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The Role of the Dal Neurons in Modulating Circadian Rhythms in Olfactory Short-Term Memory in Drosophila Melanogaster

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THE ROLE OF THE DAL NEURONS IN MODULATING CIRCADIAN RHYTHMS IN OLFACTORY SHORT-TERM MEMORY IN *DROSOPHILA MELANOGASTER*

By

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ABSTRACT

COOPER RUWE: The Role of the DAL Neurons in Modulating Circadian Rhythms in Olfactory Short-Term Memory in *Drosophila melanogaster*

Depressed short-term memory (STM) abilities during non-adaptive times of the day can significantly impact those who work occupations that require peak levels of cognitive functioning around the clock. While much work has gone into understanding the endogenous clock and circadian rhythms, there is still much to learn about the neural circuity that underlies the daily rhythms that define these regular oscillations in STM performance. The DAL neurons in the *Drosophila* brain are part of the circadian network and innervate the mushroom bodies (MBs), the species' olfactory learning center, making them compelling candidates to be involved in circadian circuitry for olfactory learning. In this thesis, I investigate the DAL neurons' role in mediating circadian rhythms in olfactory learning by examining their serotonergic synapses onto the α/β lobes of the MBs. An olfactory associative learning paradigm was used to measure and compare STM performance. Since the 5HT1A receptor was detected in the α/β lobes of the MBs, mutants for *5HT1A* are expected to lose communication between the DAL neurons and the MBs. The $5HT1A^{MB09978}$ mutants were tested against wildtype groups, and data showed the rhythm in olfactory learning was disrupted in these mutants. These results implicated the 5HT1A receptor as necessary for circadian rhythms in olfactory STM. Mutants for *5HT1B*, which was not detected in the α/β lobes, were also examined. $5HT1B^{MB05181}$ mutants retained circadian rhythms in olfactory learning, suggesting that the 5HT1B receptor does not play a role in the circadian modulation of olfactory learning. Additionally, *rutabaga* adenylyl cyclase (*rut*) was tested as a potential downstream modulator from the 5HT1A receptor. Our data confirmed that *rut* is necessary for wildtype olfactory learning but dispensable for the circadian rhythms in olfactory learning.

TABLE OF CONTENTS

LIST OF FIGURES/TABLES

Introduction

Short-term memory (STM) is an integral component of cognitive performance. STM refers to a highly accessible form of memory limited in capacity and is held in the mind temporarily; it is not completely distinguished from working memory which also holds information in an accessible state to aid in planning and performing immediate behaviors (Cowan, 2008). In this way, STM is vital in carrying out even the most basic tasks, and diminished abilities in this arena may markedly impact one's overall cognitive capabilities. Time of day can heavily influence one's STM performance which may have tremendous implications in a modern society that increasingly relies on productivity and mental acuity during times of the day to which humans are not evolutionarily accustomed (Gerstner et al., 2009; Lyons & Roman, 2009). Understanding how STM performance may have regular, daily peaks and troughs is crucial for occupations with schedules that require labor during atypical hours, such as medical professionals, transportation workers, and shift-workers (Lyons & Roman, 2009). As these professions require considerable amounts of attention and intuition, declines in STM during non-adaptive hours could impact performance and lead to decreases in safety and productivity (Lyons & Roman, 2009; Zhang et al., 2014).

These time-of-day-based variations in STM performance are governed by circadian rhythms, which are patterns of activity resulting from an endogenous clock that regulates activity levels based on an organism's inherent sense of time-of-day (Peschel & Helfrich-Forster, 2011). Circadian oscillators mediate changes in cognitive activity (including STM) independent of

fatigue and sleep deprivation (Monk & Folkard, 1978; Wright et al., 2006). The endogenous clock governs oscillations in neuronal firing patterns without regard to immediate internal or external stimuli (though the clock, in general, is entrainable by external cues such as light and temperature) (Gerstner et al., 2009). A thorough understanding of these time-based oscillations will help create more productive, safer societal institutions for those who work in professions that require peak cognitive functioning at times the human body may not be adapted to.

The circadian clocks that govern these oscillatory patterns of activity are found across all classes of organisms and work at the cellular level via core-clock genes and proteins, which function to regulate target genes rhythmically so that the rate of specific molecular processes varies temporally (Fuhr et al., 2015). Circadian clocks are cyclical and generally have a 24 hour period (Dubruille & Emery, 2008). However, they are impacted by environmental cues, which help the organisms to adjust to seasonal changes in day length and temperature (Dubruille & Emery, 2008). An organism's circadian system can be broken down into three primary components; 1) the "clock" describes the endogenous timekeeping system an organism possesses in the absence of environmental cues; 2) the input pathways help to regulate and stabilize the clock's schedule by providing information about the current temperature and light levels (amongst other potential temporal cues); and 3) the output pathways transmit this circadian information to various systems within an organism causing rhythmic patterns in activity (King & Sehgal, 2018). Because time-of-day information is relayed to the body systems, daily rhythms are present in various processes on varying scales. For example, while enzyme activities and cell metabolism are modulated by circadian clocks, so too are broader organismal-based activities such as learning and memory (Gerstner & Yin, 2010). These rhythms help the organism make efficient use of its resources by scaling down activities when they are not needed.

Drosophila melanogaster is an ideal model organism for studying the circadian modulation of short-term memory as previous research has created well-understood models of both the organism's memory and circadian systems (Hige, 2018). While it is clear that *Drosophila* STM is regulated by the endogenous circadian clock, the mechanism is still largely unknown (Lyons & Roman, 2009). Much work has gone into characterizing the molecular processes by which learning and memory occur in *Drosophila melanogaster*, and a robust model of olfactory-based associative learning has taken shape (Davis, 2005). *Figure 1* outlines the structures involved in the *Drosophila* olfactory system. Odorant detection begins on the third antennal segment and in the maxillary palps, where the sensilla detect odors via olfactory receptor neurons (ORNs) (Roman & Davis, 2001). ORNs then pass olfactory information through the antennal nerve (AN) to the antennal lobes (ALs) where each distinct family of olfactory receptors converges on their respective glomerulus (of which there are 43) via cholinergic synapses (Davis, 2005; Roman & Davis, 2001). Olfactory information then leaves the ALs via projection neurons (PNs) which bundle together to form the antennal cerebral tract (ACT) (Davis, 2005). This tract projects to two distinct regions of the *Drosophila* brain: the mushroom body neurons (MBNs) and the lateral horn (LH) (Davis, 2005). For the portion of the ACT that innervates the mushroom bodies (MBs), the PNs synapse onto the MBNs at a crowded neuropil area referred to as the calyx (C) (Davis, 2005). After receiving olfactory information from the PNs, the MBNs transmit this information through an axon bundle referred to as the pedunculus (P) back to a position dorsal from the ALs (Davis, 2005). The MBs are anatomically and functionally organized as lobes, and MBNs are classified based on these distinctions as being from the α/β lobes, the α'/β' lobes, or the γ lobe. The α/β lobes are further subdivided into three clusters of cells: posterior (p), core (c), and surface (s) (Crittenden et al., 1998). The α/β

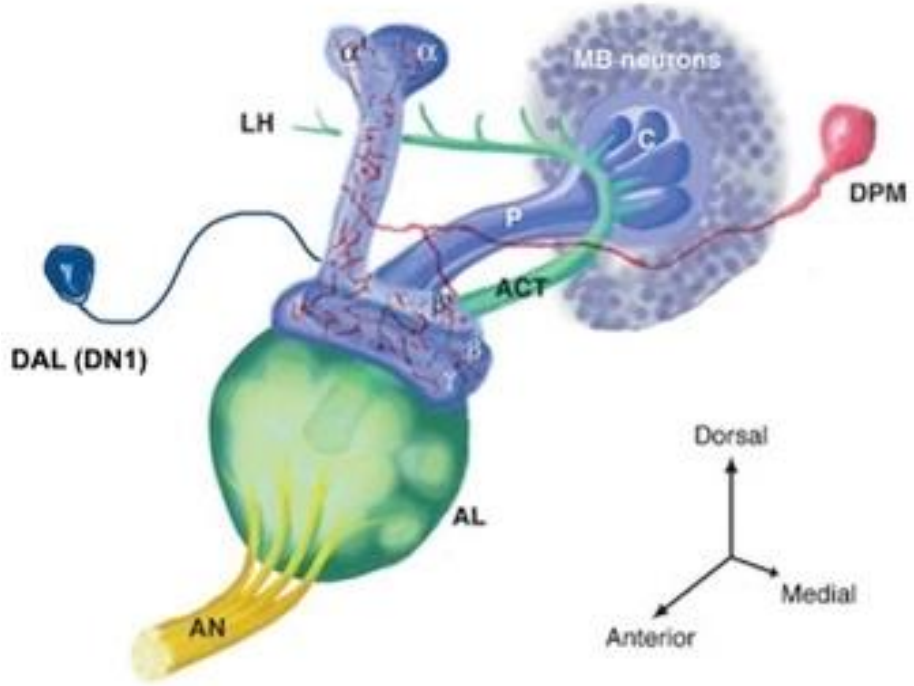

Figure 1. Model of *Drosophila* olfactory system. This model depicts the *Drosophila* brain's right hemisphere. ORNs detect odors and pass olfactory information through the AN to the ALs. Information then travels via the PNs (which bundle to form the ACT) to the MBNs and the LH. In the MBNs, the PNs synapse onto the C. MBNs then signal through the P to a point dorsal from the ALs. The MBs' lobes are labeled. The KCs are depicted in purple. Two sets of extrinsic modulators – DAL and DPM neurons – are also shown. [Taken from (Davis, 2001)]

lobes are required for olfactory, aversive memory retrieval (Krashes et al., 2007; McGuire et al., 2001). Neurons that are intrinsic to the mushroom body, Kenyon cells (KCs), are required for memory to function (Dubnau et al., 2001). These MBNs are the principal area of olfactory associative learning (Davis et al., 1995). The activities of subsets of the MBNs encode the identities of odorants, and their activity may be modulated by input from extrinsic neurons (Turner et al., 2008). Two pertinent extrinsic modulators in olfactory associative learning are the dorsal anterior lateral (DAL) neurons and dorsal paired medial (DPM) neurons (Chen et al., 2012; Davis, 2005). The DAL neurons, which innervate the posterior cells of the α/β lobes in the MB, have defined roles in consolidation of long-term memory (Chen et al., 2012; Xia et al., 2005). These neurons also express the clock proteins *per* and *tim* indicating involvement with the circadian circuit (Chen et al., 2012). The DPM neurons exclusively innervate the MBs and are required for memory storage, though the loss of function in these neurons does not markedly impact memory acquisition and retrieval (Krashes et al., 2007). Because the DAL neurons innervate the MBs and have well-defined associations with the circadian circuit, they are compelling candidates to play a role in the extrinsic circadian modulation of olfactory associative memory.

In addition to this robust olfactory associative learning model, a robust understanding of the molecular components that drive circadian oscillations in *Drosophila melanogaster* also exists (King & Sehgal, 2018). The molecular oscillator in *Drosophila* consists of a co-activator complex, CLOCK-CYCLE, that drives the expression of two genes, *period* (*per*) and *timeless* (*tim*), whose products act as co-repressors to inhibit the CLOCK-CYCLE complex (King & Sehgal, 2018). These interactions create a negative feedback loop that completes one cycle every ~24 hours due to post-transcriptional and post-translational mechanisms that delay the loop

(Zheng & Sehgal, 2012). While this rhythm is self-sustaining, external cues may be used to synchronize circadian oscillations with the environment. This process, termed "entrainment," involves the photoreceptor Cryptochrome (CRY), which, on exposure to light, binds TIM to target it for ubiquitination and degradation (Yoshii et al., 2016). In *Drosophila*, ~150 neurons expressing *per* and *tim* are classified into six groups based on neuroanatomy: large and small ventral lateral neurons (l-LNvs and s-LNvs), the dorsal lateral neurons (LNds), the lateral posterior neurons (LPNs), and three groups of dorsal neurons (DN1s, DN2s, and DN3s) [reviewed in (King & Sehgal, 2018)]. The LNvs (including both l-LNvs and s-LNvs) are identifiable by the expression of the neuropeptide Pigment-Dispersing Factor (Pdf) and appear to play a primary role in regulating rest:activity rhythms as loss of Pdf+ LNv function leads to arhythmic behavior (Helfrich-Forster, 1995; Renn et al., 1999). An additional pair of cells termed the "5th s-LNvs" and oscillators in the LNds work with the Pdf+ LNvs to coordinate a circadian network that is characterized by these two distinct sets of cells increasing locomotive activity during distinct periods: the Pdf+ LNvs in the morning and the "5th s-LNvs" and the LNds in the evening (Grima et al., 2004).

The DN1s are subdivided into anterior (DN1a) and posterior (DN1p) groups (King & Sehgal, 2018). The DN1ps integrate light, temperature, and circadian cues to promote well-defined rest:activity rhythms that define locomotor activity, sleep, and mating patterns (King & Sehgal, 2018). Similarly, the DN2s are entrained by temperature and largely control fly temperature preference throughout a day (Yoshii et al., 2010). Glial cells also express *per* and *tim* and may play a role in regulating the output of clock neurons though the mechanisms are currently unknown (Herrero et al., 2017). It should be noted that despite the well-developed understanding of the molecular mechanisms underlying clock activity and regulation in

Drosophila, there is much to be learned in regards to how the clock outputs its information and regulates the numerous systems that have display temporal oscillations in activity (King & Sehgal, 2018). One of the systems that displays this sort of rhythmic activity is the aforementioned olfactory learning system. Broadly, this study will target this *Drosophila* olfactory system and attempt to provide some level of knowledge regarding the circadian inputs to the MBs.

The neurotransmitter 5-HT functions in the *Drosophila* circadian network during entrainment (Yuan et al., 2005). The aforementioned DAL neurons are likely serotonergic and express *per* and *tim*, making them compelling candidates to be involved in outputting rhythmic information to the MBs (Chen et al., 2012). These DAL neurons synapse onto the α/β posterior neurons, which contain the 5HT1A receptor, a metabotropic 5-HT receptor (Gnerer et al., 2015; Nichols & Nichols, 2008; Shih & Chiang, 2011). The metabotropic 5HT1B receptor is expressed in the α'/β' lobes, with no detectable expression in the α/β lobes (Gnerer et al., 2015). Both of these G-protein-coupled receptors (GPCRs) may couple to the heterotrimeric G_i protein, which can inhibit certain variants of adenylyl cyclase when activated (Sadana & Dessauer, 2009). Adenylyl cyclase produces the secondary messenger cyclic adenosine monophosphate (cAMP). The cAMP molecule stimulates the activity of protein kinase A (PKA), which is active in driving a multitude of intracellular processes (Sadana & Dessauer, 2009). Thus, when an activated $G_i\alpha$ subunit inhibits adenylyl cyclase, cAMP production is slowed, and the activity of PKA and the pathways it promotes decreases. In this way, the binding of only a few GPCRs coupled to G_i can have significant impacts on overall intracellular activity. This is relevant to olfactory learning because intracellular cAMP levels are critical to the olfactory STM system (Davis et al., 1995). Notably, the adenylyl cyclase *rutabaga* (*rut*) is expressed in MBNs, and loss of function leads to

decreases in olfactory memory performance, indicating that *rut* is active in mediating olfactory learning (McGuire et al., 2003).

The DAL neurons are thought to form serotonergic synapses with the MBNs, which makes them strong candidates to be extrinsic modulators of the *Drosophila* olfactory system (Gnerer et al., 2015). The MBNs synapsing with the DALs express *5HT1A*, suggesting this modulation could occur through the inhibition of cAMP signaling if this receptor couples the heterotrimeric G_i protein. Considering that the DAL neurons express the clock genes *per* and *tim*, these neurons' role in the extrinsic circadian regulation of MBN activity becomes worthy of investigation. However, it is also worth noting that cAMP signaling in the MBs during olfactory memory formation is also impacted by other extrinsic neurons, such as the DPMs (Waddell et al., 2000).

Drosophila MBNs are not involved in regulating the organism's circadian clock. They do not express the core circadian oscillatory proteins. Yet, the activity of the olfactory system shows distinct oscillations in activity that are independent of the circadian circuit (Tanoue et al., 2004). The absence of an internal clock in the MBNs suggest they may receive time-of-day information through synaptic connections with the circadian neural circuit. The serotonergic DAL neurons innervate the α/β posterior MBNs and contain the *per* and *tim* gene products. The DAL neurons are, thus, potential candidates for the time-of-day regulation of olfactory learning. We hypothesize that the DAL neurons mediate the circadian modulation of olfactory STM through 5-HT (*Figure 2*). From this hypothesis, we predict the 5HT1A receptor coordinates rhythmic learning due to its presence in the synapse between the serotonergic DAL neurons and the α/β posterior α/β MBNs. We also predict that the 5HT1B receptor is not be involved in modulating circadian patterns of learning because it does not appear to be expressed in the α/β posterior

neurons MBNs. Because 5HT1A likely couples G_i, we expect that activated $G_i\alpha$ mediates the inhibition of Rutabaga, an adenylyl cyclase known to be expressed in the MBs. The inhibition of Rutabaga may therefore be involved in the circadian patterns of olfactory learning that have been observed in *Drosophila*, and such, we predict that loss of Rutabaga activity will lead to a loss in the circadian oscillations in short-term memory performance (*Figure 2*).

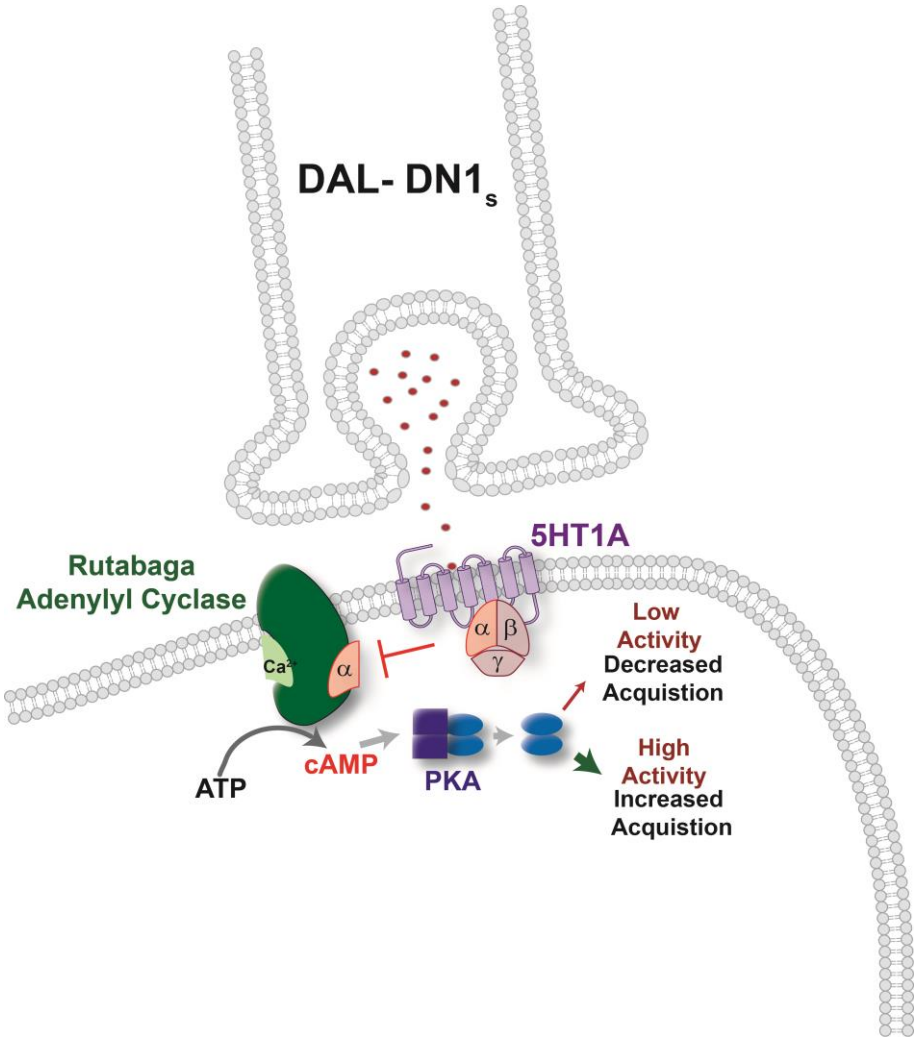

Figure 2. Proposed mechanism for the modulation of olfactory learning via 5-HT release from the DAL neurons. This diagram outlines our study's hypothesis. DAL neurons rhythmically release 5-HT into their synapses with the α/β MBNs. As 5-HT binds 5HT1A, the heterotrimeric G_i protein is activated. The $G_i\alpha$ subunit then binds Rutabaga adenylyl cyclase which inhibits production of cAMP. Lowered intracellular cAMP levels decreases activation of PKA which decreases intracellular activity and memory acquisition.

Methods

Drosophila Strains and Husbandry

Flies possessing four distinct genotypes were obtained, maintained, and assayed throughout this experiment. Canton-S (CS) flies served as a wildtype control, and all mutants were outcrossed into the Roman Lab Canton-S strain for a minimum of six generations. The *5HT1A*^{MB09978} strain and *5HT1B*^{MB05181} strain were acquired from Bloomington *Drosophila* Stock Center line. Both the *5HT1A*^{MB09978} and *5HT1B*^{MB05181} alleles are loss-of-function mutations created by the insertion of Minos elements (Bellen et al., 2011). The *rutabaga*^{2080} (*rut*^{2080}) allele is a loss-of-function mutation created by a transposable element insertion (Levin et al., 1992). *Table 1* outlines the *Drosophila* stocks used in our trials. Flies were raised on cornmeal, sucrose, and yeast agar at 25°C (Lyons & Roman, 2009). Flies were kept in LD cycles (12 hours light followed by 12 hours dark) for 2-3 days. Prior to testing, flies were kept in constant darkness (DD) for 1 day. Flies were assayed 3-7 days following eclosion. Zeitgeber Time (ZT) was used; ZT 0 represents dawn (lights come on), while ZT 12 represents dusk (lights go off) (Lyons & Roman, 2009).

Gene	Components and Genes	Stock #	Coding	Chromosome
5HT1A MB09978	w[1118]; Mi{GFP[E.3xP3]=ET1}5-HT1A[MB09978]	BDSC #27820	Gal4	Chr 2
5HT1B MB05181	w[1118]; Mi{GFP[E.3xP3]=ET1}5-HT1B[MB05181]	BDSC #24240	Gal4	Chr 2
rut2080	P{ry[+t7.2]=lArB}rut[2080]; P{w[+mC]=UAS-rut.Z}2	BDSC #9405	UAS	Chr X

Table 1. *Drosophila* mutant strains.

Olfactory Learning Paradigm

To test flies' learning and short-term memory, we employed a classical conditioning test referred to as the *Drosophila* negatively reinforced olfactory learning paradigm (Tully & Quinn, 1985). In this assay, flies are trained to associate an odor with an aversive stimulus, an electric shock. Before learning is measured, a training period must occur. In the training period, flies were exposed to one odor (the conditioned stimulus +, CS+) in the presence of an aversive electric shock (the unconditioned stimulus, US); flies were also exposed to a second odor (the conditioned stimulus -, CS-) in the absence of any additional stimuli. Odor concentrations and order of exposure (CS+ followed by CS- or vice versa) were regulated such that they did not impact fly learning. The odorants used in these trials included 4-methylcyclohexanol (MCH), 3-octanol (OCT), and benzaldehyde (BZ). Three minutes following training, flies were transferred to a T-maze to test the association between the CS+ and US. In the testing phase, flies were situated in a T-maze between two odor currents, one containing the CS+ and the other containing the CS-. If a fly had successfully learned during training, it would avoid the CS+ and enter the CS- arm of the T-maze during the testing phase. *Figure 3* depicts a generalized model of the training and testing phases. A performance index (PI) indicative of the performance for a given group of flies is given by the formula, $PI = \frac{(\#CS^{-} - \#CS^{+})}{Total\ Fly\ \#}$, where perfect learning would result in a PI of 1, and no learning would be indicated by a score of 0 (even distribution).

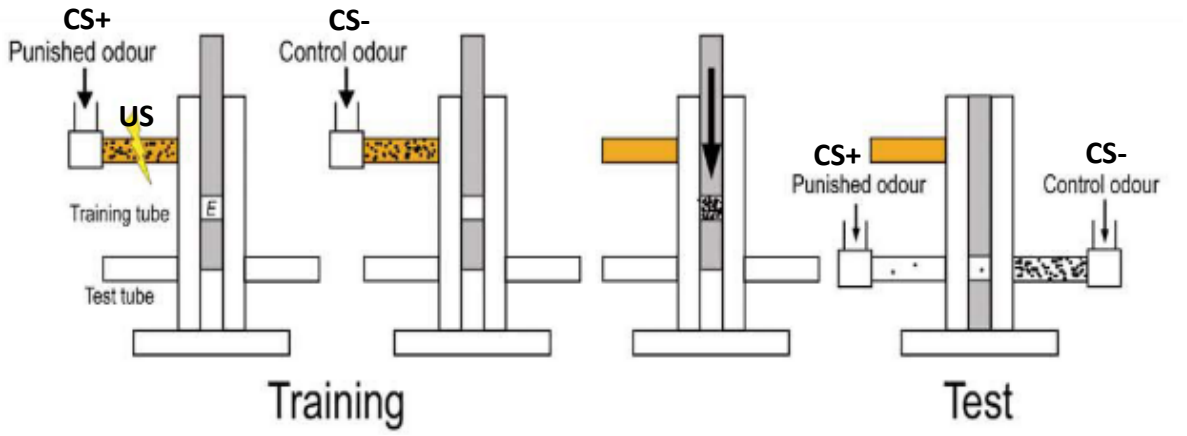

Figure 3. Negatively reinforced *Drosophila* olfactory learning paradigm. During training, flies are exposed to one odor, the CS+, in the presence of an electric shock, an aversive stimulus (US). Flies are also exposed to an odor, the CS-, in the absence of other stimuli. After training, flies are loaded into the elevator (denoted "*E*" in the figure) and lowered to a point of the T-maze where they are flanked by tubes filled with two odors, the CS+ and the CS-. Flies then disperse from the elevator into the odor filled tubes. If the Pavlovian conditioning in the training phase was successful, one would expect to see a greater proportion of flies in the CS- tube as flies would seek to avoid the CS+ which was paired with the aversive US. [Taken from (Gerber et al., 2004)]

SHORT Program

During the training stage of the olfactory learning paradigm, two distinct methods may be used: the LONG program and the SHORT program (Beck et al., 2000). In the LONG program, flies are exposed to the CS+ for one minute and are shocked twelve times at 90 volts to create an association between the CS+ and the US. However, the SHORT program exposes flies to the CS+ for only 10 seconds, and only one electric shock (90 volts for 1.25 seconds) is administered to create the association. It has been found that the LONG program overtrains flies and creates a plateau of learning, potentially masking underlying oscillations in the rate of learning. As we seek results based on circadian modulation of learning, utilizing submaximal levels of training allows for the potential observation of a higher amplitude of rhythm by removing the masking effects of overtraining (Lyons & Roman, 2009). For these reasons, we employed the SHORT program in the olfactory learning paradigm.

Data Analyses

Using the computing language "R" for data analysis, we ran a two-way ANOVA to test for significant differences in PI between our testing groups. If the ANOVA yielded a significant p-value ($p < 0.05$), we used R to run a Tukey posthoc test to determine what data showed significance. R was also used to generate box plots displaying our results.

Results

To test the hypothesis that DAL neurons affect the circadian modulation of learning through 5-HT, we first tested the prediction that the 5HT1A receptor plays an indispensable role in the circadian modulation of learning. This prediction was rooted in the knowledge that the DAL neurons expressed *per* and *tim*, clock proteins that suggested a circadian role for the neurons (Chen et al., 2012). Also, the 5HT1A receptor may be the only postsynaptic 5-HT receptor in the α/β MB lobes (Gnerer et al., 2015). Our prediction was tested by comparing $5HT1A^{MB09978}$ mutants to wildtype flies in the olfactory learning paradigm using the SHORT program for training. In the $5HT1A^{MB09978}$ mutants, learning performance was not significantly different based on genotype between the wildtype and the mutant groups ($p = 0.4501$; $F = 0.4501$) as determined by a two-way ANOVA (*Figure 4*). As seen in previous studies, learning performance in wildtype flies was significantly higher at CT13-14 compared to CT1-2 ($p = 0.0121$; $F = 7.476$), which is indicative of the rhythmic performance patterns that result from a system that is modulated by the circadian clock (Lyons & Roman, 2009) (*Figure 4*). However, the $5HT1A^{MB09978}$ mutants failed to show significant differentiation in learning based on time of day ($p = 0.832$; $F = 0.046$) (*Figure 4*). This result suggests that $5HT1A^{MB09978}$ mutants lost the circadian regulation of the olfactory system required for the oscillatory patterns of learning that are typical of wildtype flies. Currently, experiments are being performed to determine if rescuing the function of the 5HT1A receptors in these mutants returns the typical pattern of rhythmic learning. The results of these ongoing studies will be crucial in determining the role of the 5HT1A receptor in the circadian modulation of olfactory learning.

We next tested the prediction that the 5HT1B receptor would be dispensable for circadian rhythms in associative olfactory learning. In this experiment, *5HT1B*^{MB05181} mutants were compared to wildtype flies in the olfactory learning paradigm using the SHORT program in training and a two-way ANOVA test to determine significant differences. There was no significant difference ($p = 0.4389$; $F = 0.617$) between *5HT1B*^{MB05181} mutants and wildtype flies regarding learning performance as a function of genotype (*Figure 5*). Additionally, *5HT1B*^{MB05181} mutants and wildtype flies both displayed an intact rhythm in olfactory learning as olfactory learning performance was significantly different as a function of time-of-day ($p = 0.0357$; $F = 4.868$) (*Figure 5*). These results demonstrate that the 5HT1B receptor is not required for circadian modulation of olfactory learning in *Drosophila*.

Because the 5HT1A receptor in the DAL neurons is believed to be G_i-coupled, we also tested the function of Rutabaga adenylyl cyclase. As we hypothesized the 5HT1A receptor to be involved in the pathway for the circadian modulation of olfactory learning, we also hypothesized that Rutabaga adenylyl cyclase is involved in rhythmic learning. The *rutabaga*^{2080} mutants displayed significantly diminished learning compared to wildtype flies as determined via a two-way ANOVA comparing PI values ($p = 0.000499$; $F = 14.131$) (*Figure 6)*. Still, *rutabaga*^{2080} showed significant differences in learning as a function of time-of-day ($p = 0.014659$; $F = 6.456$), retaining rhythm even at a reduced performance level (*Figure 6*). This result suggests that circadian modulation via the DAL neurons does not involve *rutabaga*. This result is intriguing in conjunction with the results from the *5HT1A*^{MB09978} mutants, which implicated 5HT1A – which potentially couples G_i – as a necessity for rhythmic learning. Since the circadian STM was unaffected in *rutabaga* mutants, it seems likely that 5HT1A's role in the pathways from the DAL neurons to the MBs does not involve *rutabaga* adenylyl cyclase.

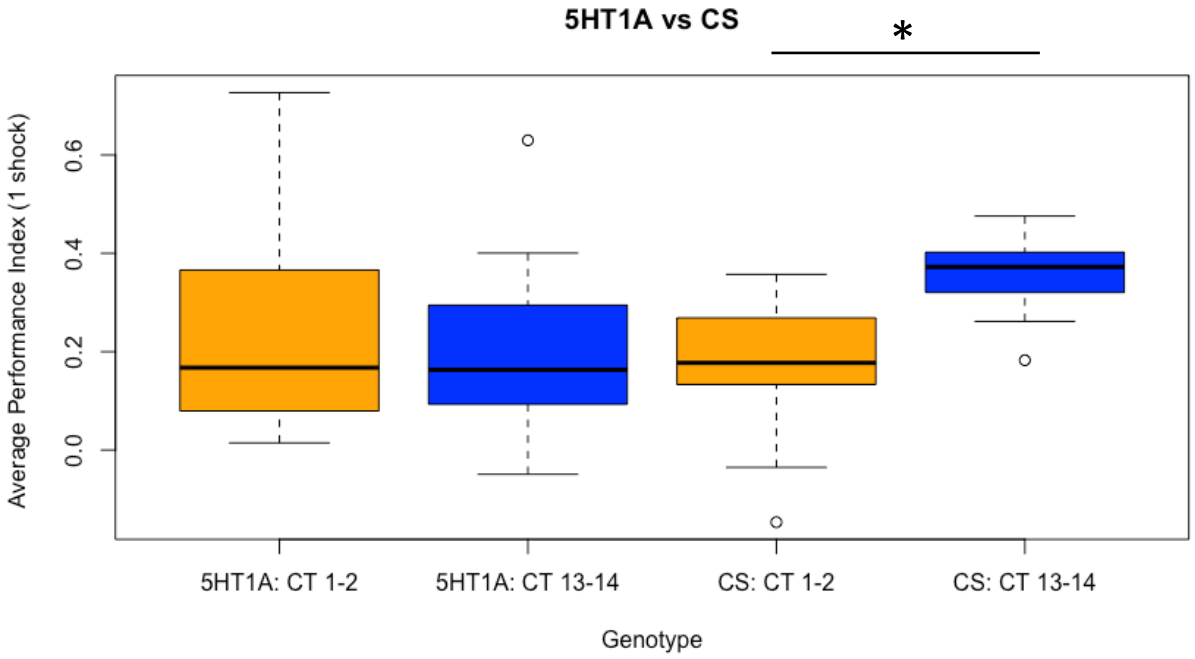

Figure 4. *5HT1A*^{MB09978} mutants vs. wildtype associative olfactory learning performance as a function of time-of-day. *5HT1A*^{MB09978} mutants displayed similar learning performance compared to wildtype flies (p = 0.4501; F = 0.4501), yet *5HT1A*^{MB09978} mutants did not display a significant difference in performance at different times of the day (p = 0.832; F = 0.046). Wildtype flies did display a difference in performance as a function of time-of-day (p = 0.0121; F = 7.476). These results suggest a role for the 5HT1A receptor in the modulation of circadian rhythms in the olfactory learning. ANOVA tests were used to determine significance.

* $p < 0.05$

n = 11

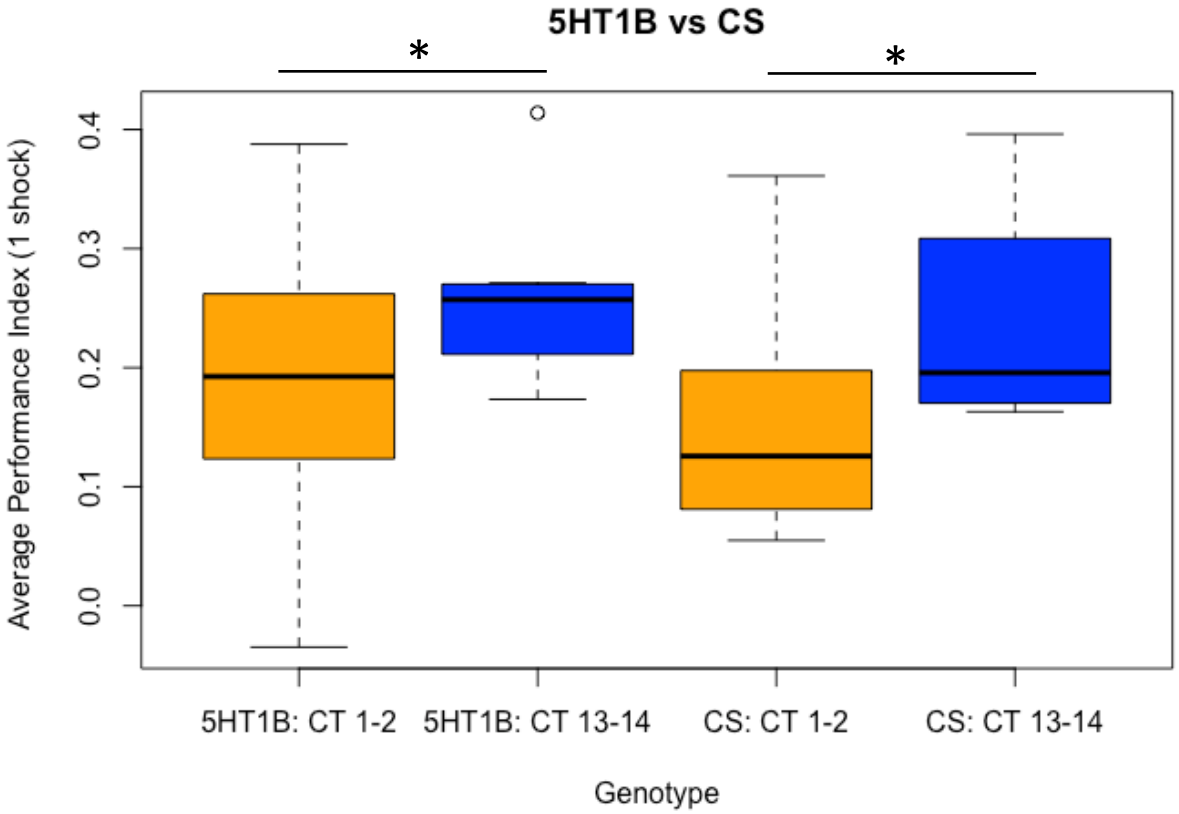

Figure 5. *5HT1B*^{MB05181} mutants vs. wildtype associative olfactory learning performance as a function of time-of-day. *5HT1B*^{MB05181} mutants displayed similar learning performance compared to wildtype flies (p = 0.4389; F = 0.617) and retained a significant difference in performance at different times of the day (p = 0.0357; F = 4.868). These results do not support a role for the 5HT1B receptor in mediating circadian rhythms in olfactory learning. ANOVA tests were used to determine significance.

* $p < 0.05$

n = 8

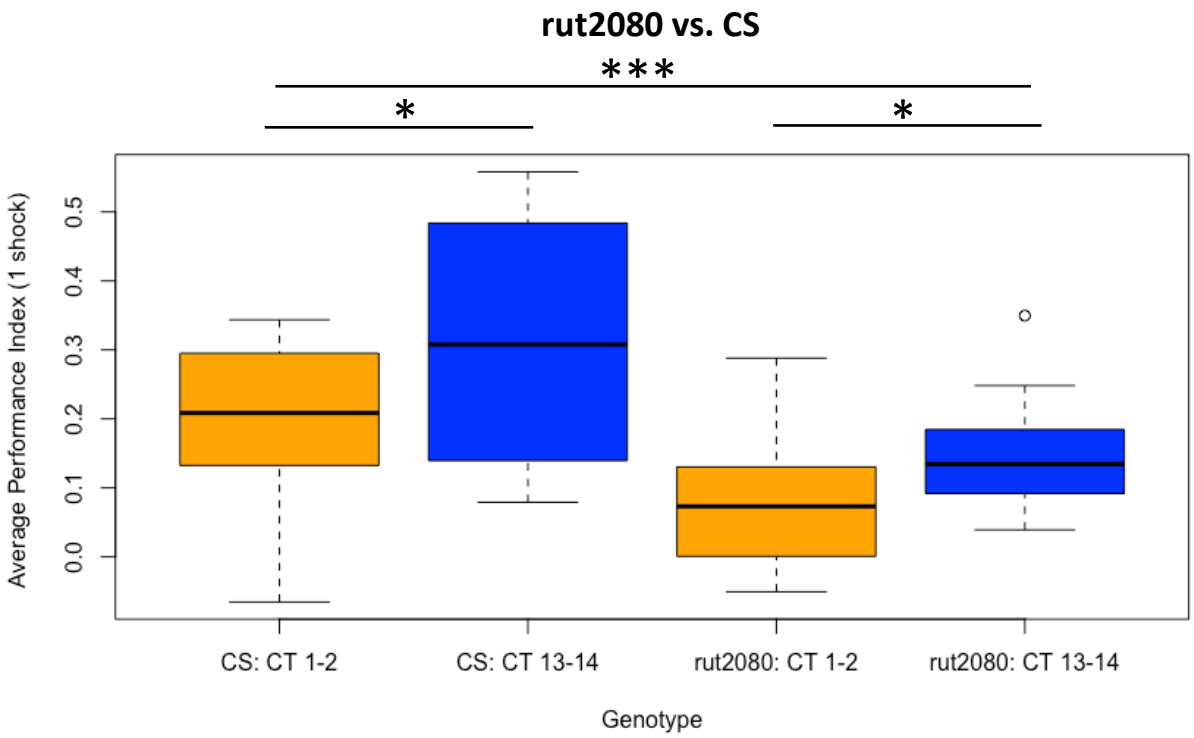

Figure 6. *Rut*^{2080} mutants vs. wildtype associative olfactory learning performance as a function of time-of-day. *Rut*^{2080} mutants performed at a significantly worse level than wildtype flies (p = 0.000499; F = 14.131) yet retained a significant difference in performance at different times of the day (p = 0.014659; F = 6.456). These results suggest that while *rutabaga* is a vital component of olfactory learning, it may not play a role in modulating circadian rhythms in the pathway. ANOVA tests were used to determine significance.

* $p < 0.05$

*** $p < 0.001$

n = 8

Discussion

This study was intended to investigate the DAL neurons' role in the circadian modulation of olfactory STM via the neurotransmitter 5-HT. Serotonin function in the synapse between the DAL neurons and the *Drosophila* MBNs was inhibited using $5HT1A^{MB09978}$ mutants. These mutants showed wildtype learning levels but lacked the temporal oscillation in performance indicative of circadian modulation. Conversely, $5HT1B^{MB05181}$ mutants did not differ from wildtype flies in STM performance. We also examined the function of Rutabaga, a potential element downstream from the 5HT1A receptor, which likely couples a G_i protein that can inhibit adenylyl cyclases. While rut^{2080} mutants showed decreased STM performance, they retained circadian rhythms. These results suggest that the 5HT1A receptor is active in the pathway that conveys time-of-day information to the MBNs. However, there was no support for the presence of the 5HT1B receptor and Rutabaga in this same pathway. More research is needed to confirm *5HT1A*'s role in the circadian modulation of MBN activity. Additionally, other elements downstream from the 5HT1A receptor should be explored just as we studied the potential downfield effector *rutabaga* to provide further clarity on the molecular mechanisms that lie beyond the 5HT1A receptor in the MBNs.

Circadian olfactory learning is impacted by 5-HT receptors in the MBs

Because the DAL neurons express the clock proteins *per* and *tim* and innervate the α/β posterior lobes of the *Drosophila* MBs – the olfactory learning center – a logical prediction

would be that the DAL neurons are involved in the modulation of rhythmic olfactory learning (Chen et al., 2012; Fropf et al., 2018). As the DAL neurons are likely serotonergic and synapse onto the α/β MB lobes, which exclusively express the 5HT1A receptor, the logic would follow that the 5HT1A receptor mediates the circadian modulation of olfactory learning by binding 5-HT (Gnerer et al., 2015). Our results support this prediction.

5HT1A^{MB09978} mutants performed at a PI value similar to wildtype flies on olfactory learning tests during the daytime. However, while wildtype flies showed a significant difference in learning as a function of time-of-day, the mutants showed no such rhythm, perhaps suggesting that the 5HT1A receptor is necessary to sustain time-of-day-based oscillations in performance. The fact that knockout of a 5-HT receptor in the MBNs prohibits rhythm in olfactory STM supports the overall hypothesis that the DAL neurons are involved in circadian modulation of olfactory learning through 5-HT.

Additionally, *5HT1B*^{MB05181} mutants displayed no significant differences in performance level or rhythm when compared to wildtype flies as predicted. The ability of the flies to maintain rhythm in olfactory learning in the absence of *5HT1B* demonstrates that this gene is not necessary for this rhythm. As such, our results support the idea that *5HT1A* is involved in the circadian modulation of olfactory STM while *5HT1B* is not involved in maintaining the rhythm in olfactory learning.

Olfactory learning depends on intracellular cAMP levels

Cyclic AMP has been demonstrated as vital in olfactory associative learning, and exploring cAMP regulation in the MBs may be crucial to understand how the circadian network

impacts olfactory learning (Davis et al., 1995). Previously, *rutabaga* had been characterized as imperative for olfactory STM, a finding that aligns with the idea that cAMP levels are crucial in olfactory learning (Davis et al., 1995; McGuire et al., 2003). If, as the previous discussion suggested, *5HT1A* is involved in establishing a rhythm in olfactory learning, an adenylyl cyclase may lie downstream of the receptor if it is coupled to a heterotrimeric G_i protein as we expect. Rhythmic 5-HT binding events could lead to rhythmic activation of the G_i protein, which could then rhythmically inhibit an adenylyl cyclase variant, establishing oscillations in intracellular cAMP concentration. Because previous studies demonstrated *rutabaga* to be indispensable for olfactory learning, we suggested that it may be an adenylyl cyclase variant that lies downstream from the 5HT1A receptor.

In our study, rut^{2080} mutants performed at significantly worse levels than wildtype groups in the olfactory learning paradigm, but, interestingly, they retained daily rhythms in activity. This finding maintains the importance of *rut* in olfactory learning and aligns with findings that intracellular cAMP levels in the MBNs are crucial in mediating olfactory learning (Davis et al., 1995; McGuire et al., 2003). However, these results fail to support the prediction that *rutabaga* played a role in modulating circadian rhythms in olfactory learning. Thus, a question arises as to the identity of the operator downstream from the 5HT1A receptor. While the potential presence of the heterotrimeric G_i protein and the importance of cAMP in olfactory memory performance suggest that this operator was an adenylyl cyclase, we do not yet know enough to rule out other configurations. Still, because rut^{2080} mutants showed significant reductions in olfactory STM performance, our results support *rutabaga*'s general role as a modulator of olfactory STM via the mediation of intracellular cAMP concentrations.

Future studies and unanswered questions

The results discussed in this study are part of ongoing research. As such, further testing may bring about data that could affect the strength and validity of our conclusions though the present data is statistically sound enough that our preliminary conclusions are not inappropriate.

Foremost, more trials will be run to ensure the validity of our data. We plan on running a genetic rescue of the *5HT1A* in $5HT1A^{MB09978}$ mutants to determine if the introduction of the functional receptor in the mutant flies recovers rhythm in olfactory STM. If the rhythm is recovered, the 5HT1A receptor's role in the circadian modulation of olfactory learning will be further supported.

The most pivotal question resulting from this study pertains to what effector lies downstream of the 5HT1A receptor if it communicates time-of-day information from the DAL neurons to the MBNs. If the 5HT1A receptor does indeed coupled G_i, one may logically suggest that this downstream effector is an adenylyl cyclase especially given the importance of intracellular cAMP levels in olfactory learning (Davis et al., 1995; Gilman, 1987). While we found *rutabaga* does not likely function in circadian pathways, other adenylyl cyclases may be worthy of investigation, especially if they have been implicated as indispensable for olfactory STM.

Another question lies upstream from the 5-HT receptors in the DAL neurons. While the presence of the clock proteins *per* and *tim* drove our prediction that the DAL neurons would provide rhythm to the MBNs, the circuity behind the DAL neurons' connections to the central clock is still unknown. While there are known spatial associations between serotonergic neurons and the s-LNvs, there is no evidence proving a connection between the s-LNvs and the DAL

neurons (Hamasaka & Nassel, 2006). This provides a compelling point for future study, and the mechanisms upstream from the DAL neurons remain essential for constructing an overall model of the pathway describing the circadian modulation of olfactory memory.

A final question lies in the potential coupling of the heterotrimeric G_i protein to 5HT1A. We expect that if G_i is coupled to 5HT1A, it too will be indispensable in mediating circadian rhythms in olfactory learning. Thus, by determining if the heterotrimeric G_i protein is necessary for rhythmic olfactory STM, we may be able to support or refute our proposition that it couples 5HT1A. The Roman lab is currently conducting this research using a CRISPR modified version of the G_i protein, which may be inhibited by the S1 subunit of pertussis toxin (PTX). A system exists that allows for the selective expression of PTX. As such, we can spatially and temporally control G_i protein inhibition via PTX expression. This study aims to determine if the G_i protein in specific brain regions is indispensable in modulating circadian rhythms in olfactory associative learning. If it is essential for rhythm, there would be further support for a 5HT1A-G_i coupling.

Conclusion

This study was conducted to better understand the mechanisms that underly the circadian clock's impact on STM. By focusing on the circadian modulation of *Drosophila* olfactory learning, we used a model organism whose olfactory memory and circadian clock systems had already been well defined. The data we obtained will help construct a model of the circuity that connects the *Drosophila* circadian clock to the MBs, the olfactory learning center. Additionally, it will inform and direct future experiments that look to build upon this model. Ideally, the

information we accumulate regarding the *Drosophila* circadian system can be translated to better understand daily rhythms in human memory. In a modern society that has ceased operating on the traditional light-dark cycles that drove our evolution, knowing how time-of-day impacts human memory is crucial. Our goal is to develop that may lead to solutions that will improve human cognitive abilities in non-adaptive hours.

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