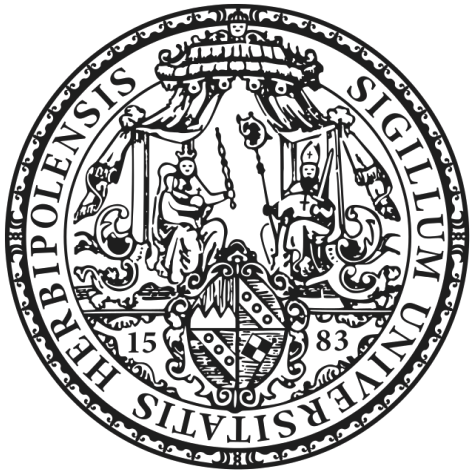
Time-odor learning in *Drosophila melanogaster*

Olfaktorisches Zeitgedächtnis bei *Drosophila melanogaster*



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1. INTRODUCTION

Animals are exposed to the daily environmental changes which affect various aspects of their lives. Some of these changes such as the seasonal modulations or the day/night transitions occur in a predictable manner. Organisms have developed a circadian clock to help them anticipate daily alterations in day/night cycles and accordingly modify their behaviors.

The mammalian timing system is composed of a master clock, located in a small region in the hypothalamus called suprachiasmatic nucleus (SCN), and subsidiary clocks in various organs and tissues (Dibner et al., 2010). The SCN influences the rhythmicity in peripheral clocks, and in-turn coordinates different behaviors via neuronal or humoral cues (Dibner et al., 2010). In flies, a set of about 150 neurons in the brain functions as the central clock, which includes the small ventral lateral neurons (s-LNvs) that act as the pacemaker cells (Helfrich-Förster, 1995). Endogenous clocks can be entrained by external signals called Zeitgeber, but can also show free-running rhythms in the absence of environmental cues. The most important Zeitgeber for the clock synchronization is the Light:Dark transition (Golombek and Rosenstein, 2010). Also, temperature, vibration and even social interactions can synchronize endogenous clocks (Levine et al., 2002; Glaser and Stanewsky, 2005, 2007; Simoni et al., 2014).

Endogenous clocks can also facilitate the adaptive usage of the time of day information. The properties of the space around an animal changes continuously on a given day. The location which has abundant resources like food or mate in the morning may not have much in the evening. The precise anticipation of such changes can help animals to forage or mate before competitors. Also, if predators become active at a certain time of day then the knowledge of the space and time can improve an animal's survival chances.

Previous studies have demonstrated that animals can remember different locations at which the food is available at distinct times of day (Mulder et al., 2013b). The present study examined the time of day related associations (TOD) in *Drosophila melanogaster* and further investigated the underlying mechanism.

1.1. Adaptive usage of the time of day information

Animals can use the time of day information provided by an endogenous oscillator to modify their behavior. Honeybees were first shown to be able to use the knowledge related to the time of day in cognitive functions. Bees can use the position of the sun as a navigational tool. However, the position of the sun changes through the day, and thus, animals need a mechanism to compensate for such variations. Studies showed that an endogenous clock can help animals to compensate for the changing position of the sun across the day (Beling, 1929; Kramer, 1950; Reppert, 2007). The endogenous clocks become non-functional in arrhythmic animals, and concurrently affects the sun-compass based navigation (Reppert, 2007). These results helped in defining the concept of a biological clock and further the understanding of its functions.

A biological clock governs the circadian rhythms and can also provide the phase information to cognitive parts of the brain, which then can be used adaptively (Gallistel, 1990). Previous studies have shown that the phase information from a clock can be tagged to a memory as a context (Mulder et al., 2013b). Such time-tagging requires the continuous consultation with an internal oscillator for the acquisition and the retrieval of the memory.

Rats trained in a passive or active avoidance paradigm show maximum memory retention when the biological time of training and testing are the same (Holloway and Wansley, 1973a; Holloway and Wansley, 1973b). This effect was proposed to be due to the circadian periodicity in the memory retention (Wansley and Holloway, 1975). Such rhythmicity in the memory retention suggests that rats may automatically time-tag every biologically significant event. Alternatively, the periodicity may be due to a unique entrainment mechanism where the time of conditioning is acting as a Zeitgeber (Gallistel, 1990; Mulder et al., 2013b). To further explore TOD dependent associations multiple cycles of training were used.

In the time-place learning (TPL) assay animals were trained to associate the location of a stimulus like food with a particular time of day. This training enables the animal to secure resources under a definite spatial and temporal setting. In TPL the entrainment of an oscillator is not sufficient to facilitate visits to multiple locations at

different times of day. Therefore, an animal has to actively use the phase information from a clock to learn multiple associations between both time and place.

First TPL studies were conducted in the honeybees, in which the bees could visit multiple locations at different times of day to collect food (Finke, 1958). The role of an endogenous oscillator in TPL was shown in the starlings, who can visit different locations to procure food at various times of day even in constant light conditions (Wenger et al., 1991). In mammals, the Long Evans rats were first shown to be able to establish time-place associations (Boulos and Logothetis, 1990). Intriguingly, the SCN lesions had no effect on the ability of rats to remember time-place associations (Boulos and Logothetis, 1990). Later studies indicated that an independent oscillator, probably a food-entrainable oscillator (FEO), might underlie TPL in rats (Mistlberger et al., 1996; Mulder et al., 2013b).

The role of a food-based oscillator indicated that the internal state of an animal might influence TPL. Only starved Wistar rats can associate the time of day with the location of the platform in the Morris water maze setup (Lukoyanov et al., 2002). Similar studies were performed with the Sprague-Dawley rats, which also showed that animals could form time-place memories only after food deprivation (Widman et al., 2000). Another group of well-fed rats demonstrated time-place associations only when their "response cost" was increased through an added weight belt (Widman et al., 2004). Response cost is the penalty associated with wrong choices in a memory test. A small cost to switch between different possibilities in a memory test has been proposed to result in defective time-place associations in rats and cichlid fishes (Boulos and Logothetis, 1990; Reebs, 1993). These studies suggested that food deprivation increases the response cost, which then can promote the adaptive usage of the time of day information (Mulder et al., 2013b).

The mechanism that underlies TPL in animals can be an ordinal timer (learning a sequence of events), an interval timer (stopwatch) or an endogenous oscillator based timer. Prior research has indicated that TPL in the mouse is promoted by an endogenous clock based system (Van der Zee et al., 2008). This clock is Cryptochrome (CRY) dependent but Period (PER) independent (Mulder et al., 2013a). The PER/CRY dimer forms the negative feedback loop in the core molecular clock in mammals.

Therefore, the oscillator for TPL has different mechanistic underpinnings than the core molecular clock and thus, may function exclusively in the context of learning and memory. The putative mechanism and the relevant oscillator that promote TPL remain elusive. Even the FEO is not entirely localized and the neural substrates are not well defined (Mistlberger, 2011).

The TOD learning in a genetically tractable organism like *Drosophila* can help to understand how the time information is generated and communicated by clocks to the brain regions responsible for the memory formation.

1.2. Learning and memory in *Drosophila melanogaster*

Flies are well suited for the genetic analysis, with their short generation time and a plethora of advanced tools. The olfactory conditioning paradigm was chosen to investigate TOD related associations in flies.

Flies can learn the association between odors and reward/punishment values (Quinn et al., 1974; Tempel et al., 1983). Flies trained to associate an odor (conditioned stimulus: CS^+) with the electric shock (unconditioned stimulus: US), showed a strong preference for the other odor (CS^-), which was presented without the shock. Other noxious stimuli like heat can also act as a negative reinforcer, and sucrose can serve as the positive US. Previous studies have shown that hungry flies can form long-lasting olfactory memories after a single presentation of an odor along with the sucrose reward (Krashes and Waddell, 2008). Therefore, the appetitive conditioning paradigm with sucrose as a reinforcer was selected to study TOD learning in flies.

In flies, odors are detected through olfactory receptor neurons (ORNs) in the antennae and maxillary palps, which send axons to the antennal lobe (AL) (Fig 1.1) (Stocker, 1994; Hildebrand and Shepherd, 1997). In the AL, ORNs form synapses onto about 150 projection neurons (PNs), which further projects to the mushroom body (MB) calyces and the lateral horns (Fig 1.1) (Stocker, 1994). The MBs in *Drosophila* comprises of approximately 2500 intrinsic neurons called the Kenyon cells (KCs). A KC neurite divides to form a dendrite-like and an axon-like branch. The dendrite-like branch arborizes in the calyx. The KC axons assemble in bundles at the base of the calyx to

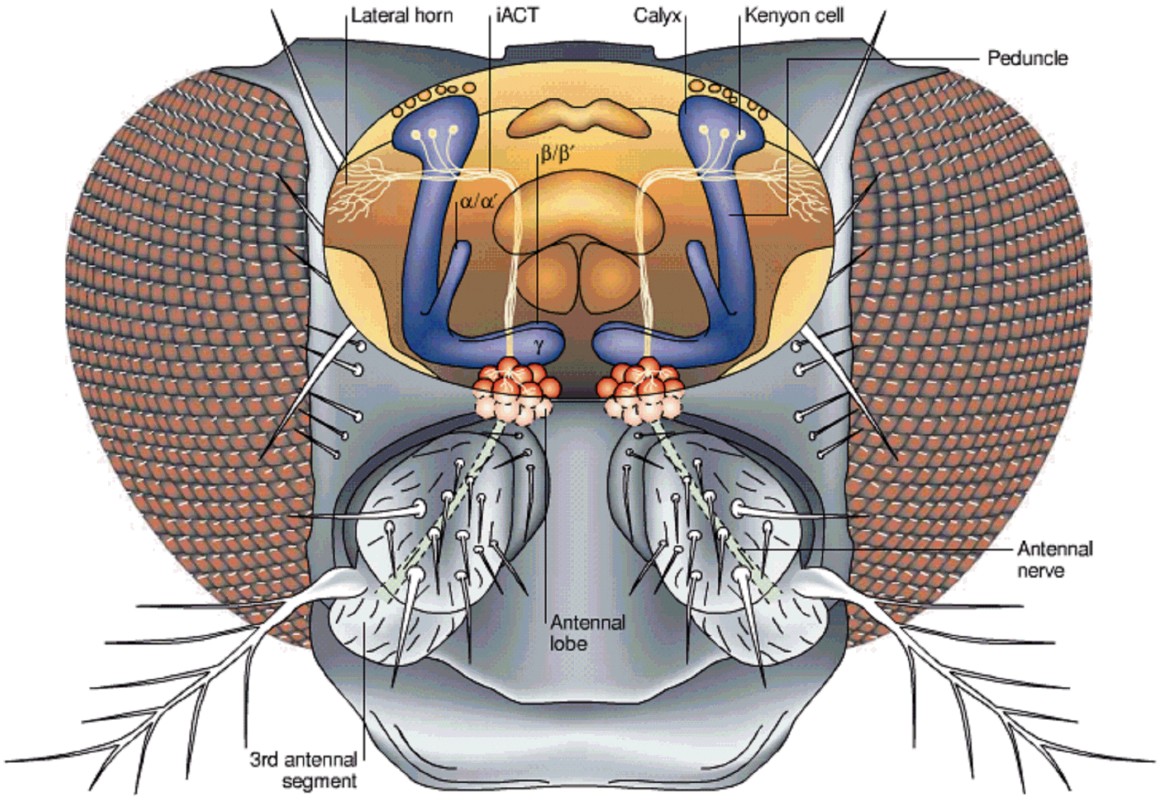

Fig 1.1. The olfactory pathway in *Drosophila melanogaster*.
Odor information is carried from the third antennal segments and maxillary palps (not shown) to the antennal lobe, where receptor fibres are sorted according to their chemospecificities in about 40 glomeruli. These represent the primary odor qualities, which are reported to two major target areas in the brain, the dorsolateral protocerebrum (lateral horn) and the calyx of the mushroom body . The inner antennocerebral tract (iACT) connects individual glomeruli to both areas. a/a', ß/ß' and γ/γ' mark the three mushroom body subsystems (Taken from Heisenberg 2003).

form stalk-like peduncles (Fig 1.1) (Fahrbach, 2006). Also, the axon-like branch further divides to form terminal arborizations in two distinct neuropils to give rise to the MB lobes: α/β, α'/β' and γ (Fig 1.1) (Crittenden et al., 1998; Strausfeld et al., 2003; Fahrbach, 2006). The PN-KC connectivity and the local GABAergic inhibition in the calyx result in a very accurate KC response to a given odor (Honegger et al., 2011).

Previous studies have shown that the MB harbors the olfactory memory traces in flies (Gerber et al., 2004). In olfactory conditioning, KC activation by an odor along with a modulatory reinforcement signal results in the strengthening of the KC and mushroom body output neurons (MBON) connection (Fig 1.2) (Heisenberg, 2003).

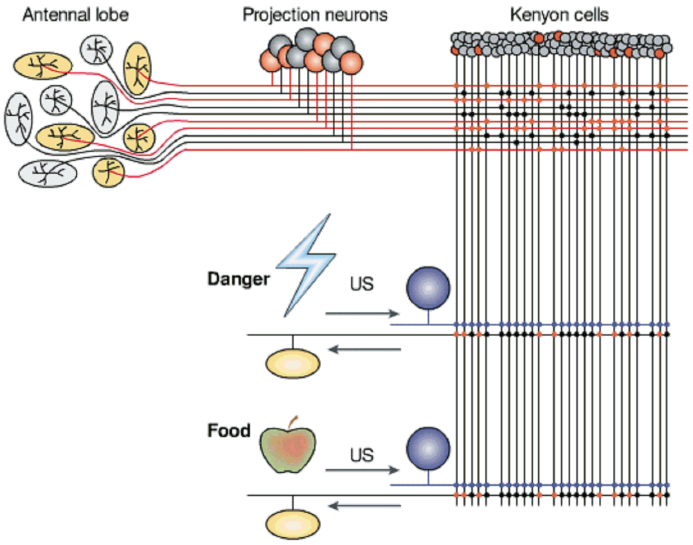

Fig 1.2. Circuit model of the odor memory.
Odors are represented in the mushroom bodies by sets of Kenyon cells. Extrinsic mushroom-body output neurons are connected to the Kenyon cells by latent synapses. The output neurons are accompanied by modulatory input neurons presenting the unconditioned stimulus to the Kenyon cells. Simultaneous arrival of the conditioned (CS) and unconditioned stimulus (US) strengthens the synapses from Kenyon cells to output neurons (Taken from Heisenberg 2003).

A set of about 100 dopaminergic neurons (DANs) in the protocerebral anterior medial (PAM) cluster provides the sucrose-based positive reinforcement signal (Burke et al., 2012; Liu et al., 2012). These neurons mostly innervate the adjacent zones on β, β' and γ lobes. The KC output projects onto 34 MBONs, and dendrites of each MBON are mostly restricted to a few DAN zones (Tanaka et al., 2008; Aso et al., 2014).

The current model for appetitive learning proposes that olfactory conditioning modifies the odor drive to the MBONs such that it depresses the avoidance MBON pathway and probably strengthens the approach pathway (Owald and Waddell, 2015). Starvation can also skew the MB network so that the MBON approach pathway is activated favorably by the trained odors (Owald and Waddell, 2015). In conclusion, learning biases the MB network by driving the KC-MBON plasticity through DANs. The reactivation of the KC-MBON during tests guides the approach or avoidance behavior based on the positive or the negative reinforcement respectively.

The question in the present study is whether *Drosophila* shows TOD related memory and if so, whether an internal oscillator can modulate the MB network so that flies show odor approach or avoidance behavior at different times of the day. Such an oscillator may affect the DANs signaling or directly strengthen the KC-MBON connectivity. To assess this flies were trained at multiple times in a day with a specific odor/reward association. The central clock in the brain or a peripheral clock may function as the internal oscillator for TOD learning in flies.

1.3. The circadian clock in flies

The first clock gene in flies, *period* (*per*), was discovered by Konopka and Benzer in 1971. The subsequent genetic screens led to uncovering of other clock genes including *clock* (*clk*), *cycle* (*cyc*) and *timeless* (*tim*) (Sehgal et al., 1994; Allada et al., 1998; Rutila et al., 1998). The molecular clock comprises of intertwined transcriptional-translational feedback loops (Darlington et al., 1998; Hardin, 2005; Dubruille and Emery, 2008). CLK and CYC form a heterodimeric complex that binds to the E-boxes of *per* and *tim*, and activates their transcription. Doubletime (DBT) and Casein Kinase 2 (CK2) phosphorylates PER, and promote its degradation via proteasomes. Phosphorylated PER is stabilized in the presence of TIM and begins accumulating in the cytoplasm. TIM phosphorylation is mediated by kinases Shaggy (SGG) and CK2 (Meissner et al., 2008). SGG and CK2 mediated phosphorylation facilitates the nuclear entry of the TIM-PER-DBT complex (Price et al., 1998; Martinek et al., 2001; Akten et al., 2003). The TIM-PER-DBT complex then blocks the transcription of its components by promoting the release of CLK/CYC from the E-boxes of *per* and *tim* (Lee et al., 1998; Lee et al., 1999; Bae et al., 2000).

Light activates Cryptochrome (CRY), a blue-light photoreceptor, that mediates the cell-autonomous degradation of TIM (Emery et al., 1998; Stanewsky et al., 1998). In the absence of TIM, PER is degraded via proteasomes and thus, allows CLK/CYC to start a new phase of *per* and *tim* transcription (Curtin et al., 1995; Price et al., 1998; Kloss et al., 2001). Therefore, light acts as the most important Zeitgeber for the clock synchronization in flies.

In the *Drosophila* brain, about 150 neurons express the molecular clock and together constitute the clock network (Fig 1.3) (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 2005). The clock network consists of the lateral and dorsal neuron clusters. The lateral neuron cluster contains three lateral posterior neurons (LPN), four pigment dispersing factor (PDF) expressing small ventral lateral neurons (s-LNvs) and four large ventral lateral neurons (l-LNvs) (Fig 1.3). This cluster also includes a PDF-negative s-LNv, and six more dorsally located dorsal lateral neurons (LNds) (Fig 1.3) (Helfrich-Forster et al., 2007; Helfrich-Förster et al., 2007; Dubruille and Emery, 2008).

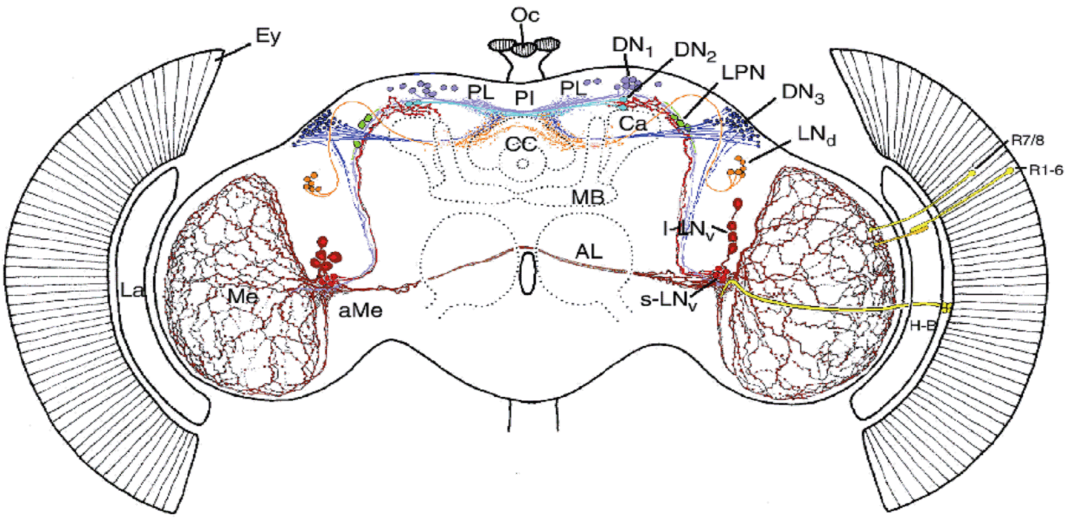

Fig 1.3. Arborization pattern of the clock neurons.
The Lateral Neurons (LNd, l-LNv, s-LNv) are shown in red/orange, the Dorsal Neurons (DN1, DN2, DN3) in blue, and the three cells in the posterior lateral brain in green (lateral posterior neurons; LPN). The s-LNv cells project toward the dorsal brain and terminate close to the Calyces (Ca) of the mushroom bodies. There, their terminals overlap with processes from the DN1 and DN2 cells that run into the pars intercerebralis/lateralis (PI, PL). Fibers from the LNd and DN3 cells terminate in close proximity to the central complex (CC) and seem to contact the DN1 and DN2 processes in the PI/PL. In the right hemisphere the light input pathways from photoreceptor cells R1-6, R7/8 of the compound eye and from the 4 H-B eyelet cells are shown. The RI-6 and R7/8 cells appear to contact the fiber network derived from the l-LNv cells on the surface of the medulla (Me) and the H-B eyelet cells putative dendritic terminals from the s-LNv and l-LNv in the accessory medulla (aMe). AL: antennal lobe; Ey: compound eye; La: lamina; MB: mushroom bodies; Oc: ocelli. (Taken from Helfrich-Förster 2005).

The PDF positive s-LNvs, the pacemaker cells, send projections to the dorsal protocerebrum and accessory medulla, which terminate in the vicinity of the MB calyces (Helfrich-Förster, 1995, 1997). The dorsal cluster of the clock consists of three groups of dorsal neurons (DN): DN1, DN2, and DN3. All the dorsal neurons innervate the dorsal protocerebrum (Fig 1.3) (Helfrich-Förster et al., 2007). Few dorsal neurons also project toward the ipsilateral accessory medulla.

In addition to the *Drosophila* brain, various organs and tissues also express the molecular clock. These secondary clocks are called peripheral oscillators. These clocks regulate rhythms in the periphery to facilitate organ specific functions (Ito and Tomioka, 2016). The role of CRY varies among peripheral oscillators. CRY can operate as a photoreceptor and a core clock component as in the clock driving the olfactory electroantennogram rhythm in the antennae (Krishnan et al., 2001). It can also function

only as a photoreceptor, for instance in the oscillator governing the cuticle deposition rhythm in epidermal cells (Ito et al., 2008). Peripheral oscillators can function cell-autonomously without any cues from the central clock in the brain as in the Malpighian tubules (Hege et al., 1997). On the other hand, the clock in oenocytes depends on the central clock for proper rhythms (Krupp et al., 2008). Light, temperature changes and feeding times can entrain peripheral oscillators.

The present study seeks to identify the appropriate oscillator that may provide the time of day information for learning and memory in flies. In rats, the central oscillator, SCN, is not involved in TPL. Thus, it would be interesting to investigate the role of the central clock in TOD related learning in flies. Alternatively, a peripheral oscillator may provide the time of day signals and promote their adaptive usage in flies.

2. MATERIALS AND METHODS

2.1. Fly rearing

Flies were raised at 25ºC and 60% relative humidity in the 12:12 h Light:Dark cycle (unless otherwise stated). Standard cornmeal-molasses medium served as the fly food and was transferred to the plastic vials and stored at 4°C until used. 3-4 days old flies were used for conditioning and were transferred to the fresh food vials 48 h before behavioral tests. For the starvation experiments, flies were kept in the plastic vials containing a thin layer of 1% agarose to prevent desiccation.

2.2. Fly strains and genetics

All fly lines were from the Wuerzburg stock collection. Fly lines used in these experiments were: *Canton-S* (wild-type control), per^{01} (Konopka and Benzer, 1971), clk^{AR} (Allada et al., 2003), $w^{+};pdf^{01}$ (Renn et al., 1999), $pdfr^{5304}$ (Hyun et al., 2005), pdf^{01};*UAS-Pdf* (Renn et al., 1999), pdf^{01};*Pdf-GAL4* (Renn et al., 1999), $pdfr^{5304}$;*UAS-Pdfr* (Hyun et al., 2005), $pdfr^{5304}$;*Clk856-GAL4* (Gummadova et al., 2009), $pdfr^{5304}$;*Pdf-GAL4/Cyo*, $pdfr^{5304}$;*Mai179-GAL4/TM6* (Grima et al., 2004) and *w*;*Clk4.1m-GAL4/TM6* (Zhang et al., 2010b).

The UAS-GAL4 system was used to target specific neurons in the fly brain. It consists of a GAL4 driver line and the UAS-responder line (Brand and Perrimon, 1993). The responder line contains an upstream activator sequence (UAS), which is cloned upstream of an effector gene. The GAL4 sequence, which encodes a transcriptional activator, is inserted at an enhancer site with tissue-specific expression (Brand and Perrimon, 1993). GAL4 binds to the UAS sequence and subsequently activates the transcription of the effector gene in a spatially restricted manner.

Genetic crosses were performed using standard techniques. For the PDF rescue, pdf^{01};*UAS-Pdf* females were crossed with pdf^{01};*Pdf-GAL4* male flies. For controls, the progeny of crosses set between pdf^{01};*UAS-Pdf* females with $w^{+};pdf^{01}$ males and pdf^{01};*Pdf-GAL4* males with $w^{+};pdf^{01}$ females were used. The expression of PDFR in limited neuronal populations was attained by crossing $pdfr^{5304}$;*UAS-Pdfr* females with

males from *pdfr*^{5304};*Clk856-GAL4*, *pdfr*^{5304};*Pdf-GAL4/Cyo*, *pdfr*^{5304};*Mai179-GAL4/TM6* and *w*;*Clk4.1m-GAL4/TM6*. Control strains were obtained by setting up crosses between the UAS- and GAL4- line males with the females of *pdfr*^{5304} mutant flies.

2.3. Behavioral assays

2.3.1. Appetitive olfactory conditioning

Olfactory conditioning experiments used two distinct odor combinations: 1. 4-Methylcyclohexanol (MCH) and 3-Octanol (OCT); 2. Ethyl acetate (ETA) and Isoamyl acetate (IAA). Odors were diluted in the paraffin oil. Appropriate dilutions were obtained by placing naïve flies in a T-maze with two odor streams from the opposite ends. The odor stream was produced with a pump that sucked in the air through the apparatus at a rate of 750 ml/min. Odor concentrations were adjusted such that naive flies distribute equally between the two odors during a choice test. Preference index was calculated as the number of flies choosing an odor divided by the total number of flies tested in the T-maze. Based on these tests following dilutions were selected for conditioning experiments: MCH-1:100, OCT-1:80, ETA-1:200 and IAA-1:100.

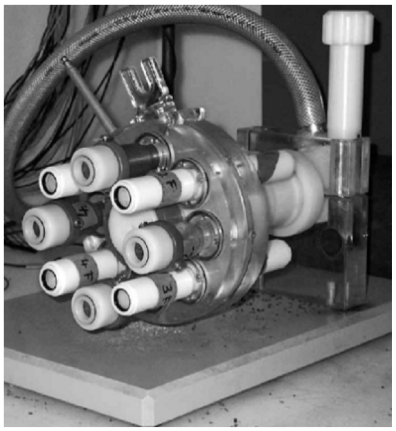

Fig 2.1. The Tully wheel. Modified T-maze apparatus with four independent T-mazes placed on a rotating disc that allows testing of four groups of flies simultaneously (Taken from Masek 2005).

Odors were delivered using 15-mm diameter cups. These cups were attached to the experimental vials during conditioning. A modified version of the T-maze apparatus was used, called Tully-wheel, which consists of 4 distinct T-mazes on a rotating disc (Fig 2.1) (Masek, 2005). This device enabled simultaneous training and testing of four independent groups of flies. All experiments were conducted in a dim red light, which is invisible to flies.

Standard Pavlovian training protocol with sucrose as the reinforcer was performed in the Tully-wheel (Tempel et al., 1983). Experiments were conducted in a control box maintained at 23-24ºC

and 70-80% relative humidity. For reward conditioning, about 150-200 flies, 3-4 days old, were starved for 16-18 h and then fed with sucrose (US) in the presence of odor A (CS^+) for 2 min. A filter paper soaked in the 2M sucrose solution served as the US. After a stream of clean air for 30 secs, flies were presented with a water-soaked and subsequently dried filter paper plus odor B (CS^-) for 2 min. This presentation was followed by another 30 secs stream of the clean air. Memory was tested by presenting flies with both odor A and odor B for 2 min in the T-maze.

The performance index (PI) was evaluated as the number of flies selecting the CS^+ odor minus the number of flies selecting the CS^- odor divided by the total number of flies. A reciprocal experimental design was used to remove the non-associative odor effects. In the reciprocal design, each PI is an average of PIs from trials in which either odor A or odor B served as CS^+.

For reversal learning experiments, 14 h starved flies were first trained in an appetitive conditioning paradigm in the morning (Zeitgeber Time 0 or ZT0). In this training, odor A (CS^+) was presented with the sucrose reward and odor B with a neutral stimulus (CS^-). These flies were then placed back in the no food vials for 6 h. Same flies were then again trained in the afternoon (ZT6) such that now odor B was used as CS^+ and odor A as CS^-. These flies were then immediately tested for the preference between odor A and odor B.

2.3.2. Time-odor learning paradigm

Two groups of 14 h starved flies were trained simultaneously to associate an odor with a reward at a particular time of day. In brief, groups of about 150-200 flies were presented with odor A (CS^+) along with sucrose and odor B without sucrose in the morning (ZT0-ZT3), and odor B (CS^+) with sucrose and odor A without sucrose in the afternoon (ZT6-ZT9) (Fig 2.2). Flies were fed for an hour after the last training of the day to keep them alive. After two cycles of the reversal training over two days, flies were tested on the third day. One group of flies was tested in the morning and the other group in the afternoon in the T-maze apparatus.

The performance index (PI_{test}) was calculated as (**mo**-**af**) / (**mo**+**af**) with **mo** specifying the number of flies preferring the odor used as CS^+ in the **mo**rning and **af** is

the number of flies selecting the odor used as CS^+ in the **af**ternoon. The morning training served as a reference for the performance index evaluation i.e. flies demonstrating the memory corresponding to the morning training will have a positive PI_{test}, while the PI_{test} will be negative for the flies showing the memory related to the afternoon training. The time of day dependent modification in memory scores was assessed using ΔPerformance index (ΔPI). It was evaluated as half of the difference between the morning and the afternoon PI_{test} in simultaneously trained groups of flies.

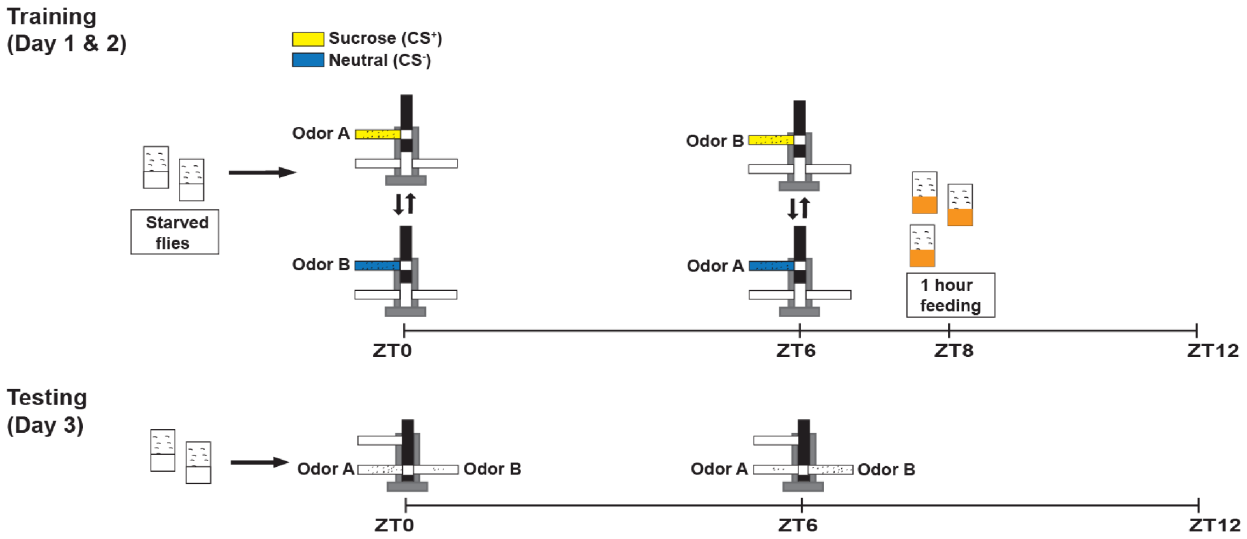

Fig 2.2. The time-odor learning paradigm.
14 h starved flies were trained with reciprocal odor combinations at two different time points, T_1 AM (Morning: ZT0-3) and T_2 PM (Afternoon: ZT6-9), in a day. Flies were fed for one hour after the second training. After two days of training, flies were tested on the third day for the time-odor memory.

2.3.3. Assay to measure the survival rate

To assess the survival rate, approximately 100 male or female flies were placed in the no food vials with only a thin layer of 1% agarose. These flies were then kept under 25ºC and 60% relative humidity. Every 8-12 h the numbers of surviving flies were counted. Flies were transferred to the fresh agarose vials every 24 h to prevent desiccation. The survival rate at each time point was calculated as the number of surviving flies divided by the total number of flies.

2.4. Statistical treatment

All measurements in figures are presented as box plots which represent the range of data, with the midpoint as median and, '+' is the mean. GraphPad Prism 6.0 was used to compare the independent groups of data. Data groups were tested for normality using Kolmogorov-Smirnov test.

Appetitive memories were compared using the student's t-test for two groups or the One-way ANOVA with Bonferroni correction for multiple groups. For time-odor learning, two-way ANOVA was used. The post hoc test compared mean performance indices (PI_{test}) corresponding to the morning and afternoon tests. Student's t-test was used to compare each PI_{test} against zero and the 'p' value for significant memories is reported in the respective figure legend. The statistical significance is demonstrated as (***) $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$; n.s. $p > 0.05$.

3. RESULTS

3.1 Appetitive conditioning at different times of day

Animals can automatically tag the time of day information to significant biological events (Gallistel, 1990). The importance of such time-tagging is supported by the periodic memory retention shown in different paradigms (Wansley and Holloway, 1975; Chaudhury and Colwell, 2002; Ralph et al., 2002; Cain et al., 2004; Cain et al., 2008; Valentinuzzi et al., 2008). I first asked whether flies are also able to use the time of day information to optimize their performance in an appetitive conditioning paradigm.

3.1.1. Long-term appetitive memory in flies

In flies, reward memories were first demonstrated by Tempel et al. (1983) using a T-maze apparatus. I used a modified apparatus, the Tully-Wheel, in which four groups of flies can be trained simultaneously (Schwaerzel et al., 2003; Masek, 2005). 4-Methylcyclohexanol (MCH) and 3-Octanol (OCT) served as odors for conditioning. I first calibrated these odors such that untrained flies distribute equally between MCH and OCT. The odors were diluted using the paraffin oil. 1:100 and 1:80 dilutions were chosen for MCH and OCT respectively. The distribution of naive flies was similar between the two odors at these dilutions (Fig 3.1A).

Following experiments tested flies for the long-term reward memory (LTM) in the Tully-Wheel. Hungry flies were introduced into the apparatus and placed in a tube with sucrose filled filter paper (Reward). Concurrently an odor stream was presented for 2 minutes followed by a second odor with a dry filter paper (Neutral). Memory test involves the introduction of flies at the choice point with simultaneous streams of two odors from the opposite directions. The performance index (PI) was calculated as the mean of sub-trials where either odor A or odor B served as CS^{+}. Wild-type flies show a robust PI when tested after 24 h following the appetitive training, reward (R) v neutral (N), in the Tully-Wheel (Fig 3.1B). The performance of flies when both the odors were presented with either a reward (R v R) or a neutral stimulus (N v N) was significantly

lower compared to R v N experiments (Fig 3.1B). These results suggest that flies can discriminate between odors based on their association with a reward.

Next, wild-type flies were starved for different durations before the appetitive training. After the training flies were returned to the no food vials and then tested after 24 h. Interestingly, PI values and durations of starvation showed a positive correlation (Fig 3.1C). Flies starved for 6 h before the training showed a robust retrieval of LTM but the PI was significantly lower than flies subjected to 18 h food deprivation (Fig 3.1C). Therefore, flies can perform in an appetitive conditioning paradigm even with low starvation periods and thus, can be trained multiple times in a day.

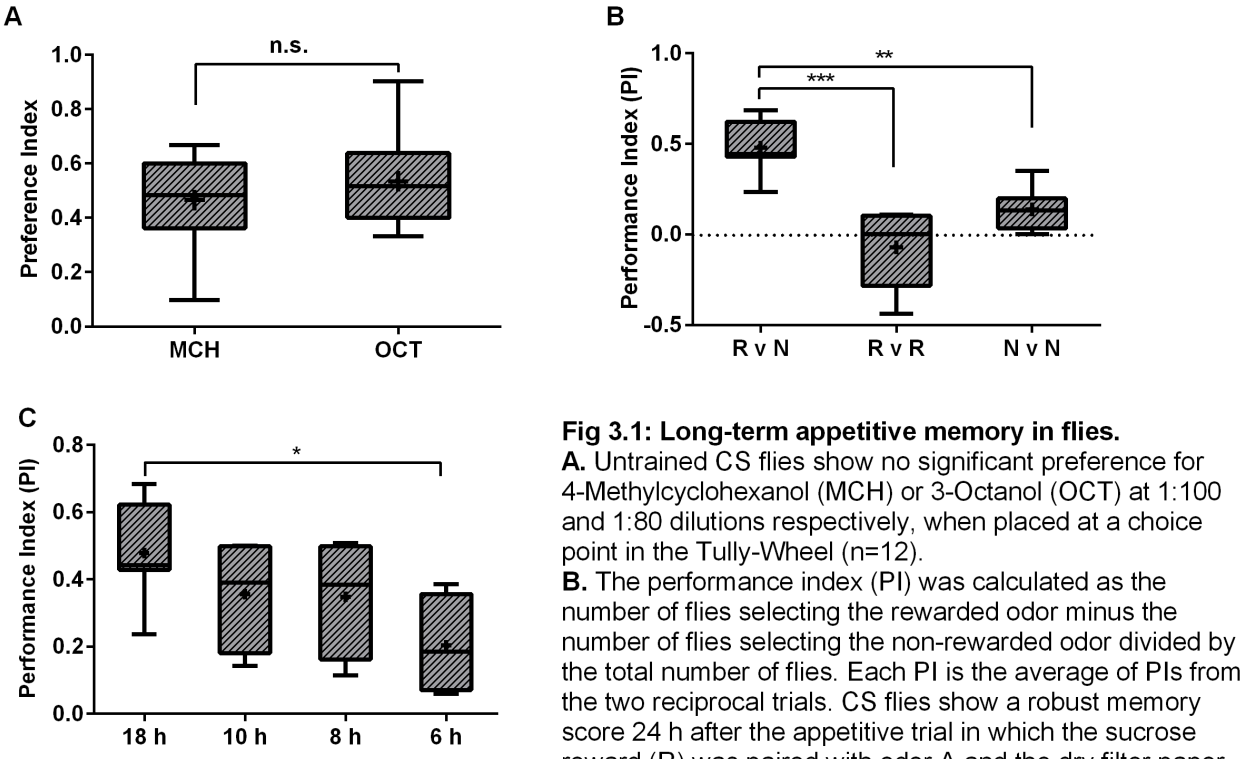

Fig 3.1: Long-term appetitive memory in flies.
A. Untrained CS flies show no significant preference for 4-Methylcyclohexanol (MCH) or 3-Octanol (OCT) at 1:100 and 1:80 dilutions respectively, when placed at a choice point in the Tully-Wheel (n=12).
B. The performance index (PI) was calculated as the number of flies selecting the rewarded odor minus the number of flies selecting the non-rewarded odor divided by the total number of flies. Each PI is the average of PIs from the two reciprocal trials. CS flies show a robust memory score 24 h after the appetitive trial in which the sucrose reward (R) was paired with odor A and the dry filter paper (Neutral-N) with odor B (n=7). Flies that are trained with both odor A and B paired with either a reward (R v R; n=5) or a neutral stimulus (N v N; n=7) show significantly low performance indices (PI).
C. The LTM PI of CS flies is significantly low, although robust when the starvation period before the trial is reduced to 6 h compared to flies that are starved for 18 h before the appetitive training ($n \geq 4$). Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

3.1.2. Influence of the time of day on the appetitive memory expression

The conditions for raising flies were the 12:12 h Light/Dark (LD), in which lights were turned on at ZT0 (Zeitgeber Time 0) and then turned off at ZT12. Flies were trained and tested in an appetitive conditioning paradigm at either ZT0 or ZT6, to assess the effect of the time of day on the memory expression.

16-18 h starved flies were trained in the apparatus at ZT0 to associate an odor with a reward. These flies were then returned to the no food vials and subsequently tested the next day at either ZT0 or ZT6, 6 h from the time of training. Intriguingly, CS flies tested at different time-points showed comparable memory scores (Fig 3.2A). Then flies were trained at ZT6 and tested the next day at either ZT0 or ZT6. PIs of CS flies were similar when tested at different times of day (Fig 3.2B). Therefore, the time of day has no effect on the appetitive memory expression. These results also indicate that flies are unable to tag the time information to every appetitive training.

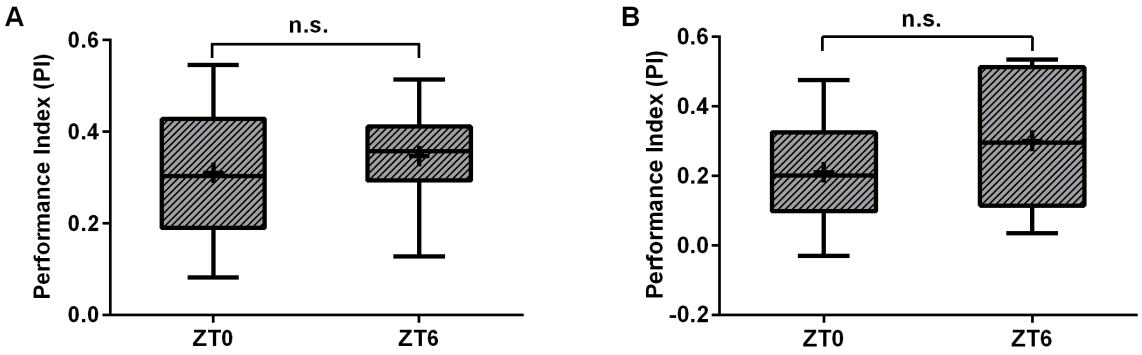

Fig 3.2: The appetitive memory performance is independent of the time of day.
A. Flies are trained at ZT0 (Zeitgeber Time 0; Lights on) and then tested the next day at either ZT0 or ZT6. The memory retrieval is similar at ZT0 and ZT6 (n=6).
B. PI scores are comparable when the flies were trained at ZT6 and then tested the next day at either ZT0 or ZT6 (n=6).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean.

3.1.3. Reversal learning in *Drosophila*

Earlier studies have demonstrated the cognitive flexibility of a brain through reversal learning experiments. Flies can change their preference for an odor based on the reward/punishment association. Previous studies have showed that flies can reverse the odor-shock association after just a single cycle of the reciprocal training (Wu et al.,

2012). Such reversal learning has also been shown with visual patterns in flies (Ren et al., 2012). Following experiments tested whether flies can reverse the odor-reward association in an appetitive training assay.

14 h starved flies were presented with odor A and the sucrose reward for 2-min, followed by 2-min of odor B with a dry filter paper. These experiments were conducted at ZT0 and then flies were returned to the no food vials. A reciprocal experiment was performed at ZT6, in which odor B was presented with sucrose and odor A without sucrose. After the second training flies were introduced at the choice point for the memory test. CS flies demonstrated a robust preference for odor B when tested immediately after the second training (Fig 3.3).

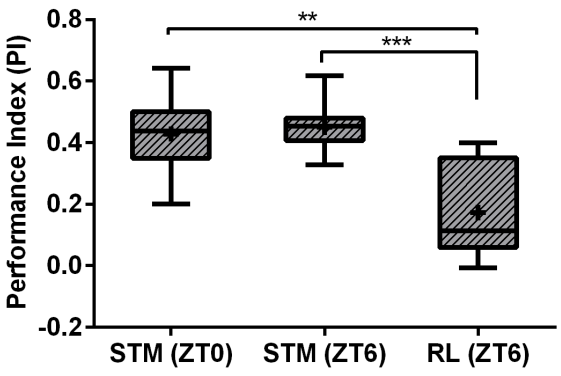

Fig 3.3: Reversal appetitive learning in flies.
Flies were presented with odor A and a reward in the morning (ZT0) and then odor B with a reward at ZT6, followed by immediate tests. CS flies show a robust PI (reversal learning: RL) related to the second training, but the memory score is considerably lower than the flies tested immediately after a single cycle of training (short-term memory: STM). The STM score is comparable between the flies trained and tested at either ZT0 or ZT6 (n=6).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**); $p < 0.001$ (***).

I also tested the short-term memory (STM) in which flies were introduced at the choice point after a single cycle of training. Interestingly, CS flies showed significantly better performance after a single training event (STM) compared to the flies that were subjected to one cycle of the reciprocal training (RL) (Fig 3.3). This difference was independent of the time of testing as STM was comparable between flies conditioned at ZT0 and ZT6 (Fig 3.3). Therefore, flies can reverse the association between a reward and an odor, but the first training is not without consequences as the memory score after reciprocal trials is considerably lower than the STM.

3.1.4. Appetitive long-term memory after a reciprocal training cycle

Following experiments investigated whether flies can use the time of day to discriminate between the two trials of a reciprocal training. First, trials examined the ability of flies to form appetitive long-term memory in the morning and the afternoon. 16-18 h hungry flies were trained at either ZT0 or ZT6 in an appetitive conditioning paradigm. Flies were then placed back in the no food vials and then tested the next day. CS flies showed comparable memory PIs when trained and tested at either ZT0 or ZT6 (Fig 3.4A). This result suggests that flies can form comparable LTM at different times of day.

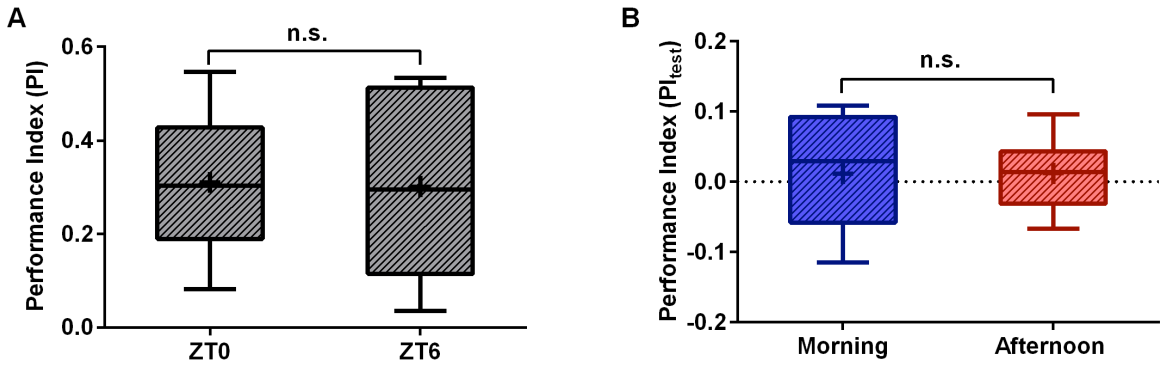

Fig 3.4: One cycle of the time of day related reversal conditioning results in low memory scores.
A. The LTM performance index is similar between the flies trained and tested at either ZT0 or ZT6 (n=6).
B. 14 h hungry flies were trained to associate odor A and odor B with the sucrose reward in the morning (ZT0) and afternoon (ZT6) respectively. The next day tests reveal no significant difference between the morning and afternoon PI_{test} values (n=8).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean.

Next experiments assessed time-odor learning in flies. Flies were starved for 14 h and then trained in the morning (ZT0-ZT3) to associate odor A with sucrose and odor B with a neutral stimulus, and subsequently again in the afternoon (ZT6-ZT9) to relate odor B with a reward and odor A with no reward. After each training flies were returned to the no food vials and were tested the next day in the morning or in the afternoon. Morning trial served as a reference for the PI_{test} evaluation, which leads to a negative value if flies prefer the odor used as CS^{+} in the afternoon. Wild-type flies showed a negligible PI_{test} related to both the morning and afternoon conditioning (Fig 3.4B). These results imply that flies are unable to use the time of day information to discriminate between trials after a single cycle of the reciprocal training.

3.2. Time-odor associations in flies

Prior studies have indicated that animals can associate the time of day with a significant stimulus. Time-place memory has been demonstrated in various animals including rats, mouse, bees and birds (Gould, 1987; Harrison and Breed, 1987; Biebach et al., 1989; Boulos and Logothetis, 1990; Zhang et al., 2006; Van der Zee et al., 2008). In the time-place learning paradigm, an animal is trained at different times of day to associate a reward such as food with both the location and the time of day. After few days of training, animals were able to modify their preference for the location according to the time of day (Zhang et al., 2006; Van der Zee et al., 2008). Previous experiments suggested that flies are unable to associate the time of day with every appetitive memory trial. The next set of experiments examined whether several days of training can induce time-odor learning in flies.

3.2.1. Time of day related appetitive memory in flies

Flies were trained using the reversal conditioning protocol as described before. After the reciprocal trial flies were kept in an empty vial for an hour and then in vials with standard fly food for 1 h feeding. This feeding was introduced to promote the survival of significant numbers of flies, which then can sustain several days of training. The same training procedure was repeated on the second day and then flies were tested on the third day. CS flies tested in the morning preferred odor associated with a reward at that time and then modified their preference in the afternoon towards the opposite odor shown by the negative PI_{test} (Fig 3.5A). The modulation of the odor preference across a day was measured with ΔPerformance Index (ΔPI), calculated as half of the difference between the morning and afternoon PI_{test} in the simultaneously trained groups of flies. A substantial ΔPI score indicates that flies can modulate their odor preference according to the time of day (Fig 3.5B). These results suggest that two cycles of the reciprocal training are sufficient to form time-odor memories in flies.

Reversal training was used to investigate time-odor learning in flies. Following experiments examined whether flies can establish the time of day related associations (TOD) without reciprocal trials. Flies were trained to associate odor A with a reward for

2-min followed by 2-min of no odor (or oil) stream along with a neutral stimulus. The same group of flies was then trained to relate odor B with sucrose in the afternoon. A second group of flies was trained to associate sucrose with odor B in the morning and odor A in the afternoon. Two days of training was followed by the third day tests with odor A and odor B in the T-maze. Each PI score was calculated as the average of the odor preference in these two groups of flies.

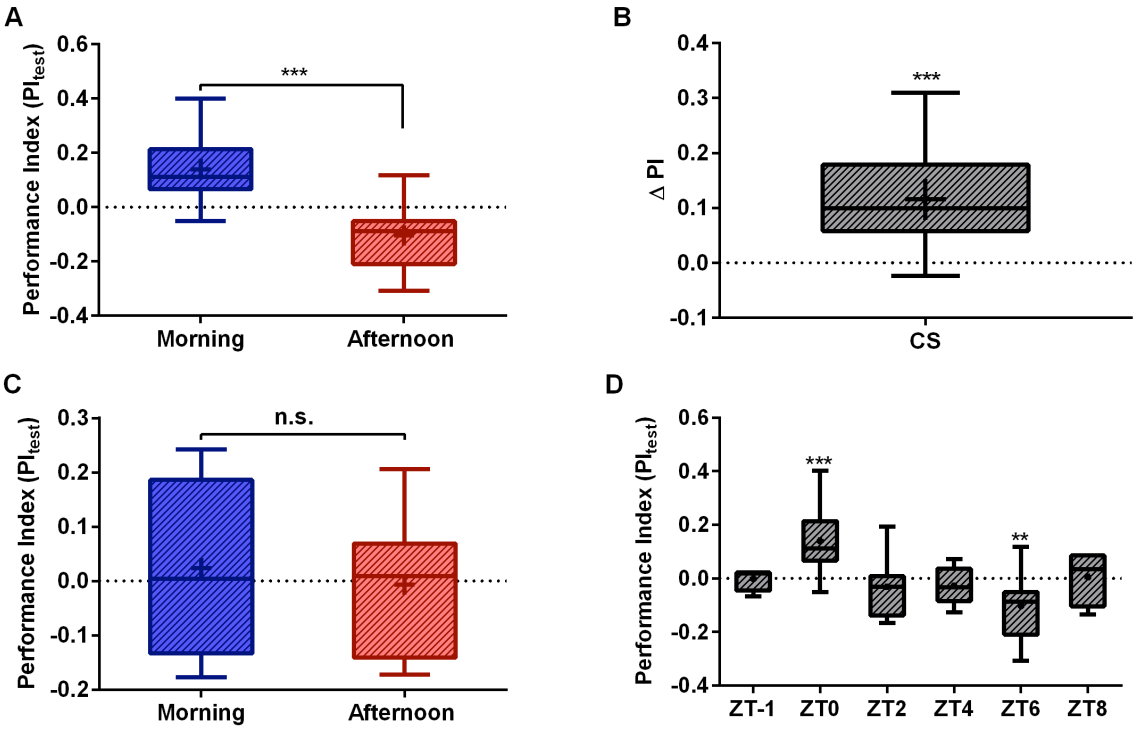

Fig 3.5: Two cycles of the reciprocal training facilitate time-odor associations.
A. Flies trained for two days with the reciprocal conditioning protocol show robust TOD related memory scores on the third day tests. PIs are calculated with respect to the rewarded odor in the morning and therefore, the morning PI_{test} is positive and the afternoon PI_{test} value negative (PI_{test} = [(mo-af) / (mo+af) with mo indicating the number of flies choosing the odor used as CS^+ in the morning and af the number of flies choosing the odor used as CS^+ in the afternoon) (n=16). Comparison of PI_{test} against zero, Morning ($p<0.001$); Afternoon ($p=0.0037$).
B. The ΔPerformance index (ΔPI) assesses the modification in the appetitive memory retrieval across the time of day. It is calculated as half of the difference between the morning and afternoon PI_{test} in concurrently trained groups of flies. CS flies show strong ΔPI score after two cycles of time-odor conditioning (n=16).
C. PI_{test} is not significant in both the morning and afternoon tests when flies are trained for two cycles with a non-reversal conditioning protocol (n=8).
D. Flies trained at ZT0 and ZT6 to associate an odor with a reward show a significant time-odor memory only when tested at ZT0 and ZT6 but not at other time points ($n \geq 4$).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**); $p < 0.001$ (***).

CS flies show no change in their preference for odors when tested in the morning and afternoon (Fig 3.5C). These results suggest that the reciprocal training is essential for establishing time-odor memories.

Following experiments examined the relative change in the performance of flies across the day after time-odor conditioning. Flies were trained with two cycles of the reciprocal training at ZT0 and ZT6 and then tested at different time points on the third day. Interestingly, flies only showed a significant memory retrieval at ZT0 and ZT6, but not when tested at other time-points (Fig 3.5D). This result suggests that the time-odor memory retrieval in flies is very precise with respect to the time of training. Another reason can be low memory scores at ZT0 and ZT6, which are significant but make the comparison of PI values between time points difficult.

3.2.2. Time-odor memory with a distinct odor combination

The time-odor memory in flies was shown using MCH and OCT. To assess whether such memory modulation is specific for the MCH-OCT combination, two new odors, Ethyl acetate (ETA) and Isoamyl acetate (IAA), were used. First, the paraffin oil was used to prepare odor dilutions for the calibration. 1:100 and 1:200 concentrations for IAA and ETA respectively showed an equal distribution of untrained flies in the T-maze apparatus (Fig 3.6A). Appetitive LTM was then examined in flies using IAA and ETA. Wild-type flies showed a significant memory PI comparable to the flies trained with MCH and OCT (Fig 3.6B). In the time-odor memory paradigm, flies trained with ETA and IAA demonstrated robust appetitive memories related to the ZT0 and ZT6 training sessions (Fig 3.6C). These results suggest that flies can form time-odor memories more generally with different odor combinations.

3.2.3. Time-odor learning with distinct time-gaps

In previous experiments flies were trained first in the morning (ZT0-ZT3) and then in the afternoon (ZT6-ZT9) with a time-gap of 6 h between trials. Time-place memory experiments from rats and bees showed that they can differentiate between multiple

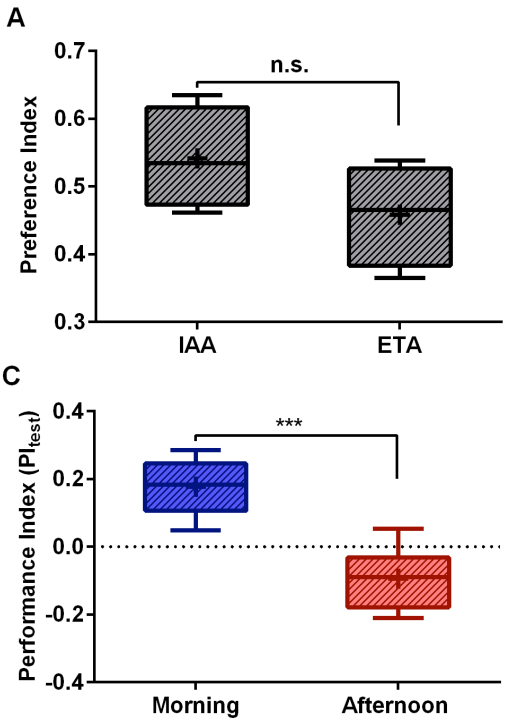

Fig 3.6: Time-odor learning with Ethyl acetate (ETA) and Isoamyl acetate (IAA).
A. No considerable preference is observed in untrained flies between ETA (1:100 dilution) and IAA (1:200 dilution) when presented at the choice point in the T-maze (n=4).
B. CS flies establish a robust appetitive LTM when tested using IAA/ETA, which is comparable to the flies examined with the MCH/OCT odor combination (n=6).
C. Flies show robust TOD related appetitive memories when trained using the IAA/ETA odorcombination in the time-odor conditioning paradigm (n=6).
Comparison of PI_{test} against zero, Morning (p=0.0097); Afternoon (p=0.05457).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.001$ (***).

trials that occur at an interval of 3 h (Zhang et al., 2006; Van der Zee et al., 2008). Also, the sun-compass orientation studies suggest that the time information might be available continuously to compensate for the changing position of the sun across a day (Beling, 1929; Kramer, 1950; Reppert, 2007).

Following experiments examined the ability of flies to form TOD related memories with different intervals between reciprocal trials. CS flies trained at ZT0-ZT3 and then at ZT8-ZT11, a gap of 8h between trials, showed a strong retrieval of TOD related memories, which was comparable to the flies trained with a 6 h gap (Fig 3.7A). Next, flies were trained with a 4 h time-gap, the initial trial at ZT0-ZT3 and the reciprocal trial at ZT4-ZT7. In contrast to 6 h or 8 h gaps, flies trained with a 4 h interval failed to show a significant appetitive memory PI_{test} related to both the morning and afternoon training sessions (Fig 3.7A). Also, CS flies trained with a 4 h break showed significantly lower ΔPI compared to those with 6h or 8 h time-gaps (Fig 3.7B).

The number of training sequences was increased to investigate whether the ability of flies to remember the reciprocal training cycles that occur at a 4 h gap

improves. Flies were trained with three cycles of reciprocal trials with a 4 h interval and then tested. CS flies show no improvement in the TOD related memory expression when tested in the morning and afternoon (Fig 3.7A). The **Δ**PI score was also low compared to the CS flies tested with a 6 h interval (Fig 3.7B). These results suggest that extending the conditioning cycles is not sufficient to establish time-odor memories with a lower time-gap between trials. Therefore, flies can only remember the appropriate odor at a given time of day when the interval between reciprocal trials is at least 6 h.

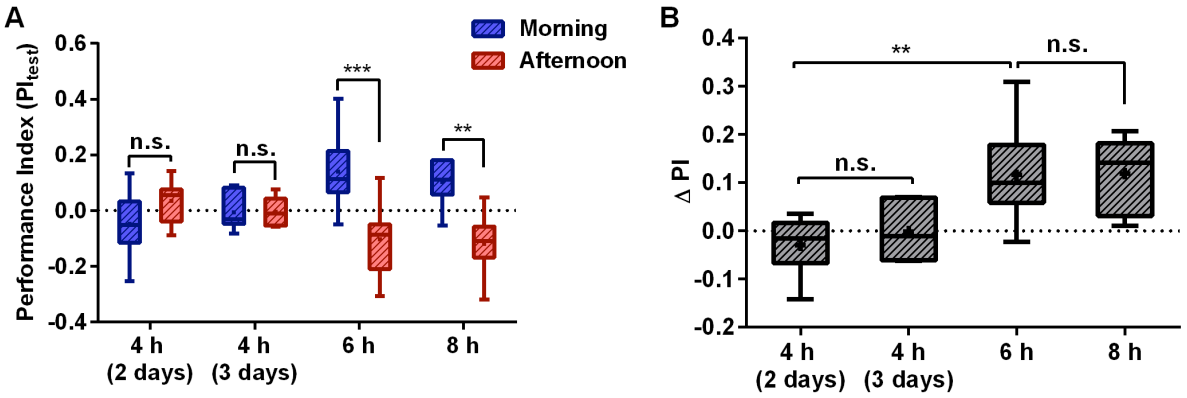

Fig 3.7: Different interval durations affect time-odor learning in flies.
A. Flies trained in the time-odor memory paradigm with either a 6 h (n=16) or a 8 h (n=6) interval between the reciprocal training demonstrate a significant memory score corresponding to both the morning and the afternoon conditioning. In contrast, a 4 h (n=8) break between two cycles of the training resulted in considerably lower PI_{test} values. Training flies with three cycles, instead of two, of the reciprocal training with a gap of 4 h do not improve the TOD related memory expression (n=7). Comparison of PI_{test} against zero for 8 h interval trial, Morning (p=0.0319); Afternoon (p=0.0159).
B. ΔPI is significantly better in flies trained with the 6h (n=16) and 8 h (n=7) intervals compared to those with a 4 h time-gap between the reciprocal training, with either two (n=8) or three (n=7) cycle of conditioning.
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**); $p < 0.001$ (***).

3.3. Internal state influences the time-odor memory formation

The hunger state can motivate an animal to perform multiple behaviors to procure food. Prior studies have shown that only starved Wistar rats, but not satiated ones, were able to associate the time with the location of the platform in a Morris water maze setup (Lukoyanov et al., 2002). The state of hunger is thought to increase the response cost

associated with a wrong choice in a memory test and thus, facilitates the TOD related memory formation (Widman et al., 2000; Widman et al., 2004). Flies were able to retrieve time-odor memories only after two cycles of the reciprocal training but not one cycle. This discrepancy might be due to the internal state of flies. Hence, following experiments examined the role of starvation in forming time-odor memories.

3.3.1. Starvation affects the survival rate in males and females differently

The resistance to starvation was assessed by placing about 100 male or female flies separately in the no food vials. A thin layer of 1% agarose was introduced in the vials to prevent desiccation. Survival rate was measured at each time point by counting the number of alive flies every 8-12 h and dividing by the total number of flies. Intriguingly, the CS female flies showed significantly better resistance to starvation compared to the male flies (Fig 3.8). These results indicate that the male CS flies are under more stress, shown by the lower survival rate, in response to food deprivation than the female flies.

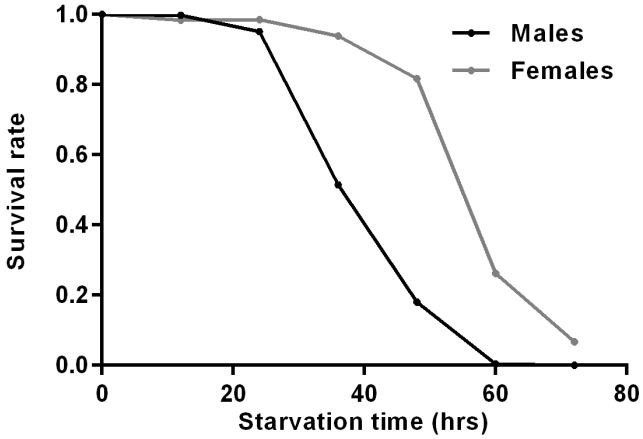

Fig 3.8: Flies show sexual dimorphism in the resistance to starvation.
Flies were kept in the no food vials, and the numbers of surviving flies were counted every 8-12 h. Survival rate evaluated as the number of surviving flies divided by the total number of flies, is significantly better in females compared to the male CS flies (n=8).

3.3.2. Prolonged starvation promotes time-odor associations

Time-odor learning was then investigated in the male and female CS flies separately. Flies were trained with two cycles of the reciprocal training and then tested on the third day morning. A significant number of flies were dead on the third day which precluded afternoon tests. The wild-type male flies showed a robust PI_{test} related to the morning presentation of odor/reward (Fig 3.9A). In contrast, the female flies were unable to show

a significant TOD related memory expression (Fig 3.9A). Also, the LTM was comparable between the male and female CS flies that were trained and tested independently (Fig 3.9B). Male flies are under greater stress due to starvation, which may promote the usage of the time as an associative cue during appetitive conditioning. Consequently, prolonged food deprivation in the female flies may increase the starvation stress and promote time-odor associations.

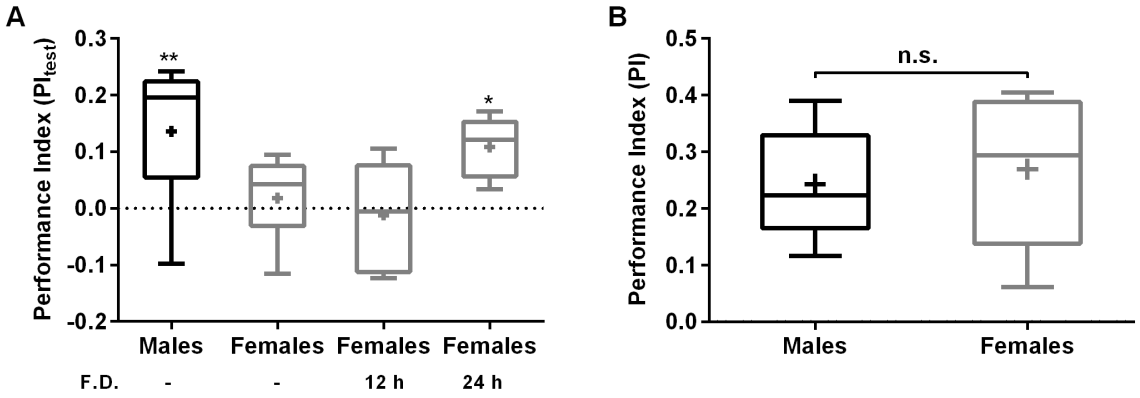

Fig 3.9: Males perform better than females in the time-odor conditioning paradigm.
A. The time-odor memory in the morning test is significant in males (n=9) but not in the female (n=8) CS flies. An extra 24 h starvation before the training improves the retrieval of the appetitive memory related to the morning trial in females (n=6). Comparison of PI_{test} against zero, Males (p=0.007); Females with 24 h extra F.D. (p=0.01). F.D. (Food Deprivation before the training).
B. The appetitive LTM PI is comparable between the male and female CS flies (n=5).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.05$ (*); $p < 0.01$ (**).

The female CS flies were subjected to additional periods of starvation before the training. Flies were starved for 14 h before the first training, and an extra period of starvation was added to it. Therefore, flies starved for additional 12 h and 24 h were famished for a total of 26 h and 38 h respectively, before the first training. The additional 24 h food deprivation led to a strong retrieval of the memory corresponding to the morning appetitive training in females (Fig 3.9A). This outcome suggests that the starvation stress is essential for time-odor associations. A greater stress can decrease the survival rate substantially and thus, increases the response cost associated with a wrong choice in a memory test which preclude food discovery.

Earlier experiments showed that flies were unable to remember time-odor associations after a single cycle of reversal trials. Following trials investigated whether the increase in the starvation stress can promote time-odor associations after a single cycle of the reciprocal training. CS flies that were subjected to an extra 24 h food deprivation before the first training demonstrated significant TOD related appetitive memories (Fig 3.10A). The **Δ**PI score was also robust, but was significantly lower compared to the flies that were trained with two cycles of the reversal training (Fig 3.10B). Therefore, high starvation stress can motivate flies to use the time of day information adaptively. Also, repetitions of conditioning cycles improve time-odor associations. These results further corroborate the correlation between the starvation stress and the response cost based performance in the time-odor learning paradigm.

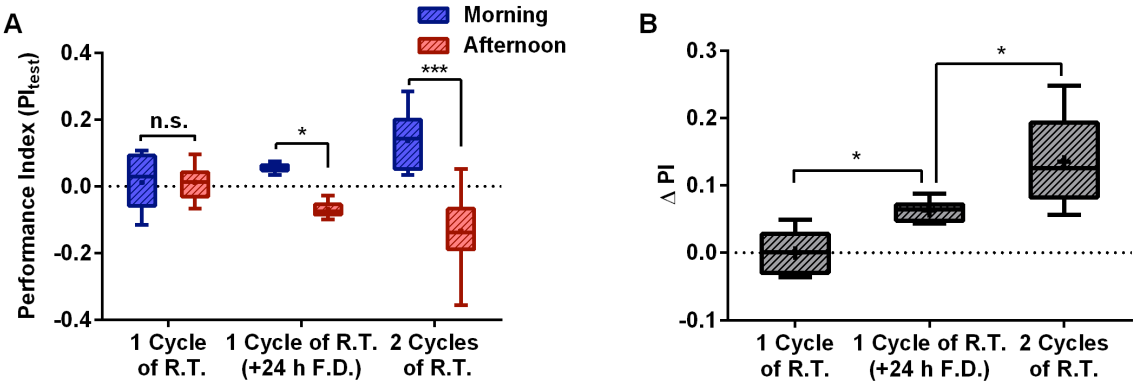

Fig 3.10: Prolonged starvation facilitates the time-odor memory formation.
A. Flies starved for an extra 24 h before the first training show significant TOD related appetitive memories after a single cycle of reversal trials (n=6). Also, two cycles of reciprocal trials result in a considerable improvement in TOD related associations (n=8). Comparison of PI_{test} against zero for 1 cycle R.T. with 24 h extra F.D. trial, Morning ($p<0.001$); Afternoon ($p<0.001$).
B. ΔPI is significantly better after two cycles of reciprocal conditioning compared to the flies that were trained for a single cycle with an added 24 h starvation before the training ($n\geq6$).
R.T. (Reciprocal Training); F.D. (Food Deprivation before training). Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.05$ (*); $p < 0.001$ (***).

3.3.3. Flies need extended starvation to form time-odor memories

Flies were subjected to the extra starvation before the first training, which resulted in robust time-odor associations. These experiments did not highlight the specific role of starvation in the acquisition and the retrieval of time-odor memories. Previous research

on the appetitive LTM suggests that satiated flies are unable to retrieve reward memories (Krashes and Waddell, 2008; Liu et al., 2012).

The role of starvation in the acquisition and the retrieval of appetitive memory was investigated in independent groups of CS flies, which were trained with a single cycle of reward-odor association. Experimental group 1 was starved before the training but then moved to the food vials after the trial for the next day tests. Flies in the Experimental group 2 were kept on the food vials before the conditioning but then transferred to the agar vials with no food after the training and were tested the next day. Another group of flies was kept on the agar vials for all experimental days. They served as the control group. As reported before, Exp. Grp 1 was unable to show a significant LTM PI (Fig 3.11). In contrast, flies that were starved after conditioning (Exp. Grp 2) showed a robust appetitive memory score (Fig 3.11). Interestingly, the PI of Exp. Grp 2 flies was significantly lower than that of the control group (Fig 3.11). These results emphasize the requirement of hunger for the retrieval of appetitive memory.

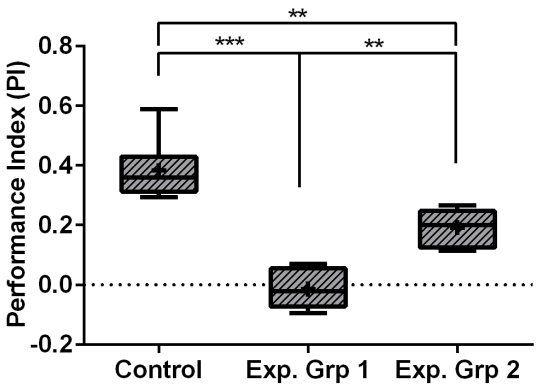

Fig 3.11: Starvation affects the retrieval of appetitive LTM in flies.
Satiated flies (Exp. Grp 1) are unable to show significant appetitive LTM PI. Flies that were starved after the training (Exp. Grp 2) can demonstrate a significant reward memory score, but are considerably compromised compared to the CS flies that are starved throughout the experiment (control) (n=6).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**); $p < 0.001$ (***).

Next, experiments examined the temporal requirement of starvation for time-odor memories. Multiple groups of flies were subjected to different feeding treatments during the training. In Experimental group 1, flies were starved before and during the two reciprocal training cycles but then moved to the food vials after the last training and then tested the next day. Flies of the Experimental group 2 were kept on the food during the morning and afternoon reversal training cycles but then moved to the agar vials after the last conditioning event, followed by the next day tests. The satiated flies of Exp. Grp. 2 were placed on the agar vials for 30 minutes before every training to induce the sucrose

ingestion. Another group of flies was starved for all the days of the experiment served as the control group. Flies that were satiated before tests (Exp. Grp 1) performed as well as the control group in establishing robust time-odor memories (Fig 3.12A). In contrast, flies that were starved only before tests (Exp. Grp 2) were unable to form time-odor memories (Fig 3.12A). Also, the **Δ**PI score of the Exp. Grp 1 flies, but not the Exp. Grp 2 flies, was robust and comparable to that of the control group (Fig 3.12B). Therefore, unlike appetitive LTM, starvation promotes the acquisition but is dispensable for the retrieval of time-odor memories.

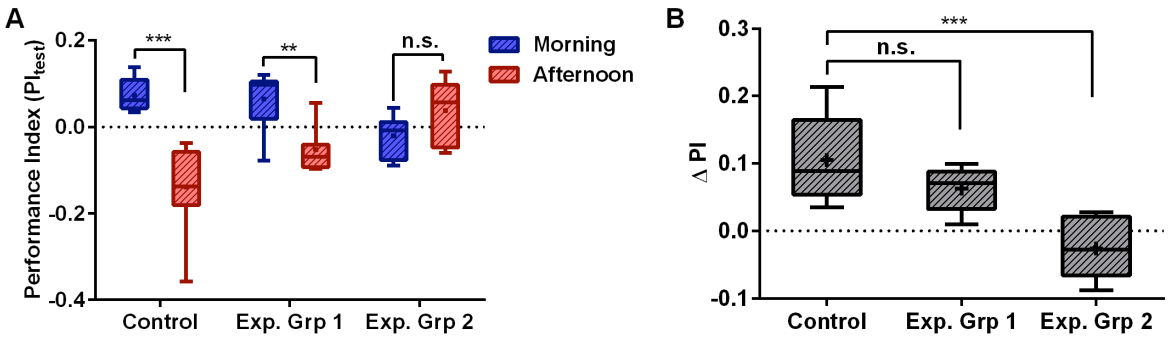

Fig 3.12: Food deprivation is dispensable for the retrieval of time-odor memories.
A. Flies starved before the training (Exp. Grp 1; n=7), but then placed on the food vials after the last conditioning event, can show robust TOD related appetitive memories. In contrast, flies that were starved only after the last training event (Exp. Grp 2; n=6) are unable to show significant time-odor memories. Control flies are food deprived for all the three days of the experiment (n=8). Comparison of PI_{test} against zero for Exp. Grp 1, Morning (p=0.0325); Afternoon (p=0.0376).
B. Exp. Grp 2, but not Exp. Grp. 1, flies displayed significantly lower ΔPI score compared to the control group (n≥6).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**); $p < 0.001$ (***).

3.4. Mechanism underlying time-odor learning in flies

For the ability of animals to form TOD related memories three different mechanisms have been proposed: ordinal timer, interval timer and circadian timer (Mulder et al., 2013b). Studies suggest that the circadian mechanism, which depends on an endogenous oscillator, can promote time-place associations in rats and birds (Biebach et al., 1991; Wenger et al., 1991; Mistlberger et al., 1996; Van der Zee et al., 2008; Mulder et al., 2013b). The ordinal timer in which animals learn the sequence of events,

and the interval timer in which animals use an external cue to measure time intervals, can also help organisms to predict the time dependent changes in the relevant stimulus (Van der Zee et al., 2008; Mulder et al., 2013b). The mechanism that mediates time-odor associations in flies was next investigated.

3.4.1. Interval or ordinal timer mechanisms

In the time-odor memory experiments flies can remember the specific odor related to the afternoon training when tested on the third day, even after skipping the morning reference. Therefore, flies do not use the ordinal timer strategy as missing the morning session had no effect on the performance of flies in the afternoon.

The role of the interval timer strategy was investigated by placing flies in different light conditions during the training or testing. Lights on/off can act as an external signal that may help flies to measure time interval between the two reciprocal trials. Therefore, flies were subjected to two cycles of the reversal training in the Light:Dark (LD) cycle and then moved into the constant darkness (Dark:Dark or DD) before tests on the third day. In these trials flies no longer have the lights 'on' cue on the third day to reset the count for the time interval.

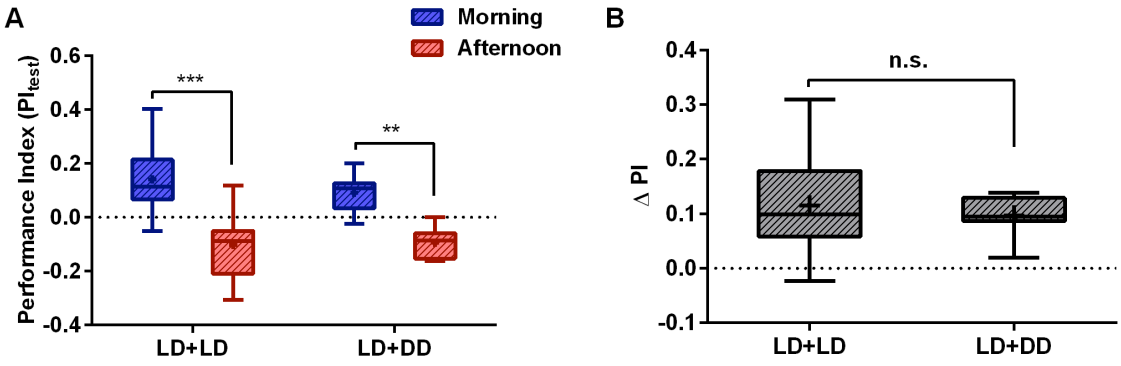

Fig 3.13: LD transitions are not necessary for the time-odor memory retrieval.
A. CS flies kept in LD settings during the training and then transferred to DD cycles before the testing, LD+DD (n=8), demonstrate robust TOD related appetitive memories comparable to the flies trained and tested in LD settings (LD+LD; n=16). Comparison of PI_{test} against zero for trials in LD+DD, Morning ($p<0.001$); Afternoon ($p<0.001$).
B. ΔPI values are comparable between CS flies trained in LD+DD (n=8) and LD+LD (n=16) conditions.
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**); $p < 0.001$ (***).

CS flies trained under LD+DD settings displayed a robust memory score corresponding to both the morning and afternoon trials (Fig 3.13A). The **Δ**PI score was also comparable to the flies that remained in LD settings during tests (Fig 3.13B). Therefore, flies do not use the interval timer mechanism to remember time-odor associations as the lack of lights 'on' signal had no effect on the TOD related memory retrieval.

3.4.2. The circadian oscillator mechanism

Following investigations examined the role of an internal oscillator in forming TOD related memories. Flies kept under constant darkness show free running rhythms that are roughly 24 h and require a functional endogenous oscillator.

Flies were trained with two cycles of the reciprocal training while in DD and then tested on the third day. Interestingly, CS flies showed a strong PI_{test} value in both the morning and afternoon tests (Fig 3.14A). Also, flies showed a robust **Δ**PI score that was comparable to the flies tested in LD settings (Fig 3.14B). These outcomes indicate a role for the clock based mechanism in establishing time-odor memories in flies.

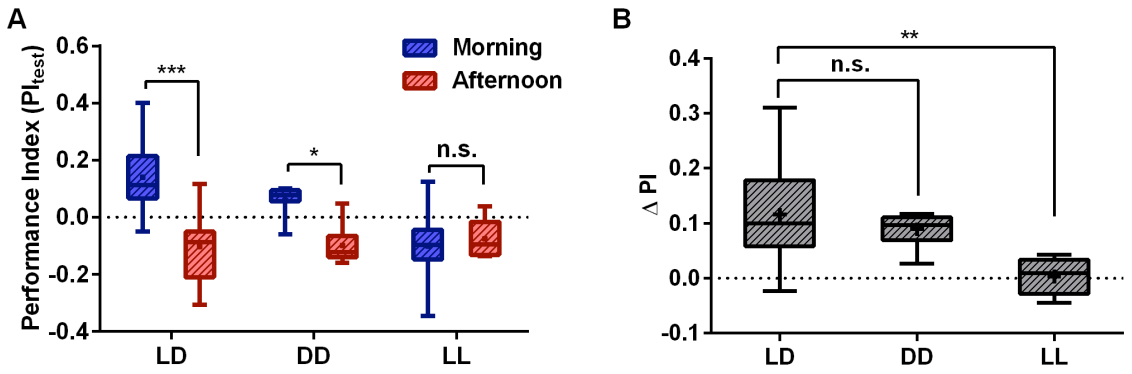

Fig 3.14: Circadian rhythms are essential for the time-odor memory formation.
A. Flies trained and tested in constant dark conditions (DD; n=7) show significant time-odor associations. In contrast, flies kept under constant light settings for the whole experiment (LL; n=7), can only retrieve the memory corresponding to the afternoon training in both the tests. Comparison of PI_{test} against zero for trials in DD, Morning (p=0.029); Afternoon (p=0.0043). For trials in LL, Morning (p=0.0375); Afternoon (p=0.0206).
B. Flies in constant darkness (DD; n=7), but not in constant light (LL; n=7), show comparable ΔPI as flies kept under LD cycles (n=16).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Circadian rhythms respond primarily to the Light:Dark cycle. Constant light (LL) settings cause a dampening of the circadian rhythms, which eventually leads to arrhythmicity in flies (Konopka et al., 1989; Matsumoto et al., 1994). Flies trained in the time-odor learning paradigm while in LL conditions were unable to retrieve the reward memory corresponding to the relevant time of day (Fig 3.14A). Interestingly, flies showed a significant preference for the odor associated with sucrose in the afternoon in both the memory tests (Fig 3.14A). Also, flies in LL settings showed a significantly lower **Δ**PI compared to flies in LD conditions (Fig 3.14B). These outcomes suggest that the arrhythmic flies fail to use the time of day information adaptively. Therefore, flies rely on an endogenous oscillator to perform in the time-odor memory paradigm.

3.4.3. Role of circadian rhythms in forming and recalling time-odor memories

The *Drosophila* clock directed circadian rhythms can be phase shifted by a brief exposure to a high-intensity light pulse. Light exposure in the night leads to the degradation of the TIM protein that either advances or delays the phase of the *Drosophila* clock (Suri et al., 1998; Yang et al., 1998; Koh et al., 2006; Tataroglu and Emery, 2014). This effect manifests as an advanced or a delayed onset of activity on the next day. The time-odor memory retrieval in flies is very precise and thus, phase shifting the clock may affect the fly's ability to remember the time of day.

Wild-type flies were trained with two reversal training cycles while in the LD cycle. After the training CS flies were subjected to a 2000 lx light pulse for 60 minutes at either ZT15 (Experimental group 1) or ZT21 (Experimental group 2), then kept in a dark chamber and subsequently tested the next day morning. CS flies showed a significant PI_{test} related to the morning training, which was comparable to the flies exposed to no light pulse before the test (Fig 3.15). Also, the time of delivery of the light pulse (ZT15 or ZT21) had no influence on the memory score as PI_{test} was comparable between the Exp. Grp. 1 and Exp. Grp. 2 flies (Fig 3.15). These results suggest that time-shifting the clock has no effect on the time-odor memory retrieval.

In the above experiment, flies were tested the next day and thus, it is possible that the exposure to the light pulse may not have affected the phase of the clock

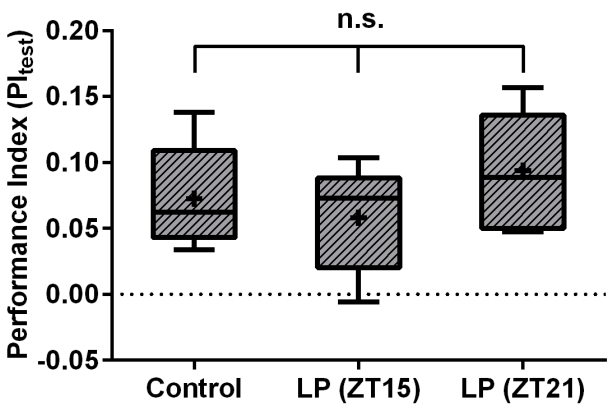

Fig 3.15: Phase shifts have no effect on the time-odor memory retrieval.
CS flies were trained for two days in the time-odor conditioning paradigm and then subjected to the 2000lx light pulse (LP) for 60 min at either ZT15 (n=5) or ZT21 (n=6). Flies were kept in the dark after the light exposure and then tested at ZT0. CS flies show robust PI in the morning test comparable to flies that were not exposed to the light pulse (Control; n=8). Comparison of PI_{test} against zero, ZT15-LP (p=0.0338); ZT21-LP (p=0.0032). Each box plot represents the range of data, with the midpoint as median, and '+' is the mean.

substantially. Therefore, to assess the requirement of functional rhythms for the retrieval of time-odor memories, flies were trained in LD but then moved to the high-intensity (2000 lx) light condition (LL) after the last conditioning, and subsequently tested the next day (Experimental group 1). Another independent group of flies was kept in the constant light during the second round of reciprocal trials but then moved to LD before tests (Experimental group 2).

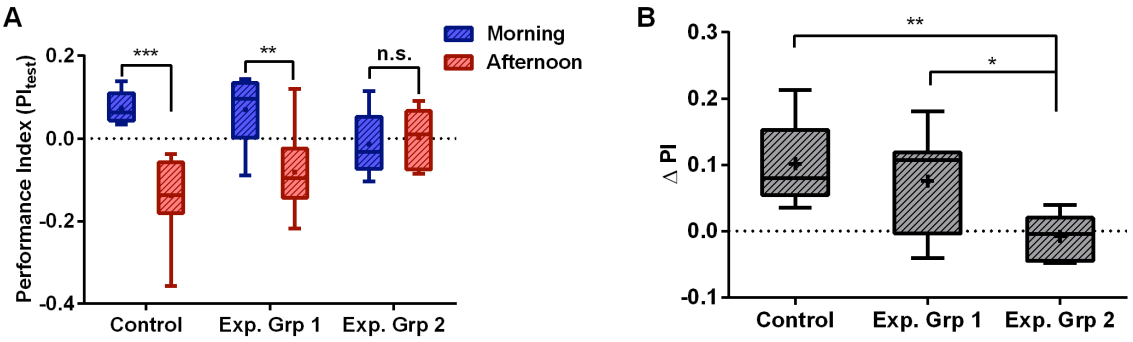

Fig 3.16: Manipulating the *Drosophila* clock does not affect the time-odor memory retrieval.
A. Flies were trained under the LD cycle but then moved to high-intensity (2000 lx) light conditions (LL) after the last training and then tested the next day (Exp. grp 1; n=9). Another group of flies were subjected to the constant light during the second cycle of reciprocal trials but then moved to LD settings before tests (Exp. grp 2; n=7). CS flies transferred to LL before the testing demonstrate strong TOD related memories, comparable to flies kept under LD cycles (Controls; n=8). In contrast, flies in the Exp. Grp. 2 show no significant memory expression. Comparison of PI_{test} against zero for Exp. Grp 1, Morning (p=0.0368); Afternoon (p=0.0425).
B. Flies in the Exp. Grp. 2 (n=7) show considerably lower ΔPI value compared to the control group (n=8) and Exp. Grp 1 flies (n=9).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Flies in LD settings for the whole experiment served as the control group. The Exp. Grp 1 flies demonstrated a strong memory retrieval related to both the morning and afternoon training sessions (Fig 3.16A). In contrast, the Exp. Grp 2 flies showed no significant memory expression (Fig 3.16A). **Δ**PI was also comparable between the Exp. Grp 1 and the control group flies, but significantly lower in the Exp. Grp 2 flies (Fig 3.16B). These results underline the need of a functional clock in forming the time and odor-preference relationship and also indicate that the rhythms might be dispensable for the memory recall.

3.5. Role of clock genes in establishing time-odor memories in flies

The *Drosophila* clock network consists of about 150 neurons each of which expresses the canonical clock machinery (Helfrich-Förster, 1995; Kaneko and Hall, 2000; Rieger et al., 2006; Shafer et al., 2006; Dubruille and Emery, 2008). It consists of a negative transcriptional feedback loop. In it the CLK/CYC heterodimeric complex acts as a transcription activator of the genes, *per* and *tim*, which then together inhibit their own transcription by blocking the activity of CLK/CYC (Konopka and Benzer, 1971; Sehgal et al., 1994; Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998; Hardin, 2005; Dubruille and Emery, 2008). The following experiments examined the role of the clock components in forming time-odor associations. The *period* null mutant per^{01} and the *clock* hypomorphic mutant allele clk^{AR} were tested in the time-odor learning paradigm.

3.5.1. Appetitive memory in per^{01} and clk^{AR} mutant flies

per^{01} flies demonstrate impaired long-term memory in the courtship conditioning and aversive memory paradigms (Sakai et al., 2004; Chen et al., 2012). Therefore, initial experiments assessed the memory of clock mutant flies in an appetitive conditioning paradigm. per^{01} and clk^{AR} mutant flies trained to associate sucrose with an odor demonstrated robust appetitive LTM, comparable to CS flies (Fig 3.17A). Next experiments examined reversal learning in clock mutant flies. Both per^{01} and clk^{AR} flies were able to remember the modified odor-reward relationship when tested after the

reciprocal trial (Fig 3.17B). These results suggest that *per*^{01} and *clk*^{AR} flies can form strong appetitive memories.

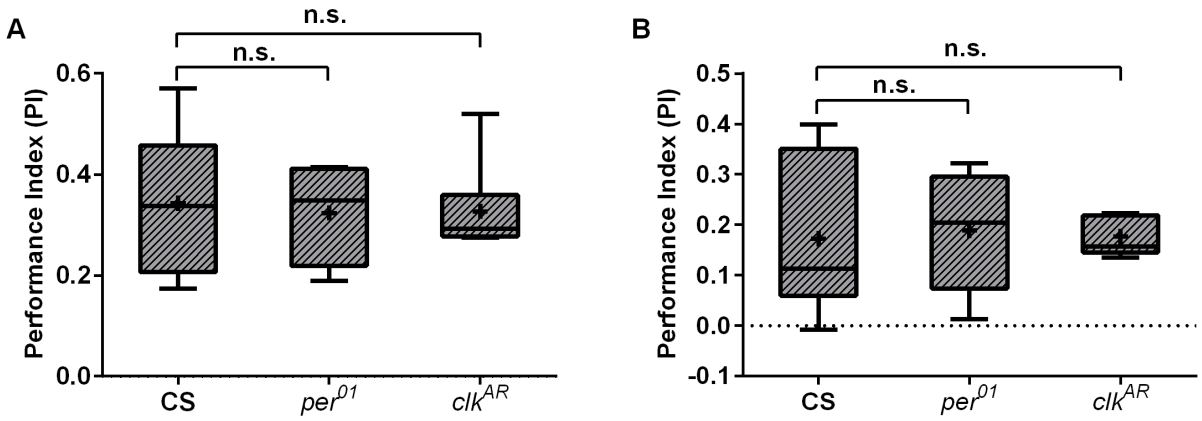

Fig 3.17: Appetitive memory in clock mutant flies.

A. Appetitive LTM PIs are comparable between clock mutant flies, *per*^{01} and *clk*^{AR}, and wild-type CS flies (n=6).

B. *per*^{01} and *clk*^{AR} demonstrate robust reversal learning similar to CS flies (n=6).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean.

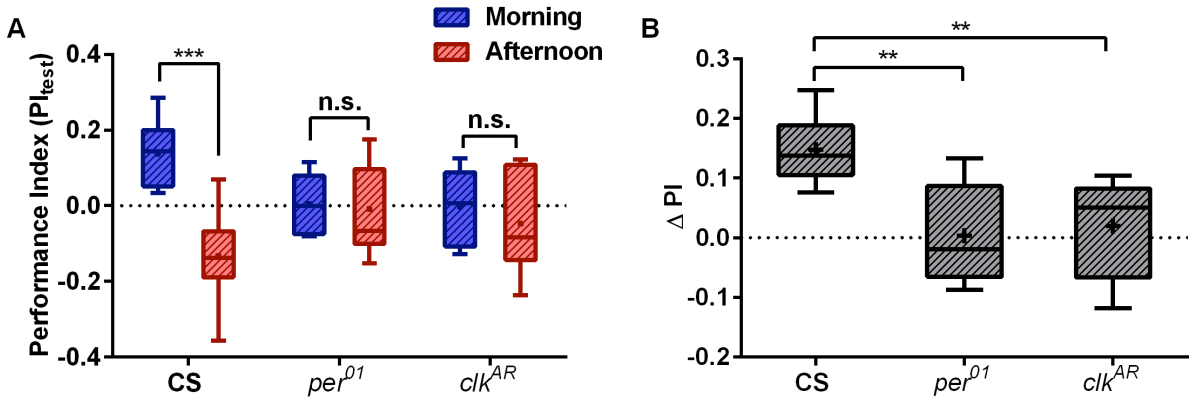

Fig 3.18: Clock mutant flies are unable to show TOD related appetitive memories.

A. *per*^{01} and *clk*^{AR} flies kept in LD conditions are unable to demonstrate significant appetitive memories when trained in the time-odor association paradigm (n=8)

B. ΔPI scores in clock mutant flies are significantly lower compared to wild-type flies (n=8).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**); $p < 0.001$ (***).

3.5.2. Clock mutant flies in LD conditions

per^{01} and *clk*^{AR} in LD conditions are unable to anticipate the daily light and dark transitions due to the compromised clock machinery (Konopka and Benzer, 1971;

Allada et al., 1998; Allada et al., 2003). *per*^{01} and *clk*^{AR} flies in trained LD settings were unable to show TOD related memories (Fig 3.18A). **Δ**PIs were also substantially lower in *per*^{01} and *clk*^{AR} mutant flies compared to CS flies (Fig 3.18B). These outcomes further imply that flies need a functional clock and cannot rely only on light/dark transitions to form time-odor memories.

3.5.3. *per*^{01} and *clk*^{AR} mutant flies in DD conditions

Following experiments examined time-odor learning in clock mutant flies when placed in constant darkness (DD). *per*^{01} and *clk*^{AR} flies in DD become arrhythmic due to impaired clocks (Konopka and Benzer, 1971; Allada et al., 2003). Clock mutant flies were trained with two cycles of the reciprocal training in LD settings but then moved to constant darkness after the last training, and subsequently tested the next day. *per*^{01} and *clk*^{AR} mutant flies in LD+DD settings showed a low PI_{test} in both the morning and afternoon tests, and were also unable to modify their odor preference (Fig 3.19A and B).

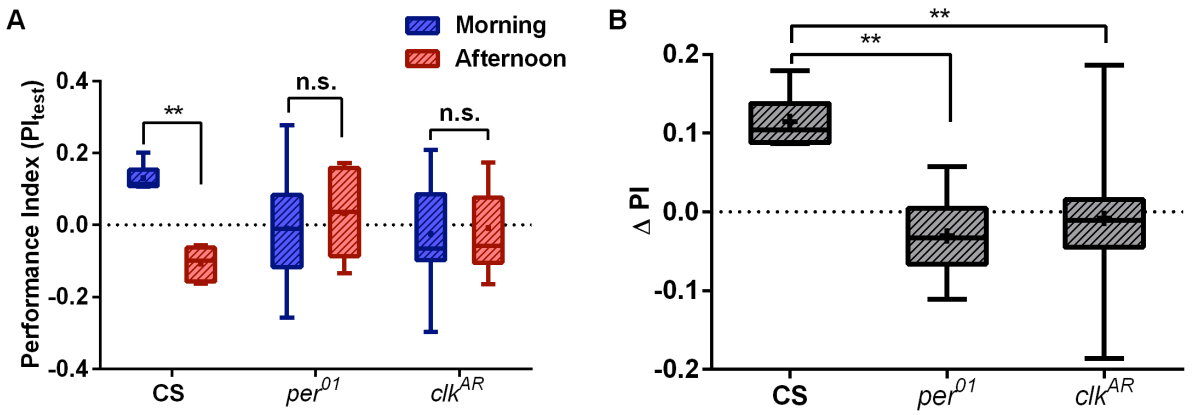

Fig 3.19: Environmental light cues do not affect time-odor associations in clock mutant flies.
A. *per*^{01} and *clk*^{AR} flies trained in LD settings and then moved constant darkness after the last training show no significant TOD related associations (n=6).
B. ΔPI scores are significantly lower in *per*^{01} and *clk*^{AR} flies compared to CS flies (n=6).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**).

per^{01} and *clk*^{AR} trained and tested in DD showed a robust memory score related to only the afternoon conditioning event in both the memory tests (Fig 3.20A). Low **Δ**PI suggests that mutant flies fail to change their odor preference across the day (Fig

3.20B). Therefore, arrhythmic flies fail to take the time of day into consideration during an appetitive reversal training and thus, only show the memory of the last conditioning event. This behavior is similar to CS flies trained in constant light in the time-odor learning paradigm.

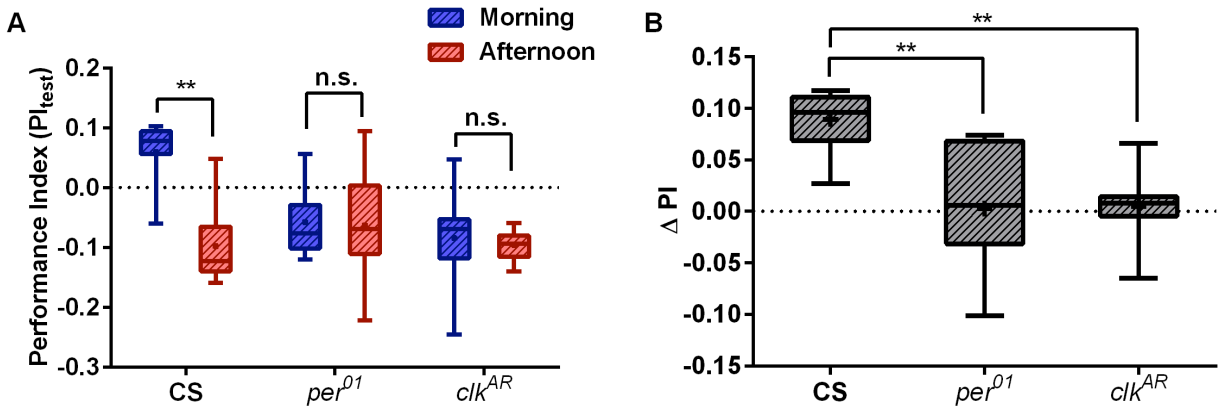

Fig 3.20: Clock mutant flies in DD settings display no TOD related modulation in appetitive memories.

A. *per*^{01} and *clk*^{AR} flies in constant darkness show a robust appetitive memory related to only the last conditioning event in both the morning and afternoon tests (n=7). Comparison of PI_{test} against zero for *per*^{01}, Morning (p=0.015); Afternoon (p=0.038). For *clk*^{AR}, Morning (p=0.006); Afternoon (p<0.001).
B. ΔPI in *per*^{01} and *clk*^{AR}, trained and tested in DD settings, is significantly lower compared to CS flies (n=7).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**).

3.5.4. Time-odor learning in *per*^{01} flies after prolonged starvation

per^{01} and *clk*^{AR} flies failed to perform in the time-odor learning paradigm. The next set of experiments examined whether prolonging the starvation period can affect the time-odor memory formation in mutant flies. *per*^{01} flies showed no significant time-odor memory retrieval even after an additional 24 h starvation before the first training (Fig 3.21A). Also, **Δ**PI score was significantly lower than wild-type flies, but comparable to the *per*^{01} mutant flies trained without an additional period of food deprivation (Fig 3.21B). Therefore, starvation based increase in the response cost is not sufficient to promote TOD related associations without a functional clock.

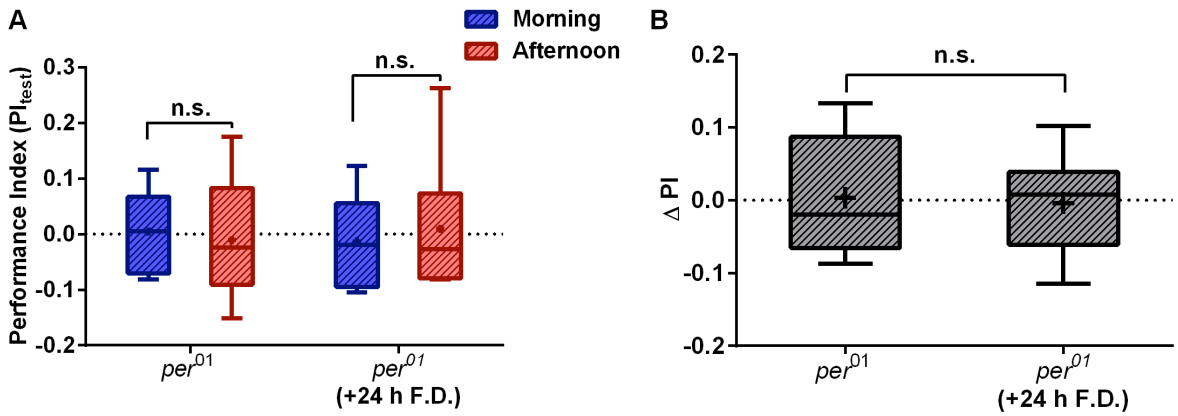

Fig 3.21: *per*^{01} flies are unable to display time-odor memories even after prolonged starvation.

A. The 24 h added starvation before the training failed to improve time-odor associations in *per*^{01} mutant flies compared to the flies with no extra food deprivation (n=6).

B. ΔPI in *per*^{01} flies subjected to an additional starvation period is comparable to *per*^{01} flies with no extra food deprivation before the training (n=6).

F.D. (Food Deprivation before training). Each box plot represents the range of data, with the midpoint as median, and '+' is the mean.

3.6. Role of the clock output in forming time-odor memories

The central question here is how the *Drosophila* clock communicates the time of day information to the mushroom bodies, the site of the appetitive memory storage. Also, the role of the central clock neurons in time-odor associations needs further investigation. One candidate that can be a link is the neuropeptide pigment-dispersing factor (PDF), the member of the family of the β-pigment-dispersing hormones, which is considered as an output factor of the *Drosophila* clock (Park and Hall, 1998; Park et al., 2000; Helfrich-Förster, 2009). The role of PDF in behaviors like locomotion, longer mating duration and geotaxis, had been previously demonstrated (Renn et al., 1999; Mertens et al., 2005; Kim et al., 2013). The following experiments examined the role of the PDF neuropeptide in forming time-odor memories.

3.6.1. Time-odor learning in *pdf* mutant flies

First, experiments assessed the appetitive long-term memory and reversal learning in *pdf*^{01}, *pdf* null mutants. *pdf*^{01} flies showed robust appetitive LTM and reversal learning, which was comparable to CS flies (Fig 3.22A and B). Also, the appetitive memory

across a day was comparable in *pdf*^{01} flies as appetitive memory PI was similar in mutant flies trained and tested at either ZT0 or ZT6 (Fig 3.22C).

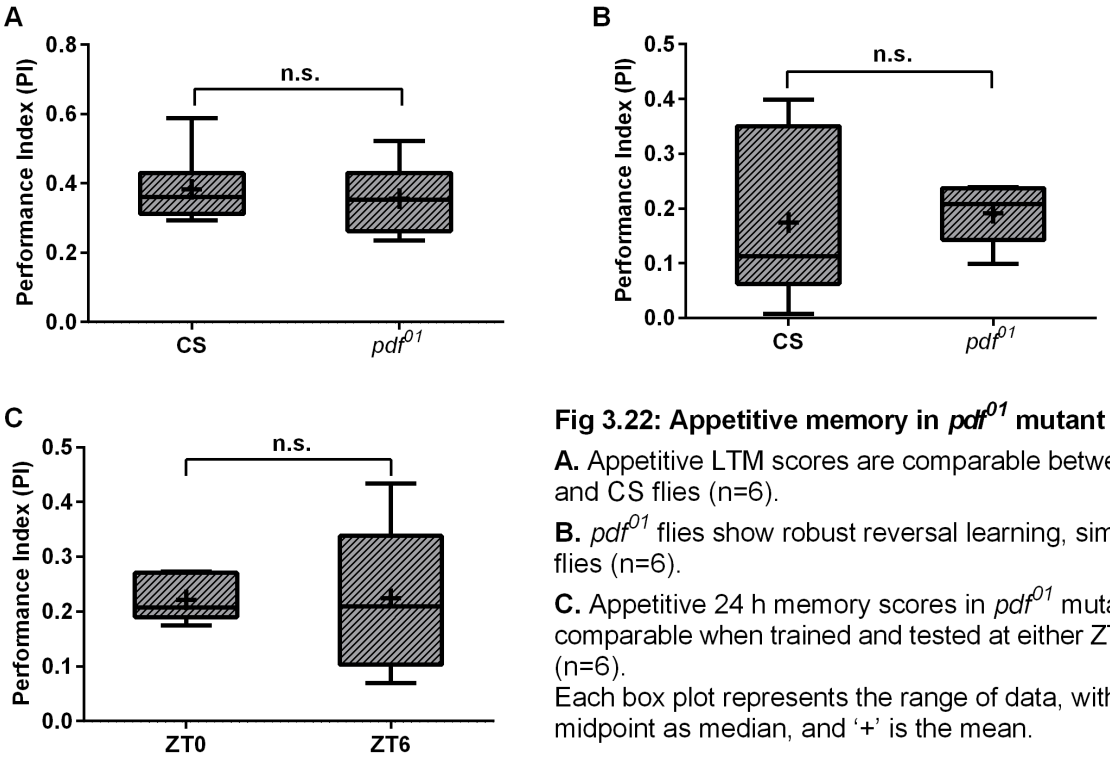

Fig 3.22: Appetitive memory in *pdf*^{01} mutant flies.

A. Appetitive LTM scores are comparable between *pdf*^{01} and CS flies (n=6).

B. *pdf*^{01} flies show robust reversal learning, similar to CS flies (n=6).

C. Appetitive 24 h memory scores in *pdf*^{01} mutant flies are comparable when trained and tested at either ZT0 or ZT6 (n=6).

Each box plot represents the range of data, with the midpoint as median, and '+' is the mean.

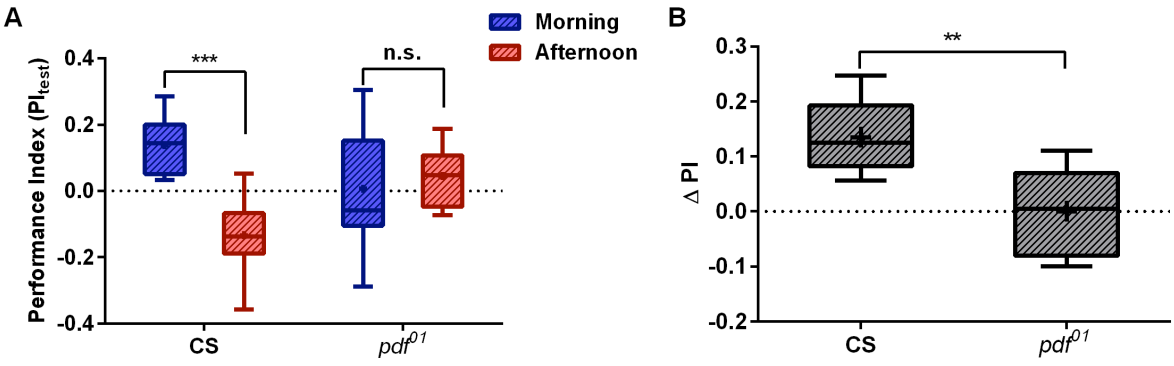

Fig 3.23: *pdf*^{01} mutant flies are unable to perform in the time-odor learning paradigm.

A. *pdf*^{01} mutant flies trained in the time-odor conditioning paradigm display negligible PI_{test} in both the morning and afternoon tests (n=8).

B. Inability to modify the appetitive memory based on the time of day is shown by significantly low ΔPI in *pdf*^{01} flies, compared to wild-type flies (n=8).

Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**); $p < 0.001$ (***).

These outcomes indicate that the *pdf*^{01} mutant flies can establish strong appetitive memories across the time of day.

Next, *pdf*^{01} mutant flies were examined in the time-odor conditioning paradigm. *pdf*^{01} flies showed a low memory score related to both the morning and afternoon training sessions (Fig 3.23A). Also, *pdf*^{01} flies were unable to modify their odor-preference across the day as ΔPI was significantly lower compared to CS flies (Fig 3.23B). These results indicate that the *pdf*^{01} flies are unable to form time-odor memories.

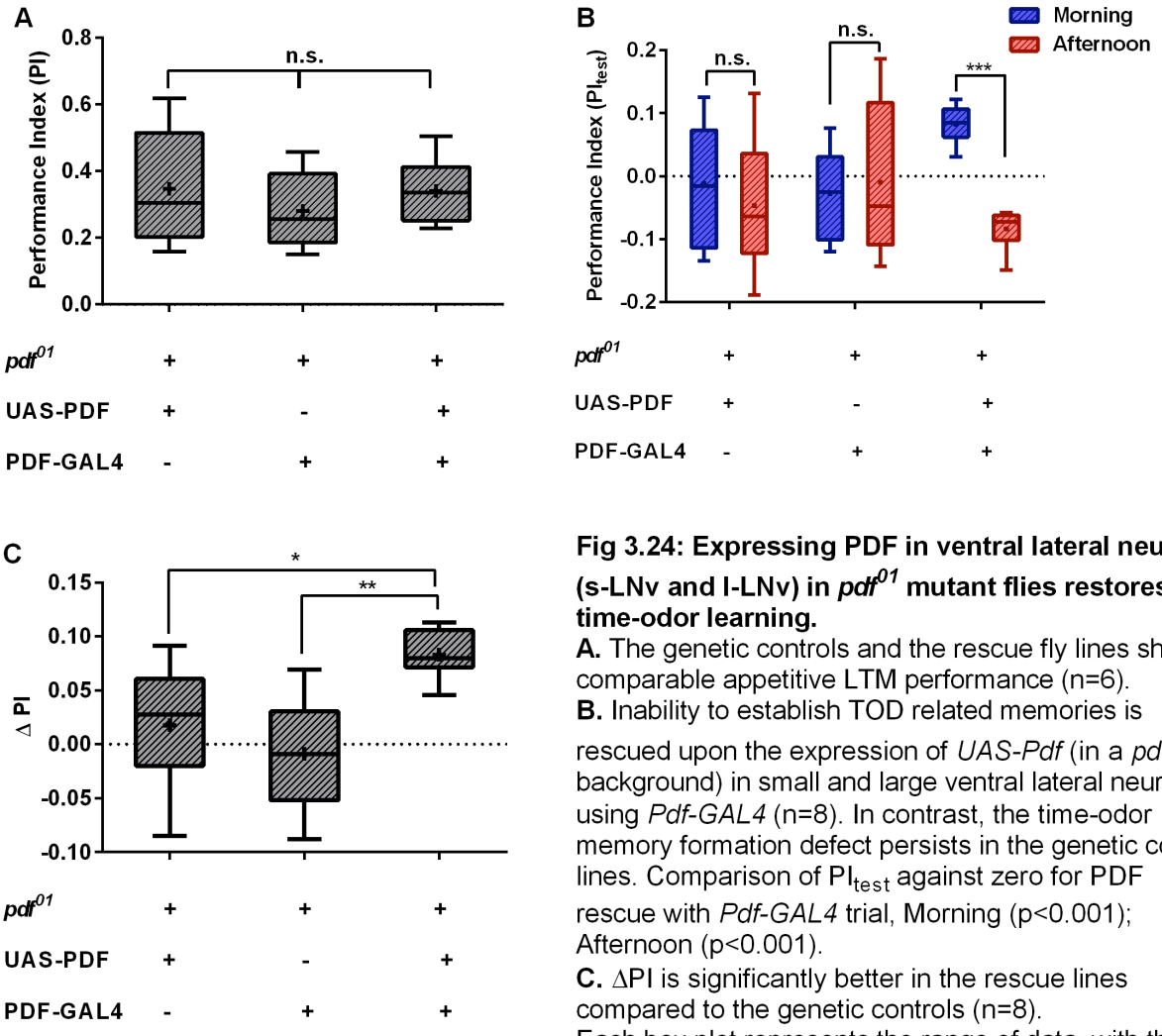

Fig 3.24: Expressing PDF in ventral lateral neurons (s-LNv and l-LNv) in *pdf*^{01} mutant flies restores time-odor learning.
A. The genetic controls and the rescue fly lines show comparable appetitive LTM performance (n=6).
B. Inability to establish TOD related memories is rescued upon the expression of *UAS-Pdf* (in a *pdf*^{01} background) in small and large ventral lateral neurons using *Pdf-GAL4* (n=8). In contrast, the time-odor memory formation defect persists in the genetic control lines. Comparison of PI_{test} against zero for PDF rescue with *Pdf-GAL4* trial, Morning ($p<0.001$); Afternoon ($p<0.001$).
C. ΔPI is significantly better in the rescue lines compared to the genetic controls (n=8).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Eight neurons per brain hemisphere, which include the s-LNvs and l-LNvs, secrete the PDF neuropeptide (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1995). The UAS-GAL4 binary system was used to express the wild-type *pdf* gene in s-LNvs and l-LNvs (*Pdf-GAL4*; (Renn et al., 1999)) neurons in a pdf^{01} mutant background. The appetitive LTM test showed no substantial difference in PIs between the genetic controls and the rescue fly line (Fig 3.24A). Flies were then trained with two cycles of the reciprocal training at different times of day. Expression of the *pdf* gene using *Pdf-GAL4* completely rescued the inability of pdf^{01} mutant flies to form time-odor memories. *Pdf-GAL4* rescue flies showed a substantial appetitive memory PI_{test} in both the morning and afternoon tests (Fig 3.24B). In contrast, pdf^{01};*UAS-Pdf*/+ and pdf^{01}; *Pdf-GAL4*/+ control flies failed to demonstrate significant memory expression (Fig 3.24B). Also, the memory modulation measured as ΔPI was significantly better in the rescue line compared to the genetic controls (Fig 3.24C). These results show that the PDF expression in s- and l-LNvs is sufficient to establish time-odor memories in flies.

3.6.2. PDF receptor signaling is essential for time-odor associations

The PDF neuropeptide activates a seven transmembrane G-protein coupled receptor (Hyun et al., 2005). The PDF receptor gene (*pdfr*) is broadly expressed in the adult fly brain, which includes the clock neurons, pars intercerebralis, ellipsoid body and optic lobes (Hyun et al., 2005; Lear et al., 2009; Im and Taghert, 2010). Flies with the $pdfr^{5304}$ mutant allele, which has all the transmembrane domains eliminated through deletion, were tested (Hyun et al., 2005). $pdfr^{5304}$ mutant flies showed no significant difference in the performance compared to CS flies in an appetitive training paradigm (Fig 3.25A). In the time-odor learning assay the $pdfr^{5304}$ mutant flies displayed a low reward memory PI_{test} in both the morning and afternoon tests (Fig 3.25B). ΔPI was also significantly lower compared to wild-type flies (Fig 3.25C). These results imply that the functional PDF signaling is necessary for the proper usage of the time as an associative cue.

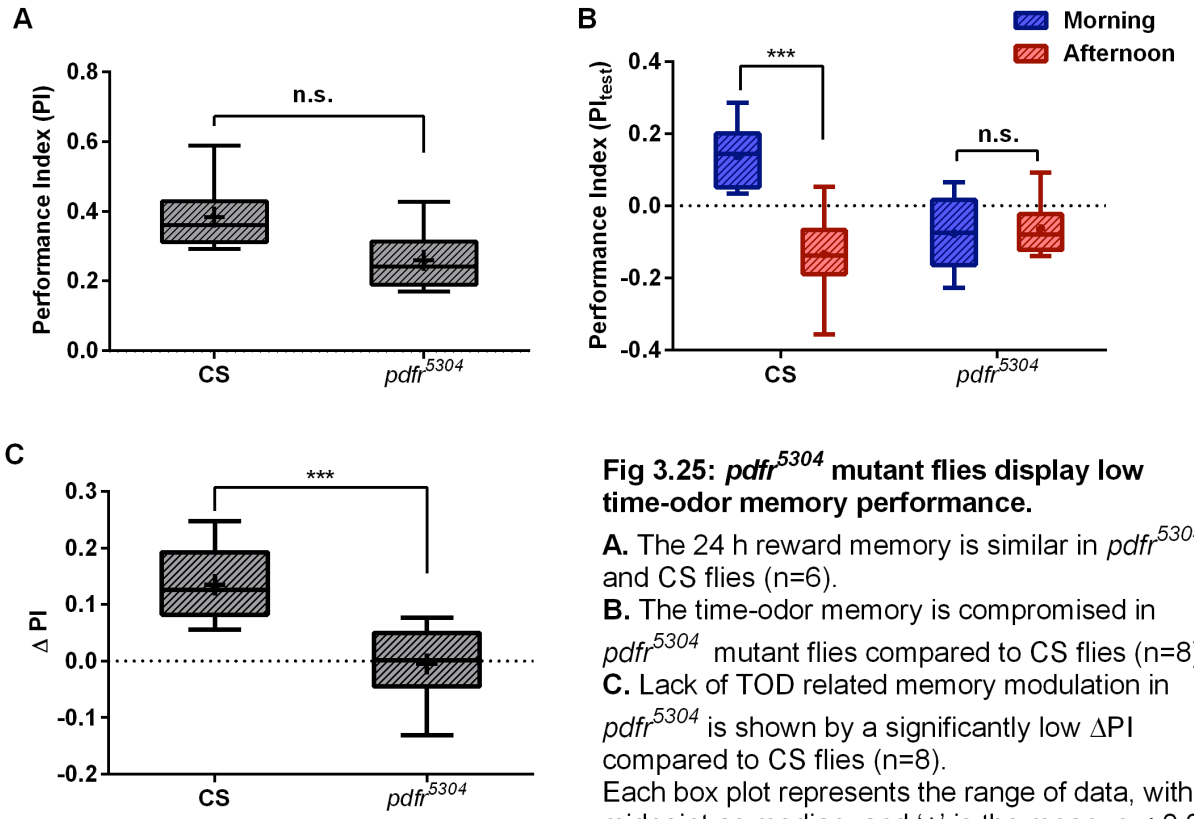

Fig 3.25: *pdfr*^{5304} mutant flies display low time-odor memory performance.

A. The 24 h reward memory is similar in *pdfr*^{5304} and CS flies (n=6).
B. The time-odor memory is compromised in *pdfr*^{5304} mutant flies compared to CS flies (n=8).
C. Lack of TOD related memory modulation in *pdfr*^{5304} is shown by a significantly low ΔPI compared to CS flies (n=8).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.001$ (***).

The UAS-GAL4 binary system was used to express the WT *pdfr* gene in a restricted neuronal population in a *pdfr*^{5304} mutant background. First, the *Clk856-GAL4* fly line, which primarily drives the expression of the *UAS-responder* in clock neurons including the lateral and dorsal neurons, was used (Gummadova et al., 2009).

In appetitive LTM trials, no substantial differences in the memory expression were observed between the genetic controls and the rescue fly line (Fig 3.26A). The rescue of the PDFR using the *Clk856-GAL4* line enabled *pdfr*^{5304} mutants to express the specific appetitive memory at the corresponding time of day (Fig 3.26B). In contrast, the control flies, *pdfr*^{5304};*UAS-Pdfr*/+ and *pdfr*^{5304}; *Clk856-GAL4*/+, were unable to demonstrate significant TOD related memories (Fig 3.26B). The rescue flies also showed comparatively better ΔPI value than the genetic control lines (Fig 3.26C). These results indicate that the PDF signaling in clock neurons is sufficient for time-odor associations. Also, the central circadian clock in the *Drosophila* brain plays a major role in the usage of the time as an associative signal.

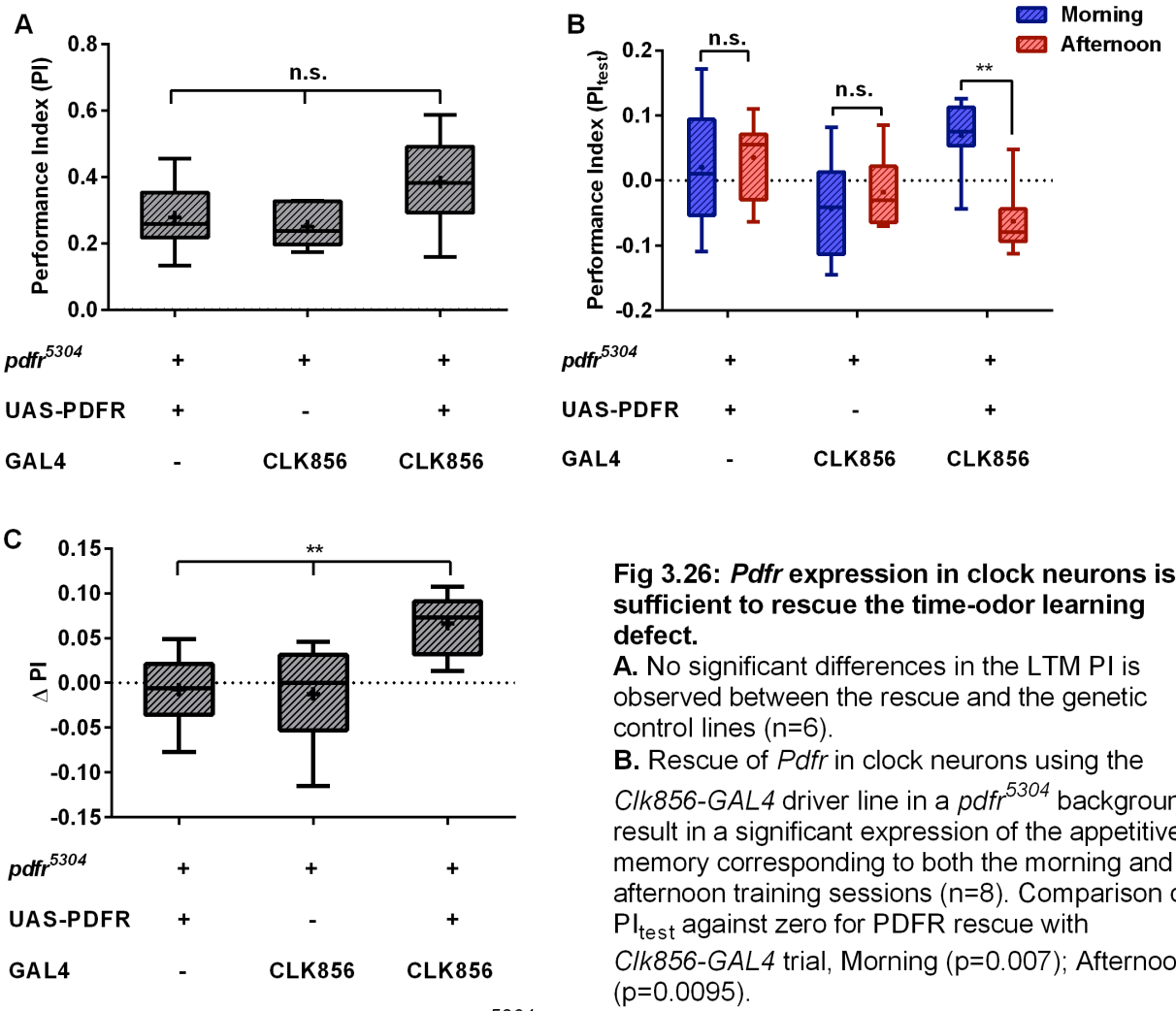

Fig 3.26: *Pdfr* expression in clock neurons is sufficient to rescue the time-odor learning defect.
A. No significant differences in the LTM PI is observed between the rescue and the genetic control lines (n=6).
B. Rescue of *Pdfr* in clock neurons using the *Clk856-GAL4* driver line in a $pdfr^{5304}$ background result in a significant expression of the appetitive memory corresponding to both the morning and afternoon training sessions (n=8). Comparison of PI_{test} against zero for PDFR rescue with *Clk856-GAL4* trial, Morning (p=0.007); Afternoon (p=0.0095).
C. ΔPI value is significantly better in $pdfr^{5304}$; *UAS-Pdfr/Clk856-GAL4* flies compared to the genetic control flies (n=8).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.01$ (**).

3.6.3. *pdfr* expression in specific neurons is sufficient for time-odor memories

The following experiments investigated if the PDF signaling is sufficient in a subset of clock neurons, or is required on a network level to maintain the clock functionality for time-odor associations. To express the *UAS-responder* in distinct subsets of clock neurons three GAL4 lines were used: *Pdf-GAL4* (s- and l-LNvs; (Renn et al., 1999)), *Mai179-GAL4* (s-LNvs and LNds; (Grima et al., 2004)) and *Clk4.1m-GAL4* (~10 DN1p; (Zhang et al., 2010b)). No significant difference in the appetitive LTM PIs was observed

between the rescue and the genetic control lines with all three genetic combinations (Fig 3.27A).

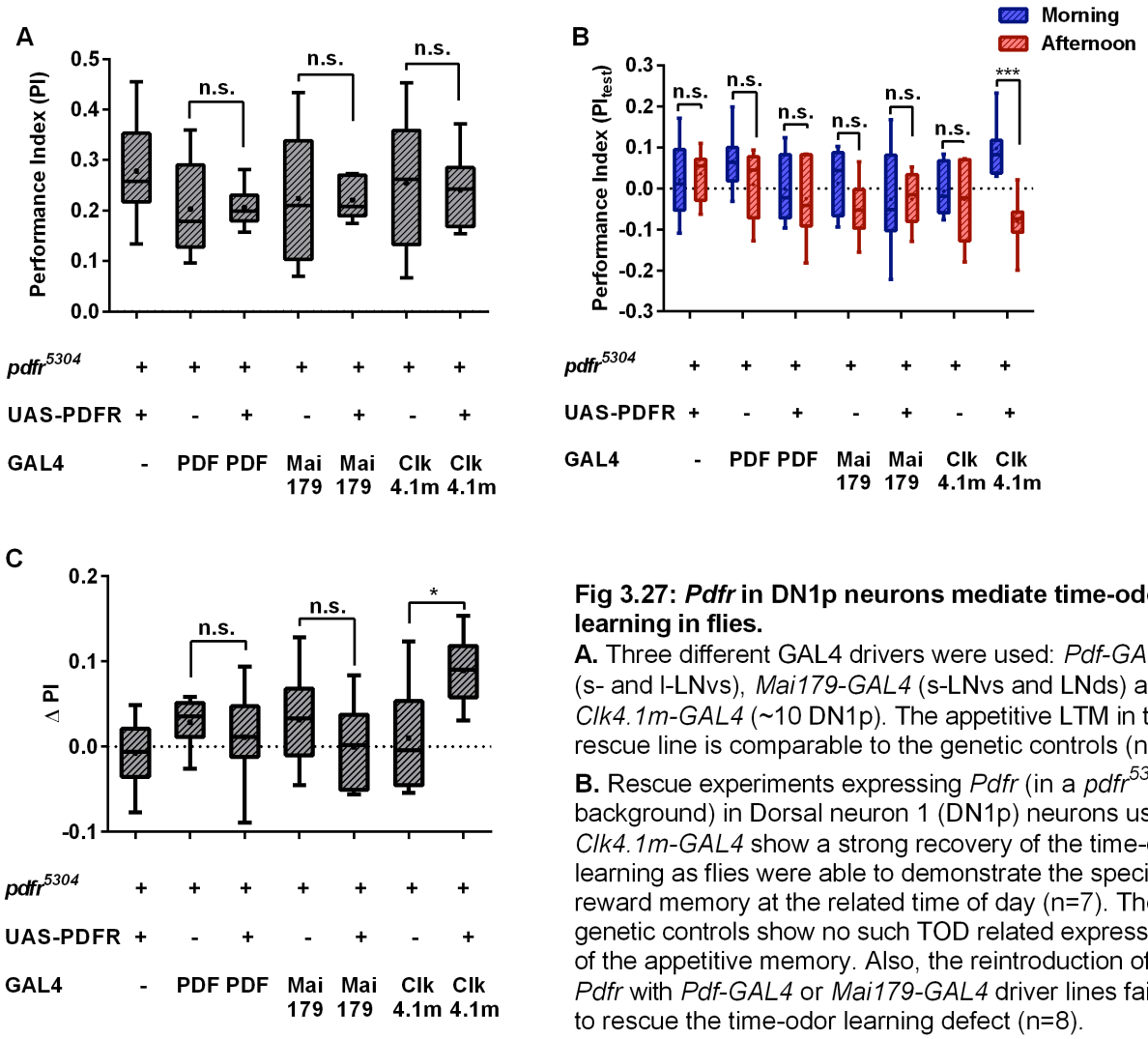

Fig 3.27: *Pdfr* in DN1p neurons mediate time-odor learning in flies.

A. Three different GAL4 drivers were used: *Pdf-GAL4* (s- and l-LNvs), *Mai179-GAL4* (s-LNvs and LNds) and *Clk4.1m-GAL4* (~10 DN1p). The appetitive LTM in the rescue line is comparable to the genetic controls (n=6).

B. Rescue experiments expressing *Pdfr* (in a $pdfr^{5304}$ background) in Dorsal neuron 1 (DN1p) neurons using *Clk4.1m-GAL4* show a strong recovery of the time-odor learning as flies were able to demonstrate the specific reward memory at the related time of day (n=7). The genetic controls show no such TOD related expression of the appetitive memory. Also, the reintroduction of *Pdfr* with *Pdf-GAL4* or *Mai179-GAL4* driver lines failed to rescue the time-odor learning defect (n=8).
Comparison of PI_{test} against zero for PDFR rescue with *Clk4.1m-GAL4* trial, Morning (p=0.0094); Afternoon (p=0.0172).

C. $pdfr^{5304}$;*UAS-Pdfr/Clk4.1m-GAL4* flies demonstrate a considerably better ΔPI compared to the genetic controls (n=7). In contrast, the rescue and the control lines with *Pdf-GAL4* or *Mai179-GAL4* drivers show no difference in ΔPI (n=8).
Each box plot represents the range of data, with the midpoint as median, and '+' is the mean. $p < 0.05$ (*); $p < 0.001$ (***).

In the time-odor learning assay the flies with the *Pdf-GAL4,* and *Mai179-GAL4* drivers showed low morning and afternoon PI_{test} values when crossed with $pdfr^{5304}$;*UAS-Pdfr* or $pdfr^{5304}$ (Fig 3.27B). In contrast, the $pdfr^{5304}$;*UAS-Pdfr/Clk4.1m-GAL4* flies can

demonstrate strong appetitive memory in both the morning and afternoon tests (Fig 3.27B). ΔPI was also significantly different between the rescue and the genetic control flies with *Clk4.1m-GAL4* (Fig 3.27C). On the other hand, the genetic control and the rescue lines with the *Pdf-GAL4* and *Mai179-GAL4* drivers showed negligible ΔPI (Fig 3.27C). These outcomes highlight the role of PDFR in about 10 dorsal neurons (DN1p) in promoting the usage of the time as an associative cue.

4. DISCUSSION

The anticipation of the environmental changes across the day can help animals to appropriate their behavior accordingly. Finding and then remembering the location of a reward such as food, territory or mates can enhance the survival of animals. However, only the information about the site of an important stimulus is insufficient as the properties of the space changes according to the time of day. For instance, the location of the food in the morning may also attract predators at other times of day. Therefore, knowledge of the space and time will benefit an animal to avoid predators and forage for the food.

In animals, the time and place associations require a functional internal circadian oscillator (Biebach et al., 1991; Wenger et al., 1991; Mistlberger et al., 1996; Van der Zee et al., 2008; Mulder et al., 2013b). However, the identity of the oscillator and the underlying mechanism that help them clocks in communicating the time information to the brain areas involved in the memory storage is not well understood. In the present study, TOD related associations were demonstrated in *Drosophila melanogaster*, a genetically tractable organism. This work also elucidates the role of the circadian rhythms generated by the central clock in the TOD memory formation.

4.1. Flies can use the time of day information adaptively

An appetitive conditioning paradigm was chosen to train the hungry flies to remember the odor/reward association. A single cycle of appetitive training is sufficient to form the protein synthesis dependent long-term memory in flies (Krashes and Waddell, 2008). In an aversive olfactory conditioning assay, the time of day of the training influences the memory formation in flies (Lyons and Roman, 2009; Fropf et al., 2014). In contrast, flies in an appetitive memory assay showed no such modulation (Fig 3.2). This suggests that starvation is a much more important determinant of the reward memory formation than the circadian rhythms in flies.

Previous reports showed that rats display maximum retention of the memory when tested at the same time of day as the training (Holloway and Wansley, 1973a;

Holloway and Wansley, 1973b). This study proposed that the effect was due to the circadian periodicity in the memory retrieval (Holloway and Wansley, 1973b). Therefore, rats can associate the time of day with the place memories automatically (Gallistel, 1990). In contrast, flies do not show such cyclic memory retrieval in an appetitive conditioning assay (Fig 3.2).

The periodic memory retention may not be due to the learned association between a stimulus and the time of day. The other explanation can be that the presentation of a stimulus during the training may act as a Zeitgeber, which entrains the memory output like freezing in rats. In contrast, time-place learning in which an animal relates different resource locations with a particular time of day must involve more than just the entrainment of an oscillator (Mulder et al., 2013b). In this case, animals should actively discriminate between different choices based on the time of day. Interestingly, flies were able to relate the time of day with appetitive trials when trained with two cycles of the reciprocal training at two distinct times during the day (Fig 3.5). After the training flies showed a significant difference in their odor-preference between the morning and afternoon tests. This result gave the first evidence that an animal can associate the time of day with reward based odor memories.

Interestingly, reciprocal trials are essential for establishing TOD associated memories (Fig 3.5). Flies were unable to remember the time-odor associations when trained in a non-reversal manner (Fig 3.5). In reversal learning, flies tested immediately after the training show a robust memory retrieval corresponding to the second conditioning. In this case, flies do not completely forget the first training as the memory after a single conditioning event is significantly better than the reversal learning PI (Fig 3.3). This outcome indicates that in the reversal learning assay flies may form interrelated memory traces. Consequently, in certain circumstances flies may show a strong preference for the odor used as CS^+ in the first training, for instance, if it signals the availability of the food. Such modulation in preference might be the case in time-odor learning in which flies prefer a specific odor based on the odor/reward association at a certain time of day. In contrast, flies trained in a non-reversal manner establish two independent memory traces that can be recalled at any time of day and thus, are unable to demonstrate time-odor memories.

Studies of the sun-compass orientation have shown that animals can use the time of day information to compensate for the changing position of the sun (Reppert, 2007). These studies suggest that the time information might be used in a continuous manner to promote the navigation in a certain direction. In contrast, flies are only able to remember reciprocal trials that are presented at an interval of at least 6 h duration but not with a 4 h gap (Fig 3.7). This outcome indicates that flies may use the time of day information in a domain-wise manner and the length of each domain is about 6 h.

The domain based time information might be used such that different time-domains signify time-gaps larger than 6 h. For instance, the 'time-domain 1' represents ZT0-ZT6 and the 'time-domain 2' is for ZT6-ZT12 and so on. When both the conditioning cycles are in the 'time-domain 1', separated by a gap of less than 6 h, then the flies are unable to store the reciprocal trials separately. In contrast, when flies are subjected to two trials with a 6 h or larger interval, then the first trial is associated with the 'time-domain 1' and the 'time-domain 2' relates to the second trial. Such distinct time-domains can help flies to form TOD related memory traces. It is important to note that the beginning of a time-domain is dependent on the time of training. If the first training occurs at ZT1 then the 'time-domain 1' is from ZT1-ZT7. Even in other paradigms like the sun compass orientation, such domain based time information might be in use but only with a smaller length for each time-domain. No case has been described in which the time of day is stored in broad time-domains. This finding asks for a more detailed characterization in flies.

The memory retrieval was significant only when flies were tested at the same time as trained in the morning and afternoon (Fig 3.5). Flies use the 6 h time-domains to form TOD related memory traces. In contrast, the memory score related to a particular time of day is not robust in the whole 6 h domain range during tests. Taking the conditioning as it is and moving the test in smaller steps from ZT0 to ZT8 suggests that the conditioning cycle sets the time at which the memory retrieval is robust, indicating that the time resolution is quite good (Fig 3.5). Therefore, flies may use time-domains as an associative cue during memory acquisition but may not rely on the domain based time information for the retrieval of the TOD related memory.

The internal state of an animal can influence its ability to perform in the TOD related memory paradigm. Starvation had been proposed to motivate animals to use the time information as an associative cue (Widman et al., 2000; Widman et al., 2004). The male flies, which show lower resistance to starvation than females, can form robust time-odor memories (Fig 3.9). In contrast, females are only able to establish time-odor associations after an extended period of starvation before time-odor learning (Fig 3.9). Increased starvation may motivate flies to reduce the number of wrong choices in the TOD related memory assay. These wrong choices preclude food discovery and thus, can further exacerbate the starvation stress.

Alternatively, food deprivation may promote the synchronization of an internal oscillator in which the delivery of the sucrose reward may act as the Zeitgeber. In satiated flies, the external light is a dominant signal that entrains internal clocks. Prolonged food deprivation may make the food cues more pertinent. A food-entrainable oscillator (FEO) had been proposed to underlie time-place learning in rats (Mistlberger et al., 1996; Mulder et al., 2013b). No FEO has been shown in flies but the role of a food dependent oscillator in time-odor associations cannot be ruled out.

Time-odor memories can be recalled even if the flies are satiated (Fig 3.12). In contrast, the satiated flies are unable to retrieve the appetitive LTM after a single cycle of conditioning (Fig 3.11). This discrepancy points towards an intriguing possibility that the retrieval mechanism of the time-odor memory may differ from the odor memory formed after a single cycle of appetitive training.

4.2. Mechanism underlying time-odor associations in flies

Different mechanisms can help animals to change their choice in a memory paradigm according to the time of day. The circadian strategy in which animals receive the time information from an internal oscillator can promote time-place associations (Van der Zee et al., 2008; Mulder et al., 2013b).

An external cue such as the Light:Dark transition can help flies to count the time intervals. Lights ‘on/off’ can signal the start/stop/reset of the time counting and can help flies to associate a specific time-interval with every appetitive trial. For instance, flies

may learn to prefer odor A short after the ‘lights on’ and choose odor B after a relatively longer delay. This approach is called the interval timer strategy. To assess if this mechanism is involved in time-odor learning, flies were trained in LD conditions but then moved to DD before tests. Flies demonstrated robust TOD related appetitive memories comparable to the flies left in LD before tests (Fig 3.13). These results suggest that flies do not use an interval timer strategy and thus, may employ a circadian strategy to establish time-odor memories.

Manipulation of the external light conditions provides further evidence for the circadian strategy. In constant darkness, flies demonstrate free running rhythms and can perform as well as the flies in LD conditions in the time-odor learning paradigm (Fig 3.14). In contrast, flies in constant light become arrhythmic and consequently, demonstrated no modulation in the memory performance according to the time of day (Fig 3.14). These outcomes indicate that flies rely on an endogenous oscillator to form time-odor memories. An internal oscillator can provide flies with the information about the current time of day, help them to store different memories according to TOD related associations, and facilitate correct choices in tests (Mulder et al., 2013b).

The present study suggests that different internal oscillators may facilitate the time-odor memory acquisition and the retrieval respectively. Flies subjected to a brief pulse of a high-intensity light in the dark phase demonstrate phase shifts that manifest into an advance or a delay of the peak locomotor activity (Suri et al., 1998; Yang et al., 1998). Flies show no change in the time-odor memory expression when subjected to a high-intensity light pulse in the dark phase before tests (Fig 3.15). Also transferring the flies to constant light after the last training had no effect on the performance in the time-odor learning paradigm (Fig 3.16). These manipulations affect the rhythms generated by the light dependent central oscillator but show no discernible change in the retrieval of time-odor memories. In contrast, flies subjected to constant light during the second round of reciprocal training were unable to show TOD related appetitive memories (Fig 3.16). This result indicates that a light-dependent oscillator is essential for the acquisition of the time-odor relationship. Also, the retrieval of time-odor memories rely on a different oscillator, which may function independently of the light.

In *Drosophila* the light entrainment is mediated by Cryptochrome (CRY), a blue light-sensitive protein, and inputs from the visual system which includes eyes, ocelli and Hofbauer-Buchner eyelets (Helfrich-Förster et al., 2001; Rieger et al., 2003). Therefore, the CRY-negative neurons in the *Drosophila* central clock are the prime candidates, but the probable role of a peripheral oscillator for the time-odor memory retrieval cannot be ruled out.

4.3. Neurogenetic underpinnings of time-odor memories

The mammalian clock consists of a transcriptional-translational feedback loop where CLOCK and BMAL1 heterodimer acts as a transcription activator for Period (PER) and Cryptochrome (CRY), which then, in-turn, blocks the CLOCK/BMAL1 activity (Ko and Takahashi, 2006). The time-place learning is impaired in the clock gene *Cry* double knockout mice (*Cry*$1^{-/-}$*Cry*$2^{-/-}$) (Van der Zee et al., 2008). In contrast, the *Per1/Per2* double knockout mice showed wild-type like performance in the time-place learning paradigm (Mulder et al., 2013a). Therefore, the endogenous oscillator facilitating time-place learning in mice is *Cry* dependent but does not need the *Per* gene. These results point towards an oscillator that may function specifically for learning and memory (Mulder et al., 2013a).

In flies, the transcription-translation feedback loop consists of the CLK/CYC heterodimeric complex activating *per* and *tim* transcription, which then together block CLK/CYC activity (Dubruille and Emery, 2008). In the time-odor memory assay, per^{01} and clk^{AR} mutant flies demonstrate compromised TOD related appetitive memories (Fig 3.18). Also, per^{01} and clk^{AR} flies in constant darkness showed a strong preference for only the afternoon trained odor in both the tests (Fig 3.20). This behavior is similar to the CS flies in LL conditions, which suggest that arrhythmic flies display memories related to only the last trained event. Thus, the oscillator promoting time-odor learning in flies is more general than in mammals and requires *per* and *clk* genes.

In mice, the identity of the internal oscillator that enables the use of the time of day information adaptively is less well understood. The suprachiasmatic nucleus (SCN), the master clock in the brain is light-entrainable and requires the *Per* and *Cry* but is

dispensable for time-place associations in rats (Boulos and Logothetis, 1990; Mistlberger et al., 1996; Dibner et al., 2010). A food-entrainable oscillator (FEO) has been proposed to underlie time-place associations but the locus and the neural substrates of the FEO are not well defined (Mistlberger, 2011).

In *Drosophila* the central circadian clock comprises of about 150 neurons. The most studied output factor of the *Drosophila* clock is a neuropeptide pigment-dispersing factor (PDF), which is secreted by four s-LNvs and four l-LNvs per brain hemisphere (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1995). The pdf^{01} mutant flies failed to perform in the time-odor learning paradigm (Fig 3.23). This outcome indicates that the 16 PDF positive clock neurons are involved in forming time-odor memories. This possibility was further confirmed by expressing the WT *pdf* in these 16 clock neurons in pdf^{01} mutant flies, which resulted in the complete rescue of the inability to form time-odor memories (Fig 3.24). These results also implicate the central clock oscillator in forming TOD related memory traces.

PDFR shows strong expression in the clock neurons, pars intercerebralis, optic lobes and the ellipsoid bodies (Lear et al., 2009). Intriguingly, no PDFR expression was reported in the mushroom bodies (MB), the site of the appetitive memory trace for odors in the fly brain (Lear et al., 2009; Im and Taghert, 2010). Therefore, the PDF neuropeptide may not directly provide the time information to the MB cells to form time-odor memories. This hypothesis was further investigated with the UAS-GAL4 binary system. Experiments demonstrated that PDFR expression in the clock neurons (*Clk856-GAL4*) is sufficient to rescue the time-odor learning defect of $pdfr^{5304}$ mutant flies (Fig 3.26). These results further emphasize that PDF signaling has an indirect role in time-odor associations.

On a network level, the PDF neuropeptide acts as a coupling factor between clock neurons and maintains the clock speed. To further delineate the role of PDF signaling, the UAS-GAL4 system was used to sub-localize the PDFR neurons involved in time-odor associations. Rescue experiments suggest that the PDFR in about 10 dorsal neurons (DN1p), is sufficient for establishing time-odor memories in flies (Fig 3.27). This outcome indicates that the PDF neuropeptide, secreted by the s- and l-LNvs,

activates the PDFR in DN1p neurons, which then probably communicate with the downstream circuits to establish TOD related memory traces.

The function of the dorsal neurons is less well comprehended. Prior studies have suggested that the DN1s integrate circadian and environmental factors to influence the circadian rhythms (Lear et al., 2009; Zhang et al., 2010a; Zhang et al., 2010b). DN1s are also able to drive the rhythmic locomotor activity under certain settings (Murad et al., 2007). A recent model demonstrated the circadian output circuit for the locomotor behavior (Cavanaugh et al., 2014). It showed that the pacemaker neurons s-LNvs projects through the DN1s (likely signals through the PDF) to the pars intercerebralis, which then modulates the locomotor activity through the release of DH44 (Diuretic hormone 44) (Cavanaugh et al., 2014).

Therefore, an output circuit involving the s-LNvs and DN1 cells is probably providing the time of day signal to the downstream neurons to form time-odor memories. Further studies may investigate the identity of downstream circuits that communicate the time of day information to the MB neurons.

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6. SUMMARY

Endogenous clocks help animals to anticipate the daily environmental changes. These internal clocks rely on environmental cues, called Zeitgeber, for synchronization. The molecular clock consists of transcription-translation feedback loops and is located in about 150 neurons (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 2005). The core clock has the proteins Clock (CLK) and Cycle (CYC) that together act as a transcription activator for *period* (*per*) and *timeless* (*tim*) which then, via PER and TIM block their own transcription by inhibiting CLK/CYC activity (Darlington et al., 1998; Hardin, 2005; Dubruille and Emery, 2008). Light signals trigger the degradation of TIM through a blue-light sensing protein Cryptochrome (CRY) and thus, allows CLK/CYC to resume *per* and *tim* transcription (Emery et al., 1998; Stanewsky et al., 1998). Therefore, light acts as an important Zeitgeber for the clock entrainment. The mammalian clock consists of similarly intertwined feedback loops.

Endogenous clocks facilitate appropriate alterations in a variety of behaviors according to the time of day. Also, these clocks can provide the phase information to the memory centers of the brain to form the time of day related associations (TOD). TOD memories promote appropriate usage of resources and concurrently better the survival success of an animal. For instance, animals can form time-place associations related to the availability of a biologically significant stimulus like food or mate. Such memories will help the animal to obtain resources at different locations at the appropriate time of day. The significance of these memories is supported by the fact that many organisms including bees, ants, rats and mice demonstrate time-place learning (Biebach et al. 1991; Mistlberger et al. 1997; Van der Zee et al. 2008; Wenger et al. 1991). Previous studies have shown that TOD related memories rely on an internal clock, but the identity of the clock and the underlying mechanism remain less well understood. The present study demonstrates that flies can also form TOD associated odor memories and further seeks to identify the appropriate mechanism.

Hungry flies were trained in the morning to associate odor A with the sucrose reward and subsequently were exposed to odor B without reward. The same flies were exposed in the afternoon to odor B with and odor A without reward. Two cycles of the

reversal training on two subsequent days resulted in the significant retrieval of specific odor memories in the morning and afternoon tests. Therefore, flies were able to modulate their odor preference according to the time of day. In contrast, flies trained in a non-reversal manner were unable to form TOD related memories. The study also demonstrates that flies are only able to form time-odor memories when the two reciprocal training cycles occur at a minimum 6 h interval.

This work also highlights the role of the internal state of flies in establishing time-odor memories. Prolonged starvation motivates flies to appropriate their search for the food. It increases the cost associated with a wrong choice in the T-maze test as it precludes the food discovery. Accordingly, an extended starvation promotes the TOD related changes in the odor preference in flies already with a single cycle of reversal training. Intriguingly, prolonged starvation is required for the time-odor memory acquisition but is dispensable during the memory retrieval.

Endogenous oscillators promote time-odor associations in flies. Flies in constant darkness have functional rhythms and can form time-odor memories. In contrast, flies kept in constant light become arrhythmic and demonstrated no change in their odor preference through the day. Also, clock mutant flies per^{01} and clk^{AR}, show compromised performance compared to CS flies when trained in the time-odor conditioning assay. These results suggest that flies need a *per* and *clk* dependent oscillator for establishing TOD related memories. Also, the clock governed rhythms are necessary for the time-odor memory acquisition but not for the retrieval.

Pigment-Dispersing Factor (PDF) neuropeptide is a clock output factor (Park and Hall, 1998; Park et al., 2000; Helfrich-Förster, 2009). pdf^{01} mutant flies are unable to form significant time-odor memories. PDF is released by 8 neurons per hemisphere in the fly brain. This cluster includes the small (s-LNvs) and large (l-LNvs) ventral lateral neurons. Restoring PDF in these 16 neurons in the pdf^{01} mutant background rescues the time-odor learning defect. The PDF neuropeptide activates a seven transmembrane G-protein coupled receptor (PDFR) which is broadly expressed in the fly brain (Hyun et al., 2005). The present study shows that the expression of PDFR in about 10 dorsal neurons (DN1p) is sufficient for robust time-odor associations in flies.

In conclusion, flies use distinct endogenous oscillators to acquire and retrieve time-odor memories. The first oscillator is light dependent and likely signals through the PDF neuropeptide to promote the usage of the time as an associative cue during appetitive conditioning. In contrast, the second clock is light independent and specifically signals the time information for the memory retrieval. The identity of this clock and the underlying mechanism are open to investigation.

7. Zusammenfassung

Die endogenen circadianen Uhren helfen Tieren, die täglichen Veränderungen der Umwelt zu antizipieren. Diese internen Uhren stützen sich auf externe Umweltreize, sogenannte Zeitgeber, die den Tagesrhythmus vorgeben. Im Fliegengehirn bilden etwa 150 Neuronen die zentrale innere Uhr (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 2005). Diese Neuronen exprimieren die molekulare Uhr, die aus Transkriptions-Translations-Feedback-Schleifen besteht. Die Uhr besitzt die Proteine Clock (CLK) und Cycle (CYC), die zusammen die Transkription von *period* (*per*) und *timeless* (*tim*) aktivieren. PER und TIM bilden dann ein Heterodimer um die Transkription von *clk* und *cyc* zu blockieren (Darlington et al., 1998; Hardin, 2005; Dubruille and Emery, 2008). Lichtsignale lösen den Abbau von TIM durch das für blaues Licht sensitive, 'Sensing Protein Cryptochrome' (CRY) aus, daß wiederum CLK und CYC freisetzt um die *per* und *tim* Transkription wieder aufzunehmen (Emery et al., 1998; Stanewsky et al., 1998). Daher wirkt Licht als wichtiger Zeitgeber. Die innere Uhr der Säuger besteht aus ähnlich miteinander verflochtenen Rückkopplungsschleifen.

Die internen Uhren ermöglichen und erleichtern Verhaltensveränderungen in einer Vielzahl von Situation, entsprechend der Tageszeit. Zudem wird die Information den jeweiligen Speicherorten im Gehirn bereit gestellt, um zeitbezogene Gedächtnisbildung zu ermöglichen. Zeitabhängige Gedächtnisbildung sorgt für eine angemessene Nutzung der Ressourcen und sichert gleichzeitig das Überleben des Tieres. Zum Beispiel können Tiere Zeit-Ort-Assoziationen im Zusammenhang mit der Verfügbarkeit einer biologisch wichtigen Ressource, wie Nahrung oder Paarungspartnern bilden. Solche Assoziationen helfen dem Tier Ressourcen an verschiedenen Orten, abhängig von der Tageszeit, zu erschließen. Die Wichtigkeit dieser Fähigkeit wird durch die Tatsache gestützt, daß zum Beispiel Bienen, Ameisen, Ratten und Mäuse ein zeitlich abhängiges Ortgedächtnis bilden können (Biebach et al. 1991; Mistlberger et al. 1997; Van der Zee et al. 2008; Wenger et al. 1991). Frühere Studien haben gezeigt, daß zeitbezogene Erinnerungen auf einer internen Uhr beruhen. Die genaue Identität dieser Uhr und die zugrunde liegenden Mechanismen sind jedoch nicht ausreichend bekannt. In der vorliegenden Studie wird gezeigt, daß Fliegen in der

Lage sind ein zeitabhängiges olfaktorisches Gedächtnis zu bilden. Zudem wird versucht die zugrunde liegenden molekularen Mechanismen zu identifizieren.

Hungrige Fliegen werden zu verschiedenen Tageszeiten konditioniert verschiedene Gerüche mit einer Saccharose-Belohnung zu assoziieren. Morgens ist Geruch A mit Zucker gepaart während Geruch B ohne Zucker präsentiert wird, am Nachmittag ist Geruch B belohnt, Geruch A nicht. Dieses reziproke Training wird an zwei aufeinander folgenden Tagen durchgeführt. Am dritten Tag werden die Fliegen entweder am Morgen oder Nachmittag auf ihre Geruchspräferenz zwischen A und B getestet. Die Fliegen modulieren ihre Geruchspräferenz abhängig von der Tageszeit. Im Gegensatz dazu sind Fliegen, die nicht mittels eines reziproken Trainings konditioniert wurden, nicht in der Lage, ein zeitabhängiges olfaktorisches Gedächtnis zu bilden. Die Ergebnisse zeigen auch, daß Fliegen nur dann in der Lage sind zeitbezogene Erinnerungen zu bilden, wenn die beiden reziproken Trainingszyklen mindestens 6 h voneinander getrennt durchgeführt werden.

Die Arbeit ebeleuchtet zudem die Rolle des internen Zustands der Fliegen im Kontext des zeitabhängigen olfaktorischen Gedächtnisses. Länger andauernder Hunger motiviert die Fliegen stärker ihre Suche nach Nahrung zeitlich anzupassen. Schon ein Zyklus reziproken Trainings reicht für die Bildung Zeit-spezifischen Geruchsgedächtnisses aus. Die Erhöhung der Kosten, die mit einer falschen Wahl in einem T-maze-Test verbunden ist, kann offenbar zeitabhängige Änderungen der Geruchspräferenzen in Fliegen begünstigen. Erstaunlicherweise begünstigt der Hunger speziell die Gedächtnisbildung, ist jedoch für den Test nicht erforderlich.

Endogene circadiane Oszillatoren werden für das zeitabhängige olfaktorische Gedächtnis der Fliegen gebraucht. Fliegen, die im Dauerdunkel gehalten wurden, zeigen rhythmisches Verhalten so wie zeitbezogenes olfaktorisches Gedächtnis. Im Gegensatz dazu sind im Dauerlicht aufgezogene Fliegen arrhythmisch und zeigen kein Zeit-spezifisches Geruchsgedächtnis. Zudem sind auch die arrhythmischen Mutanten per^{01} und clk^{AR} in der Zeit-Geruchskonditionierung gestört. Diese Ergebnisse legen nahe, daß Fliegen einen *per*- und *clk*-abhängigen Oszillator benötigen, der von externen Lichtsignalen abhängig ist, um ein zeitabhängiges olfaktorisches Gedächtnis

zu bilden. Außerdem wird der durch die innere Uhr vorgegebene Rhythmus nur während der Gedächtnisbildung und nicht für das Abrufen des Gelernten benötigt.

Pigment dispersing factor (PDF) ist ein Neuropeptid, das von Neuronen der inneren Uhr gebildet wird (Park and Hall, 1998; Park et al., 2000; Helfrich-Förster, 2009). Die pdf^{01}-Mutante ist nicht in der Lage ein signifikantes zeitbezogenes olfaktorisches Gedächtnis zu bilden. PDF wird von jeweils einer Gruppe von 8 Neuronen pro Hemisphäre, die die kleinen und großen ventral-lateralen Neuronen umfaßt, sezerniert. Die Wiederherstellung der Expression von PDF in diesen 16 Neuronen im pdf^{01} Mutanten Hintergrund, rettet das zeitabhängige olfaktorische Gedächtnis. Das PDF-Neuropeptid aktiviert einen sieben-Transmembran-G-Protein-gekoppelten Rezeptor (PDFR), der weit verbreitet im Fliegenhirn exprimiert wird (Hyun et al., 2005). Diese Studie zeigt, daß die Expression von PDFR in ~ 10 dorsalen Neuronen (DN1p) für eine robuste zeitabhängige olfaktorische Gedächtnisbildung in Fliegen ausreicht.

Zusammenfassend läßt sich sagen, daß Fliegen verschiedene endogene Oszillatoren benutzen um ein zeitabhängiges olfaktorische Gedächtnis zu bilden und abzurufen. Der erste Oszillator ist lichtabhängig und wahrscheinlich durch das PDF-Neuropeptid vermittelt. Es ermöglicht die Verwendung der Information 'Zeit' als assoziatives Signal während der appetitiven Konditionierung. Im Gegensatz dazu ist die zweite Uhr lichtunabhängig und vermittelt speziell die Zeitinformation für die Gedächtnisabfrage. Die Identität der zweiten Uhr und der zugrunde liegende Mechanismus sowie die zugrunde liegende Kommunikation zwischen den Neuronen, bedarf weiterer Untersuchungen.

8. APPENDIX

8.1. Affidavit

I hereby declare that my thesis entitled: **„Time-odor learning in *Drosophila melanogaster*"** is the result of my own work.
I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.
Furthermore I verify that the thesis has not been submitted as part of another examination process neither in identical nor in similar form.

Eidesstattliche Erklärung
Hiermit erkläre ich an Eides statt, die Dissertation: „ **Olfaktorisches Zeitgedächtnis bei *Drosophila melanogaster*"**, eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, den

_____

Unterschrift

8.2. Curriculum Vitae

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8.3. Publications and Conference contributions

Publications

Chouhan, NS, Wolf, R, Helfrich-Förster, C and Heisenberg, M (2015). Flies can remember time of day. Curr. Biol. 25, 1-6.

Conference contributions

Chouhan, NS, Wolf, R and Heisenberg, M (2016). Time-odor learning in *Drosophila melanogaster*. Poster presentation delivered at “European neurobiology of *Drosophila* conference” at Crete, Greece.

Chouhan, NS, Wolf, R and Heisenberg, M (2015). Time-odor learning in *Drosophila melanogaster*. Poster presentation delivered at “Neurobiology of *Drosophila*” at Cold Spring Harbor Laboratory, USA.

Chouhan, NS, Wolf, R and Heisenberg, M (2014). Time-odor learning in flies. Poster presentation delivered at “Learning and memory: a synthesis of bees and flies” at Janelia Farm Research Campus, USA.

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