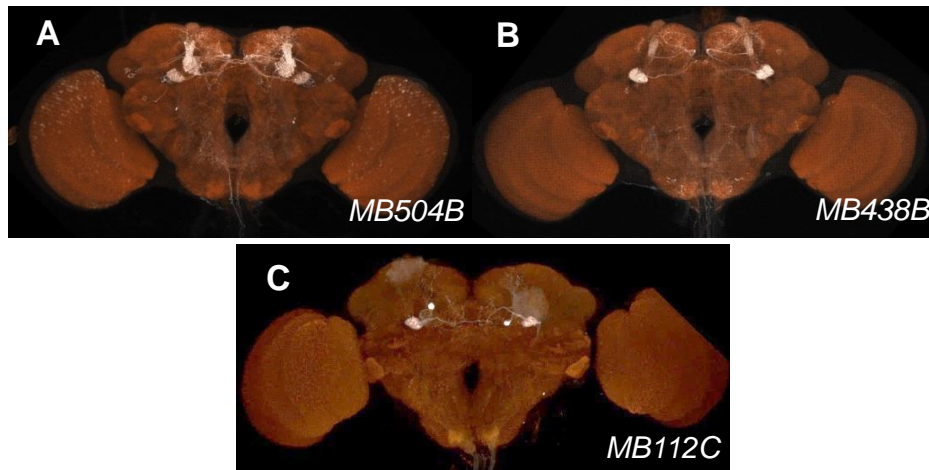
On top is the expression pattern depicted (red label = impaired in aversive conditioning; blue label = impaired in appetitive conditioning). Grey intensity reflects expression strength in different output neuron subsets (e.g. darker grey = stronger expression).



**Figure 31: MB-sub-compartments  $\gamma$ 4-5 seem to be important for appetitive conditioning.**

(A-B) Expression pattern of dopamine Split-GAL4-lines that show impairment in appetitive visual conditioning. (A) *MB315C-Split-GAL4* labels dopamine neurons of the PAM cluster innervating the  $\gamma$ 5-lobe sub-compartment. (B) *MB042B-Split-GAL4* labels dopamine neurons of the PAM Cluster innervating the  $\gamma$ 1-5-lobe sub-compartments. (C-G) Expression pattern of potential output Split-GAL4-lines that show impairment in appetitive visual conditioning. (C-D) *MB210B-Split-GAL4* and *MB011B-Split-GAL4* label  $\gamma$ 5 and  $\beta$ '2 output neurons. (E-F) *MB052B-Split-GAL4* and *MB542B-Split-GAL4* label V2 ( $\alpha$ 2) output neurons. (G) *MB434B-Split-GAL4* labels a  $\gamma$ 4 output neuron. (H) Circuit model of appetitive visual short-term memory.

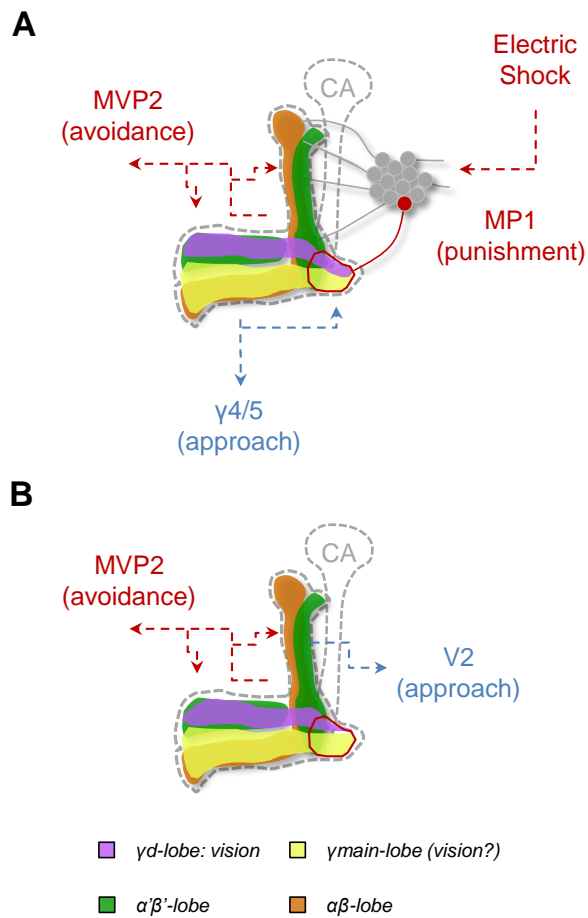
Visual information is conveyed to the  $\gamma$ d-lobe neurons of the MB KCs. Output of these KCs is locally modulated by a specific subset of dopamine neurons (PAM:  $\gamma$ 4/5) to form aversive memories.  $\gamma$ 4/5 and V2 neurons mediate output to the surrounding protocerebrum and back to the  $\gamma$ 1 lobe sub-compartment of the MBs. (CA = Calyx).



**Figure 32: MB-sub compartment  $\gamma$ 1 is important for aversive conditioning.**

**(A-B)** Expression pattern of dopamine and output Split-GAL4-lines that show impairment in aversive visual conditioning, respectively. *MB504B-Split-GAL4* labels MP1, MV1, V1 and the neuron projecting to the tip of the  $\alpha$ -lobe. *MB438B-Split-GAL4* labels the MP1 and the V1 neuron. **(C)** Expression pattern of MB-output Split-GAL4-line that shows impairment in aversive visual conditioning. *MB112C-Split-GAL4* labels the MVP2 neuron. **(D)** Circuit model of aversive visual short-term memory. Visual information is conveyed to the  $\gamma$ d-lobe neurons of the MB KCs. Output of these KCs is locally modulated by a specific subset of dopamine neurons (PPL1: MP1 (V1)) to form aversive memories. MVP2 neurons mediate memory output to the surrounding protocerebrum and back to the  $\alpha/\beta$  lobes of the MBs. (CA = Calyx).





**Figure 33: Possible MB feedback loops in visual appetitive and aversive memory circuits.**

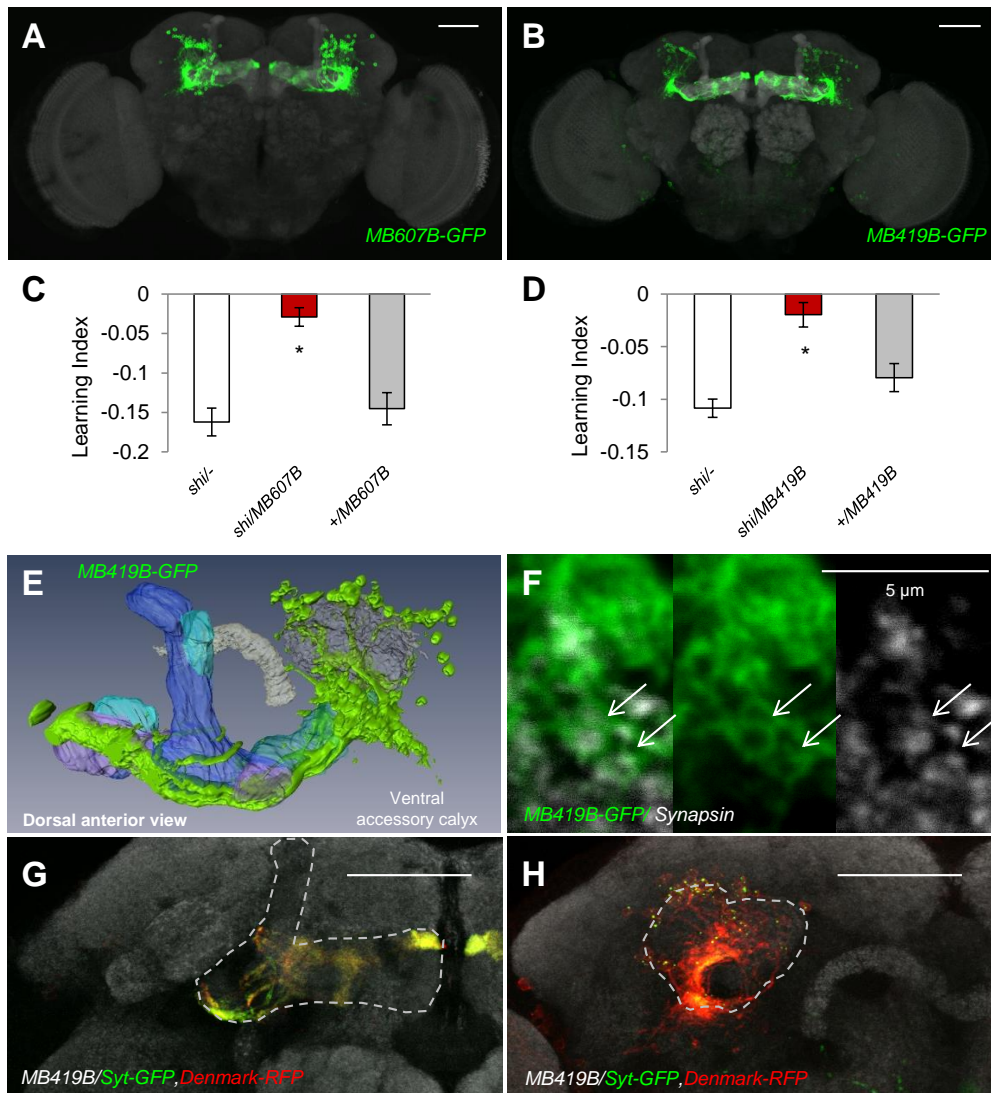
**(A)** Appetitive  $\gamma$ 4 output neurons could possibly modulate KCs and output neurons involved in the aversive learning circuit in the  $\gamma$ 1 sub-compartment. **(B)** MVP2 output neurons that are required for aversive learning could modulate V2 output neurons that are required for appetitive memory. (CA = Calyx).

### 3.8 Visual information representation in the MB

#### **$\gamma$ d KCs form a ventral accessory calyx (VACA)**

The  $\gamma$ d-lobe of the MB labeled by *419B-Split-GAL4* and *355B-Split-GAL4* is required for visual aversive learning (Figure 25, Figure 34B,D,E). To further confirm the requirement, an additional line was tested, that very specifically and strongly labels the  $\gamma$ d-lobe, *MB607B-Split-GAL4* (Figure 34A,C). Blocking the output of these neurons revealed again a strong impairment in visual learning. Thus, the  $\gamma$ d-lobes play a major role in visual conditioning with aversive stimuli. Since no phenotype was found with olfactory conditioning (Figure 26), the  $\gamma$ d-lobe neuron could be specifically required for processing of non-olfactory stimuli or even only for processing of visual stimuli.

Interestingly the  $\gamma$ d KCs possess a special protrusion antero-ventral to the primary calyx. This protrusion possesses typical claw-like structures (Figure 34F) and is stained by a postsynaptic marker (Figure 34G,H), thus this region marks the ventral accessory calyx (VACA, (Aso et al. 2009; Butcher et al. 2012)). Possessing such an exceptional input-region further supports the idea that input from sensory systems other than olfactory reach through it to the MB. Similar arrangement was shown for accessory calyces in other insects (Farris 2008).

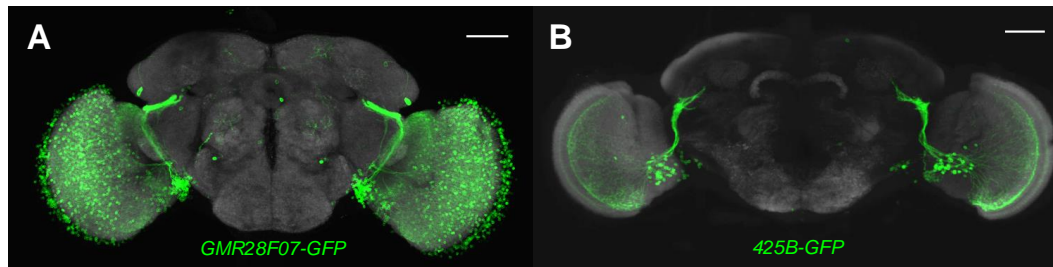


**Figure 34: yd KCs possess a specific ventral accessory calyx (VACA).**

**(A)** Expression pattern of *MB607B-Split-GAL4* that specifically labels yd neurons. **(B)** Expression pattern of *MB419B-Split-GAL4* that specifically labels yd neurons. **(C)** Blocking output of *MB607B-Split-GAL4* with *shi<sup>ts</sup>* reveals impairment in aversive visual conditioning (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ ),  $n = 8-12$ . **(D)** Blocking output of *MB419B-Split-GAL4* reveals impairment in aversive visual conditioning (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ ),  $n = 18-60$ . **(E)** 3D reconstruction of yd neurons in *Split-GAL4* line *MB419B*. Green color labels expression pattern of *MB419B*, blue and grey colors label other lobes of MB, calyx and protocerebral bridge, respectively. **(F)** Dendritic claw in the VACA of the yd KCs. Overlap with presynaptic terminals is indicated with white arrows. **(G-H)** *Split-GAL4* line *MB419B*; presynaptic terminals are labeled by *Syt-GFP*; postsynaptic terminals are labeled by *Denmark-RFP*. **(G)** yd-lobes preferentially contain presynaptic terminals. MB medial and part of vertical lobes is outlined. **(H)** yd ventral accessory calyx preferentially contains dendrites. Primary calyx is outlined. Scale-bar represents 50  $\mu$ m if not otherwise stated. Bars and error bars represent mean and SEM, respectively.

## VPNs project to the $\gamma$ d accessory calyx– Anatomy

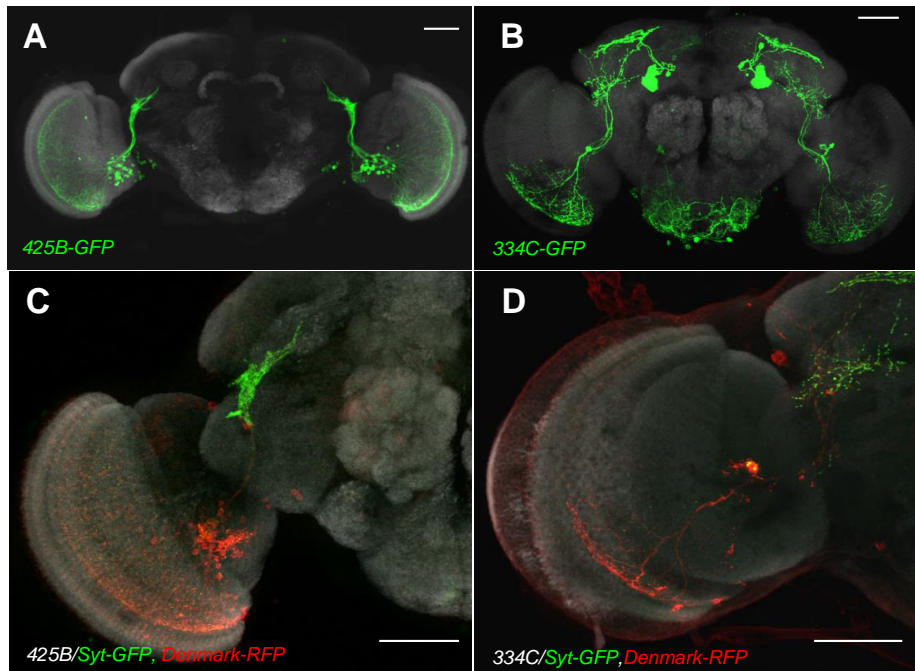
The identification of the specific dendritic ventral output region of the  $\gamma$ d-lobes allowed anatomical screening for possible visual projection neurons among specific Split-GAL4 lines (Figure 35).



**Figure 35: Generation of specific Split-GAL4 lines labeling VPNS from Generation1-GAL4 lines.**

- (A) Unspecific GAL4-line *GMR28F07* expresses in different types of VPNS and other cells in the optic lobes.  
(B) More specific *425B-Split-GAL4* labels only few types of VPNS in the optic lobes. Background is stained with Synapsin. Scale-bar represents 50  $\mu$ m.

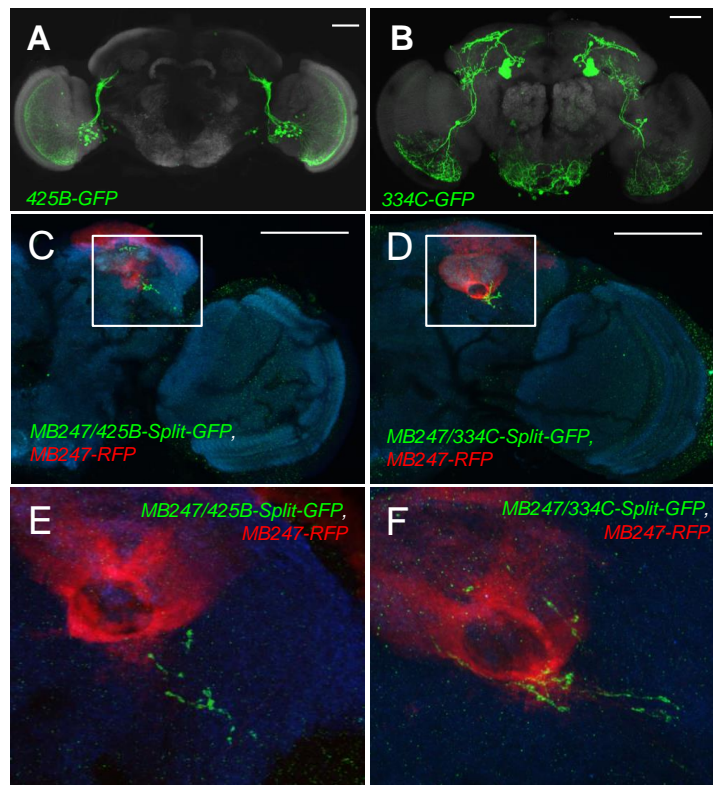
Two promising candidate lines with expression in optic lobe neurons had been detected based on anatomical expression: *MB425B-Split-GAL4* and *MB334C-Split-GAL4* (Figure 35, Figure 36). They possess projections to the central brain region around the ventral accessory calyx. Double staining with Denmark-RFP (labels postsynaptic regions) and Synaptotagmin GFP (labels presynaptic regions) revealed that the VPNS receive their main input inside the medulla of the optic lobe and that they provide output to the central brain and potentially to the  $\gamma$ d VACA. If the VPNS indeed connect with the VACA, this would be the first time that direct connection between the optic lobes and the MB could be shown.



**Figure 36: VPNs receive information in the optic lobes and provide output to the central brain.**

**(A)** Expression pattern of *425B-Split-GAL4* **(B)** Expression pattern of *334C-Split-GAL4*. **(C)** *425B-Split-GAL4* possesses prominent dendritic regions in the medulla and presynaptic terminals in the protocerebrum close to the  $\gamma$ d accessory calyx. **(D)** *334C-Split-GAL4* possesses prominent dendritic regions in the medulla and presynaptic terminals in the protocerebrum close to the  $\gamma$ d accessory calyx. Background is stained with Synapsin. Scale-bar represents 50  $\mu$ m.

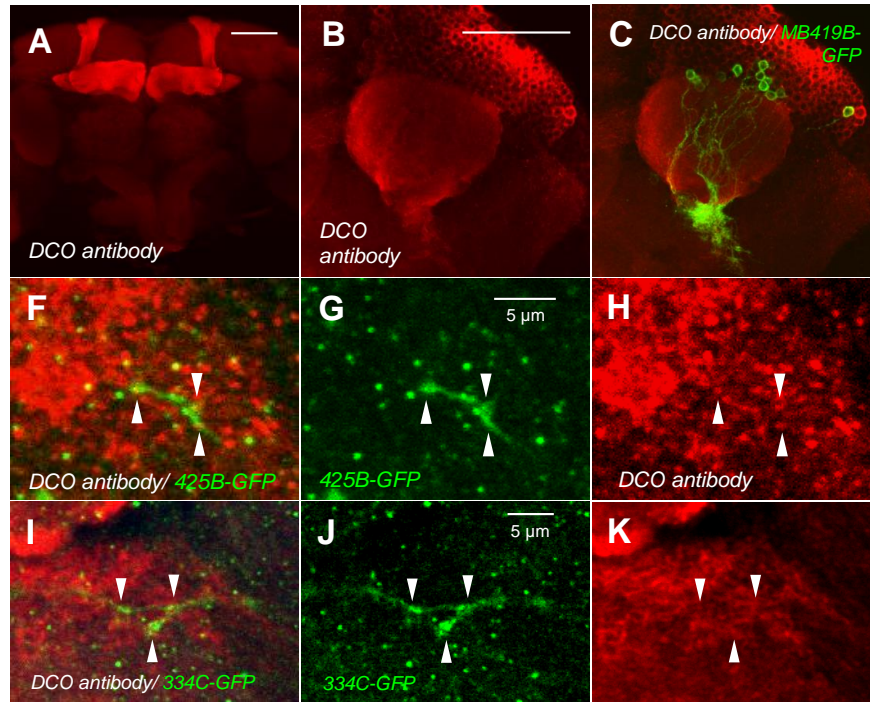
The axons of the VPNs and the dendrites of the  $\gamma$ d-lobe both project to a similar region in the protocerebrum. To see if they indeed could be connected a few different approaches were employed. To form synaptic connections the axons and dendrites have to come very close together. By expressing one half of a Split-GFP protein under the control of a MB-driver and the other half of the Split-GFP protein under the control of a VPN driver I could visualize such potential connections (Figure 37A,B). Only at sites where the neurons come very close together a functional GFP-protein can be formed. Indeed performing this method with both VPN-lines, respectively, I detected a reconstituted GFP-signal in the expected region in the protocerebrum (Figure 37C-F). This result is a first hint that the neurons indeed connect at some time point during development and/or adulthood.



**Figure 37: Reconstituted GFP expression reveals close contact between VPN axons and VACA dendrites.**

(A) Specific Split-GAL4 line *425B* labeling only few types of VPNs. (B) Specific Split-GAL4 line *334C* labeling only a single type of VPNs. Background is stained with Synapsin. (C+E) Split-GFP labeling of *MB247* and *425B* leads to reconstituted GFP signal in the  $\gamma$ d-accessory calyx region. (D+F) Split-GFP labeling of *MB247* and *334C* leads to reconstituted GFP signal in the  $\gamma$ d-accessory calyx region. Background is stained with n-Cad. Scale-bar represents 50  $\mu$ m.

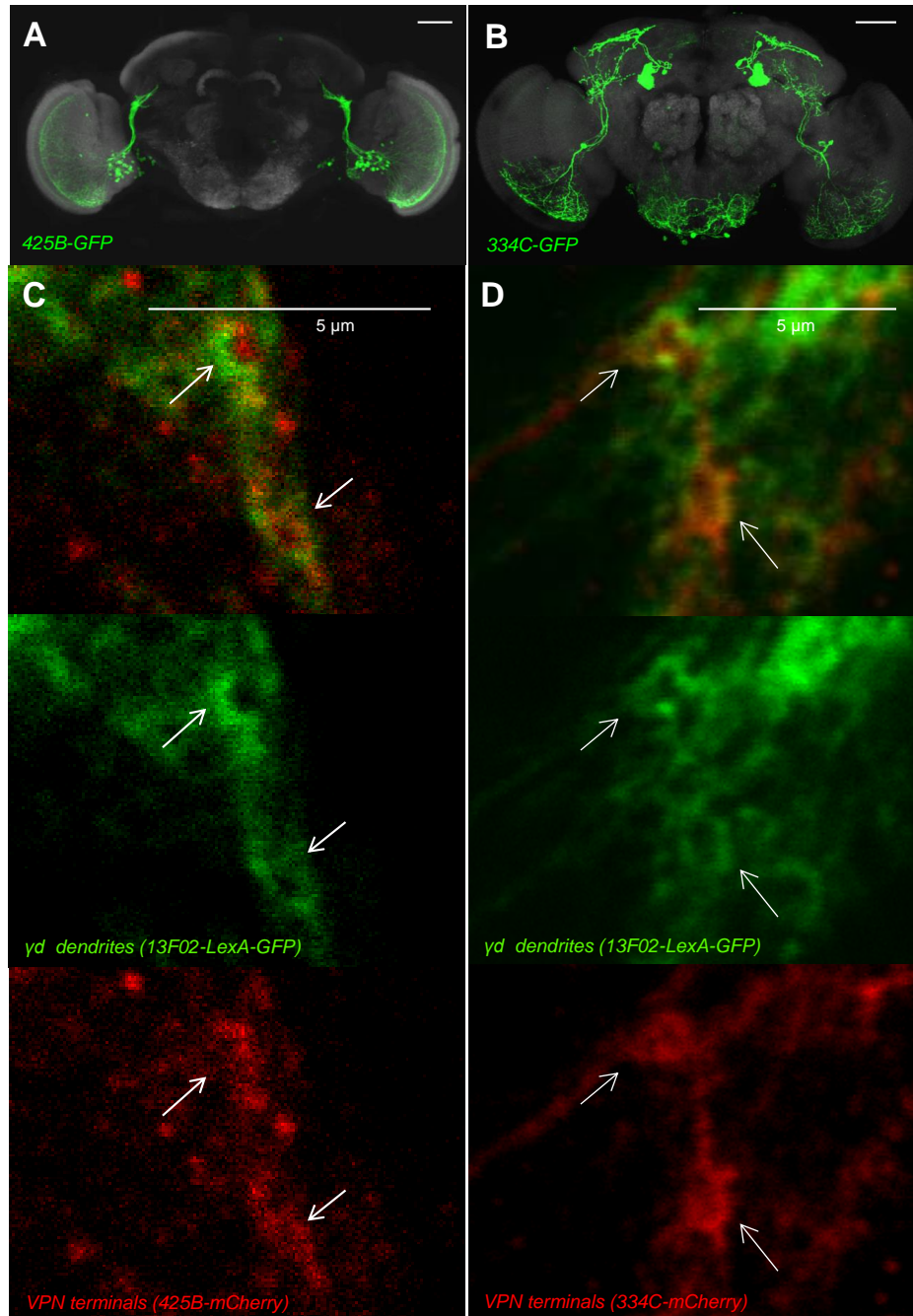
To collect further evidence for a possible connection I performed double labeling with a specific protein that is highly enriched in the MB (DCO = catalytic subunit of the PKA, Figure 38A-C). Therefore I visualized the VPN neurons with an UAS-GFP-effector. The DCO-antibody was visualized via RFP-channel. The DCO protein shows very prominent expression in the ventral accessory calyx and possible overlap between VPNs and the DCO staining can be found in the region of interest, the VACA (Figure 38F-K).



**Figure 38: VPN axons overlap with DCO staining in the VACA.**

**(A-C)** DCO antibody preferentially labels the MBs including the  $\gamma$ d-KCs stained by *419B-Split-GAL4*. **(F-G)** DCO antibody staining overlaps with the VPN expression in *425B-Split-GAL4*. **(I-K)** DCO antibody staining overlaps with the VPN expression in *334C-Split-GAL4*. Scale-bar represents 50  $\mu$ m.

In a third approach to test for connection between VPNs and the  $\gamma$ d calyx, double labeling of the VPN Split-GAL4s and a MB-LexA lines was performed (Figure 39A-B, *13F02-LexA*). The Split-GAL4 lines were visualized via a RFP-effector, respectively, whereas the LexA-line was visualized by expressing a GFP protein. Also here overlap in expression pattern in the VACA region can be found in single slices of the confocal recording (Figure 39C-D). All these results strongly suggest that there is a possible connection between the VPNs and the ventral accessory calyx. Thus, the MB could receive direct visual input from the optic lobes.



**Figure 39: VPN axons overlap with MB-dendrites of the VACA.**

**(A)** Expression pattern of *425B-Split-GAL4*. **(B)** Expression pattern of *334C-Split-GAL4*. **(C)** Double labeling of *425B-Split-GAL4* and *MB-LexA (13F02)* reveals potential overlap in the VACA region. **(D)** Double labeling of *334C-Split-GAL4* and *MB-LexA (13F02)* reveals potential overlap in the VACA region. Scale-bar represents 50 μm if not otherwise stated.

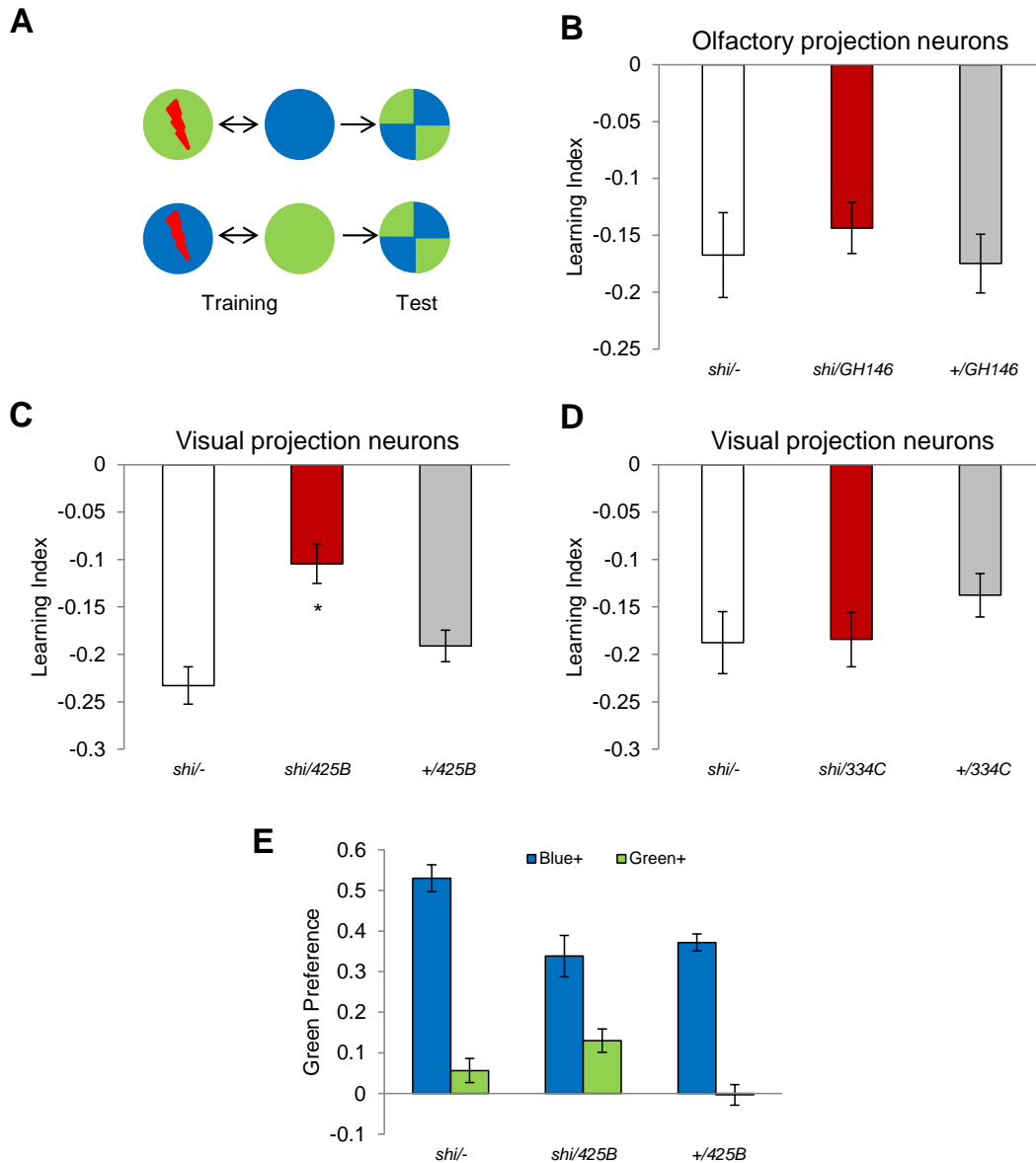


## Functional properties of the VPNs

To perform olfactory conditioning, olfactory input that is conveyed to the primary calyx by described olfactory projection neurons is critical (Pitman et al. 2011). These olfactory projection neurons are not required for aversive visual color learning (Figure 40AB). Thus, specific VPNs should convey visual information to the MB. The anatomical data already suggests a role of the here presented VPNs in mediating visual stimuli from the optic lobes to the MBs. Thus, the VPN neurons could contain visual information for formation of associative memory formation inside the MBs.

### *Color conditioning*

I blocked the output of the VPN-Split-GAL4 lines with expressing *shi<sup>ts</sup>* and tested them with different visual conditioning protocols. Testing flies with the normal color learning protocol, where they have to discriminate between green and blue stimuli (Figure 40A), revealed a strong impairment when blocking the output of the neurons expressed in the Split-GAL4 line *425B* (Figure 40C). Visual stimulus discrimination remained intact (Figure 40). Blocking the output of neurons expressed in Split-GAL4 line *334C* under the same conditions did not lead to a decrease in memory formation (Figure 40D).

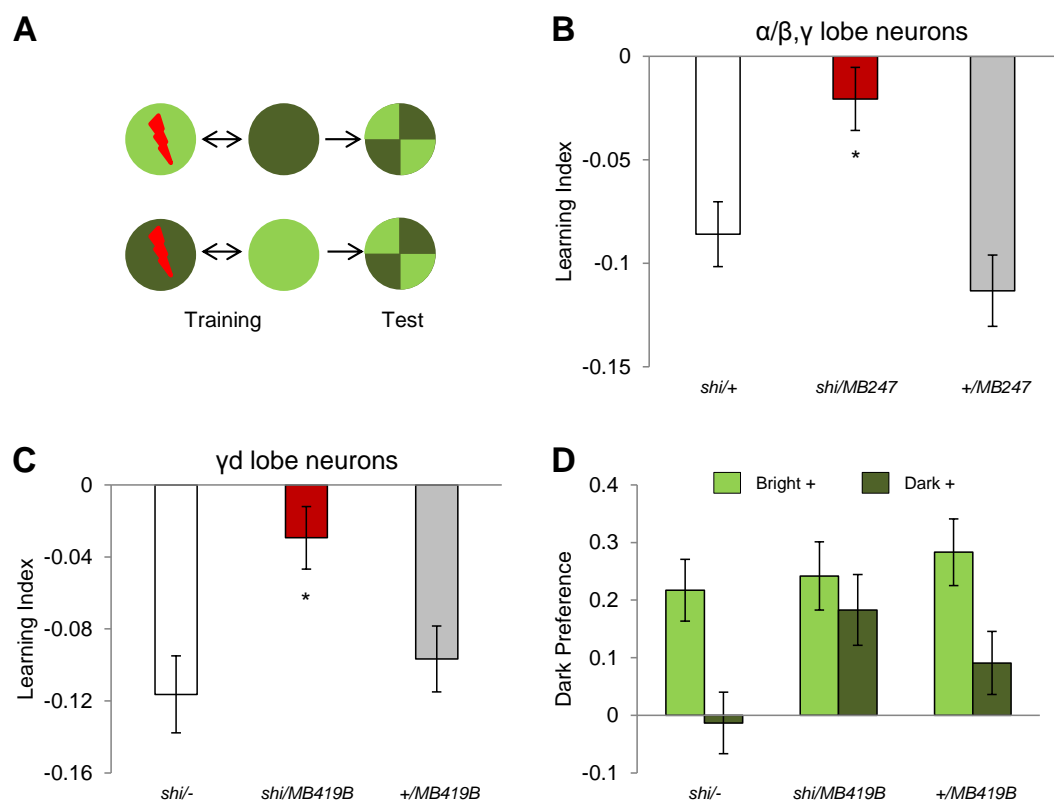


**Figure 40: Specific subsets of VPNs but not OPNs are required for visual color memories.**

**(A)** Scheme of aversive color conditioning protocol. **(B)** Olfactory projection neurons labeled by *GH146-GAL4* are not required for visual color learning (one-way ANOVA,  $p > 0.05$ ),  $n = 8$ . **(C)** VPNs labeled by *425B-Split-GAL4* are required for color learning (one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.01$ ),  $n = 10-12$ . **(D)** VPNs labeled by *334C-Split-GAL4* are not required for color learning (one-way ANOVA,  $p > 0.05$ ),  $n = 9$ . **(E)** The green and blue bars represent the punished stimulus during training in both reciprocals (Green+ and Blue+). Blocking VPNs labeled by *425B-Split-GAL4* does not impair green preference in test. Deviation from zero shows bias in visual choice, thus animals are able to discriminate visual stimuli in test. Bars and error bars represent mean and SEM, respectively.

## Intensity conditioning

Our visual stimulus tested can not only contain information about color cues but also information about light intensity. These two distinct features of our stimulus are commonly processed in parallel neural systems and mediate different perceptual functions (Livingstone & Hubel 1988; Gegenfurtner & Kiper 2003; Osorio & Vorobyev 2005). A previous study showed that conditioning WT flies with differential intensities of either blue or green (1:10 ratio) resulted in significant intensity discrimination in sugar conditioning (Schnaitmann et al. 2013).

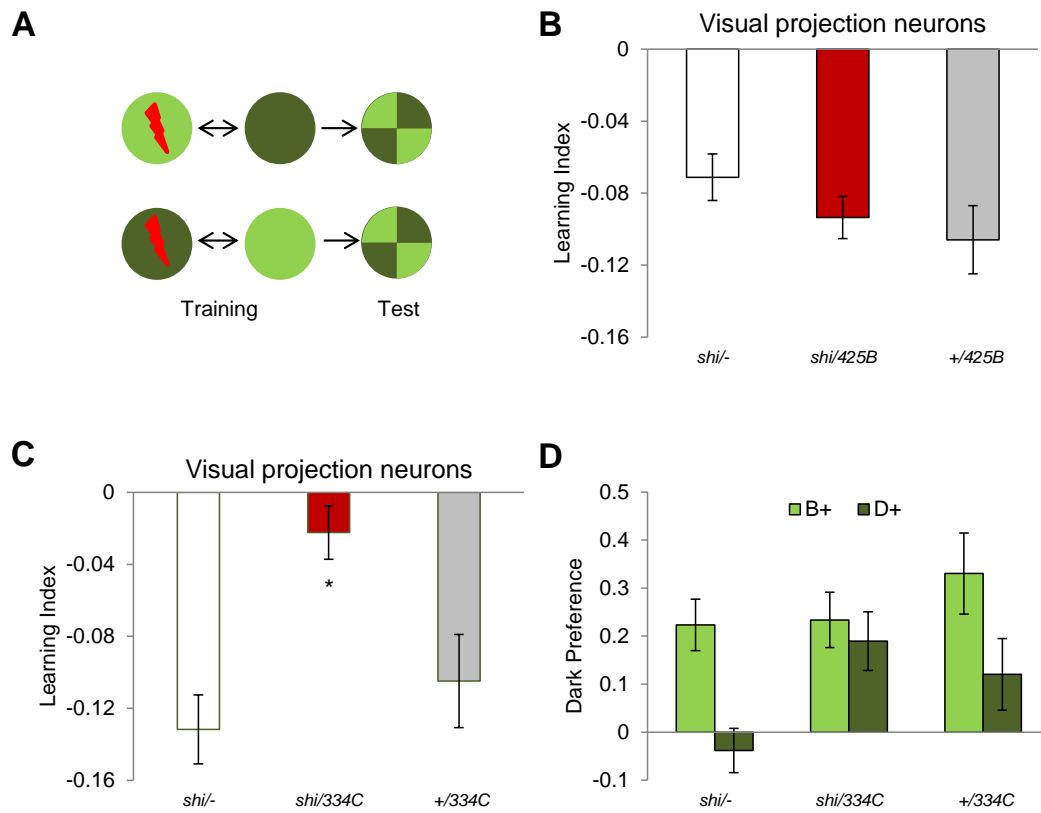


**Figure 41:  $\gamma$ d lobes of the MB are required for green intensity conditioning.**

**(A)** Scheme of aversive green intensity conditioning protocol. **(B)** Blocking output of several MB lobes impairs green intensity learning. (*MB247-GAL4*, one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ ,  $n = 5-9$ ). **(C)** Blocking output of MB  $\gamma$ d-lobes impairs green intensity learning. (*MB419B-Split-GAL4*, one-way ANOVA, *post-hoc* pairwise comparisons,  $p < 0.05$ ,  $n = 9-11$ ). **(D)** Visual stimulus preference after aversive intensity conditioning. The green dark and bright bars represent the punished stimulus during training (Bright+ and Dark+). Blocking  $\gamma$ d-lobes leaves dark preference intact. Deviation from zero shows bias in visual choice, thus animals are able to discriminate visual stimuli in test. Bars and error bars represent mean and SEM, respectively.

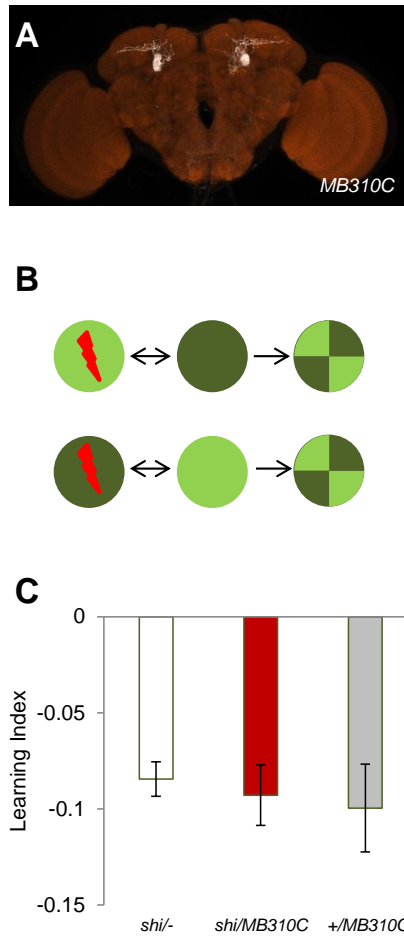
I could show that this kind of visual intensity learning is also MB-dependent (Figure 41A). Blocking the output of several MB-GAL4-lines (*201y/MB247*) led to strong impairment in green intensity memory (Figure 41B). Interestingly blocking the output of the yd-lobe is also sufficient to impair the memory (Figure 41C, *MB419B*), however, leaving discriminability of visual stimuli intact (Figure 41D).

Therefore I also tested the VPN-candidate lines for intensity conditioning. Blocking the output of neurons labeled by *425B-Split-GAL4* did not impair the intensity memory (Figure 42A-B). However, blocking the output of the neurons labeled by *334C-Split-GAL4* led to strong impairment in green intensity memory (Figure 42C) but not visual stimulus discrimination (Figure 42D). *334C-Split-GAL4* additionally labels the MB  $\alpha 1$  output neuron. To exclude that the phenotype in intensity conditioning is due to the expression outside the optic lobe I tested a Split-GAL4 line specific for this MB-output neuron (*MB310C*). Blocking the output of this neuron had no effect on intensity conditioning (Figure 43). Thus, there seems to be a functional segregation in requirement of the VPN neurons. Probably different cell types are labeled by the different candidate lines. The VPN cell type included in *425B-Split-GAL4* seems to be preferentially required for processing chromatic information, whereas the VPN cell type labeled by *334C-Split-GAL4* seems to be preferentially required to process achromatic stimuli.



**Figure 42: Differential requirement of VPNs for green intensity conditioning.**

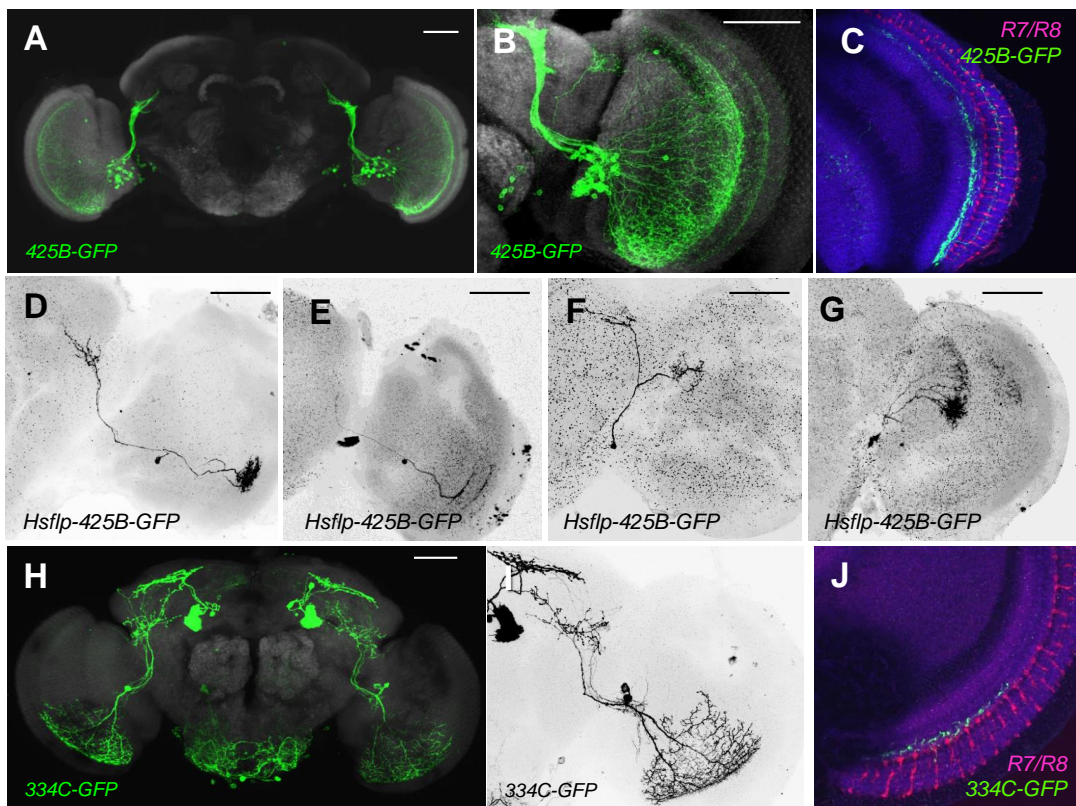
**(A)** Scheme of aversive green intensity conditioning protocol. **(B)** Blocking output of VPNs labeled by *425B-Split-GAL4* does not impair green intensity conditioning (one-way ANOVA,  $p > 0.05$ ),  $n = 11-13$ . **(C)** Blocking output of VPNs labeled by *334C-Split-GAL4* impairs green intensity conditioning (one-way ANOVA, *post-hoc* pairwise comparisons,  $p > 0.05$ ),  $n = 8-10$ . **(D)** The dark and bright green bars represent the punished stimulus during training in both reciprocals (Bright+ and Dark+). Blocking VPNs labeled by *334C-Split-GAL4* does not impair dark preference in test. Deviation from zero shows bias in visual choice, thus animals are able to discriminate visual stimuli in test. Bars and error bars represent mean and SEM, respectively.



**Figure 43: The  $\alpha 1$  output neuron is not required for aversive green intensity learning.**

**(A)** *MB310C-Split-GAL4* shares expression of the  $\alpha 1$  MB-output neuron with VPN line *334C-Split-GAL4*. **(B)** Scheme of aversive green Intensity conditioning protocol. **(C)** Blocking output of neurons labeled by *MB310C-Split-GAL4* does not impair green intensity conditioning (one-way ANOVA,  $p > 0.05$ ),  $n = 8-11$ ). Bars and error bars represent mean and SEM, respectively.

## Input properties of VPNs



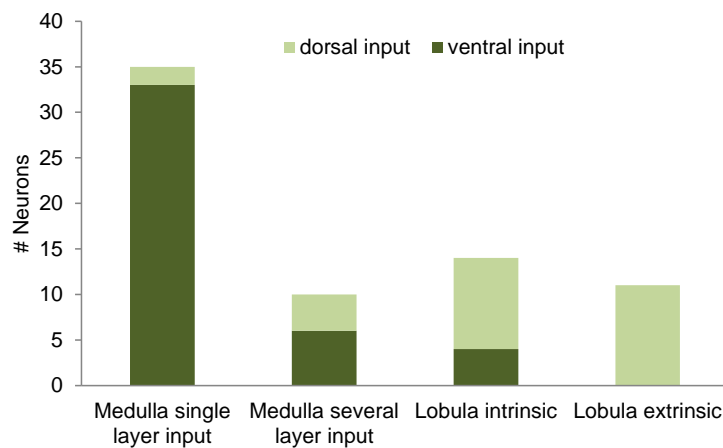
**Figure 44: Differential dendritic input and projections to the central brain for different VPN cell types.**

(A-B) Expression pattern of *425B-Split-GAL4*. In the optic lobe, prominent expression in different layers of the medulla can be seen. The ventral part of the medulla shows most abundant expression. Background is stained with Synapsin. (C) Double labeling of *425B-Split-GAL4* and antibody for R7/R8 axons. Neurons labeled in *425B-Split-GAL4* seem to receive input preferentially in layer M8 of the medulla, but also layer M3/4. Background is stained with n-Cad. (D-G) Single neurons of *425B-Split-GAL4* (heat-shock flip-outs). Two different cell types were found in medulla (D-E) and lobula (F-G), respectively. Background is stained with n-Cad. (D) Medulla type I neuron that has clear projection to the VACA region possesses a relatively small receptive field and innervates a single layer in the medulla (probably M8). (E) Medulla type II neuron that shows weak projection to the central brain and receives input from several layers in the medulla. (F) Lobula type I neuron that has projections to the central brain however anterior to the VACA. (G) Lobula type II neurons that are intrinsic to the lobula. (H-I) *334C-Split-GAL4* contains a single VPN cell type that expands its dendrites over the ventral half of the medulla. Background is stained with Synapsin. (J) Double labeling of *334C-Split-GAL4* and antibody for R7/R8 axons. VPNs labeled by *334C-Split-GAL4* show prominent projection to the VACA and seem to innervate preferentially layer M7 (and M6) in the medulla. Background is stained with n-Cad. Scale-bar represents 50  $\mu\text{m}$ .

The results from the conditioning experiments strongly suggest that different cell types are labeled by the two candidate VPN lines. Therefore comparing the anatomy of the dendrites in the optic lobes of the two lines could endorse such claim.

*Possible color input: 425B*

The neurons in the Split-GAL4 line *425B* receive strong input from several Medulla layers in the optic lobe. Double staining with R7/R8 antibody (mouse mAb24B10 (Zipursky et al. 1984)) shows that strongest input is provided by layer M8, however additional input could be received from layers M3/M4 and M1. Such differential input could also be required for chromatic information processing. Since the Split-line *425B* labels not only a single cell type in the optic lobe, we performed a heat-shock flip out (hsflp-out) during development to be able to stain single cells (average amount of cells stained in 47 optic lobes: 1.5). Indeed at least 3 different neuronal cell types were found in the optic lobes; weakly labeled lobula intrinsic and extrinsic neurons and the strongly labeled medulla neurons projecting into central brain. Variation in the dendritic input region of the medulla neurons could even suggest that there are two different cell types labeled.



**Figure 45: *425B-Split-GAL4* preferentially labels a specific medulla VPN neuron.**

Different cell types that are labelled by *425B-Split-GAL4* receive input either from ventral or dorsal part of the optic lobe. Performing single cell (1-2 cells labeled) staining via heat shock flip-out during development led to preferential labeling of medulla extrinsic neurons that receive input from a single medulla layer, n = 47 (optic lobes analyzed).

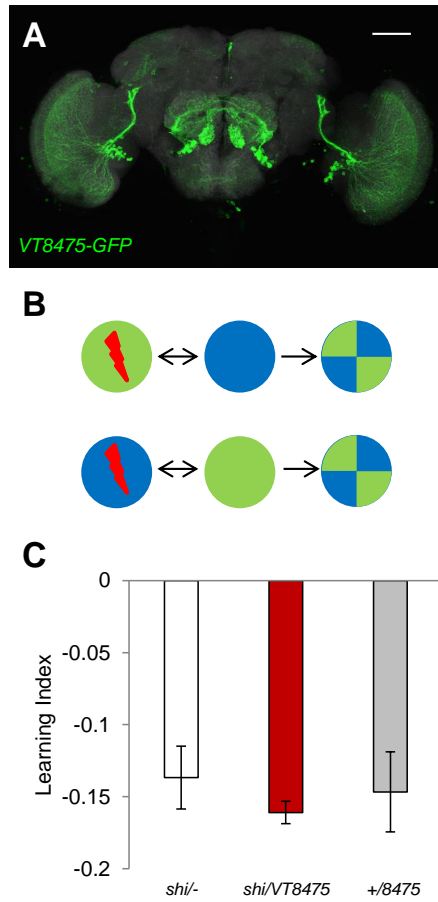


Final analysis of the heat-shock flip out experiments shows that medulla extrinsic neurons that receive input from a single layer seem to be most abundant. These neurons preferentially receive input from the ventral part of the medulla. The other three cell types were less abundant and also did not show preference for ventral expression. The lobula extrinsic neuron only receives input from the dorsal part of the optic lobe. Single cell staining of the most prominent medulla extrinsic neurons further showed that their dendritic region is rather small, suggesting that they are probably receiving input from ~5 columns (20° of the visual field) in the medulla. Together the neurons in *425B* extend over the whole visual field, however the most prominent expression can be found in the ventral region of the medulla. Testing a different GAL4-line (*VT8475*, VDRC Vienna; Figure 46) that seems to label anatomically similar neurons as in *425B* that express uniformly across the medulla did not impair color memory. Thus, it could be possible that specific ventrally expressing neurons provide the visual input to the MB. The neurons labeled by *VT8475* do also not project to the MB. Their axonal protrusions invade the protocerebrum but do not extend until the ventral accessory calyx region.

#### *Possible intensity input: 334C*

The Split-GAL4 line *334C* labels one cell type that also has its dendritic regions inside the medulla. Double staining with R7/R8 antibody (mouse mAb24B10 (Zipursky et al. 1984)) shows that strongest input is provided by layer M6/M7. Interestingly the dendritic region of this neuron covers only the ventral half of the optic lobe.

Thus, there are several differences in the anatomy of the different VPNS that could lead to functional segregation in visual information processing such as receiving input from different layers in the medulla and size of dendritic region of a single neuron. However, there are also similarities such as strong ventral expression, which could be a requirement for visual processing in our specific behavioral paradigm.



**Figure 46: A different type of VPNs is not required for color learning.**

**(A)** *VT8475-GAL4* expression pattern. Specific VPNs are labelled that do not possess connection to the MB calyx nor preferential ventral expression. **(B)** Scheme of aversive color conditioning protocol. **(C)** Blocking output of neurons labeled by *VT8475-GAL4* does not impair color conditioning (one-way ANOVA,  $p > 0.05$ ),  $n = 8$ ). Bars and error bars represent mean and SEM, respectively.

## 4. Discussion

### 4.1 Summary of results

Using a single training device for visual learning with exchangeable modules for electric shock as punishment (Schnaitmann et al. 2010) and sucrose as reward (Schnaitmann et al. 2010), respectively, allows to contrast appetitive and aversive visual memories (Figure 9). In both visual setups the conditioned stimulus is applied via the same LED light source to a group of freely walking flies, comparable to the odor application in both olfactory conditioning paradigms (Tully & Quinn 1985; Schwaerzel et al. 2003). For memory assessment in olfactory and visual conditioning, distribution of flies is taken as measurement. Therefore, the only variable in both setups is the presentation of either electric shock as punishment or sucrose as reward, respectively, which again allows high comparability between both of them. I found different afferent and efferent MB neurons are employed for visual aversive and appetitive conditioning, respectively (Figure 27, Figure 28, Figure 29, Figure 30). However, the same lobe requirement inside the MB applies for both visual training procedures (Figure 24, Figure 25). Contrasting the required MB learning circuit for visual learning to the olfactory learning circuit I found that shared dopamine neurons mediate at least aversive reinforcement information between the two modalities, whereas distinct roles apply for defined sets of Kenyon Cells (KCs). Visual input is conveyed to a specific subset of KCs, the  $\gamma$ d-lobe. These cells have a specific dendritic region ventral to the primary calyx of the MB (Figure 34). Information about color and intensity of the visual stimulus is mediated via different visual projection neurons (VPNs) from the medulla to the ventral accessory calyx of the  $\gamma$ d KCs, respectively (Figure 40, Figure 42). Therefore, in my thesis I present a new visual learning circuit that includes the MB as a pivotal element. For the first time I can show that the MB is involved in classical visual conditioning in *Drosophila* and that visual information is directly conveyed via specific VPNs from the medulla to the MB.

## 4.2 Advantages of the newly established visual conditioning assay

### Visual vs. Olfactory setup: A parametric comparison

Earlier parametric studies using the visual conditioning paradigm already showed similar conditioning effects compared to studies in the olfactory conditioning paradigm. In appetitive conditioning, increasing the amount of training trials improves memory performance (Schnaitmann et al. 2010). The same is true for olfactory conditioning (Kim et al. 2007). In aversive conditioning, application of increasing shock intensities leads to an increase in memory performance and reaches a plateau around 30-40V (Tully & Quinn 1985) (Figure 11C) (Diploma thesis Katrin Vogt, 2009). Also the persistence of the acquired memory seems to be longer after appetitive conditioning than after aversive conditioning in both paradigms (Tempel et al. 1983; Schnaitmann et al. 2010) (data not shown (Diploma Thesis, Katrin Vogt, 2009)). Thus, applying the same reinforcers revealed similar properties of memory acquisition and retention in visual and olfactory conditioning.

### High throughput

Establishing an aversive visual learning paradigm that employs electric shock as punishment not only allows comparison with the aversive olfactory conditioning assay but also automation of the conditioning procedure. Visual and electric shock stimuli can be controlled by a single software application. Therefore high throughput in experiments can be reached by multiplication of the setups (Figure 10). Especially during the MB screening project, for which I performed experiments with the aversive setup at the Janelia Farm Research Campus (HHMI, Ashburn, Virginia, USA) it was very helpful to be able to use an automated setup. In *Janelia*, 20 setups could be run in parallel and up to 100 experiments could be performed during a single day. This throughput rate is rather unique for a *Drosophila* learning paradigm. In the new visual setups, the task that the flies have to perform is rather simple compared to the flight simulator where the fly has to

fly continuously. With this setup, a large amount and variety of flies can be tested, also at high temperature. Additionally, it is possible to vary the visual conditioning task, in terms of protocol (operant vs. classical, see 2.5, 3.4) or visual stimulus presented (color vs. intensity, see 2.5, 3.8).

### **4.3 Conditioning with variable ISI**

The presented classical visual aversive conditioning paradigm in *Drosophila* allows application of color and electric shock stimuli automatically during experiments. Thus, exact timing of CS and US exposure is possible, which is an enormous advantage when training flies with different inter-stimulus intervals (ISIs). Furthermore, in the recent visual learning assay we can exclude that any CS residuals in the experimental setup can manipulate learning results as for example in olfactory conditioning (Galili et al. 2011). Exposing flies to visual learning protocols with variable ISIs revealed that they are indeed able to form positive and negative memories with aversive reinforcement; similar to what was found in olfactory conditioning (Tanimoto et al. 2004). Notably, both kinds of visual conditioning regimen can be performed within the same behavioral setup similar to olfactory conditioning; however, using the visual learning paradigm I can employ a further CS modality that is also widely used in studies with mammals and humans. Mostly visual stimuli are used when training mammals, monkeys or humans in trace or relief conditioning (mammals (Rogan et al. 2005); monkeys (Belova et al. 2007); humans (Seymour et al. 2005; Andreatta et al. 2010)).

#### **Trace conditioning**

Flies form an association and significantly avoid the CS+, even if it precedes the US in the training, leaving a gap of around twenty seconds between the two stimuli. Thus, flies can not only keep a sensory trace about olfactory stimuli, but also about visual stimuli. Using the same reinforcing stimuli and an almost identical stimulus application protocol it is possible to compare trace conditioning in both modalities. The

underlying mechanism could be very similar since similar CS/US requirements apply; the duration of the gap matters and prolonged duration of the gap further impaired memory formation in a linear way (Figure 14, Figure 15)(Galili et al. 2011). Overlap of the CS+ and US is important; however, the CS+ should always precede the US (Burman & Gewirtz 2004; Rescorla 1988). It is necessary to extend the length of CS application over a critical duration (more than 5s). Prolonged CS application however does not affect memory score (15s versus 25s) also similar as in olfactory conditioning (Figure 15)(Galili et al. 2011). The fact that flies are able to learn about a visual stimuli trace although the stimulus probably becomes less salient if presented with a gap, leads to the conclusion that such visual information seems to be indeed very important for flies. If flies would preferentially rely on sensory cues from the environment other than visual stimuli, one would expect that they should not be able to keep a visual information trace and thus would be not able to perform the trace learning task. To keep a sensory information trace is probably rather costly since it can require prolonged activation of neural circuits (Niven & Laughlin 2008; Chittka & Niven 2009). Thus, my results imply that fruit flies heavily use visual information from the environment to adapt their behavior in future events.

### **Relief conditioning**

Employing the same setup but reversing the order of CS and US, now the US preceding the CS+, leads to significant positive memory scores also in visual conditioning. Flies associate the CS+ with the end of punishment and a positive experience as in olfactory relief conditioning (Figure 14)(Tanimoto et al. 2004; Yarali et al. 2008; Murakami et al. 2010). Thus, I am the first one to show that flies can perform relief conditioning also with visual stimuli. As suggested from results in olfactory conditioning (Yarali et al. 2008) I applied few electric shocks (three shocks of 1 s duration) with rather low intensity (60 V AC) per trial during training. Higher shocks seem to increase positive valence of the CS+, but do not lead to diminished negative valence whereas less exposure to shocks implies lower positive valence but it is also easier to overcome the negative valence. Thus, the right balance between positive and negative valence should be found to detect the relief learning effect (Franklin et al. 2013). Also high number of training trials seems to be preferable to induce relief learning, at least 4-6 trials (Tanimoto et al. 2004; Yarali et al. 2008), thus applying eight training trials indeed not only gave good performance in visual delay, but also trace conditioning (Figure 13).

## ISI conditioning

The sensory pathways underlying trace and relief conditioning should depend on the stimulus modality. Also saliency of the CS+ is probably not only affected by duration of the gap but probably also by the identity of the conditioned stimulus (Pavlov 1927). Thus, trace and relief conditioning studies using different CS are not necessarily comparable. However, overall ISI learning effects look similar when comparing visual and olfactory conditioning results in *Drosophila*. This could be the case because both paradigms employ the same US application of electric shock. Going a step further and comparing the ISI effect across different phyla; insects, mammals, primates and humans (Bitterman et al. 1983; Rogan et al. 2005; Belova et al. 2007; Seymour et al. 2005; Andreatta et al. 2010), reveals that also here similar effects were found even though different CS and US were used. Thus, the cellular mechanisms for keeping the CS+ trace may be related across different modalities and animals. To identify the underlying mechanisms of trace and relief conditioning in *Drosophila* could help us to further understand how we process traumatic experiences.

A similar trace and relief learning effect in visual and olfactory conditioning already supports the idea of a common neural mechanism underlying these two tasks, respectively. MBs were suggested to play a role in keeping the trace in olfactory conditioning (Shuai et al. 2011). Since I can show that MBs are also involved in visual delay conditioning (Figure 16) and indeed seem to receive information about visual stimuli (Figure 40, Figure 42), they could represent a common relay station for different sensory stimuli. Information about both stimuli is mediated to the calyx and further processed in specific lobes of the MB (Figure 23, Figure 26 Figure 40). The same dopamine neurons are required to modulate visual and olfactory cues in the MB lobes (Figure 28)(Aso et al. 2012). Thus, a similar mechanism could be applied across modalities inside the MBs of the fly.

#### **4.4 Visual conditioning with wild-type flies**

Performing different parametric and wild-type studies in the new visual learning paradigm revealed that there are similar memory acquisition effects comparing appetitive and aversive visual and olfactory conditioning, respectively (Tully & Quinn 1985; Tempel et al. 1983; Schnaitmann et al. 2010; Vogt, Schnaitmann, et al. n.d.). Furthermore, comparing trace and relief memories in both modalities gives a hint that there is high similarity in stimulus application and memory formation between visual and olfactory conditioning assays. Thus, I am confident to further compare these two paradigms in the following studies.

#### **4.5 Visual classical learning circuit vs olfactory classical learning circuit**

##### **MB is also required for formation of visual memories**

In olfactory conditioning it is clearly shown that the MB plays a pivotal role in memory formation and storage. In contrast, in classical visual conditioning until now MBs were shown to be dispensable (Wolf et al. 1998). I can clearly show that MBs indeed are important for the new classical visual learning task. In both visual assays, appetitive and aversive conditioning, blocking the output of the MBs with rather broadly labeling GAL4-lines ( $\alpha/\beta/\gamma$ -lobes) strongly impaired visual short-term memories (Figure 16). Flies were not able to form significant memory. Necessity of the overall MB output during training and test further implies that visual memory formation and storage could be implemented in the MBs (Figure 21, Figure 22). In olfactory conditioning it was also shown that MB output is necessary during memory formation and retrieval; however, different subdivisions of the MB mediate these different tasks. A feedback loop between  $\alpha/\beta$  and  $\alpha'/\beta'$ -lobes was suggested to stabilize memory (Krashes et al. 2007; Dubnau et al. 2001; McGuire et al. 2001). However, it needs to be further investigated if a similar feedback loop mechanism exists in visual conditioning. Blocking output of neurons labeled by the more specific MB-GAL4 line *201y* revealed that these neurons are only required during test (Figure 21). Thus, indeed there seems to be different cell populations required for



memory formation and retrieval also in visual conditioning. Interestingly, presynaptic terminals in the calyx were only found for  $\alpha/\beta/\gamma$ -lobes neurons but not for  $\alpha'/\beta'$ -lobes neurons (Christiansen et al. 2011). Thus, one possibility to form a recurrent network within the  $\alpha/\beta/\gamma$ -lobes could be via a KC:KC interaction in the calyx.

#### **4.6 Visual classical learning circuit vs visual learning circuit in the flight simulator**

##### **Comparison of the behavioral protocol**

Using a parallel experimental design identified the common requirement of the MBs in visual and olfactory memories. Performing conditioning experiments in the flight simulator paradigm, the central complex (CC) (Liu et al. 2006; Pan et al. 2009) and not the MB (Wolf et al. 1998) was shown to be involved in visual learning. Several differences between our paradigm and the flight simulator could account for these results (Table 2), for instance different protocols were used to train the flies. In the flight simulator flies need to perform a training regime that includes an additional operant component, namely they can avoid punishment during training. This is different to our new paradigm which does not involve a choice during training and flies are trained purely classical. Thus, we wanted to exclude that solely changing the training task could lead to requirement of MBs. Another difference in protocol is that flies in the flight simulator are exposed to same stimuli in training and test. It was shown that changing the background color of visual stimuli between training and test leads to requirement of the MB for learning (Liu et al. 1999). In our paradigm we have a change in visual stimulus since we subsequently present a single color in training, but two colors at a time in parallel during test which could possibly account as context change (Figure 11). We controlled for these differences like inclusion of an operant component in training and context (CS) change between training and test and could exclude that they are responsible for requirement of the MB in our learning task (Figure 18). Interestingly, inclusion of an operant component did not improve overall memory scores, what would have been expected from a former study (Heisenberg et al. 2001). Operant behavior was shown to facilitate the formation of classical associations in the flight simulator. An explanation could be that the test situation in the new paradigm was changed from four choice points (four quadrants) to

one choice point in the arena (2 halves). Fewer encounters with a choice point could have made it harder for the flies to choose between the two visual stimuli in test. Since the operant component during training also allows flies to escape the reinforcer, most of them were probably less exposed to the punishment and therefore not able to form a strong association (Quinn et al. 1974).

Even though we controlled for context change and operant component of the flight simulator protocol, other differences that could lead to differential results are; distinct conditioned stimuli (pattern vs. color learning), different presentation of the visual stimuli (ventral vs. frontal) or usage of different reinforcers (laser vs. electric shock). A very substantial difference between the two visual conditioning paradigms is also the behavioral task the fly has to perform (single flying fly vs. walking mass assay). Therefore, memory paradigms have to be as similar as possible to allow circuit comparisons and comparisons between the newly established visual paradigm and the flight simulator should be avoided.

### **Requirement of the central complex**

The CC was shown to be required and sufficient for pattern memory formation in visual aversive conditioning in the flight simulator (Liu et al. 2006; Pan et al. 2009). However, in the new color learning paradigm a functional CC is only required to form associations during training but not to retrieve them during test (Figure 19). Thus, the CC does not seem to be the place of memory storage such as it is possibly the case in pattern learning (Zhang et al. 2013; Pan et al. 2009; Liu et al. 2006). Since blocking the output of these cells during test did not impair memory retrieval, one can also exclude that CS information is presented in the CC (Table 3). The phenotype would rather suggest that the US-pathway could be impaired; however, shock avoidance is intact (see Appendix, Table 8). Interestingly the GAL4-line employed, *c205*, labels a specific neuron that could have direct connection to the MB vertical lobes, and is innervating the upper layer of the fan shaped body (F5 neuron (Young & Armstrong 2010; Li et al. 2009)). Thus, possible interaction during training between MB and CC could be necessary for memory formation. The CC was also shown to affect sleep rhythm (Q. Liu et al. 2012) and fixation in flies (Xiong et al. 2010). Therefore, blocking CC output during training could probably have led to an attention deficit in flies such that they were not able to master the association task. Such attention mechanism could be dispensable once the memory is formed.

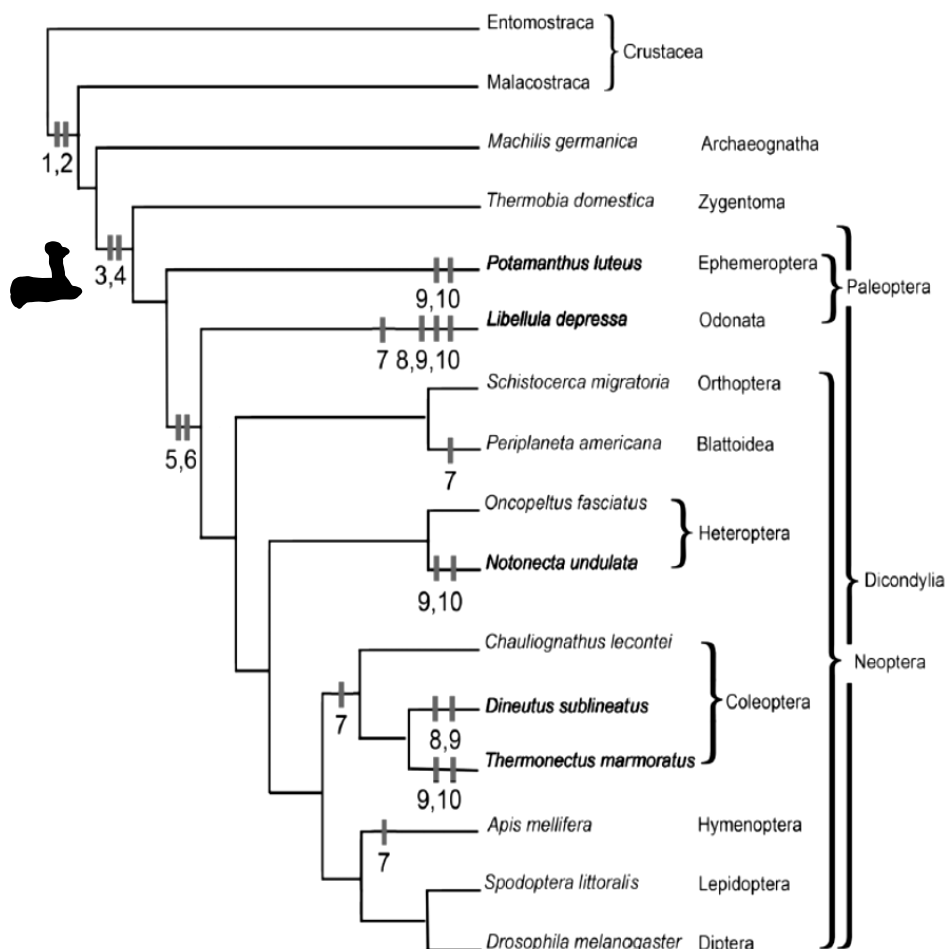
## ***rut* requirement**

In olfactory memory the adenylatecyclase *rut* is thought to be the coincidence detection protein for olfactory and reinforcement cues. Testing flies with the *rut*<sup>1</sup> mutation leads to decreased memory scores (Zars et al. 2000). This implies that memory formation with olfactory cues not fully depends on the *rut* pathway but can also be achieved via other biochemical pathways (Gilgamesh protein (Tan et al. 2010)). In the classical visual conditioning paradigm the memory impairment employing the same *rut*<sup>1</sup> flies is complete, thus it seems as if other biochemical mechanisms are probably not exploited for memory formation as in olfactory learning (Figure 20). A similar effect was found in visual pattern conditioning in the flight simulator (Pan et al. 2009). Rescuing the mutant defect in the MBs restores the memory formation in olfactory conditioning but not visual conditioning (Figure 20). However, rescuing the mutant defect in the CC also does not recover the visual color memory defect as found for visual pattern learning. These results could suggest that the *rut*-protein pathway could be additionally employed in other neuropiles, such as the optic lobes, for processing visual stimuli such as color or intensity. Thus, one cannot exclude that *rut*, amongst other functions, also acts as a coincidence detector in the MB similarly as in olfactory conditioning or in the central complex. In the  $\gamma$ -lobe of the MB, the catalytic subunit of the PKA-protein (DCO), which is part of the cAMP-cascade, is indeed highly enriched. Further experiments should be done to identify additional neuropiles that are enriched in *rut* pathway proteins and also are necessary for visual learning.

## **4.7 Evolutionary function of the MBs**

Especially in *Drosophila* the MB is mostly known for processing olfactory information, even though it is involved in many other tasks the fly has to perform. However, in other protostomes, the widespread function of this neuropil is more accepted. MBs or MB-like structures can be found in different phyla and subphyla of the *Protostomia*, as for example in *Annelida* (Heuer & Loesel 2008; Loesel & Heuer 2010; Tomer et al. 2010), *Chelicerata* (horseshoe crab (Fahrenbach 1979)), *Onychophora*

(Velvet worms (Strausfeld et al. 2006)), *Myriapoda* (*Diplopoda* (Strausfeld 1998)) and of course *Hexapoda* (insects). However, due to the many temporal gaps in the protostomes pedigree, it is not clear if MBs evolved several times independently or if many other phyla and subphyla secondarily lost them. Amongst insects, most recent studies suggest that already the *Zygentoma* possessed MBs with calyces (Farris 2005), whereas more ancient insects, like *Crustacea* and *Archaeognatha* only possessed olfactory glomeruli which supplied the lateral protocerebrum, however lacked MBs (Strausfeld et al. 2009). Thus, MBs indeed seemed not to have originated as special olfactory processing



**Figure 47: Phylogenetic tree of the class Insecta.**

Numbers indicate modification, innovation or loss during evolution: 1. Olfactory glomeruli. 2. Glomeruli supply lateral protocerebrum. 3. Mushroom body with calyx. 4. Calyx supplied by olfactory and gustatory glomeruli. 5. Double calyx. 6. Parallel subdivisions of the lobes. 7. Visual inputs to calyx. 8. Modality substitution at calyces. 9. Loss of olfactory glomeruli. 10. Loss or diminution of calyces. From Strausfeld,

2009

neuropiles, they probably allowed better processing of sensory stimuli overall. Although the dendritic region of the KCs, the calyx, seems to be highly important for odor detection and processing in most species, evolutionary pressure should also have been highly selective for sensation of other senses, such as tactile and vibration information (Hill 2001). In *Odonata*, that do not possess antennal lobes, visual input is perceived by a reduced calyx. Thus, modality substitution, subtraction and probably also expansion at the calyx were possible and happened probably several times during evolution.

Indeed many insects (*Neoptera* and *Zygentoma* (Farris 2005)) possess different afferent inputs to the calyx, either via non-directional connections from proto-, deutocerebrum or tritocerebrum (Farris 2008) or directly from antennal lobe or even optic lobes. In addition to sensory afferents, even neuromodulatory input and feedback elements can project to the calyx (Mobbs 1982; Hammer 1993; Grünewald 1999; Li & Strausfeld 1999). Such representation of different modalities inside the calyx is probably rather costly but can be advantageous since it could allow multi-modal-processing. Another interesting point is that in anosmic insects that lost their calyces or never possessed them, different sensory inputs can be processed in the MBs via afferents to peduncle and lobes (Strausfeld et al. 2009). Here modulation of local circuits between afferents and efferent neurons on the KC lobes is possible. The lobe projections of the KCs were indeed shown to be pre- and postsynaptic to extrinsic neurons and to one another (Schuermann & Klemm 1973; Ito et al. 1998; Strausfeld 2002). Overall these different afferents connecting to the lobes and the calyces can contain sensory information about visual (Gronenberg 1986; Mobbs 1982; Gronenberg & Lopez-Riquelme 2004), tactile (Schildberger 1984), acoustic (Li & Strausfeld 1997), gustatory (Schröter & Menzel 2003; Farris 2008) and olfactory information (Ito et al. 1998; Strausfeld 2002; Yasuyama et al. 2002). Thus, from evolutionary origin until now, the MB indeed rather seems to be a collection and modulation center of multi-sensory stimuli than a simple olfactory sensory processing center.

## 4.8 The visual learning circuit

Due to the usage of specific Split-GAL4 lines it is possible to further describe the intrinsic and extrinsic neurons of the MB that are employed for the visual learning task.

### Intrinsic neurons

Subdivision of the MB into different longitudinal lobe structures suggests that MBs may comprise of several parallel and isolated networks that may support different computational functions (Yang et al. 1995; Crittenden et al. 1998; Strausfeld et al. 1998). Comparing requirement of such different KC subsets for immediate aversive and appetitive visual and olfactory conditioning showed that most probably  $\gamma$ -lobes are employed for formation of these memories (Figure 23)(Blum & Dubnau 2010; Trannoy et al. 2011; Isabel et al. 2004; Qin et al. 2012; Vogt, Schnaitmann, et al. n.d.). Thus, overall the  $\gamma$ -lobes of *Drosophila* seem to support rather basic memory functions (olfactory STM (Zars et al. 2000)), courtship conditioning (15min (Keleman et al. 2012)). Interestingly, during the development of the fly, these KCs are born first, already in the embryo. *Drosophila* larvae are already capable of learning olfactory cues employing this minimal subset of embryonic born KCs (Pauls et al. 2010; Lee et al. 1999). During pupae stadium  $\gamma$ -KCs undergo reorganization and only provide a medial lobe in the adult fly, suggesting a simpler processing circuit than the other MB subdivisions which comprise of a medial and vertical lobe structure. Moreover, the  $\gamma$ -KCs possess higher amount of synaptic connections (claws) per neuron in the calyx than the other types of KCS and the  $\gamma$  lobes are more intensely invaded by extrinsic neurons than the other lobes (Ito et al. 1998), suggesting that they receive massive input, undergo strong modulation and could provide most output.

Especially for visual learning, a specific subset of the MB  $\gamma$ -lobe is required to form appetitive and aversive memory, the  $\gamma_d$ -lobe (Figure 24, Figure 25 Figure 26.). This specific subset is comprised of about 70 KCs (Aso et al. 2009). Their dendrites form a distinct input region outside the main calyx of the MB, the ventral accessory calyx (VACA). For olfactory memory formation this KC subset is not required, however the remaining  $\gamma_{main}$ -lobe needs to be intact (data not shown). Thus, both, olfactory and visual information, are present in the  $\gamma$ -lobes, however probably in different compartments. One

cannot surely exclude that visual information is present in the  $\gamma_{\text{main}}$ -lobe. Interestingly, most of the flight simulator experiments employed MB mutants or flies with chemically ablated MBs (HU). Since we found a specific, rather small sub-compartment of the MB  $\gamma$ -lobe to be necessary for visual learning, there is the possibility that ablation and mutation did not affect this KC subset.  $\gamma_d$  KCs were shown to be amongst the first, embryonic born KCs that are not affected by chemical HU ablation, since this substance is fed in larval state (Pauls et al. 2010; Lee et al. 1999). Also structural defects by mutation varied between flies and often did not fully ablate MB lobes. Using the GAL4/UAS system it is possible to obtain reproducible and strong blockade or activation of specific cell subsets.

Parallel processing of different modalities is an efficient solution, since both modalities can be modulated by common neurons, but still can be processed independently and more rapidly. Hence, functional segregation of different MB-lobes could be a conserved mechanism, since it is also found for the  $\alpha'/\beta'$ -lobes, which are clearly dispensable for visual learning but highly important for olfactory conditioning in *Drosophila* (Figure 23, (Blum & Dubnau 2010; Trannoy et al. 2011)). Response of  $\alpha'/\beta'$ -lobes KCs is the strongest among all KC subsets when presenting olfactory stimuli during electrophysiological recording (Turner et al. 2008). Interestingly, also in honeybees, ants and cockroaches, olfactory and visual sensory modalities are represented in two separate domains of the calyx, the collar and lip, respectively (Grünewald 1999; Ehmer & Gronenberg 2002; Nishino et al. 2012).

Formation of  $\alpha/\beta$ -lobes only takes place rather late during fly development, during pupae stadium (Lee et al. 1999) and therefore they form the core of the peduncle (Tanaka et al. 2008). Thus, their effect on basic STM learning would be expected to be minor, which is at least the case for visual conditioning, where no consistent defect was found (Figure 23). The role in visual learning of  $\alpha/\beta$ -lobes of adult *Drosophila* could entail further modulation of different sensory memories, as several studies in the flight simulator suggest (blocking fast formation of operant memory (Brembs & Wiener 2006; Brembs 2009); saliency discrimination (Tang & Guo 2001; Zhang et al. 2013); context change: (Liu et al. 1999)). This hypothesis is also supported by their role in olfactory conditioning where  $\alpha/\beta$  lobes are preferentially needed for memory consolidation and long term memory formation (Krashes et al. 2007).  $\alpha/\beta$  lobes thus could be the place where presentations of different sensory stimuli overlap and multi modal memories could be formed.

Identification of a specific subset of KCs required especially for visual memory formation provides a starting point in the search for the complete visual appetitive and aversive visual learning circuits including corresponding input and output neurons.

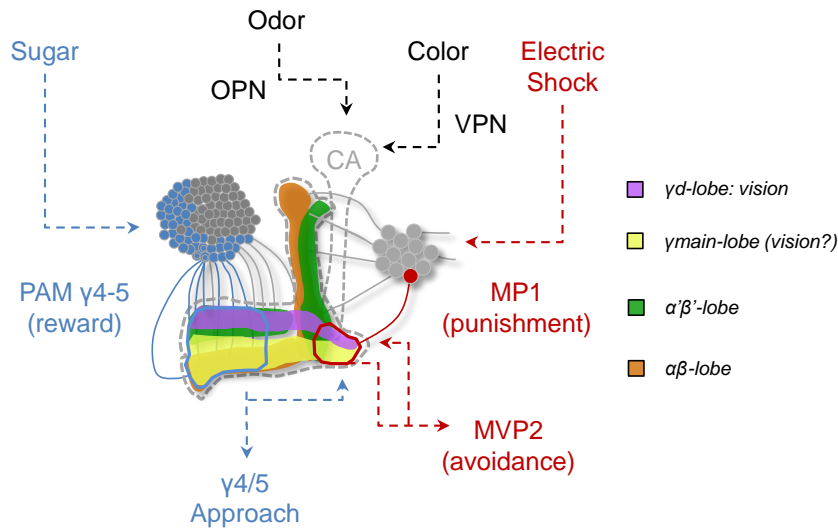
### **Extrinsic neurons**

In addition to the subdivision into different lobe structures in the fly, one can also detect further sub-compartments along the lobes, leading to transverse divisions defined by dendrites and axons belonging to extrinsic neurons (Strausfeld et al. 2003; Tanaka et al. 2008). For example, aminergic neuromodulatory cells providing information to the lobes have already been described in many insects (Honey bee (Schuermann & Klemm 1973), Locust (Homberg 2002)). Even such sub-compartments, sometimes innervated by single neurons of a cell type, can serve a specific computational function. During the MB-Screening project visual and olfactory memories were tested using the same extrinsic Split-GAL4 lines (Table 10, Table 11 and Table 12). The application of the same appetitive and aversive reinforcers and the similar task requirement for olfactory and visual memories allows comparing underlying reinforcement and output circuits. For olfactory memory middle term memory (MTM, retention of 2h) was tested whereas for visual learning short term memory (STM, retention of 2min) was tested.

### **Aversive learning circuit**

The same clusters of dopamine neurons are required and sufficient for driving aversive visual memories and olfactory memories, respectively (Vogt, Schnaitmann, et al. n.d.). Similar to olfactory learning (Aso et al. 2012; Claridge-Chang et al. 2009), neurons in the PPL1 cluster are necessary and sufficient for aversive visual memory formation. Rescue of dopamine receptor in the MB showed that these dopamine neurons project to the MB (Vogt, Schnaitmann, et al. n.d.). Further specific dissection revealed that especially the two MP1 neurons seem to be necessary for short term memory formation in both modalities (Figure 28)(Aso et al. 2012; Vogt, Schnaitmann, et al. n.d.).





**Figure 48: Circuit model of appetitive and aversive visual short-term memories.**

Output of  $\gamma_d$  KCs is locally modulated by specific subsets of dopamine neurons (PAM:  $\gamma_4/5$ , PPL1: MP1) to form appetitive or aversive memories, respectively. MVP2,  $\gamma_4/5$  and V2 neurons mediate output to the surrounding protocerebrum and provide potential feedback loops to the MBs. Olfactory information is conveyed to the primary calyx by OPNs. Visual information is conveyed to the VACA via specific VPNs.

These neurons innervate a subsection of the  $\gamma$ -lobe of the MB in the medial lobe heel, called  $\gamma_1$  (Figure 48). Also potential aversive memory retrieval in visual and olfactory conditioning could be mediated via a specific MB-output neuron that actually receives input from the same  $\gamma_1$  region in the  $\gamma$ -lobes as the MP1 input-neuron (Figure 30). Interestingly, this neuron, called MVP2, not only projects outside the MB but also back to the  $\alpha/\beta$  lobes inside the MB (Tanaka et al. 2008). This anatomical aspect indeed could support a feedback circuit inside the MB that could be used for consolidation of aversive memories or modulation of other memory components (Figure 33).

The aversive conditioning circuit is shared amongst two different modalities; even though different memories were tested, 2h vs. 2min, exactly the same input and output neurons are required in olfactory and visual memory. Since electric shock is a very potent but not naturally occurring aversive reinforcer, one would not expect a specifically evolved memory circuit for the different sensory modalities. Probably only different reinforcers can lead to requirement of different neurons, as for example laser, heat, chemical or shaking stimuli.

## **Appetitive learning circuit**

In appetitive conditioning, dopamine PAM cluster neurons play crucial roles in both olfactory and visual memories (C. Liu et al. 2012; Burke et al. 2012; Vogt, Schnaitmann, et al. n.d.). Rescue of dopamine receptor in the MB showed that these dopamine neurons project to the MB (Vogt, Schnaitmann, et al. n.d.). Further analysis in visual aversive learning suggests that specific sub-compartments of the  $\gamma$ -lobe, the  $\gamma 5/\gamma 4$  compartments, are responsible for STM memory formation (Figure 27, Figure 48). However, the input candidate-lines were only tested in primary screening and no further control experiments had been performed. For olfactory middle-term-memory (MTM) conditioning different sub-compartments seem to be necessary (data not shown). Since sucrose is a natural stimulus there could have evolved specific circuits for different modalities. However, the fly brain is rather small and it is rather costly to develop different learning circuits for a single reinforcer across different modalities. Another study can indeed show that in olfactory STM (2 min) input to the  $\gamma 4, \gamma 5$  sub-compartments also seems to be employed, whereas other compartments found to be necessary during the olfactory screening are rather involved in long-term memory (LTM) formation (data not shown; Yamagata et al., in prep.). Thus, also for visual and olfactory STM a shared circuit could exist. The differential requirement of input and potential output neurons for appetitive visual and olfactory memory in the screening can be explained by employing parallel memory formation circuits for olfactory STM and LTM. One trial conditioning, as it was performed in the olfactory screening in appetitive learning can indeed lead to formation of LTM in olfactory learning (Krashes & Waddell 2008). The  $\gamma 4$  output neuron that was found to be required in visual appetitive learning interestingly projects back into the  $\gamma$ -lobe of the MB (Figure 29). Interestingly the output of this neuron overlaps with the  $\gamma 1$  compartment that is required for aversive learning. Thus, modulation of the aversive learning pathway inside the MB is possible here (Figure 33, Figure 48).

Afferents to and efferents from the KC axonal region in *Drosophila* indeed segregate specific compartments that seem to have also functional segregation in visual learning inside the MB lobes ( $\gamma 1$  = aversive conditioning,  $\gamma 4/ \gamma 5$  = appetitive conditioning). Overall, this data further strongly supports that the MB is the center for coincidence detection also in visual learning. I could show that information about both appetitive and aversive reinforcements are conveyed via specific dopaminergic neurons.

Also in humans, dopamine neurons are involved in information processing of appetitive and aversive stimuli (Matsumoto & Hikosaka 2009).

### *Partially shared output circuits*

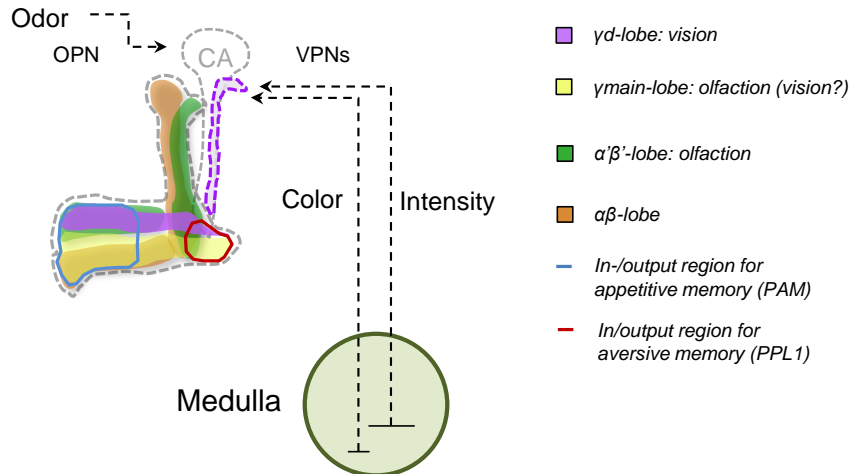
Such specific subdivisions inside the lobes make it possible to perform many different computations in a very densely packed and rather small insect neuropil. In other insects, MB output neurons were already shown to provide different kinds of information. Multimodal information is provided by efferents with dendrites inside and also outside the MB lobes (honey bee (Erber 1978), cricket (Schildberger 1984), cockroach (Li & Strausfeld 1997; Li & Strausfeld 1999)). In cockroach, other efferents were found that connect to protocerebral regions with few providing descending collaterals (Li & Strausfeld 1997). The honeybee possesses a unique output neuron (Pe1 (Mauelshagen 1993; Rybak & Menzel n.d.)) which shows response plasticity during learning and memory. In *Drosophila* there seem to be two protocerebral regions close to the MB-lobes where several efferent neurons target preferentially (data not shown). However, the functions of these output clusters are not clear yet. There seems to be no direct connections to descending neurons (Ito et al. 1998). Specification of MB-output neurons required for different sensory memories could help to identify target neurons further downstream of MB-output neurons in the future. Not only subdivision into lobes and sub-compartments allows a huge variety of combinatorial outputs, but also there seems to be different feedback loops of output neurons projecting back to the MB lobes (e.g.  $\gamma_4 \rightarrow \gamma_{1/2}$  in line *MB434B*, Figure 33). Such KC-KC or feedback connections could be important elements for circuits that are capable of processing multiple complex behavioral tasks (Arena et al. 2013).

## 4.9 Visual information circuit

Results from the MB screening project strongly suggest that the MB is the convergence center of US-information and visual information in our visual conditioning task. Thus, visual input should be provided to and represented in the required intrinsic cell population, the  $\gamma$ d KCs.

### Input to the $\gamma$ d ventral accessory calyx (VACA)

The newly described ventral accessory calyx is formed by a small subpopulation of the  $\gamma$ -lobe KCs, ~70  $\gamma$ d-KCs (Aso et al. 2009; Butcher et al. 2012; Vogt, Knapek, et al. n.d.).  $\gamma$ -lobe KCs were shown to consist of a specific class of neurons, the clawed class II KCs in the primary calyx (honeybee (Strausfeld 2002), *Drosophila* (Butcher et al. 2012; Lee et al. 1999)). However, it is not clear if the  $\gamma$ d-KCs subpopulation provide the same type II KCs, since the VACA was only poorly described so far (Aso et al. 2009; Butcher et al. 2012). In contrast to the  $\gamma$ d VACA, the dorsal accessory calyx of *Drosophila* was already described in detail in terms of anatomy (Tanaka et al. 2008; Aso et al. 2009; Butcher et al. 2012). A subset of late born  $\alpha/\beta$  KCs,  $\alpha/\beta$ p, forms this specific accessory calyx that does not receive direct olfactory input from projection neurons like the primary calyx. In other insects, physically separated calyx sub-regions termed accessory calyces, have been described consisting of another specific subtype of neurons, the class III KCs (Farris 2005; Farris 2008). Common features of these cells that often perceive gustatory input from the tritocerebrum are the following; they are smaller than the primary calyx, they are located post-ventral to the primary calyx, sometimes also integrated in the primary calyx (*Lepidoptera*: (Pearson 1971), specific Y-tract (Ali 2009)) and they have an early birthdate. Since they are born early in development they often have their cell-bodies and axons at the outer perimeter of the primary calyx, peduncle or lobes, respectively (Schuermann & Klemm 1973; Malaterre et al. 2002; Farris & Strausfeld 2003).



**Figure 49: Circuit model of visual information processing in the central brain.**

Visual and olfactory information is probably conveyed to distinct sets of KCs. Olfactory input to the primary calyx via OPNs is well characterized. Visual input is conveyed from different layers in the medulla to the MB VACA formed by the  $\gamma$ d KCs. Output of KCs, representing olfactory and visual information is locally modulated by the different subsets of dopamine neurons (PAM, PPL1) in the MB lobes to form appetitive and aversive memories.

Almost all these points also account for the newly described ventral accessory calyx in *Drosophila*. The  $\gamma$ d KCs cover probably less than 5% of the total amount of KCs and thus form a rather small accessory calyx (Figure 34). Their cell bodies indeed form the outer layer in the mass of Kenyon cell bodies and their projections wrap around the peduncle and the anterior part of the medial lobes as found in other insects (Malaterre et al. 2002; Farris & Strausfeld 2003; Sjöholm et al. 2005), suggesting that they were first born KCs, even earlier than the  $\gamma$ main KCs (Figure 34, Figure 49). Thus, one could conclude that also *Drosophila* possesses class III KCs that receive input from the optic lobes. If they also receive input from the tritocerebrum still has to be investigated. MBs are necessary for gustatory learning (Masek & Scott 2010), thus gustatory information input could probably be mediated to the KCs also via an accessory calyx. Since several different insect lineages possess these characteristic class III KCs it is suggested that all insects possess the genetic information to form these accessory calyces, however not all of them make use of it (Farris 2008). Establishing an accessory calyx could be the first step to integrate sensory information other than olfactory in the MB. In several insects that belong to the infraclass *Neoptera*, direct connection from the optic lobes to the MB calyces could be detected via anatomical studies in social *Hymenoptera* (Ehmer &

Gronenberg 2002; Gronenberg & Lopez-Riquelme 2004; Paulk & Gronenberg 2008) cockroach (Strausfeld & Li 1999; Nishino et al. 2012) or *Coleoptera* (Lin & Strausfeld 2012). By identifying similar direct visual input from optic lobes to the MB calyx in *Drosophila*, which also belongs to the infraclass *Neoptera*, one could suggest that the ancestral organization of at least the *Neopteran* MB could also have consisted of visual and olfactory input from antennal lobes and optic lobes, perhaps supported by the genetic bauplan of accessory calyces. Expansion of the brain is however rather costly (Niven & Laughlin 2008; Chittka & Niven 2009) and would not be maintained if not needed. Some members of the *Neopteran* could have secondarily lost either both inputs or only one of them. There is e.g. the water-living whirligig beetle (Lin & Strausfeld 2012) that still has similar MB and calyx structure as found in terrestrial species but lacks antennal lobes. This beetle only possesses connections between calyx and optic lobes. Other *Neoptera* only possess olfactory input provided to the calyx, just as previously thought in *Drosophila* (Ito et al. 1998). Thus, many insects that do not heavily rely on visual or olfactory information do not possess specific visual or olfactory projection neurons to the MB calyx. Although olfactory information processing is very prominent in the *Drosophila* MB, the flexible modulation of visual input thus also seems to be highly important.

### **Visual information pathway**

Reinforcement signals are projected to the MBs which are required during training and test in visual conditioning (Figure 22, Figure 48). The missing piece of the puzzle is the visual input to the MBs. By identifying that the  $\gamma$ d-lobe of the MB is especially needed for visual learning, a starting point for searching for visual projection neurons was set (Figure 26).  $\gamma$ d-KCs form a specific dendritic region outside the main calyx, the so-called ventral accessory calyx (VACA). Indeed I could show that there are visual projection neurons that are directly connecting the medulla in the optic lobe with this ventral region of the MB calyx (Figure 36, Figure 37, Figure 38 and Figure 39). These connections are required for different kinds of visual aversive memory, respectively. A previous study showed that in visual appetitive conditioning flies can discriminate stimuli that not only differ in their wavelength but also stimuli that only differ in their intensity (Bright Green/Blue vs. Dark Green/Blue (Schnaitmann et al. 2013)). The stimuli that are presented during the standard color conditioning protocol probably

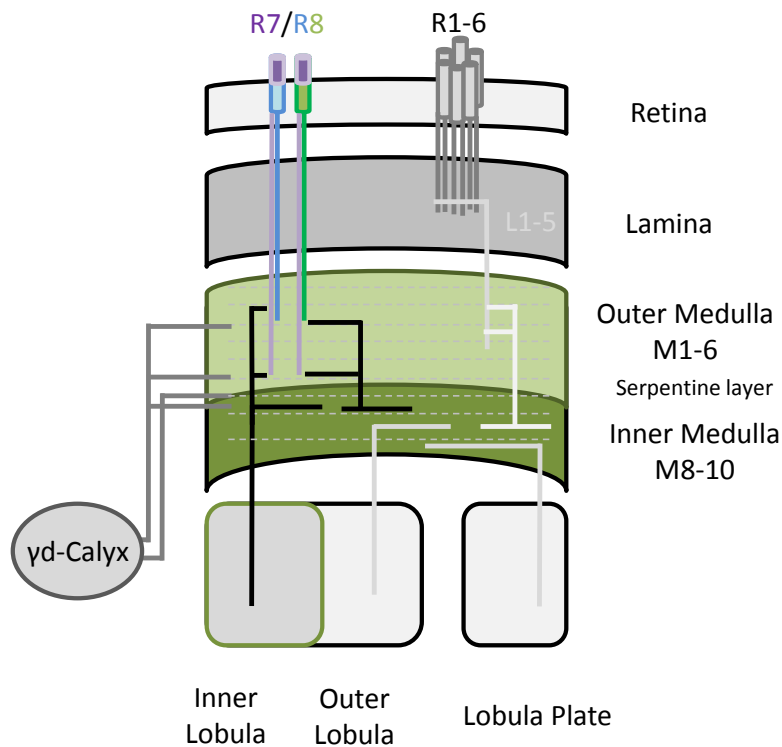
include both properties. However, when introducing intensity inversion between training and test during color learning; training with dark-blue/bright-green and test with bright-blue/dark-green, and *vice versa*, the choice priority still resides on the color cue (Schnaitmann et al. 2013). These results demonstrate that discrimination in the classical visual learning paradigm with chromatic stimuli is based on spectral composition of conditioned stimuli and not solely on achromatic stimuli. According to this I found separate VPNS required for intensity and color conditioning (Figure 40, Figure 42).

#### **4.10 Visual stimuli processing in the optic lobe**

Both newly described types of VPNS receive their input in the medulla of *Drosophila* and project directly in to the central brain, to the ventral accessory calyx of the MB (Figure 36). In the medulla they innervate specific layers and thus probably receive different kind of information from upstream neurons (Figure 44).

##### **Visual information processing in the Medulla**

Most visual information processing is possibly happening in the most prominent optic neuropil, the medulla. Overall the more superficial layers of the medulla (outer) contain less processed information (Morante & Desplan 2008). Motion information is received from the lamina; color information however is directly received from photoreceptors R7/8 to the outer medulla (M1-M6). R8 axons from the retina innervate medulla layers M3/M4, whereas R7 axons from the retina innervate M6. Thus, VPN neurons labeled by Split-GAL4 line 334C could receive direct input from R7 in medulla layer M6 (Figure 44). Since they are not required for learning of chromatic stimuli this less processed information from the outer medulla could be sufficient to discriminate intensity differences. L3 neurons from the lamina overlap and could possibly interact with R8 neurons in medulla layer M3 (Jacob et al. 1977). L3 possesses wide-field medulla efferent terminals endowing broad-band input from R1-6 (Strausfeld & Lee 1991; Gao et al. 2008).



**Figure 50: Visual sensory circuit in the optic lobe and projections to the central brain for visual conditioning.**

Visual signals are perceived via eight different photoreceptors in the retina. R1-6 project to the lamina, from where L1-5 transmit the perceived signal to the medulla layers M1-5. R7-8 directly project to the medulla layer M3/4 and M6. Inside the medulla, several extrinsic and intrinsic transmedullar neurons (Mi, Mt, Tm, Tmy) modulate the visual information and also mediate it to further neuropiles like lobula and/or lobula plate.

Additionally, VPNs mediate color and intensity information for visual conditioning from different medulla layers (M3/4/8 or M6/7, respectively) to the VACA of the  $\gamma$ d-KCs, respectively.

Even though the medulla receives only small amount of input from retina, many inter- and output-neurons per column are present (Morante & Desplan 2008). Several transmedullar (Tm) neurons connect inner and outer medulla, thus these neurons could be involved in early processing of color information (Figure 50; Tm16, Tm20, Tm28, TmY9 and TmY4 are connecting M3 with M8, whereas TmY13 and TmY14 connect M4 to M8 (Takemura et al. 2013). Like this, medulla layers M3, M4 and M6 are all connected to M8 (inner Medulla). Similar as in the antennal lobe in the olfactory pathway these interneurons could also provide inhibitory or excitatory modulation (Chou et al. 2010). To be able to differentiate colors, color-opponent cells that process input from different photoreceptors are necessary. Tm5 and Tm9 were suggested as such since they receive direct input from R7/R8 and also indirect input from R1-6 in the medulla and provide



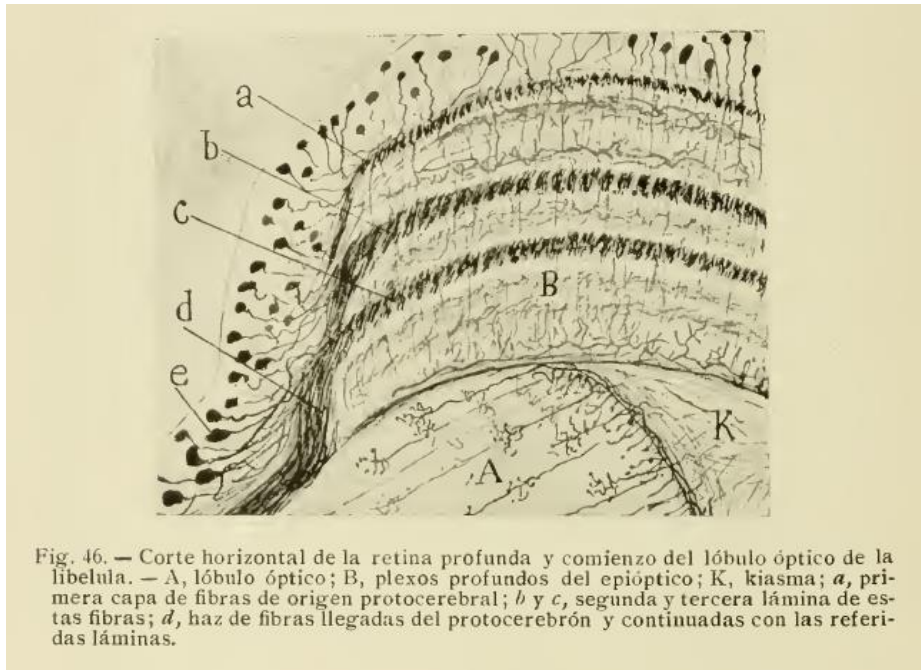
output not only to the lobula but also to layer M8 in the medulla (Gao et al. 2008). Thus, M8 is a possible site of trichromatic interaction. Also VPN Split-GAL4 line *425B* receives strongest input from the medulla layer M8, therefore it could indeed be possible that these neurons convey processed information about chromatic stimuli to the MB (Figure 44). Also in other insects like honeybee and bumblebee (review (Dyer et al. 2011)) a similar mechanism can be found. In the medulla of the optic lobes of honeybees, several neurons have been described that respond differentially to different wavelength stimuli and thus allow the description of a color opponency model in the honeybee (Medulla (Hertel & Maronde 1987; Kien & Menzel 1977) Model (Backhaus 1991)). Hence, the inner deeper layers of the medulla contain more processed information (Morante & Desplan 2008) and the overall complex neuronal architecture of the medulla suggests that already further processed information can be provided at a very early stage in the visual processing pathway.

### **Anatomy of medulla intrinsic neurons**

Generally, neurons in the medulla vary strongly in their anatomy and based on this can be classified to being either part of a vertical (columnar) or horizontal (non-columnar) pathway. The size of visual field inputs differs between such interneurons, for example in columnar and non-columnar transmedullar neurons (Tm cells in Diptera, (Strausfeld & Lee 1991; Takemura et al. 2013)). Overall, columnar interneurons (e.g Tm1) have very small, dense and homogenous dendritic fields. Non-columnar interneurons however have broad and very fine arborizations extending into many neighboring columns. Thus, they possess less spatial resolution, but collect information from different ommatidia (pale and yellow) and could retain better color differentiation (Morante & Desplan 2008). Testing the VPN candidate lines, I found not only color specific projection neurons but also intensity specific neurons. Interestingly, the anatomy of these neurons could reflect their function. Most of the neuron candidates for conveying color information indeed receive input from a rather large visual field compared to columnar neurons. The dendrites seem to expand over ~5 ommatidia (20° of the visual field). Thus, the spatial resolution provided would probably not be sufficient for motion vision or small object detection, however fine color discrimination should be possible. All the color VPNs in the medulla at least receive input inside the inner medulla layer M8, where already processed and integrated color information is available. Also in

honeybees, neurons projecting from the inner medulla to the MB possess strong response to color stimuli and even contain color opponency neurons (see review (Dyer et al. 2011)). The VPNs necessary for intensity conditioning possess an even larger dendritic field; they receive information from the whole ventral half of the medulla. Such input pattern does not provide high spatial resolution which however should also not be required for light intensity detection. The input layers occupied in the medulla seem to be M6-M7. Less processed information is available in the outer medulla e.g. in the honeybee outer medulla no color opponency neurons were found. Interestingly, in the honeybee, VPNs from outer medulla do not project to the MB, but mainly directly to the posterior protocerebrum ((Paulk et al. 2009) for review see (Dyer et al. 2011)). Such differential processing of different attributes of a visual stimulus (e.g. color and intensity) can also be found in the primate visual system (Livingstone & Hubel 1988).

The first descriptions of the different cell types in the fly optic lobe were done by applying Golgi staining (Figure 52, Cajal & Sanchez 1915; Fischbach & Dittrich 1989). A more recent study focused on the medulla and reconstructed different cell types from electron microscopy data (Takemura et al. 2013). A few cell types have been described in the medulla that could resemble the ones possibly responsible for conveying color and intensity information to the MB. Already Cajal y Sanchez (1915) described neurons connecting the medulla and the central brain in insects (Figure 51). The VPN cells described in this project could be similar to medulla tangential neurons (Mt cells) from the outer optic anlage (Meinertzhagen 1973; Hofbauer 1979). They are established first and differentiate earlier than columnar neurons. Their cell bodies are outside and anterior to the medulla, similar as in the VPNs, e.g. Mt4 with the cell body anterior to lobula, covers a big part of the visual field and gets most input from M8 (also Mt7, Mt8, Mt10 and Mt11; (Fischbach & Dittrich 1989; Takemura et al. 2013)). Even though there is already such detailed description of many types of neurons, the newly described VPNs could not have been reliably identified in previous studies. Unfortunately, less interest was devoted to the projection of the different output neurons into the central brain of the fly. Especially medulla output neurons are understudied, probably since lobula and lobula plate are rather thought to provide major output connections to the protocerebrum (Otsuna & Ito 2006), and to the anterior optic tract (AOT (Fischbach & Lyly-Huenerberg 1983; Strausfeld 1976)). Thus, it could likely be that the new VPNs in *Drosophila* have been described before concerning their expression in the medulla but not in the central brain.



**Figure 51: Horizontal section of the medulla and a small part of the lobula of the dragonfly.**

(A) Lobula, (B) Medulla, (K) inner optic chiasm, (a) first layer of neurons originating in the protocerebrum, (b) and (c) second and third layer of such neurons, (d) strand of neurons that arrive from the protocerebrum and continue in the referred layers of the medulla, (e) cell bodies of neurons connecting medulla and protocerebrum. From Cajal y Sanchez, 1915; p.90

### Visual information processing beyond Medulla

Third order neurons that collect more elaborate visual information already integrated by other Tm cells carry this processed visual information to downstream targets outside the medulla. M8 as potential part of the color pathway is further connected to the inner layer of the lobula. In the potential motion-pathway, M9 and M10 are connected to outer lobula layers or lobula plate. Thus, there is again a segregation happening at the output of the medulla to lobula and lobula plate (Strausfeld & Lee 1991). In contrast to the described color pathway via the lobula to the central brain, the newly described VPNs are conveying information already from the outer and inner medulla to the central brain (M7, M8; see Figure 50). This suggests that there could be two separate pathways for color processing. In honey bees and ants, there are also different tracts conveying information from different sub-compartments of the optic lobes to the protocerebrum (Ehmer & Gronenberg 2002; Gronenberg & Lopez-Riquelme

2004). A more complex processing mechanism could be necessary for pattern discrimination learning, the most used visual stimulus in the flight simulator other than color discrimination learning. Additional computation of the visual information according to the task could thus recruit the CC as a higher brain center after further modulations in the lobula. Also in human working memory there is a differentiation in brain region requirement between different attributes of a visual stimulus. Information processing about recognition (“What”) and location (“Where”) of an object is performed in different brain regions (Rottschy et al. 2012).

#### **4.11 Advantage of an early visual information pathway to the central brain**

The VPNs that provide visual information to the MB receive their information at an early point in the visual processing pathway, in the medulla. This is an interesting point that could make them especially suitable for association learning. A probably minimal amount of synapses is actually employed for processing the visual information (2-3 synapses), similarly as in olfactory conditioning (2-3 synapses; (Heisenberg 2003)). In vertebrates, information that passes from retina to cortex is contaminated along the way with increasing noise, thus decreasing the temporal precision of the signal (Kumbhani et al. 2007). In invertebrates, the information provided by VPNs that bypass the lobula, etc., should contain less noise and would therefore be more suitable for establishment of an associative memory. The connection between the medulla and the MB via the VPNs in *Drosophila* seems to be similar to the more prominent medulla-calyx connections that are found in honeybees (Ehmer & Gronenberg 2002), bumble bees (Paulk & Gronenberg 2008) and ants (Gronenberg 1999; Ehmer & Gronenberg 2004). Indeed, in honeybees the VPNs that project from inner medulla to the MBs not only respond to specific chromatic stimuli, but additionally possess a specific temporal response pattern (Paulk et al. 2008; Dyer et al. 2011). Also medulla recordings in the locust reported highly precise spike timing (Osorio 1987). Such high spike timing precision that was found in the medulla of locusts and bees is a prerequisite for spike timing dependent plasticity (STDP, found in KCs of locusts (Cassenaer & Laurent 2007) and honeybees: (Szyszka et al. 2005)). Thus, VPNs that could provide a direct connection between medulla and the MB could be especially equipped to support

learning mechanisms since they could be able to convey an increased amount of precise information.

### **Different pathway for naïve visual preference**

The visual projection neurons do not seem to provide information that is needed for naïve behavioral performance in *Drosophila*. Even when the output of the VPNs or the MB is blocked, flies can still differentiate between different intensities and colors, but are not able to form an association with the US (Figure 16, Figure 40 and Figure 42). Similarly, cockroaches that are trained in a maze are not able to find a hidden target when blocking the MBs, but still can see and detect a visible target. Thus, in insects there seem to be different pathways providing information for naïve visual behavior and learned visual behavior, similar as in the olfactory sensory system (Tanaka et al. 2004). In flies, there are two pathways that seem to be involved in color perception. The connection from medulla to MBs seems to be responsible for providing visual information for associative conditioning, whereas the other pathway from medulla to lobula and then later to the central brain, optic glomeruli or descending neurons could be used for mediating naïve behavior. One neuron, collecting information about dim UV light was indeed shown to be necessary for naïve UV preference (Dm8 (Gao et al. 2008)). Dm8 is an amacrine-like intrinsic neuron in the medulla that is pooling information from several R7 inputs in M6 and giving output to Tm (5/9) neurons which project to the lobula. Further VPN cell types were described to be specifically required for phototaxis responses of light with different wavelengths (Otsuna et al. 2014). The MC61 neurons are specially required for response to violet and green light, whereas the LT11 neurons are specifically required for response to blue light. These neurons receive input from medulla and lobula and project to the AOT and the protocerebrum, respectively.

#### **4.12 Effect of ventral presentation of visual stimuli**

In the newly established visual conditioning setups, the trained visual stimulus is presented to the flies from beneath. Interestingly, the VPNs that I found necessary for

both intensity and color learning have either exclusively or preferably strong expression in the ventral part of the medulla. Thus, these VPNs could be especially required to collect information about ventral stimuli, such as they are presented in the new visual conditioning setups, and project it to the central brain. The ventral VPNs for color learning indeed form connections with the  $\gamma$ -calyx dendrites (VACA). In the *425B-Split-GAL4* line impaired in color learning, there are also VPNs labeled that project from the dorsal medulla to the protocerebrum (Figure 44). Employing the additional GAL4-line *VT8475* that uniformly labels a different kind of VPNs over the whole medulla however did not lead to impairment in color learning (Figure 46). This implies that the specific enrichment of VPNs in the ventral area of the medulla could indeed be responsible for color information collection in this specific paradigm. Additionally the neurons that project from the dorsal part of the medulla to the central brain labelled in the *MB425B-Split-GAL4* do not possess as prominent connections to the MB VACA as the ventrally projecting neurons.

Not only during development there is a differentiation in dorso-ventral growth in insects (for review see (De Robertis 2008)), also during adulthood there seems to be ventral-dorsal differentiation in the optic lobes. In *Drosophila* polarization vision, there is different and independent functions of the photoreceptors in the ventral and dorsal optic lobe (Wernet et al. 2012). Flies respond to direct polarized light when presented dorsally via specialized photoreceptors in the dorsal rim area. Interestingly colored polarized light can be hardly detected via these dorsal receptors but a strong response is seen to ventral stimulation of colored polarized light perceived by unspecialized photoreceptors. In nature, the ventral area probably receives indirect polarized light from shiny surfaces such as leaves or water. Furthermore flies can associate visual stimuli depending on the height of the stimulus in their frontal visual field (Yang & Guo 2013). Best learning performance was found when stimuli were presented in the lower or upper part of a screen depending on the background illumination, respectively. This could suggest that there are two independent ventral and dorsal systems for visual stimulus processing, respectively. During normal visual conditioning in the flight simulator, stimuli are mostly presented in the frontal field, thus the identified ventral VPNs could either not be activated by these stimuli or the visual information could still be processed by a dorsal color pathway. In honeybees, visual stimuli that are presented ventrally seem to be processed differently. Color cues can be learned when presented ventrally, however pattern cues cannot (Giger & Srinivasan 1997). Also honeybees mastered to recognize colored landmarks on the floor that marked a sucrose reward (Cheng et al. 1986).

On the anatomical level, the provided visual input from lobula and medulla is separately presented not only in specific calyx regions in the honeybee MB, but also there is further distinction between lower and upper medulla regions (Ehmer & Gronenberg 2002). Same is also true for ants (Ehmer & Gronenberg 2004; Gronenberg & Lopez-Riquelme 2004). Another very extreme example is the aquatic whirligig beetle; it possesses four eyes, two dorsal eyes, above the water surface, and two ventral eyes below the water surface (Lin & Strausfeld 2012). The dorsal and ventral optic lobe is separated into two laminae and two medullae, but shares the same lobula (Lin & Strausfeld 2013). Thus, in this insect, specific selective pressure could have led to strong differentiation between the dorsal and ventral system.

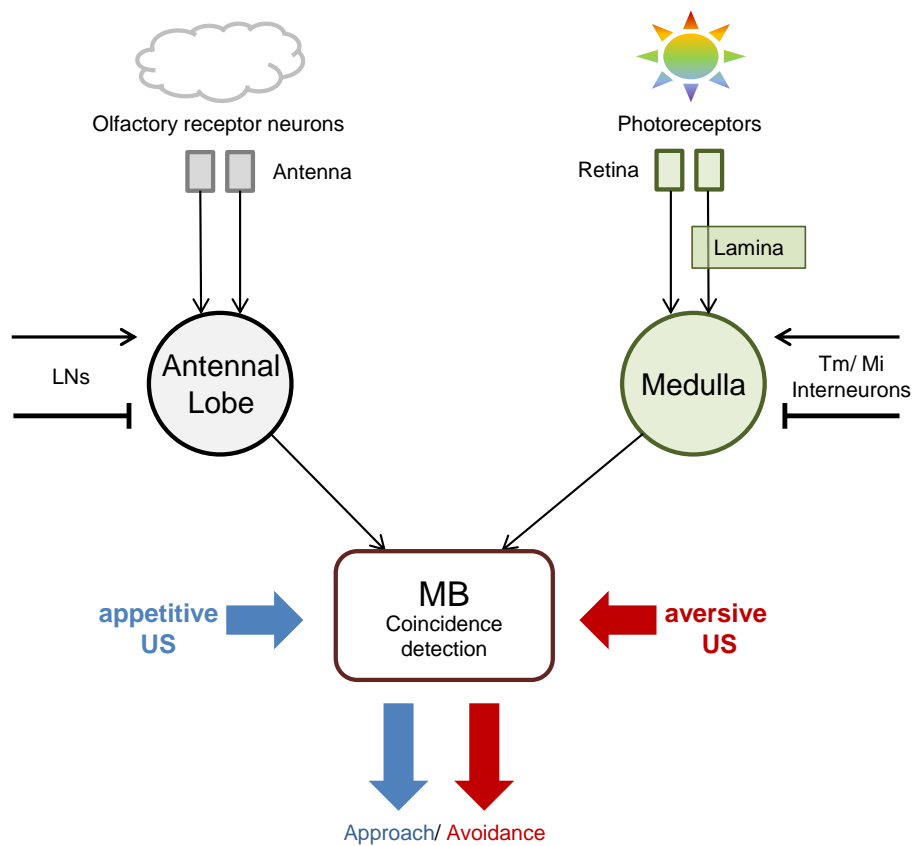
All these findings could lead to the assumption that insects possess a common pattern of ventral and dorsal differentiation inside the optic lobes. A specific but not exclusive requirement of the ventral visual field for color perception could be reasonable since in nature information about colors can be available beneath the insect such as e.g. flowers for foraging bees or fruits for feeding flies.

## 5. Summary

### 5.1 Functional advantages of a multi-sensory integration center

Walking flies can perform classical visual learning tasks, like appetitive and aversive color and intensity learning, respectively. For visual memory formation and storage, they use the same neuropil, the MB, as already described in olfactory conditioning (Figure 52). Visual input to the MB is provided by a direct neural connection with the optic lobes. All my findings strongly suggest the MBs as a site of convergence of reinforcement and conditioned visual stimuli, similar as in olfactory STM formation (Qin et al. 2012; Gerber, Tanimoto, et al. 2004). Furthermore, MB requirement is not restricted to olfactory and visual classical conditioning, but there is also requirement e.g. for gustatory STM (Masek & Scott 2010) and courtship conditioning (15 min (Keleman et al. 2012)). Thus, in *Drosophila*, I suggest the MB as a site for coincidence detection across several different sensory modalities. Employing one neuropil across associative conditioning tasks with different modalities could not only allow modulation of a specific sensory stimulus by a reinforcer, but probably also modulation amongst different sensory information. It would indeed be interesting to also test MB requirement with further sensory stimuli in a simple association paradigm (e.g. auditory conditioning (Menda et al. 2011) or magnetic sense conditioning (Gegear et al. 2008)). Such centralization of similar brain functions (appetitive/ aversive immediate associative memory) across different sensory modalities (visual/ olfactory/ gustatory, etc.) indeed is a widely used and economic circuit design. During fear conditioning in rats different visual and auditory memories are established in the amygdala, respectively (Campeau & Davis 1995). Converging inputs of different stimuli into one multisensory area also have been well described in humans and primates (Beauchamp et al. 2008; Schroeder & Foxe 2002). These multisensory areas could at least provide information necessary to form multi-modal concepts. A shared circuit architecture, as I propose it for the *Drosophila* MBs, is a huge advantage since costs for establishing similar circuits in different brain areas can be avoided and multi-modal memories could be formed easily. Especially in insects, which possess a limited amount of cells in the brain, neural reuse should be an efficient mechanism to still be able to perform many complex tasks (Arena et al. 2013).





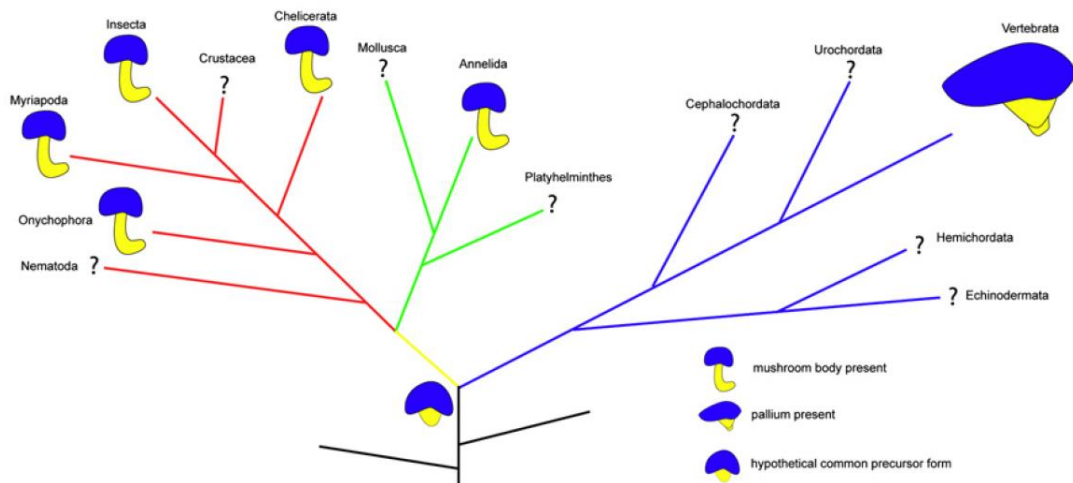
**Figure 52: Comparison between the visual and the olfactory learning pathway.**

Sensory stimuli are perceived via receptor neurons. Odors are perceived via olfactory receptor neurons in the antenna whereas visual stimuli are perceived via photoreceptors in the retina. Sensory information is mediated to and processed in second order neuropiles. Olfactory information is conveyed to the antennal lobe and modulated by local interneurons. Visual information is conveyed to the medulla and can be modulated by diverse intrinsic or transmedullar interneurons. Sensory information for associative learning is conveyed to the MBs. Visual and olfactory information is conveyed to the MB either via VPNS or OPNS, respectively, where both can be modulated by the same appetitive and aversive reinforcement neurons. Output neurons of the MB provide information for behavioral output.

## 5.2 Parallel evolution of vertebrate higher brain centers and invertebrate higher brain centers

MBs can be involved in computation of various sensory inputs, in relaying information to the protocerebrum and in comparison of ongoing and past stimuli (Erber et al. 1987). Many insect species probably secondarily lost MBs or just possess vestigial MBs due to the costly maintenance of such higher brain centers (Chittka & Niven 2009; Niven & Laughlin 2008). Modulation of visual information inside the MB also seems to be a rather costly task since rather few members of the *Neoptera* still possess or developed direct neural connection to the optic lobes. However, although this specific ability for visual processing seems to come along with some cost, it also seems to provide the insects with new advantageous possibilities (Farris 2013). *Hymenoptera*, *Periplaneta* (Bell et al. 2007) and *Drosophila* (Lachaise et al. 1988) have in common that they have a generalist lifestyle. Such a generalist or even parasitoid lifestyle indeed requires performance of more complex tasks with higher flexibility and faster adaptation than living an inflexible specialist lifestyle. *Drosophila* probably relies on chromatic or achromatic visual cues like color and intensity to find and explore new food sources. In other insects it was shown that there is indeed an advantage by integrating not only olfactory or tactile information, but also visual information when for example foraging across different food patches (grasshoppers (Bernays & Wrubel 1985), blowfly (Conlon & Bell 1991; Fukushi 1985)). Thus, for *Drosophila*, as a generalist feeder (Lachaise et al. 1988), increased flexibility in finding new food sources due to additional sensory integration can only be an advantage. Comparing the MB of *Drosophila* with these of other insects indeed shows that the MB architecture resembles their lifestyle. For example ants that mostly stay in their nest receive less visual input and possess a reduced visual input area in the MB since they rather rely on odor cues. On the other hand, honeybees that possess very large and differentiated visual input areas in the MBs need to be able to remember specific landmarks during flight to identify different foraging areas and thus probably also require more storage space for visual information (see review: (Chittka & Niven 2009)). Also for longer living insects, like cockroaches, possessing a large MB (175,000 KCs = 20% of all neurons (Bell et al. 2007)) to store visual information about a food source could be an advantage compared to relatively short-living *Drosophila* which possesses smaller MBs (2,000 KCs = 2% neurons (Aso et al. 2009)). The size of the MBs could thus not only reflect the complexity of the task an

insect has to perform but the amount of information that it needs to store. The larger and more complex brains probably also were the prerequisite for sociality in honeybees or ants (Farris 2013). *Drosophila* thus seems to be an intermediate in insect evolution between rather ancient insects possessing diminished calyces with restricted sensory input and advanced insects like hymenoptera with increased calyces and prominent multi-sensory input. Fruit flies possess high complexity inside their brain with different sub-compartments and are able to perform multi-modal or even social learning (Sarin & Dukas 2009; Zhang et al. 2013); however they also have a short life and rather simple generalist lifestyle that does not require complex memory tasks as in *Hymenoptera* or *Periplaneta*. Thus, it is indeed interesting to study *Drosophila* in the way to understand how more complex behaviors evolved in insects and what mechanisms are employed and exploited. The overall factors that played a role in establishing higher brain complexity in invertebrates could have been; novel food gathering behaviors across multiple or patchy food sources, a greater reliance upon vision (as a part of food acquisition) and a general capacity for learning, innovation and behavioral flexibility (Farris 2013). Interestingly, these are the same evolutionary pressures that apply for vertebrates.



**Figure 53: Phylogenetic distribution of mushroom body in *Protostomes* and pallium in *Deuterostomes* with a less complex hypothetical common precursor form in *Bilateria*.**

From Turner et al., 2010

There is already some evidence that indeed the MBs of insects and the vertebrate higher brain centers could have evolved from a common and rather advanced neuropil in their protostome-deuterostome ancestor (*Prebilateria*). It was shown that the different higher brain centers have conserved brain topology, meaning they employ a conserved pattern mechanism and built up similar neuron types during development (Tomer et al. 2010, Figure 54). The same evolutionary pressure could have brought up similar processing steps in homologous brain regions across vertebrates and invertebrates. For example, KC subpopulations could be the functional equivalent of cortical areas that get modulated by specific afferents (cortex (Kaas 1995; Finlay 2001)). Also the increased brain size due to more folding of the vertebrate brain could have functional similarity with double calyces in the MB. MBs were also compared to the thalamus in fish, which is important in attention and decision making (Hanstroem 1928), with hippocampus in mammals, since same learning related molecules have elevated expression (for review see (Kandel & Abel 1995)), with olfactory cortex, due to the position of the MB being the next relay station after the antennal lobe or with the cerebellum, due to similar architecture of intrinsic and extrinsic cells (Farris 2011). Thus, by understanding the relatively simple brain circuit architecture in insects that evolved under shared evolutionary pressures as vertebrates, we could also get a step closer to understand how the human brain circuit solves similar processing steps.

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## 7. Appendix

### Controls

<b>Crosses</b>	<b>Sugar preference Mean +/- SEM</b>	<b>Shock avoidance Mean +/- SEM</b>
<i>shi/+</i>	0.569 +/- 0.039	-0.359 +/- 0.034
<i>shi/201y</i>	0.444 +/- 0.039	-0.401 +/- 0.033
<i>+/201y</i>	0.649 +/- 0.064	-0.353 +/- 0.033
<i>shi/+</i>	0.569 +/- 0.039	-0.387 +/- 0.029
<i>shi/MB247</i>	0.535 +/- 0.018	-0.297 +/- 0.038
<i>+/MB247</i>	0.575 +/- 0.048	-0.384 +/- 0.028
<b>CS</b>	Not tested in conditioning	-0.501 +/- 0.033
<i>rut</i>	Not tested in conditioning	-0.607 +/- 0.051
<i>shi/+</i>	Not tested in conditioning	-0.508 +/- 0.048
<i>shi/c205</i>	Not tested in conditioning	-0.383 +/- 0.056
<i>+/c205</i>	Not tested in conditioning	-0.478 +/- 0.046
<i>shi/+</i>	0.548 +/- 0.024	-0.366 +/- 0.031
<i>shi/MB010B</i>	0.526 +/- 0.061	-0.393 +/- 0.052
<i>+/MB010B</i>	0.591 +/- 0.048	-0.353 +/- 0.040
<i>shi/-</i>	0.569 +/- 0.039	-0.493 +/- 0.0312
<i>shi/MB010B</i>	0.526 +/- 0.061	-0.483 +/- 0.0590
<i>+/MB010B</i>	0.591 +/- 0.048	-0.451 +/- 0.0173
<i>shi/MB364B</i>	Not tested for preference	-0.379 +/- 0.0494
<i>+/MB364B</i>	Not tested for preference	-0.486 +/- 0.0357
<i>shi/MB152B</i>	Not tested in conditioning	-0.454 +/- 0.0484
<i>+/MB152B</i>	Not tested in conditioning	-0.385 +/- 0.0693
<i>shi/M009B</i>	0.689 +/- 0.0667	-0.503 +/- 0.0644
<i>+/MB009B</i>	0.738 +/- 0.0169	-0.362 +/- 0.0439
<i>shi/M355B</i>	Not tested for preference	-0.388 +/- 0.0408
<i>+/MB355B</i>	Not tested for preference	-0.376 +/- 0.0864
<i>shi/M419B</i>	Not tested for preference	-0.291 +/- 0.0264
<i>+/MB419B</i>	Not tested for preference	-0.354 +/- 0.0568

<b>Crosses</b>	<b>Sugar preference Mean +/- SEM</b>	<b>Shock avoidance Mean +/- SEM</b>
<i>shi/M504B</i>	No conditioning phenotype	-0.403 +/- 0.0669
<i>+/MB504B</i>	No conditioning phenotype	-0.344 +/- 0.0619
<i>shi/M438B</i>	No conditioning phenotype	-0.434 +/- 0.0344
<i>+/MB438B</i>	No conditioning phenotype	-0.399 +/- 0.0822
<i>shi/M112C</i>	No conditioning phenotype	-0.489 +/- 0.0367
<i>+/MB112C</i>	No conditioning phenotype	-0.489 +/- 0.0400
<i>shi/M262B</i>	No conditioning phenotype	-0.493 +/- 0.0783
<i>+/MB262B</i>	No conditioning phenotype	-0.453 +/- 0.0202
<i>shi/-</i>	0.380 +/- 0.0275	No conditioning phenotype
<i>shi/MB210B</i>	0.498 +/- 0.0415	No conditioning phenotype
<i>+/MB210B</i>	0.573 +/- 0.0372	No conditioning phenotype
<i>shi/M011B</i>	0.394 +/- 0.0576	No conditioning phenotype
<i>+/MB011B</i>	0.519 +/- 0.0469	No conditioning phenotype
<i>shi/M434B</i>	0.355 +/- 0.0495	No conditioning phenotype
<i>+/MB434B</i>	0.499 +/- 0.0526	No conditioning phenotype
<i>shi/M052B</i>	0.293 +/- 0.0331	No conditioning phenotype
<i>+/MB052B</i>	0.660 +/- 0.0514	No conditioning phenotype
<i>shi/M542B</i>	0.273 +/- 0.0487	No conditioning phenotype
<i>+/MB542B</i>	0.688 +/- 0.0347	No conditioning phenotype
<i>shi/-</i>	Not tested in conditioning	-0.360 +/- 0.0575
<i>shi/MB607B</i>	Not tested in conditioning	-0.370 +/- 0.0522
<i>+/MB607B</i>	Not tested in conditioning	-0.362 +/- 0.0488

**Table 8: Sugar preference and shock avoidance of lines with impaired visual memories.**

No significant defect in naïve sugar preference is detected among the experimental groups and the corresponding control groups (one-way ANOVA,  $p > 0.05$ ),  $n = 4-8$ . No significant defect in naïve shock avoidance is detected among the experimental groups and the corresponding control groups (one-way ANOVA,  $p > 0.05$ ),  $n = 4-10$ .

Crosses	Appetitive conditioning in permissive temperature Mean LI +/- SEM	Aversive conditioning in permissive temperature Mean LI +/- SEM
<i>shi/-</i>	Not tested in permissive temperature	-0.173 +/- 0.0181
<i>shi/MB010B</i>	Not tested in permissive temperature	-0.181 +/- 0.0569
<i>shi/MB364B</i>	Not tested in permissive temperature	-0.127 +/- 0.0217
<i>shi/MB152B</i>	Not tested in restrictive temperature	-0.158 +/- 0.0343
<i>shi/M009B</i>	Not tested in permissive temperature	-0.185 +/- 0.0442
<i>shi/M355B</i>	Not tested in permissive temperature	-0.183 +/- 0.0283
<i>shi/M419B</i>	Not tested in permissive temperature	-0.185 +/- 0.0389
<i>shi/M504B</i>	No conditioning phenotype	-0.236 +/- 0.0381
<i>shi/M438B</i>	No conditioning phenotype	-0.138 +/- 0.0233
<i>shi/M112C</i>	No conditioning phenotype	-0.208 +/- 0.0525
<i>shi/M262B</i>	No conditioning phenotype	-0.263 +/- 0.0416
<i>shi/-</i>	0.114 +/- 0.0258	No conditioning phenotype
<i>shi/MB210B</i>	0.161 +/- 0.0459	No conditioning phenotype
<i>shi/M011B</i>	0.161 +/- 0.0386	No conditioning phenotype
<i>shi/M434B</i>	0.133 +/- 0.0170	No conditioning phenotype
<i>shi/M052B</i>	0.163 +/- 0.0505	No conditioning phenotype
<i>shi/M542B</i>	0.123 +/- 0.0262	No conditioning phenotype
<i>shi/-</i>	Not tested in restrictive temperature	-0.117 +/- 0.0264
<i>shi/MB607B</i>	Not tested in restrictive temperature	-0.124 +/- 0.0360
<i>+/MB0607B</i>	Not tested in restrictive temperature	-0.150 +/- 0.0282

**Table 9: Appetitive and aversive conditioning at permissive temperature**

No significant defect in appetitive memory performance at permissive temperature could be detected among the experimental groups and the corresponding control group (one-way ANOVA,  $p > 0.05$ ),  $n = 4-25$ . No significant defect in aversive memory performance in permissive temperature could be detected among the experimental groups and the corresponding control group (one-way ANOVA,  $p > 0.05$ ),  $n = 7-13$ .

Description of MB Split-GAL4 lines

Split-GAL4 line	AD	AD site	DBD_in_attP2	y <sub>d</sub>	y <sub>main</sub>	α'/β'a	α'/β'm	α'/β'p	α/βp	α/βs	α/βc	calyx (CSD)	AC (DGI/DAL)	calyx (MB-C1)
MB010B	13F02	attP40	52H09	■	■	■	■	■	■	■	■			
MB152B	19B03	attP40	26E07	■	■	■	■	■	■	■	■			
MB364B	13F02	attP40	21B06	■	■	■	■	■	■	■	■			
MB009B	13F02	attP40	45H04	■	■	■	■	■	■	■	■			
MB028B	26E07	attP40	16H11	■	■	■	■	■	■	■	■			
MB355B	12E03	attP40	26E07	■	■	■	■	■	■	■	■			
MB419B	26E07	attP40	39A11	■	■	■	■	■	■	■	■			
MB131B	13F02	attP40	89B01	■	■	■	■	■	■	■	■			
MB417B	26E07	attP40	29G11	■	■	■	■	■	■	■	■			
MB005B	13F02	attP40	34A03	■	■	■	■	■	■	■	■			
MB370B	13F02	attP40	41C07	■	■	■	■	■	■	■	■			
MB461B	35B12	attP40	26E07	■	■	■	■	■	■	■	■			
MB418B	26E07	attP40	30F02	■	■	■	■	■	■	■	■			
MB463B	35B12	attP40	34A03	■	■	■	■	■	■	■	■			
MB008B	13F02	attP40	44E04	■	■	■	■	■	■	■	■			
MB371B	13F02	attP40	85D07	■	■	■	■	■	■	■	■			
MB185B	52H09	attP40	18F09	■	■	■	■	■	■	■	■			
MB477B	44E04	attP40	26E07	■	■	■	■	■	■	■	■			
MB465C	37D04	VK00027	51B02	■	■	■	■	■	■	■	■	■	■	■
MB460B	34E09	attP40	45E06	■	■	■	■	■	■	■	■	■	■	■
MB380B	17A04	attP40	65D07	■	■	■	■	■	■	■	■	■	■	■

Table 10: Split-GAL4 lines labeling KCs and calyx associated neurons



Split-GAL4 line	AD	AD site	DBD_in_attP2	Y1 ped (MB-MVP1)	Y2 $\alpha$ '1 (MV1)	$\alpha$ '12 $\alpha$ 2 (MB-V1)	$\alpha$ 3	$\alpha$ '3	Y12-Y4	Y3	Y4	Y5	$\beta$ 1(MB-MVP1)	$\beta$ 1p ped	$\alpha$ 1(MVP1)	$\beta$ sp2(M3)	$\beta$ 2cp	$\beta$ '1	$\beta$ '2a	$\beta$ '2m	$\beta$ '2p	PPL2ab	
MB 504B	52H 03	attP4 0	TH																				
MB 502B	52H 03	attP4 0	14E 06																				
MB 438B	40B 08	VK00 027	23C 06																				
MB 060B	82C 10	attP4 0	72B 05																				
MB 439B	52G 04	VK00 027	94B 10																				
MB 296B	30E 08	attP4 0	11C 07																				
MB 065B	30G 08	attP4 0	TH																				
MB 058B	30E 11	attP4 0	22B 12																				
MB 308B	58E 02	attP4 0	93A 09																				
MB 304B	24H 08	attP4 0	53F 03																				
MB 042B	58E 02	attP4 0	22E 04																				
MB 196B	65B 09	attP4 0	81E 11																				
MB 040B	58E 02	attP4 0	18D 09																				
MB 188B	71D 08	VK00 027	49C 12																				
MB 195B	58E 02	attP4 0	93G 08																				
MB 316B	15B 01	attP4 0	26F 01																				
MB 312B	58E 02	VK00 027	48H 11																				
MB 194B	58E 02	attP4 0	29G 04																				
MB 315C	53C 03	attP4 0	24E 12																				
MB 043B	58E 02	attP4 0	32D 11																				
MB 213B	65B 09	attP4 0	51D 04																				
MB 047B	15B 01	attP4 0	27G 01																				
MB 299B	71D 08	attP4 0	92B 02																				
MB 301B	TH	attP4 0	72B 05																				
MB 025B	65B 09	attP4 0	11F 03																				
MB 087C	58E 02	attP4 0	10G 03																				
MB 109B	73H 08	VK00 027	40B 08																				
MB 032B	80G 12	attP4 0	53H 03																				
MB 83B	TH	attP4 0	42H 01																				

Table 11: Split-GAL4 lines labeling dopamine MB-input neurons

Split-GAL4 line	AD	AD site	DBD_in_attP2	calyx(CP1)	γ1 ped (MVP2)	γ3β'1 (γ4)	γ4->γ12	β1(MV2)	γ5β'2a(M4-6)	β2 β'2a(M4-6)	β'2mp(M4-6)	β'1	α1	γ2α'1	α'2(V4)	α3(V3)	α2(V2α)	α2p	α'3(V2α'3amp)	α'1(V2α'1)
MB 242A	64F07	su(Hw)at tP8	57C10	■																
MB 112C	25D01	attP40	19F09		■															
MB 083C	12C11	attP40	14C08			■														
MB 434B	14C08	attP40	15B01				■	■												
MB 298B	58E02	attP40	36B06				■													
MB 433B	21D02	VK00027	12C11					■												
MB 011B	82C10	attP40	26F01						■											
MB 210B	30E08	attP40	53C10							■										
MB 074C	30E11	attP40	82C10								■									
MB 399B	82C10	attP40	50B03									■								
MB 002B	21D02	attP40	22C12										■							
MB 057B	33E02	VK00027	50A05											■						
MB 310C	20G03	attP40	19F09												■					
MB 077B	58E02	attP40	11A03													■				
MB 051B	58E02	attP40	31F06														■			
MB 082C	71D01	attP40	58F02															■		
MB 018B	52G04	VK00027	17C11																■	
MB 093C	70B10	attP40	19F09																	■
MB 052B	71D08	attP40	11F03																	■
MB 552B	61H03	VK00027	28E09																	■
MB 549C	76F05	attP40	23C12																	■
MB 050B	24E12	attP40	52H01																	■
MB 080C	58E02	attP40	37E10																	■
MB 542B	93D10	VK00027	13F04																	■
MB 027B	TH	attP40	26F01																	■
MB54 3B	76F05	attP40	32G08																	■

Table 12: Split-GAL4 lines labeling potential MB-output neurons

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My thanks to Prof. Dr. Mark Hübener who, as my PhD supervisor, gave me helpful advices regarding the progress of my projects. I am also thankful for advices from my thesis committee members Prof. Dr. Bertram Gerber, Prof. Dr. Axel Borst and Dr. Ilona Kadow.

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Many thanks as well to our collaborators Dr. Gerry M. Rubin and Dr. Yoshinori Aso of the Janelia Farm Research Campus (Ashburn, Virginia) who provided transgenic fly-lines, supported my stay at the JFRC to perform behavioral experiments and organized the MB screening project. Being part of this screening project helped on collecting critical data for my project.

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## Non-self-contributions

### Conditioning setups

Christopher Schnaitmann designed the appetitive and aversive visual conditioning setup. Stephan Prech from the electronic workshop of the MPI of Neurobiology and Christopher Schnaitmann built the two setups, including the expanded setup for the screening project. Christopher Schnaitmann and I developed the conditioning protocol for the appetitive setup.

### Olfactory conditioning

All olfactory experiments presented in this thesis were performed by other members of the Tanimoto lab. Stephan Knapek tested *rut* flies in olfactory conditioning (Figure 20). Kristina Dylla tested MB-block flies in olfactory conditioning during her master thesis (Figure 23). Toshiharu Ichinose took part in the screening project and tested all 80 Split-GAL4 lines in 2-hour olfactory conditioning. Additionally he tested *MB419B-Split-GAL4* in 2-min olfactory conditioning (Figure 26).

### Visual conditioning

Kristina Dylla also tested part of the MB-block lines in temperature shift experiments during aversive visual conditioning during her master thesis (Figure 21, Figure 22).

### Immunohistochemistry

Stephan Knapek dissected and stained most of the fly brains that are presented in the thesis and recorded confocal data (Figure 34, Figure 35, Figure 36, Figure 37, Figure 39, Figure 44 and Figure 46). Anja B. Friedrich performed DCO staining and R7/R8 double staining (Figure 38, Figure 44). Confocal pictures and expression patterns of the Split-GAL4 lines were provided by Flylight, JFRC and Yoshinori Aso.

# Curriculum Vitae

## Katrin Vogt

### Personal Information

Date of birth: April 9<sup>th</sup> 1985  
Citizenship: German  
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Languages: German, English, Spanish, French

### Education

2009-2014 Graduate student in the lab of Dr. Hiromu Tanimoto (Behavioral Genetics, junior group, MPI of Neurobiology, Munich, Germany) on “Dissecting the visual learning circuit in *Drosophila*” under the supervision of Prof. Dr. Mark Huebener

2013 Collaboration with Janelia Farm Research Campus (Virginia, USA), overall three months stay to perform experiments for a collaboration project

2009 Diploma in Biology with grade average 1.0 (summa cum laude)

2008-2009 Diploma thesis in the lab of Dr. Hiromu Tanimoto (Behavioral Genetics, junior group, MPI of Neurobiology, Munich, Germany) on “Visual aversive learning in *Drosophila melanogaster*” under the supervision of Prof. Dr. Alexander Borst (MPI of Neurobiology, Munich, Germany) and Prof. Dr. Erich Buchner (University Würzburg, Germany)

2004-2009 Study of Biology, University of Würzburg, Germany

## Teaching experience

- 2010 Supervision of master student Kristina Dylla (University of Konstanz) on “Role of mushroom bodies in *Drosophila* associative learning”
- 2010-2013 Supervisor of yearly undergraduate practical course in genetics and neurobiology (LMU, Munich)
- 2013 Theoretical and practical supervision of undergraduate student Fabian Stamp for three month working on “Appetitive visual learning in *Drosophila*”

## Publications

- 2010 Appetitive and aversive visual learning in freely moving *Drosophila*. Schnaitmann C, **Vogt K**, Triphan T, Tanimoto H (2010). Front Behav Neurosci 4: 10
- 2011 The Similarity between Odors and Their Binary Mixtures in *Drosophila*. Eschbach C, **Vogt K**, Schmucker M, Gerber B (2011). Chem Senses
- 2014 Shared mushroom body circuits underlie visual and olfactory memories in *Drosophila*.  
**Vogt K\***, Schnaitmann C\*, Dylla KV, Knapek S, Aso Y, Rubin G, Tanimoto H (2014). eLife  
\*equal contribution
- 2014 Event timing in visual conditioning leads to different memory traces in *Drosophila*.  
**Vogt K**, Tanimoto H (in preparation).
- 2014 *Drosophila* visual memory in the MBs depends on specific visual input from the optic lobes.  
**Vogt K**, Knapek S, Friedrich AB, Aso Y, Rubin G, Tanimoto H (in preparation).
- 2014 MB Anatomy and Screening Paper in collaboration with Janelia Farm Research Campus  
Aso Y, ....., **Vogt K**, Schnaitmann C, ....., Rubin G (in preparation).

## Invited Talks

- 2013 Prof. Bertram Gerber lab (Leibniz Institute, Magdeburg, Germany) on “Dissecting the visual learning circuit in *Drosophila*”

## Conference talks and poster

- 2009 Oral presentation: Regional *Drosophila* Meeting in Muenster: “Classical aversive conditioning with visual stimuli”
- 2010 Poster: Neuroethology Conference in Salamanca, Spain: “A new behavioral assay for aversive visual learning in *Drosophila melanogaster*”
- 2011 Poster: Meeting of the German Neuroscience Society in Göttingen, Germany: “Aversive visual learning in *Drosophila melanogaster*”
- 2011 Lab visit: Prof. Bruno van Swinderen (Queensland Brain Institute, Brisbane, Australia)
- 2011 Poster: QBI/MCN Meeting in Brisbane, Australia: “Aversive visual learning in *Drosophila melanogaster*”
- 2012 Oral presentation: Behavioral Ethology Conference in Lund, Sweden: “Aversive visual learning in *Drosophila melanogaster*”
- 2012 Poster: Neurofly conference in Padua, Italy: “Dissecting the visual learning circuit in *Drosophila melanogaster*”
- 2012 Oral presentation: “Learning and memory in insects: new insights from honeybee and *Drosophila*” conference in Konstanz, Germany: “Dissecting the visual learning circuit”
- 2013 Poster: “Insect vision” conference in Janelia Farm Research Campus, Virginia, USA: “Dissecting the visual learning circuit in *Drosophila melanogaster*”
- 2013 Poster: “The Nervous System of *Drosophila melanogaster*. From Development to Function” conference in Freiburg, Germany: “Shared mushroom body circuits for visual and olfactory memory in *Drosophila*”
- 2013 Oral presentation: “Munich Fly retreat” in Ringberg, Tegernsee, Germany: “Visual learning in *Drosophila*”



## Declaration (Eidesstattliche Versicherung)

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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## Erklärung

Hiermit erkläre ich, \*

- dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.
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