CONNECTING THE CIRCADIAN CLOCK WITH CHEMOSENSATION

A Dissertation

by

ABHISHEK CHATTERJEE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Biology

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Approved by:

Chair of Committee,	Paul Hardin
Committee Members,	Mark Zoran
	Ginger Carney
	Gladys Ko
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ABSTRACT

Connecting the Circadian Clock with Chemosensation. (May 2011)
Abhishek Chatterjee, B.Sc. (Hons.), Presidency College; M.Sc., University of Calcutta
Chair of Advisory Committee: Dr. Paul Hardin

Chemoreception is a primitive sense universally employed by organisms for finding and selecting food, rejecting toxic chemicals, detecting mates and offspring, choosing sites for egg-laying, recognizing territories and avoiding predators. Chemosensory responses are frequently modulated based on the internal environment of the organism. An organism's internal environment undergoes regular changes in anticipation and in response to daily changes in its external environment, *e.g.*, light-dark cycle. A resettable timekeeping mechanism called the circadian clock internally drives these cyclical changes with a ~24 hour period. Using electrophysiological, behavioral and molecular analyses, I tested *where* and *how* these two conserved processes, *viz.*, the circadian timekeeping mechanism and the chemosensory pathway, intersect each other at organismal and cellular levels.

The presence of autonomous peripheral oscillators in the chemosensory organs of *Drosophila*, prompted us to test whether chemosensory responses are under control of the circadian clock. I found that local oscillators in afferent (primary) chemosensory neurons drive rhythms in physiological and behavioral responses to attractive and aversive chemical signals. During the middle of the night, high level of G protein-coupled receptor kinase 2 (GPRK2), a clock controlled signaling molecule present in chemosensory neurons, suppresses tastant-evoked responses and promotes olfactory responses. G-protein mediated signaling was shown to be involved in generating optimal response to odorants. Multifunctional chemosensory clocks exert control on feeding and metabolism. I propose that temporal plasticity in innate behaviors should offer adaptive advantages to flies.

DEDICATION

This work is dedicated to creators whose music, films and words let me breathe when I feel trapped.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Paul Hardin, for his guidance and encouragement. He has been a greatt mentor, and a perfect gentleman. My committee members, Dr. Mark Zoran, Dr. Ginger Carney, and Dr. Gladys Ko offered me constructive support. I have always received generous help from Dr. Zoran throughout the course of this research. I also want to extend my gratitude to Dr. Gregg Roman who provided me reagents for the G protein experiments. Many teachers from the good old city of Calcutta contributed to the development of my scholastic interests.

Thanks also go to my friends - Nandina, Jayeeta and Vikram, and colleagues – Xin, Yixiao, Jerry, Mahesh, Paul, Shintaro, Partha and Wangjie, and the department faculty and staff for making my time at Texas A&M University a unique experience. Thousands of flies were sacrificed for my research – I will continue to wonder at their liveliness.

I am indebted to religion for trying to teach me how to look at lives from a macroscopic perspective. Finally, thanks to Arnab for giving me the opportunities to experience overpowering emotions, to Payel for her unwavering loving attachment, my mother for instilling into my subconscious “the audacity of hope”, my father for his conviction that I am going to win the Nobel prize, and my brother for being my protector from childhood.

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INTRODUCTION

Prologue

The circadian clock controls daily rhythm in gene expression, which is subsequently translated into rhythms in physiology, metabolism and behavior. By allowing the internal metabolic processes to run in accordance with external zeitgeber cycles, the circadian oscillators offer critical survival advantages. Rhythmic chemosensory responses are one of the major outputs of the circadian clock in insects and mammals. The molecular and cellular mechanisms by which the chemosensory system and the circadian clock operate are well known in *Drosophila*. Ease and efficiency of using neurogenetic tools, and the similar principle of organization of the chemosensory system and the circadian oscillator between mammals and *Drosophila* make the fruit fly an ideal model system to analyze the connections between the clock and chemoreception at behavioral, physiological, genetic and molecular levels. The main objective of the proposed research in this dissertation is to identify how the circadian pacemaker alters daily responsiveness to odorants and tastants. Previous research suggested that the clock modulates components of the chemosensory signal transduction pathway in *Drosophila*. The molecular cascade that underlies chemoelectrical signal transduction is poorly characterized in insects. Therefore, I carried out additional experiments to elucidate the olfactory signaling pathway of *Drosophila*.

To determine the connection between chemoreception and the clock, it is essential to know beforehand (a) how does the circadian oscillator function, (b) how do flies detect

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This dissertation follows the style of Cell.

odors and tastants, and subsequently mount appropriate behavioral response against these important chemical cues. These will be briefly discussion in the “introduction”.

First, single-unit recordings and electroantennograms will be carried out to probe the role of G proteins in olfactory reception (Chapter II). Then electrophysiological techniques will be employed to understand clock control over unitary action potentials from individual chemosensory neurons (Chapter III). Using various transgenic manipulations I will investigate how the oscillator influences single unit responses (Chapter III). Finally, the role of clock in regulating gustatory behavior and feeding will be studied (Chapter IV). I will identify the pacemaker cell that drives rhythm in appetitive behavior and also determine the key molecule that links taste with the circadian oscillator (Chapter IV). The similarity in chemosensory system organization and feeding behavior in flies and mammals, as well as diurnal changes in chemosensory sensitivity in humans, suggest that our results are relevant to the situation in humans.

Circadian clocks operate via feedback loop oscillators

Circadian clocks are endogenous timekeeping mechanisms allowing most organisms to anticipate daily events and hence to organize their physiology and behavior in a proactive rather than a responsive manner. Circadian clocks oscillate with an approximately 24-hr period in a temperature-compensated manner, persist without environmental time cues but entrainable by environmental zeitgebers (light, temperature, food, social interaction), and presumably confer a selective advantage. The clock controls a plethora of behavioral, physiological, metabolic and molecular processes in organisms ranging from bacteria and fungi to humans. Clock deficiencies are associated with abnormal sleep wake cycles (*e.g.* Familial Advanced Sleep Phase Syndrome), epilepsy, cerebrovascular disease, mood disorders, diabetes, cancer, etc. Disorders coupled with a dysfunctional clock emphasize the clinical importance of understanding

the molecular organization of the circadian system, which includes input pathways for entrainment, the core oscillator, and output pathways that generate overt rhythms.

We have gathered substantial knowledge about the molecular architecture of the core oscillator that underlies rhythmic gene expression. A highly conserved feature of circadian clocks is that they are composed of cell-autonomous transcriptional feedback loops that include both positive and negative elements which regulate cyclical gene expression (Bell-Pedersen et al., 2005). In *Drosophila melanogaster*, the core circadian feedback loop is composed of the positively acting basic-helix–loop–helix (bHLH) PER-ARNT-SIM (PAS) partners CLOCK (CLK) and CYCLE (CYC), which bind to E-box (CACGTG) enhancer elements and stimulate transcription of *period (per)* and *timeless (tim)* in a time-dependant manner (Fig. 1), along with other key clock and downstream effector genes (Hardin, 2005). CLK-CYC binding to upstream and/or intronic E-boxes promotes chromatin modifications (acetylation of histone H3-K9, and trimethylation of histone H3-K4) that aid RNA polymerase II action (Taylor and Hardin, 2008). In the late afternoon/early evening, PER and TIM begin to accumulate in the cytoplasm and eventually interact to form a complex that enters the nucleus in the middle of the night. As PER is produced it is phosphorylated by DBT (doubletime) and CK2 (casein kinase 2), which leads to its degradation. TIM binds to, and stabilizes, phosphorylated PER, which remains bound to DBT. PER is also stabilized by PP2a (protein phosphatase 2a), which removes phosphates that were added to PER. The TIM-PER-DBT complexes are phosphorylated by SGG (shaggy), which, in concert with phosphorylation by CK2, promotes their transport into the nucleus. TIM-PER-DBT complexes then bind to CLK-CYC, and inhibit *per* and *tim* transcription by (a) CLK-mediated recruitment of PER to circadian promoters leading to the nighttime decrease of CLK/CYC activity, and (b) sequestration of CLK in a strong, approximately 1:1 PER-CLK off-DNA complex (Menet et al., 2010). PER and CLK are then destabilized, via DBT phosphorylation, and degraded, whereas cryptochrome (CRY)-dependent TIM degradation (at least in response to light) is triggered by tyrosine phosphorylation. The accumulation of non-

phosphorylated (or hypophosphorylated) CLK leads to another cycle of *per* and *tim* transcription (Hardin, 2005).

Post-translational regulatory mechanisms are thought to modulate the stabilities, activity levels, and subcellular localization of clock components which in turn affect the timing of their action in the daily cycle. Multiple levels of posttranslational controls are built into these systems, presumably to delay the cycles so that they take a full 24 hr and maintain robust amplitude of cycling from the transcription of clock components all the way to physiological outputs. In addition, these controls provide mechanisms by which the clock can be reset by environmental inputs.

In the *Clk* feedback loop (Fig. 1), CLK-CYC heterodimers bind to E-boxes and activate Vrille (Vri) and PAR Domain Protein 1ε (Pdp1ε) transcription. VRI accumulates in parallel with its mRNA, binds to promoters and inhibits Clk transcription. PDP1ε accumulates in a delayed fashion and supplants VRI from V/P boxes to derepress Clk transcription. However, the primary activator of Clk transcription has not yet been identified. PDP1ε levels also control rhythmic locomotor output in a direct fashion (Benito et al., 2007). Accumulation of non-phosphorylated (or hypophosphorylated) CLK leads to heterodimerization with CYC and another cycle of *vri* and *pdp1ε* transcription (Hardin, 2005).

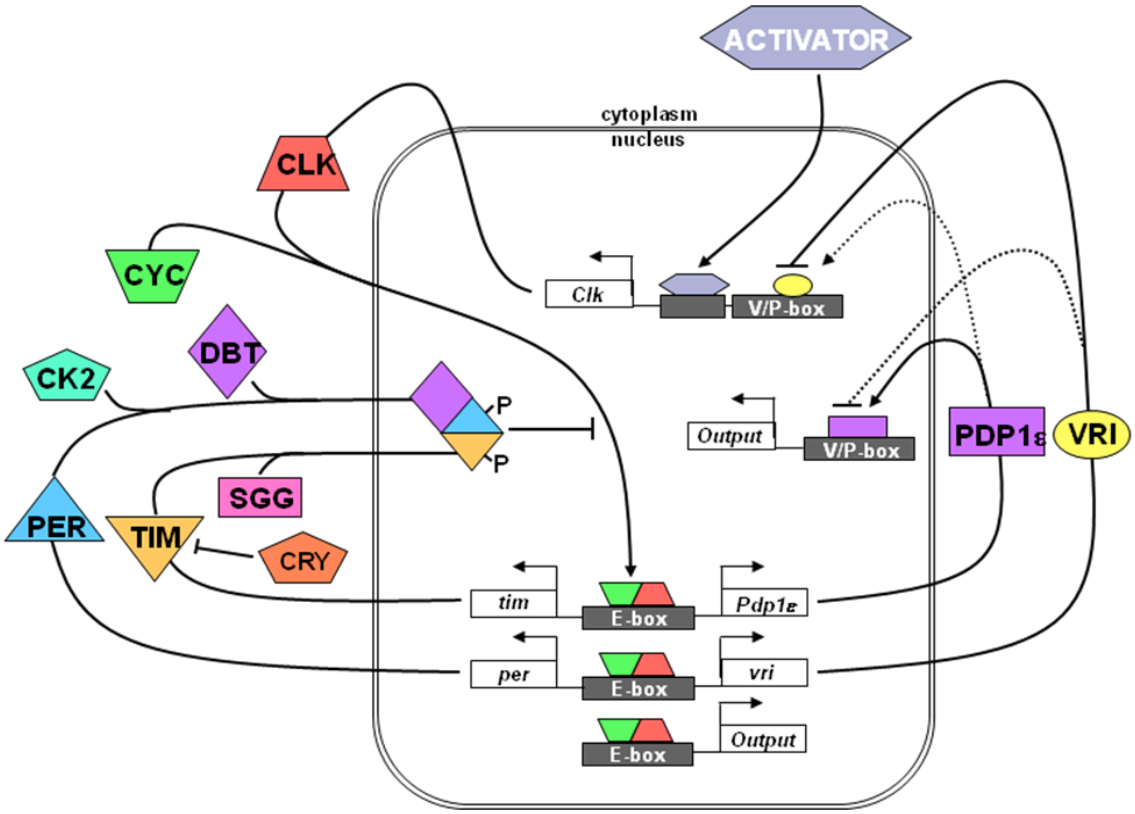

Fig. 1: Model of the transcriptional feedback loops that keep circadian time in *Drosophila* (adapted from Benito et al., 2007).

Clock influences physiological and behavioral outputs

Based on functionality, the circadian clocks are divided into two major categories, namely central pacemaker and peripheral circadian clocks. The central pacemaker located within the suprachiasmatic nucleus (SCN) of humans maintains synchrony between the different peripheral clocks via neuronal and humoral cues. Almost every human cell, *e.g.*, hepatocytes, cardiomyocytes, pancreatic β cell, etc. possesses an intracellular circadian clock, capable of altering both cellular and organ function over the course of the day (Young and Bray, 2007). Central and peripheral clocks do not expresses a completely overlapping set of clock-controlled genes, and additionally the

phases of gene expression rhythm differ between them. The importance of both central and peripheral clocks in generating biological rhythms and their interactions with tissue-relevant signaling is increasingly evident (Giebultowicz and Kapahi, 2010).

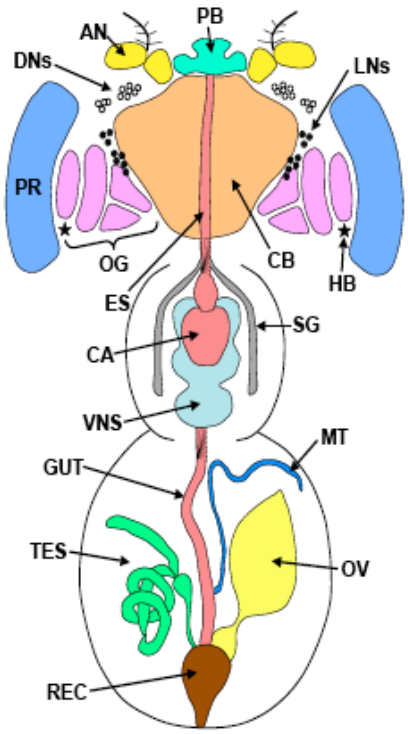

Fig. 2: Distribution of clock-gene expressing tissues in *Drosophila* body. PB=proboscis, AN=antenna, DNs=dorsal neurons, LNs=lateral neurons, OG=optic ganglia, CB=central brain, HB=Hofbauer-Buchner eyelet, ES=esophageus, SG=salivary gland, CA=cardia, VNS=ventral nervous system, MT=malpighian tubule, GT=gut, TS=testis, OV=ovary, RC=rectum (Bell-Pedersen et al., 2005).

Circadian oscillators are present in a variety of tissues throughout the head, thorax and abdomen of *Drosophila* (Fig. 2). In fact all clock gene expressing tissues displayed rhythmic expression except for the ovary (Hardin, 1994). Experiments using *per*-driven luciferase reporter gene showed that these oscillators operate autonomously and are directly light entrainable (Plautz et al., 1997).

Although circadian oscillators are found in many tissues (Fig. 2), only few rhythmic outputs have been identified in *Drosophila* adults. The most extensively studied rhythmic output is locomotor behavior, *e.g.*, rest/activity cycles. A group of 4-5 small ventral lateral neurons (sLN_vs) in each hemisphere of the brain are both necessary and sufficient to drive robust activity rhythms (Renn et al., 1999). Many sensory systems are regulated by the circadian clock. Daily rhythms in neuronal sensitivity/activity have been reported for visual (Barth et al., 2010), auditory (Lotze et al., 1999), and electroceptive (Stoddard et al., 2007) systems of animals. *Drosophila* larvae elicit rhythmic phototaxic behavior - strength of these responses peak at dawn, clock mutants show altered photophobicity, and the larval pacemaker neurons act on Bolwig's organ to filter visual sensitivity (Mazzoni et al., 2005).

Chemosensation is also under control of the internal clock in fruit flies. Spontaneous electrical activity of olfactory receptor neurons (ORNs), odor-induced physiological responses (EAG) of antenna and odor-driven chemotactic behavior of adult flies show circadian oscillations (Krishnan et al., 1999; Krishnan et al., 2008; Zhou et al., 2005). Circadian rhythms in odor-evoked physiological responses have been described also in human, mouse, cockroach and moth (Granados-Fuentes et al., 2006; Merlin et al., 2007; Nordin et al., 2003; Page and Koelling, 2003). Daily changes in taste sensitivity are known in human, blowfly, moth and fruit fly (Nakamura et al., 2008; Hall, 1980; Simmonds et al., 1991; Chatterjee et al., 2010). Rhythms in chemosensory ability are often effected by local clocks in chemosensory tissues. Interestingly, the influence of the chemosensory clock may extend beyond modulation of chemosensation, *e.g.*, feeding is under circadian clock control in *Drosophila*, with regulatory contributions from clocks in chemosensory neurons and the fat body (Xu et al., 2008; Chatterjee et al., 2010). The chemosensory clock offers an advantageous model for probing the molecular clockwork and network property of peripheral oscillators. A peripheral oscillator in the epidermis regulates rhythms in cuticle deposition (Ito et al., 2008). In the visual system, two

classes of interneurons in the first optic neuropil (lamina), the monopolar cells L1 and L2, show rhythmic circadian changes in the shape and size of their axons (Pyza and Gorska-Andrzejak, 2008). Circadian rhythms in immunity, short-term memory formation, eclosion, sleep-wake cycle, male sex drive, and egg-laying behavior of adult female flies have been documented (Allada and Chung, 2010). Genome-wide circadian expression profiling studies have revealed potential connections between circadian clocks and many aspects of carbohydrate, amino acid, lipid, and protein metabolism, as well as detoxification (Giebultowicz and Kapahi, 2010). The clock in fat body plays a major role in these processes (Xu et al., 2008).

Functional anatomy of the *Drosophila* chemosensory system

Sensory systems - touch, hearing, vision, taste, smell - map features of the external world into internal representations in the brain that ultimately allow animals to navigate their environments (Laissue and Vosshall, 2008). Chemosensation in the fruit fly, *Drosophila melanogaster*, is crucial for a variety of behaviors, including associative learning, courtship, foraging, egg-laying, avoiding predators and toxins, and flight (Iyengar et al., 2010; Montell, 2009).

In the *Drosophila* olfactory system, olfactory sensory neurons (OSNs, also known as olfactory receptor neurons or ORNs) located in the antennae and maxillary palps (Fig. 3) send axons to the antennal lobe in the central brain. Ciliated endings of ORN dendrites are present in sensory organs called sensilla, where they are exposed to the environment, and the different types of sensilla respond to different types of odorants. The design of all chemosensory sensillae is similar in principle. Gustatory sensilla are present in the form of taste hairs and smaller taste pegs on the labella of the proboscis, legs, anterior wing margins and ovipositor (Fig. 3); internal sensilla occur on the pharyngeal part of proboscis. The sensillar lymph contains odorant-binding proteins (OBPs) and

chemosensory proteins (Che) secreted by non-neural accessory cells present in the sensilla (Starostina et al., 2009). OBPs are thought to facilitate the transfer of apolar odorants in the aqueous lymph to membrane-associated odorant receptors, and are essential intermediaries in pheromone detection and insect-host plant interactions (Arya et al., 2010). Olfactory sensilla can be distinguished morphologically from thermo- and hygro-sensitive sensilla by the presence of many small pores on the sensillar wall, which are believed to allow access to odors (Laissue and Vosshall, 2008). A total of about 410 sensilla (containing ~1300 ORNs) cover the antenna, while the maxillary palp has about 60. A sensillum houses the dendrites of 1-4 ORNs (Fig. 3). Three distinct morphological and functional classes of olfactory sensilla are present: club-shaped basiconic sensilla, long and pointed trichoid sensilla and short, peg-shaped coeloconic sensilla. The different sensilla types are distributed on the antennal surface in a stereotyped maner. Large basiconic sensilla are clustered at the medial face of the antenna and they sense food odors and carbon dioxide (a stress pheromone in flies), coeloconics are interspersed but concentrated in the center (sacculus) of the antenna and are used to smell water vapor, ammonia, acids and putrescine, while the pheromone-sensing trichoid sensilla are arranged in diagonal bands across the lateral face of the antenna (Laissue and Vosshall, 2008).

The external taste hairs are structurally analogous to trichoids, but the internal taste papillae resemble basiconics (Isono and Morita, 2010). Taste hairs in the proboscis are of three types: short hairs contain four GRNs (gustatory receptor neurons) and are very sensitive to bitter compounds, intermediate hairs have only two GRNs, long hairs with four GRNs respond well to sugars.

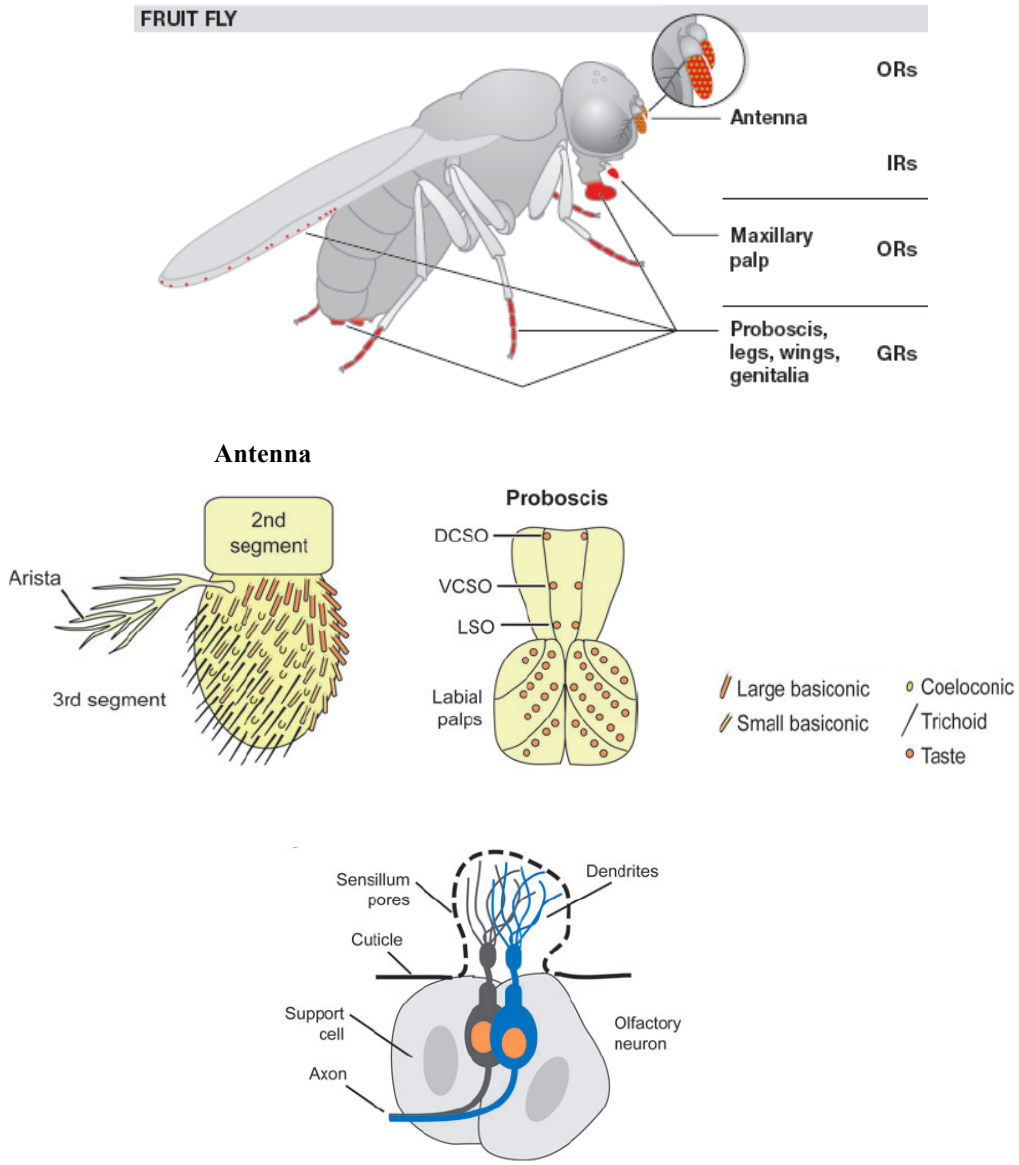

Fig. 3: The chemosensory organs of flies. The upper panel shows the peripheral location of chemosensory organs. Abbreviations: GRs, gustatory receptors; IRs, ionotropic receptors; ORs, odorant receptors. The middle panel depicts the schematics of the exterior surface of the olfactory organ, antenna and the gustatory organ, proboscis. Abbreviations: LSO, labral sense organ; VCSO, ventral cibarial sense organ; DCSO, dorsal cibarial sense organ. The bottom panel shows the schematic of a typical olfactory sensillum, housing two ORNs (upper panel - Silbering and Benton, 2010; middle and bottom panels - Vosshall and Stocker, 2007).

A typical taste hair is made up of 7-9 cells: a mechanosensory neuron (MSN), 2-4 GRNs, a trichogen cell that secrets proteins to build the shaft of the taste hair, a tormogen cell that produces the socket cuticle material, a thecogen cell that wraps the soma of the neurons, and lastly a glia that ensheaths the neuronal axons (Isono and Morita, 2010). The bipolar GRN sends a single thin dendrite into the sensillum that has a terminal pore through which tastants come into contact with the GRNs. The four GRNs in a sensilla may be categorized into sugar-sensing S neuron, water/hypoosmolarity-responsive W neuron, one L1 neuron that responds to low concentration of monovalent cations in salt solution, and a L2 neuron that detects bitter and high salt concentration. S and W neurons can promote feeding/drinking behavior, which is inhibited by the L2 neuron. Twelve gustatory receptor (GR) genes including *Gr66a* are expressed in the L2 cell, whereas 8 GR genes including *Gr5a* are expressed in the S neuron. *Gr66a* labels only bitter (L2) neurons, whereas *Gr5a* is expressed in most sugar (S) neurons.

Just as in the mammalian olfactory system, most *Drosphila* ORNs individually express one of approximately 50 functional odor receptors in adults, in addition to the highly conserved co-receptor OR83b. But most GRNs express more than one GR. The contrasting design of GR expression in GRNs as compared to OR expression in ORNs, hints at a wider ligand-spectrum and restricted discriminatory ability of GRNs (Isono and Morita, 2010). ORN axons expressing the same OR converge on the same glomerulus in the antennal lobe (Vosshall and Stocker, 2007). Nearly every OR has been mapped for its expression in specific sensilla and for its corresponding ORN axon projection to a specific glomerulus (Couto et al., 2005). With one exception, all ORs are expressed in basiconic and trichoid ORNs, while a family of proteins related to ionotropic glutamate receptors detects odors in coeloconic ORNs.

Chemical stimuli detected by the chemosensory receptors are converted into electrical signals in the ORNs and GRNs (Kaupp, 2010). ORs are believed to be the sole determinant of the odor responses in a given ORN. The activity of ORNs, either

excitation or inhibition, provides behaviorally relevant information about odorants such as their identity, concentration, and source (Iyengar et al., 2010). The odor-signal is processed in the glomeruli of the antennal lobe by circuits consisting of both PNs (projection neurons) and local interneurons (LiNs), before being further transmitted to the kenyon cells (KCs) of mushroom bodies and the lateral horn (LHN) of the protocerebrum (Huang et al. 2010). PNs relay the olfactory information "vertically" from ORNs to mushroom bodies and the protocerebrum, whereas inhibitory LiNs (iLiNs) and excitatory LNs (eLiNs) provide lateral connections among different glomeruli that presumably endow PNs with variable spatial and temporal coding capabilities (Huang et al. 2010). LHN responses are highly selective and therefore suitable for driving innate behaviors, whereas KCs provide a more general sparse representation of odors suitable for forming learned behavioral associations (Luo et al. 2010). Functional feedback from KCs to PNs and LNs mediated by the βγ-lobes of MB modulates of olfactory information processing in a top-down fashion (Hu et al. 2010). The GRNs and MSNs project to the thoracic ganglion and the subesophageal ganglion (SOG) in the ventral brain. The projection of GRNs is organotopic, *i.e.*, segregated based on their original location in different peripheral taste organs (Rajasekhar and Singh, 1994). The projection of GRNs is also chemotopic – axons of phagostimulatory Gr5a neuron and phagoinhibitory Gr66a neuron terminate in SOG in a non-overlapping manner (Wang et al., 2004).

Physiological activity of the afferent chemoreceptor neurons encodes the first step in chemosensory processing. Both in terms of anatomy and physiology the GRNs and ORNs bear considerable resemblance. Chemosensory receptors convert chemical information into an electrical response known as the receptor potential, a graded membrane depolarization that acts to either increase or decrease the basal firing rate of the neuron. Traces of action potentials could be recorded extracellularly from the dendritic regions as single units (Chatterjee et al., 2009; Chatterjee et al. 2010). A recording electrode placed in the desired sensillum captures voltage changes due to the

firing of action potentials by the neuron. For ORNs the base of a sensillum is impaled with a sharpened recording electrode, but for GRNs the distal tip of a sensillum is brought in contact with a recording electrode. Because the sensillum may contain more than one chemosensory neuron, the resulting trace represents the activity of all the neurons housed within the same sensillum. Often, it is possible to distinguish the different chemosensory neurons based on their spike waveforms. Odor-evoked field potential of the olfactory organ could also be easily recorded as electroantennogram (EAG) (Krishnan et al., 1999). The graded receptor pontential contributes to the EAG response. Single-sensillum recording is by far a better technique than the EAG because it offers higher spatiotemporal resolution.

Single unit recordings on a fly strain with a mutation in the Or22a/b gene, in which odor-evoked responses in the ORN where the receptor is expressed is abolished without eliminating the ORN itself (the ‘empty neuron' paradigm) has yielded significant and comprehensive insight into the peripheral sensory map (Hallem et al., 2004). This has been a medium-throughput tool for OR de-orphanization, and demonstration of the fact that ORs are responsible for the spontaneous activity and response dynamics of ORNs (Hallem et al., 2004). Electrophysiological studies *in vivo* have been complemented by whole-cell patch clamping, outside-out patch clamping and Ca^{2+} imaging experiments in cell culture: a subset of insect ORs and GRs can in fact be functionally expressed in *Drosophila* S2 cells, moth Sf9 cells, human embryonic kidney 293 (HEK293) cells, HeLa cells and *Xenopus laevis* oocytes (Pellegrino and Nakagawa, 2009; Chyb et al., 2003). The functional characterization of insect ORs in heterologous expression systems has provided several new insights into the molecular mechanism of odor reception, including functional interaction between OR subunits, novel signaling properties of insect ORs, etc. (Pellegrino and Nakagawa, 2009).

Chemosensory behaviors elicited by the fruit flies can also be quantitatively analyzed. Proboscis extension reflex (PrER), shown in Fig. 4, and two-choice food preference tests

are commonly used to assay gustatory behavior (Amrein and Thorne, 2005). The proboscis extension reflex is a direct, robust and all-or-none measure of taste response of specific GRNs. The neural circuit involved in this reflex-like behavior is currently largely unknown. Phagoattractants such as sucrose, trehalose and dilute salt solution stimulate a large increase in the frequency of proboscis extension in hungry flies. The reduction in proboscis extension can measure the effects of phagodeterrents. In the two-choice feeding assay overall perception of food is measured at the organismal level. Two substrates are colored with 'tasteless' non-metabolizable dyes, allowing rapid examination of the ingested food by scoring the color of the midgut (Amrein and Thorne, 2005). A feeding preference index of zero signifies neutrality, negative preference scores are recorded for phagodeterrents and positive indices are elicited by phagoattractants. Odor-mediated osmotropotactic behavior can be measured by T-maze assays (Zhou et al., 2005). This assay calculates a preference index after flies are given a choice of being in one chamber containing an odorant or in the other chamber containing air.

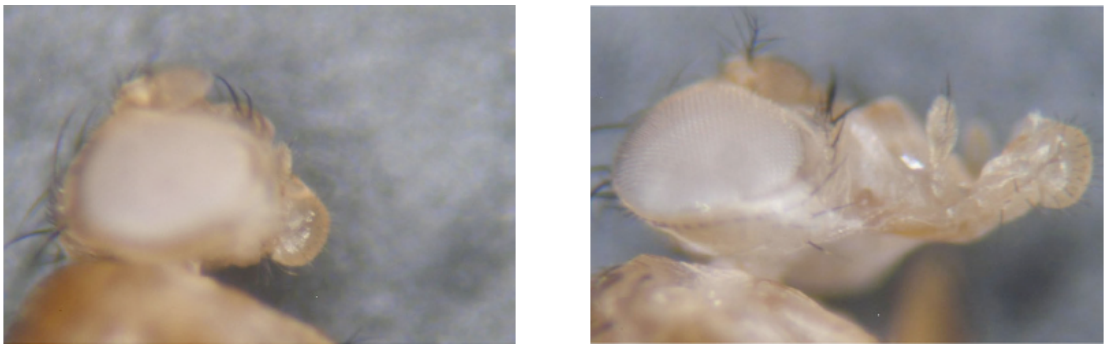

Fig. 4: Proboscis extension reflex (PrER) in *Drosophila* (Isono and Morita, 2010).

Chemosensory signal transduction

Lipid soluble, volatile (low molecular weight) odorants are bound in the mammalian mucus by small globular (8-stranded barrel) OBPs, which help to concentrate the odor. Mammalian ORs, a class of 7-TM (transmembrane) GPCRs, are present on ciliary membranes of ORN dendrites. Mouse has ~1300 and humans have ~900 OR genes. Each odor binds to 2-6 OR subtypes. Each OR binds a range of related odors with varying affinities: some ORs are generalists some are specialists. Hypervariable regions in TM 3, 4 and 5 helices as well as in the N terminal region of ORs lead to binding specificity. One mammalian ORN generally expresses one OR. This plan of organization bears similarity with the olfactory system of fruit flies.

The GPCR undergoes conformational changes upon odor binding. In the activated state, the OR sequences needed for interaction with G-proteins are exposed. Gαolf is a Gs like heterotrimeric G protein present in ORNs. Gαolf segregates from βγ dimer on interacting with activated GPCRs, and traverses through the lipid bilayer to activate a membrane bound 12-TM adenylate cyclase (AC3). AC3 generates cAMP from ATP. Increase in intracellular cAMP concentration opens CNGCs on the ciliary membrane, allowing influx of Ca^{2+} and Na^{+}. This causes membrane depolarization. Ca^{2+}can also amplify depolarization by opening anoctamin 2, a Ca^{2+}-sensitive Cl^{-} channel, leading to efflux of Cl^{-} ions. This way the graded receptor potential (RP) develops and electrotonically spreads. If the RP is sufficiently large (generator potential) it causes generation of AP in the axon hillock of ORNs. Later, Ca^{2+} is pumped out of ORNs by Na^{+}/Ca^{2+} exchangers, and Ca^{2+}-ATPases present in the cilia and dendritic knobs, thus maintaining Ca^{2+} homeostasis and returning the cell to electrical neutrality. Adaptation is achieved rapidly when Calcium-binding protein CaM (calmodulin) binds to CNGCs and reduces their affinity for cyclic nucleotides. Calcium-CaM also activates CaMKII which phosphorylates AC3 to decrease its catalytic activity, and activates an enzyme called PDE (phosphodiesterase) that transforms cAMP to 5'AMP. GPRKs and arrestin help in

receptor internalization and deactivation (https://www.qiagen.com/geneglobe/pathwayview.aspx?pathwayID=332).

In the worm *C. elegans*, a family of GPCRs act as ORs. Here a single ORN expresses multiple ORs. The AWA and AWC chemosensory neurons mediate chemotaxis towards attractive odorants, while the ASH, AWB and ADL neurons detect aversive odorants. Odor binding activates the Gα protein ODR-3 to decrease intracellular cGMP levels in the AWC neuron by regulating the guanylyl cyclases, ODR-1 and DAF-11. Calcium levels decrease due to closing of the cGMP-gated calcium channels TAX-2 and TAX-4. G-protein signaling initiates long-lasting olfactory adaptation by promoting the nuclear entry of EGL-4, and once EGL-4 has entered the nucleus, processes such as PUFA activation of the TRP channel OSM-9 may dampen the output of the AWC neuron (O'Halloran et al., 2009). In lepidopterans, response to pheromones are elicited at least in part via a metabotropic PLCβ-dependent signal transduction cascade (Stengl, 2010). However, rapid production of phosphoinositide (PI) metabolites in response to odorants in moth olfactory neurons is not sufficient to prove that these second messengers are direct mediators of olfactory signal transduction. For example, they may underlie long-term homeostatic responses to neuronal activity (Ha and Smith, 2009). Volatile pheromones, nevertheless, increase cellular IP_3 concentration that causes Ca^{2+} influx in ORNs. Opening of Ca^{2+} -dependent ion channels is believed to cause depolarization (Flecke et al., 2006). Ca^{2+}-activated Cl^- current in moth ORNs is involved in ORN repolarization corresponding to the falling phase of receptor potential (Pezier et al., 2010). Strong pheromone stimuli, which are possibly perceived upon direct contact with the female, activate a receptor-guanylyl cyclase and increase cGMP levels leading to olfactory adaptation (Stengl, 2010). Termination of olfactory signaling may in part be driven by odor degrading enzymes (ODE) secreted into the sensillar lymph by non-neural cells (Vogt, 2005). Surprisingly, insect ORs have been shown to mediate olfactory signal transduction via an ionotropic mechanism (Sato et al., 2008).

Drosophila seven transmembrane odorant receptors (ORs) were recently found to have inverted membrane topology compared to typical GPCRs, with their N terminus facing the cytoplasm rather than the extracellular space (Benton et al., 2006; Lundin et al. 2007). Additionally, *Drosophila* ORs also require OR83b, another seven transmembrane OR-family protein highly conserved in insects, as an obligate coreceptor and chaperone (Larsson et al., 2004). Specific domains in the third cytoplasmic loops of ORs, have been implicated to interact with the third loop of OR83b (Benton et al., 2006). OR/OR83b complexes form ligand-gated nonselective cation channels (Sato et al., 2008; Wicher et al., 2008), a striking difference to GPCRs in worms and vertebrates that rely on second messengers to activate ion channels. Recently, a new class of odorant receptors, called IRs (ionotropic receptors), that are related to ionotropic glutamate receptors, have been discovered (Benton et al., 2009). IRs contain divergent ligand-binding domains that lack glutamate-interacting residues.

When stimulated in heterologous cells with brief puffs of odorants, insect ORs exhibited transient current responses with a simple waveform characterized by a short delay (≤30 ms), a rapid rise and a slower decay to baseline (Sato et al., 2008; Wicher et al., 2008). One model (Sato et al., 2008) suggests that the ORs form an ion channel that is opened directly in response to the binding of odorants. The heteromeric receptor consists of a unique OR (OrX) and a co-receptor (OR83b). This model (Fig. 5) does not specify the location of the channel pore. Additionally, this model does not address the possibility of feedback or modulatory mechanisms. An alternative model (Wicher et al., 2008) suggests that there are two pathways by which odor-induced electrical response can be generated. In the direct pathway, odorant binding directly opens a channel formed by the OR83b subunit, generating a fast and short depolarization; in the indirect pathway (Fig. 5), activation of a Gs and an adenylyl cyclase leads to cyclic AMP production. Upon binding of cAMP to OR83b, the channel opens and generates slow and prolonged depolarizing currents.

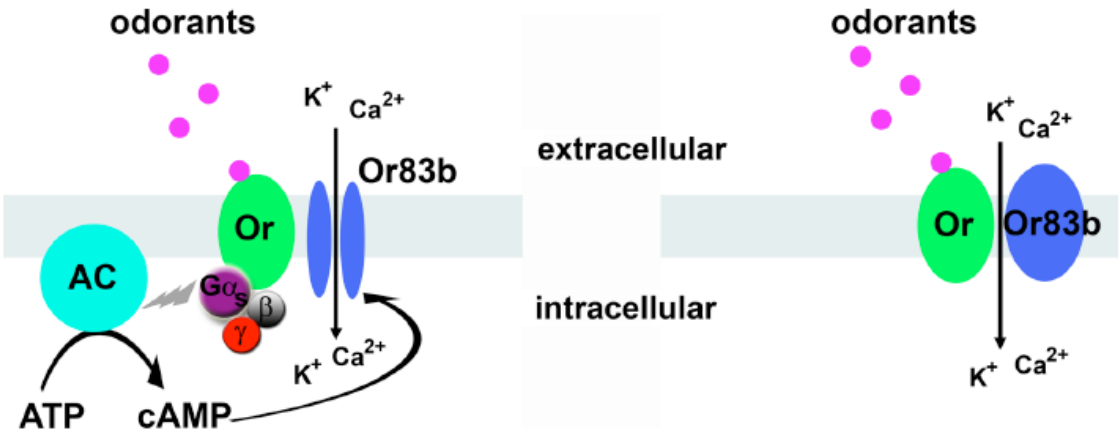

Fig. 5: Models of odor-reception in *Drosophila*. Left model (Wicher et al., 2008) proposes that ORs are GPCRs; OR83b forms the odor-activated and cyclin nucleotide-gated cation channel. Right model (Sato et al., 2008) proposes that odorants directly gate the OR/OR83b receptor complex in an exclusively ionotropic manner (Ha and Smith, 2009).

The dual activation model (Nakagawa and Vosshall, 2009) posits that OR/OR83b orchestrate an intial ionotropic response by rapidly fluxing cations including calcium. The influx of calcium could lead to a slower metabotropic response to sensitize the OR/OR83b complex by increasing the open probability of the receptor upon modulation by cyclic nucleotides (Nakagawa and Vosshall, 2009). Cyclic nucleotides could act indirectly by activating kinases that brings about post-translational modification of the OR/OR83b complex. The OR/OR83b complex may directly interact with G proteins, uncharacterized membrane receptors co-stimulated by OR/OR83b activation might trigger G-protein signaling, alternatively, an exclusively intracellular signaling network may directly stimulate G proteins (Nakagawa and Vosshall, 2009).

Mammals can distinguish among 10,000 odors but only 5 taste modalities are present. Salty and sour tastes are sensed in a GR-independent manner. Na^+ (salty taste) can directly diffuse through amiloride sensitive Na^+ channel and cause depolarization. Transient receptor potential (TRP) family ion channels detect sour taste. Additionally H^+ (sour taste) can directly inhibit a hyperpolarizing K^+ current to cause depolarization.

Sweet taste is detected by T1R2/T1R3, bitter by T2R and umami is detected by T1R1/T1R3 GPCRs. GPCRs undergo conformational changes on tastant binding and in turn activate a G protein called Gustducin whose alpha subunit stimulates the membrane bound phospholipase C β2 (PLC). IP3 (Inositol triphosphate) and DAG (diacylglycerol) are produced from PIP2 by PLC. An increase in intracellular [IP3] causes release of Ca^{++} from intracellular stores. This event, through an unknown mechanism, opens the TRP channel TRPM5 resulting in depolarization of the cell. A subset of sweet tastants act through cAMP. cAMP-dependent protein kinase phosphorylates a K^+ channel. In the resting state this K^+ channel brings in hyperpolarizing current. Its closure by cAMP cascade results in depolarization of the cell. A subset of bitter tastants (*e.g.* CaSO4) directly block a hyperpolarizing K^+ current (Chandrashekar et al., 2006).

Drosophila GRs and ORs belong to the same family of 7-TM domain proteins, share amino acid motifs near the C terminus and the 7^{th} TM helix, and are evolutionarily related. Among all the Ors, Or83b is structurally most similar to GRs. Even the worm, *C. elegans*, contains a few functional insect-like GR genes. GRs are 350-550 amino acid long proteins with very low sequence homology among each other. In addition to GRs, taste in flies may be mediated by (a) TRP channels (*e.g. painless*) that respond to a few noxious and bitter compounds in a PLC-dependent pathway, (b) conventional GPCRs (*e.g. DmXR* detects the insecticide $_L$-canavaline), (c) amiloride-sensitive epithelial Na^+ channels (*e.g.*, *pickpocket 19*, *pickpocket 11* are involved in salt perception; *pickpocket 28* is required for water taste), and (d) possibly IRs (Isono and Morita, 2010). Sugar receptors seem to be heterodimers – GR5a~GR64f complex detects trehalose and glucose, GR64a~GR64f complex senses sucrose, maltose etc; whereas bitter receptors are composed of at least three GRs (Isono and Morita, 2010). Our understanding of the molecular mechanism of gustatory signal transduction in insects is at best fragmented. Water reception involves Ca^{2+} signaling and the participation of calmodulin (CaM) plus protein kinase C (PKC) (Meunier et al., 2009). For sugar-sensation, a number of G

protein subuits and Gα classes, adenylyl cyclases, and IP3 receptor (itpr) have been implicated (Bredendiek et al., 2010).

Role of G-protein signaling in chemoreception

Heterotrimeric G proteins transduce the signals from G protein–coupled receptors (GPCRs), the largest receptor family in the animal kingdom (Pierce et al., 2002). The heterotrimeric G proteins exist as complexes of the GDP-bound α-subunit and the β- and γ-subunits during the resting state. On ligand activation, GPCRs act as guanine nucleotide exchange factors and catalyzes the substitution of GDP for GTP on the Gα-subunit. This leads to dissociation of the trimeric G protein complex into the GTP-loaded Gα and the βγ-heterodimer. Both components of the initial complex can interact with downstream effectors. Signal specificity is mainly represented by the α-subunits; 16 genes for the α-subunits are present in the human genome, and six in *Drosophila* (Malbon, 2005).

It is not yet known whether G proteins are involved in insect olfactory transduction. In vivo experiments are inconclusive: individually disrupting *G$_{\alpha}$s*, *G$_{\alpha}$q*, *G$_{\alpha}$o* and *G$_{\gamma}$30A* had no significant effect on OR-mediated electrophysiological responses (Yao and Carlson, 2010), but other groups reported that mutations in *G$_{\alpha}$q* reduce the sensitivity of antennal neurons to several odors (Kain et al., 2008), and expression of *G$_{\alpha}$q* RNAi in ORNs led to decreased odor-evoked behavioral responses (Kalidas and Smith, 2002). Additionally, there is considerable previous evidence for both cAMP (Gomez-Diaz et al., 2004) and inositol 1,4,5-trisphosphate (IP3) signaling in *Drosophila* olfactory reception (Gomez-Diaz et al., 2006). *G$_{\alpha}$s* is a prominent modulator of cAMP signaling, whereas phospholipid signaling is controlled by *G$_{\alpha}$q*. Mutants in the gene *stambhA* (*stmA*), which encodes a putative phosphatidylinositol 4,5 bisphosphate-diacylglycerol lipase, exhibit a significant reduction in the amplitudes of odor-evoked responses recorded from the

antennal surface of adult *Drosophila* (Kain et al., 2009). *IP3 kinase1,* which reduces the levels of its substrate IP3 by converting it into inositol 1,3,4,5-tetraphosphate (IP4), showed expression in olfactory sensory organs. Overexpression of the *IP3Kinase1* gene resulted in abnormal behavioral and neuronal responses to certain odorants (Gomez-Diaz et al., 2006). Olfactory responses from the adult antenna are significantly reduced in *Drosophila* mutants of *dgq* gene (which encodes $G_{\alpha}q$), a phospholipase Cβ ortholog, (*plcβ21c*), and a diacylglycerol kinase, (*rdgA*) (Kain et al., 2008). Overexpression of the *dnc* (dunce) gene encoding phosphodiesterase that increases intracellular cAMP levels resulted in abnormal behavioral responses to some odorants (Gomez-Diaz et al., 2004). Mutations in *rut* (rutabaga gene encoding an adenylyl cyclase) and *dnc* altered the onset kinetics of electrophysiological responses (Martin et al., 2001). In lobster olfactory cells, the cAMP system mediates hyperpolarization and inhibitory responses, and the IP3 pathway leads to depolarization and excitatory responses (Fadool and Ache, 1992). The presence of two main transduction cascades with opposing effects in olfactory neurons probably acts as a coding mechanism to generate differential messages.

$G_{\alpha}o$ is the most abundant G protein in the central nervous system of flies and mammals (Jiang and Bajpayee, 2009). The downstream effectors of $G_{\alpha}o$ are much less understood compared with the effectors of *Gi* and *Gs* which inhibit or stimulate adenylyl cyclases, respectively. *Drosophila G-oα47A* gene, also known as *brokenheart*, encodes 2 protein isoforms of $G_{\alpha}o$, generated by alternative splicing (Yoon et al., 1989). The isoforms are composed of 354 amino acids, are 40 kD in size, but differ in seven amino acids in the N-terminal region (Yoon et al., 1989), and they can functionally complement each other (Bredendiek et al., 2010). Based on sequence similarity, FLYBASE reported that $G_{\alpha}o$ has GTP binding and GTPase activity. Within the nervous system of fruit flies, this protein is expressed in antennal nerve, ocellar nerve, lamina, and brain cortex (Wolfgang et al., 1990). The protein occurs both in the plasma membrane and cytoplasm of cells (Wolfgang et al., 1990). Presence of consensus sequences for myristoylation and palmitoylation on $G_{\alpha}o$ suggests that the protein is anchored to the plasma membrane by

lipid modifications (Bredendiek et al., 2010). *$G_{\alpha}o$* has been implicated in a multitude of developmental and physiological functions in *Drosophila*, such as Wnt/frizzled signaling (Katanaev et al., 2005), development of sensory organs (Katanaev and Tomlinson, 2006), cardiac development (Fremion et al., 1999), and associative learning within mushroom body (Ferris et al., 2006). In flies, G protein subunits *$G_{\alpha}s$*, *$G_{\alpha}q$*, *$G_{\alpha}o$* and *$G_{\gamma}1$* are involved in sugar reception (Bredendiek et al., 2010 ; Ishimoto et al., 2005; Kain et al., 2010 ; Ueno et al., 2006). *$G_{\alpha}s$*, *$G_{\alpha}i$*, *$G_{\alpha}q$*, *$G_{\alpha}o$*, *$G_{\beta}f$*, and concertina (*cta*); *$G_{\beta}5$* and *$G_{\beta}13F$*; *$G_{\gamma}1$ and $G_{\gamma}30A$* are expressed in *Drosophila* antenna (Boto et al., 2010). *$G_{\alpha}o$*-RA transcript is expressed in the third antennal segment, but not in maxillary palps, yet *$G_{\alpha}o$*-RB transcript is expressed in both the olfactory organs of flies (Boto et al.,2010). In mosquitoes too, *$G_{\alpha}o$* is expressed in the odorant receptor neurons (ORNs) of antenna, suggesting the functional involvement of G_o in insect olfaction (Rutzler et al., 2006). *$G_{\alpha}o$* regulates several cellular effectors, including ion channels, enzymes, and even small GTPases to modulate cellular function (Jiang and Bajpayee, 2009).

Circuits underlying appetitive behavior

Growth, survival, and reproductive requirements of any organism are met by feeding. Animals have evolved specialized feeding habits depending on their specific metabolic needs and external sensory inputs. Food consumption is associated with body weight regulation and caloric intake. Most animals consume food in discrete bouts called meals, and total food intake is a function of both meal size and meal frequency. Signals that control meal size and frequency may regulate (a) initiation, (b) maintenance, and (c) termination of feeding. In a hungry mammal, the sight, smell and taste of food initiate feeding. Stimulation of the stretch receptors on the distended stomach wall signals cessation of feeding. Evaluation of the caloric content of ingested food, as it is absorbed from the small intestine, can also contribute to meal termination (Al-Anzi et al.). Feeding behavior in insects involves (a) detection of food, (b) initiation of ingestion, and

(c) consumption of meals. Based on olfactory cues flies land on a potential food source; as it stands on the food, the tarsal (leg) taste sensilla are used to check palatability; if the fly is hungry it will stick out its proboscis to begin feeding while simultaneously sampling the taste and texture of food using pharyngeal and labral sensory hairs; food gets temporarily stored in the collapsible crop. In larger species of musciod flies (*e.g.*, *Phormia regina*) (a) habituation of GRN response (frequency), (b) activation of the abdominal stretch receptors, and (c) activation of the cells monitoring foregut activity contribute to termination of feeding (Edgecomb et al., 1994).

The nervous system plays a crucial role in feeding. It evaluates the amount of available fuel and accordingly regulates food intake. In mammals, hypothalamus and brain stem modulate feeding and energy expenditure. Regulation of feeding behavior in a fly may happen at multiple levels – *e.g.*, willingness for ingestion, meal frequency, meal volume, food storage, defecation rate, etc. The feeding behavior of wild-type flies displays a few consistent features. (i) Flies have a significant preference for sucrose (carbohydrate) over yeast (protein). (ii) Flies fed on diluted (sugar conc. ≤ 25 millimoles/litre) food solutions compensate for the dilution of their nutrients, as they increase their intake and get the same amount of nutrients as flies fed on more concentrated solutions. The increase in feeding happens at the levels of meal frequency, crop size and rate of defecation – all of which increases with decreasing sugar conc. (iii) If the sugar concentration of the food is high (≥ 50 millimoles/litre), compensation is not observed. Not surprisingly, with increasing sucrose concentration up to 100 millimoles/litre, chances of a fly's survival also increases (Edgecomb et al., 1994; Vigne and Frelin, 2010). Food-deprived flies upregulate their willingness for ingestion, meal frequency and meal volume; food consumption in these flies proportionally increases with increasing sugar concentration in the given food (Edgecomb et al., 1994). Food-deprived flies have heightened chemosensory sensitivity – viz., they can elicit behavioral response to even 20 micromolar sucrose (Rodrigues, 1978). Unless the sucrose concentration is > 5

millimoles/litre, ingestion is primarily driven by 'thirst' in food-deprived flies, and food intake in such scenario offers negligible nutritional benefit (Edgecomb et al., 1994).

In *Caenorhabditis elegans* the pharyngeal pumping rate serves as an indirect measure of food intake. But the opaque body of adult fly precludes this possibility. By allowing flies to feed on semisolid medium colored with a non-absorbable, non-metabolizable dye and subsequent spectrophotometric quantitation feeding can be recorded. But variable retention time of the dye in crop may offer misleading information on food intake (Wong et al., 2009). *Drosophila* feeding behavior can also be monitored by radioactive labeling *e.g.* [α-32P]dCTP, [α-32P]dATP, [14C]sucrose of the medium. High specificity and sensitivity of radiolabeling permits measurement of steady-state food consumption in *ad lib* fed flies (Carvalho et al., 2006). Using a caplillary tube to offer liquid food (CAFE assay) enable the direct and simple measurement of how much liquid food has been consumed over longer periods of time without any need for killing the flies (Ja et al., 2007). Both meal frequency and size can be tracked using this assay, but the unnatural setting and reduced survival of flies in CAFE paradigm are of concern. An alternative is to use the proportion of time a fly spends having its proboscis protruded out, as a proxy for feeding (Wong et al., 2009). The disadvantage here is the possibility that proboscis extension is not tightly coupled with food consumption – *e.g.*, a fly may extend its proboscis just to evaluate a potential food without ingesting it. Scientists are yet to develop a sensitive assay capable of automated long-term recording of food intake in flies.

Studies in mammalian systems have provided important insight into the relationships between food intake, energy output, and fat deposition. Regulation of metabolism involves the intestine (digestion and absorption of food), adipose tissue (storage of excess fat), the liver (de novo fatty acid synthesis, amino acid synthesis, breakdown as well as synthesis of glycogen, gluconeogenesis), and the brain (monitoring of the metabolic state of the body). *Drosophila* midgut is the site for food digestion and

nutrient absorption. Nutirents that do not get immediately used are sent to the fat body, which metabolizes and stores large reserves of glycogen and lipid, thus acting like the mammalian liver and white adipose tissue (Kaun and Heberlein, 2009). Specialized clusters of cells called oenocytes perform hepatocyte-like functions in lipid processing, *i.e.*, metabolizing lipids during starvation (Kaun and Heberlein, 2009). Based on humoral signals emitted by the metabolic tisuues, the CNS modulates physiological and behavioral outputs to maintain the fly's energy homeostasis. In mammals, control of caloric intake by the brain occurs through hypothalamic nuclei that regulate eating and metabolism. Surgical ablation or overstimulation of these nuclei affects feeding behavior and body weight. In flies, silencing of *fruitless*-expressing neurons and *c673a-Gal4* neurons increases fat storage (Al-Anzi et al., 2009). Both mammals and flies use conserved signaling pathways to affect carbohydrate, lipid, and energy homeostasis, as well as food intake (Kaun and Heberlein, 2009). For example, NPY (NPF in flies), neuromedin-U (hugin in flies), FOXO and the insulin signaling pathway all seem to function similarly in flies and mammals (Kaun and Heberlein, 2009). In *Drosophila*, as in mammals (in contrast to worms which do not have dedicated fat-storing cells), regulation of fat storage by adipocytes is coordinated by molecular components whose sequences and functions are also conserved between insects and mammals (Al-Anzi et al., 2009). These include the perilipin/Lsd-2 protein, which is required for the formation of lipid storage droplets, the Brummer (Bmm) lipase required for catabolizing triacylglycerol, and the cytochrome P450 Cyp4g1, a regulator of fat storage (Al-Anzi et al., 2009).

CHAPTER II

$G_{\alpha}o$ CONTRIBUTES TO OLFACTORY RECEPTION*

Background

Most animals rely on olfaction for foraging, predator and toxin avoidance, and social interactions. Odorants are detected by 7-transmembrane receptors, which normally transduce olfactory signaling by activating G-proteins. However, recent work in the fruit fly *Drosophila melanogaster* demonstrates that insect odorant receptors (ORs) act as ligand gated (Sato et al., 2008; Wicher et al., 2008) and cyclic nucleotide gated (Sato et al., 2008; Wicher et al., 2008) cation channels, and thus do not function as traditional G-protein coupled receptors. The G_{α} protein(s) responsible for inducing the production of cyclic nucleotides *in vivo* that activate cation channels formed by OR-complexes have not been identified, although G_q has been implicated in *Drosophila* olfactory transduction (Kain et al., 2008). Another G_{α} protein, G_o, is expressed in the odorant receptor neurons (ORNs) of antenna from *Drosophila,* the silk moth *Bombyx mori,* and the mosquito *Anopheles gambae*, suggesting the functional involvement of G_o in insect olfaction (Miura et al., 2005; Rutzler et al., 2006; Wolfgang et al., 1990). Although definitive immunohistochemical proof for dendritic localization of G_o in olfactory sensilla is lacking, previous studies could not rule out the possibility of G_o expression in ORN dendrites.

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In *Drosophila*, the S1 subunit of pertussis toxin (PTX) selectively ADP-ribosylates G_o, thereby inhibiting G_o signaling (Hopkins et al., 1988; Thambi et al., 1989). I have employed existing and newly developed tools for controlling the spatial and temporal expression of PTX to investigate how G_o inactivation affects physiological responses to odorants (Ferris et al., 2006; Fremion et al., 1999). Loss of G_o signaling in ORNs reduced the amplitude and enhanced the termination of EAG responses independent of odor type or concentration, and decreased odor-induced spike frequency in individual ORNs. These results demonstrate that G_o is involved in modulating olfactory responses in *Drosophila*.

Results

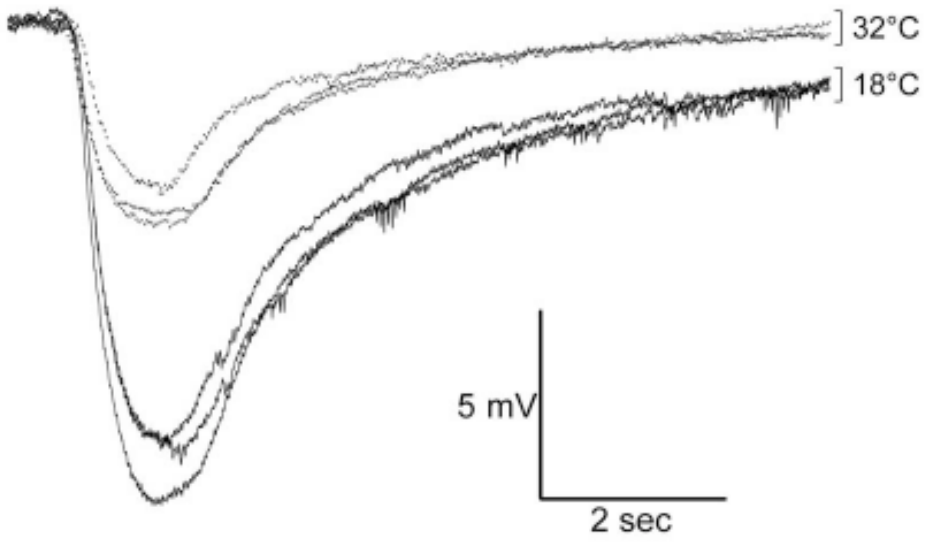

Fig. 6: PTX reduces the amplitude of ethyl acetate induced EAG responses. EAG traces evoked by the application of 10^{-4} ethyl acetate in flies at temperatures that restrict (18°C, black lines) or permit (32°C, gray lines) PTX expression.

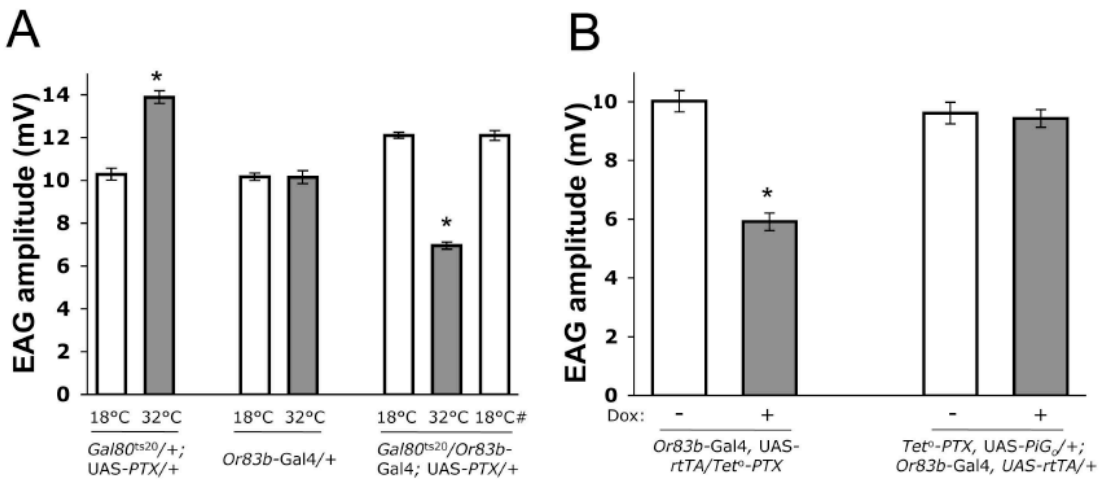

Fig. 7: Inhibition of heterotrimeric G_o signaling reversibly reduces the amplitude of 10^{-4} ethyl acetate evoked EAG responses. (A) EAG responses from *Gal80*^{ts20}/+; UAS-PTX/+ and *Or83b*-Gal4/+ control strains do not decrease (p>0.5) at 32°C compared to 18°C. *Gal80*^{ts20}/*Or83b*-Gal4; UAS-PTX/+ flies have a significantly (p<0.0001) higher EAG amplitude in the absence of PTX expression before heat induction (18°C) or after recovery from heat induction (18°C#) than in the presence of PTX expression (32°C). (B) EAG responses from *Or83b*-Gal4; UAS-*rtTA*/*Tet*°-PTX flies are significantly (p<0.0001) higher in the absence of PTX expression (dox -) than in the presence of PTX expression (dox +). EAG responses from flies that express PTX-insensitive G_o (PiG_o) in ORNs (*Tet*°-PTX, UAS-PiG_o/+; *Or83b*-Gal4, UAS-*rtTA*) are not different (p>0.7) whether PTX expression in ORNs is induced (dox +) or uninduced (dox -). For each genotype and treatment, at least 12 EAG recordings from minimum 6 flies were analyzed. Asterisks denote a significant (p < 0.05) change. All values are mean ± S.E.M.

To determine whether G_o signaling mediates olfactory responses, EAG measurements were carried out on flies in which the widespread olfactory receptor neuron driver *Or83b*-Gal4 was used to drive UAS-PTX in ORNs (Wang et al., 2003). Conditional expression of PTX was achieved using the *Gal80*^{ts20} TARGET system; at 18°C, functional *GAL80*^{ts20} binds to and inhibits GAL4 and at 32°C GAL80^{ts20} is inactivated thus allowing PTX expression (McGuire, 2003). At 32°C, *Or83b* promoter driven GAL4 was free to drive the transcription of PTX and inactivate *Drosophila* G_o (Ferris et al.,

2006). As a result, Gal80^{ts20}/Or83b-Gal4; UAS-PTX/+ flies, which show a ~12 mV EAG amplitude to 10^{-4} ethyl acetate at 18°C, produce a significantly ($p<0.0001$) decreased EAG amplitude of ~7 mV at 32°C (Fig. 6, Fig. 7A). This result demonstrates that PTX-sensitive G_o is needed for high amplitude EAG responses, suggesting that G_o is involved in generating receptor potential.

To insure that the observed decrease in EAG amplitude did not arise from cell damage and/or cell death, I placed temperature-treated flies at 18°C for 24 hours and measured EAG responses. These flies regained normal EAG amplitude of ~12 mV, demonstrating that the effect of PTX is reversible (Fig. 7A). Moreover, EAG responses evoked by 10^{-4} ethyl acetate in *Gal80*^{ts20}/+; UAS-PTX/+ and *Or83b*-Gal4/+ control strains did not show a decreased ($p>0.5$) amplitude when the temperature was increased from 18°C to 32°C (Fig. 7A), thus decreased amplitude does not result from an increased temperature. Temperature did induce a moderate increase in EAG amplitude in *Gal80*^{ts20}/+; UAS-PTX/+ control flies, but this is likely due to the *Gal80*^{ts20} transgene genetic background since *Gal80*^{ts20}/+ flies displayed a modest increase in EAG amplitude when temperature was increased to 32°C (Fig. 8).

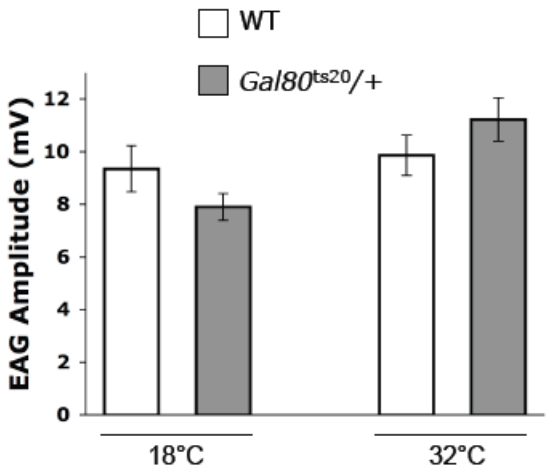

Fig. 8: Genetic background of *Gal80*^{ts20} transgene causes a temperature-dependent increase in EAG amplitude.

To confirm that PTX suppressed EAG amplitude, PTX was conditionally expressed in ORNs by combining the Gal4/UAS and tetracycline (Tet)-inducible Tet-On transactivator (Tet-On TA) systems (Stebbins et al., 2001). Or83b-Gal4 was used to drive expression of UAS-rtTA (reverse tetracycline transactivator) in ORNs. In the presence of the tetracycline analog doxycyline, rtTA binds to the tet-operator (tet^o) and activates transcription of the tet^o-PTX transgene. Upon addition of doxycyclin, PTX expression suppressed ($p<0.0001$) EAG amplitude by ~40% (Fig. 7B).

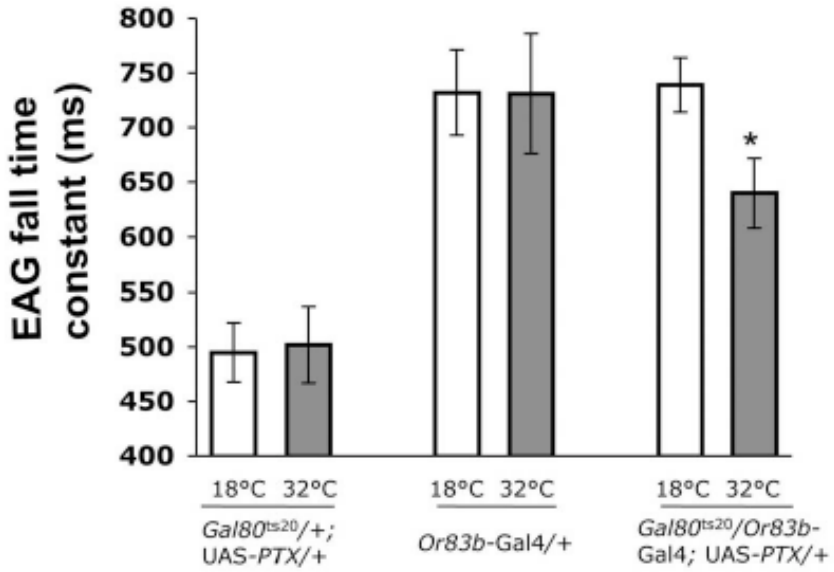

Fig. 9: G_o activity is required for the perdurance of EAG responses. The EAG fall time constant in *Gal80*^{ts20}/+; UAS-PTX/+ and *Or83b*-Gal4/+ control strains is not different ($p>0.8$) at 18°C and 32°C. *Gal80*^{ts20}/*Or83b*-Gal4; UAS-PTX/+ flies have a significantly ($p<0.01$) longer EAG fall time constant in the absence of PTX expression (18°C) than in the presence of PTX expression (32°C).

To insure that PTX suppressed EAG amplitude by inhibiting G_o, a PTX insensitive G_o (PiG_o) was expressed along with PTX in ORNs. Doxycycline-induced PTX expression did not affect ($p>0.7$) EAG amplitude in flies expressing PiG_o in ORNs, demonstrating

that PiG_o completely rescued the action of PTX on endogenous G_o (Fig. 7B). These results map the effects of PTX to G_o and confirm that G_o signaling contributes to olfactory responses.

To investigate the effect of PTX on EAG dynamics, I looked at fall time constant (τ_f) as a measure of the termination kinetics of EAG responses. Fall time constant is the time necessary to recover one-third of the maximal EAG amplitude after stimulation. This parameter is independent of amplitude, and unlike amplitude τ_f remains relatively unaffected by small changes in electrode placement (Alcorta, 1991). Upon stimulation for 500 ms with 10^{-4} ethyl acetate, τ_f was significantly ($p<0.01$) lowered in the *Gal80*^{ts20}/*Or83b*-Gal4; UAS-PTX/+ flies at 32^{o}C compared to that at 18^{o}C, whereas the two control strains showed no effect ($p>0.8$) of temperature on τ_f (Fig. 9). For a given odorant, τ_f decreases if either the concentration of the odorant or its delivery duration is reduced (Alcorta, 1991). Inhibition of G_o resulted in faster termination kinetics typically seen in control flies upon application of a 10-fold lower dose of odorant. Since inactivation of G_o shortened τ_f, it can be argued that transduction of odor-information in the antenna was impaired in absence of G_o. Our observation that G_o is needed for the persistence of the electrophysiological response *in vivo* corroborates the *in vitro* results that implicate G-protein mediated signal amplification in prolonged odor signaling (Wicher et al., 2008).

Odor-induced EAG responses are thought to mainly consist of the summation of receptor potentials of many ORNs in close proximity to the recording electrode (Ayer and Carlson, 1992). However, it is difficult to correlate EAG responses with single cellular processes that occur when individual ORNs respond to odorants.

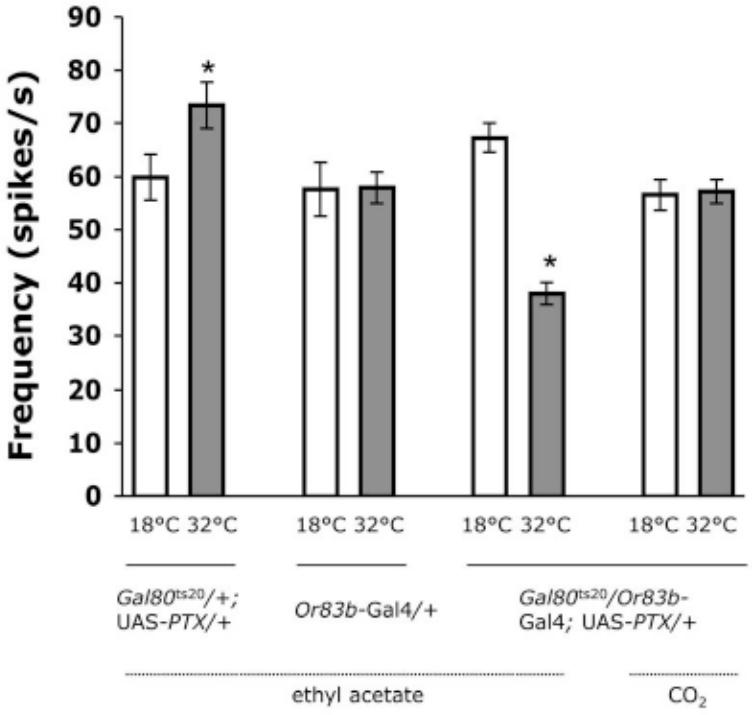

Fig. 10: G_o inhibition reduces odor-evoked firing frequency. For each fly strain, CO_2 and a 10^{-4} dilution of ethyl acetate were used to evoke spike activity from ab1C or ab1A neurons respectively. Spike frequency in *Gal80*^{ts20}/+; UAS-PTX/+ and *Or83b*-Gal4/+ control strains do not decrease ($p>0.5$) at 32°C compared to 18°C. *Gal80*^{ts20}/*Or83b*-Gal4; UAS-PTX/+ flies have a significantly ($p<0.0001$) higher ethyl acetate evoked ab1A spike frequency in the absence of PTX expression (18°C) than in the presence of PTX expression (32°C), whereas CO_2-induced single unit responses in the ab1C neuron was not unaffected ($p>0.8$).

The limited resolution of EAGs can be overcome by recording single unit responses from individual sensilla. In contrast to EAG responses, single unit recordings consist of spikes that represent extracellularly recorded action potentials of individual ORNs in the sensillum (Hallem et al., 2004). To investigate the role of G_o at the level of single cell physiology, I performed single-sensillum recording on ab1 sensilla whose 'A' neuron (*e.g.* the neuron producing the largest 'A' spike) is known to robustly respond to ethyl acetate (de Bruyne et al., 2001). Expression of PTX significantly ($p<0.0001$) reduced the

ethyl acetate-evoked firing frequency of ab1A spikes (Fig. 10). However, the spontaneous firing frequency did not ($p>0.2$) change, indicating that inactivation of G_o did not alter the physiology of uninduced resting membrane. The same sensillum houses the CO_2-sensing ab1C neuron (Jones et al., 2007), which does not express *Or83b*-driven PTX. CO_2-induced single unit responses are not affected by *Or83b*-driven PTX in ab1C neurons (Fig. 10), thus confirming the specificity of our gene expression system. The reduction in ethyl acetate induced spike frequency was not a mere physical response caused by increase in temperature because the two control strains did not show any decrease ($p>0.5$) in firing frequency in response to increased temperature.

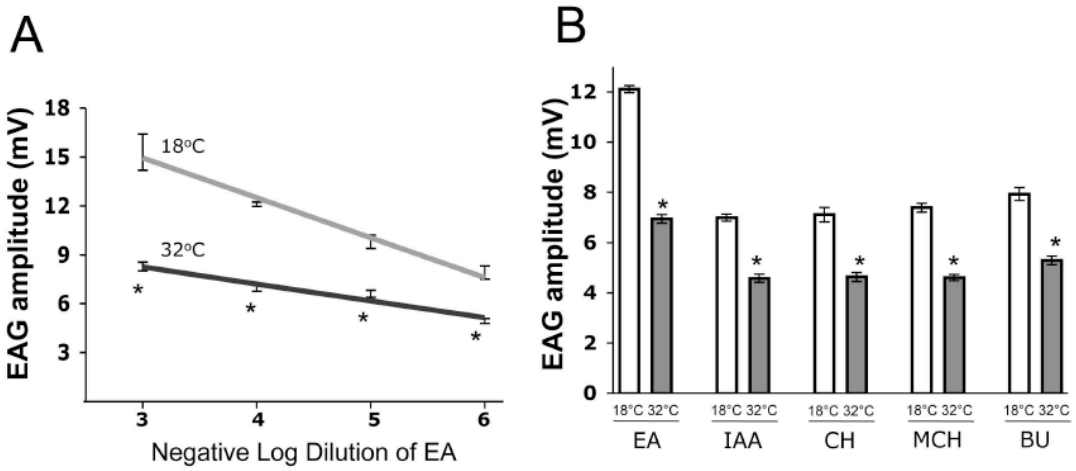

Fig. 11: G_o signaling is required for normal EAG responses to diverse odorants. (A) EAG responses evoked by four different concentrations of ethyl acetate (EA) in *Gal80^{ts20}/Or83b-Gal4*; UAS-PTX/+ flies are significantly ($p<0.0001$) higher in the absence of PTX expression (18°C) than in the presence of PTX expression (32°C). (B) EAG responses evoked by a 10^{-4} concentration of ethyl acetate (EA), a 10^{-4} concentration of isoamyl acetate (IAA), a 10^{-4} concentration of cyclohexanone (CH), a 10^{-4} concentration of 4-methylcylcohexanol (MCH), and a 10^{-3} concentration n-butanol (BUT) in *Gal80^{ts20}/Or83b-Gal4*; UAS-PTX/+ flies are significantly ($p<0.0001$) higher in the absence of PTX expression (18°C) than in the presence of PTX expression (32°C).

Inhibition of G_o signaling lowered the odor-induced frequency of ab1A spikes and odor-evoked EAG response by an equivalent amount, *i.e.*, 40-45% reduction in response. Taken together, these results reveal that G_o plays an important role in olfactory reception within the *Drosophila* ORNs.

To determine whether inhibition of G_o signaling impairs olfactory responses only at certain concentrations of ethyl acetate, I recorded EAG responses in both PTX expressing and PTX non-expressing *Gal80*^{ts20}/*Or83b*-Gal4; UAS-PTX/+ flies exposed to various concentrations of ethyl acetate (Fig. 11A). PTX was found to repress EAG responses over a 1000-fold range of stimulus intensities ($p<0.0001$); although the degree of repression was slightly higher at high concentrations of ethyl acetate. This effect was in contrast with the odor-intensity dependent effect of dG_{q3}RNAi in behavioral response of *Drosophila* to odors (Kalidas and Smith, 2002). Odor sensitivity was compared by noting the increase in odor concentration that is needed in G_o-compromised flies to elicit EAG responses as high as that in flies with unaffected G_o (see Methods). Comparison of the two dose-response curves reveals that PTX mediated suppression of EAG response is associated with a ~470 fold difference in sensitivity to ethyl acetate (Fig. 11A).

I next determined whether G_o contributes to the detection of odorants by other classes of sensilla. I chose a small panel of odorants, which included two acetates (ethyl acetate, isoamyl acetate) perceived by basiconic sensilla, one ketone (cyclohexanone) known to activate a single class of coeloconic sensilla, an alcohol (4-methyl-cylcohexanol) that is detected by trichoid and coeloconic sensilla, and another alcohol (n-butanol) that is detected by basiconic and coeloconic sensilla (Benton et al., 2009; Clyne et al., 1997; de Bruyne et al., 2001; Yao et al., 2005). Our odor panel contained both attractants (*e.g.* ethyl acetate at 10^{-4} concentration) and repellents (*e.g.* 4-methyl-cyclohexanol at 10^{-4} concentration). EAG recordings revealed that PTX expression significantly ($p<0.0001$) repressed EAG amplitudes to all five odorants tested (Fig. 11B). In each case, the EAG amplitude was reduced by 38 ±5 percent. These results suggest that G_o plays a role in

olfactory signaling across multiple classes of sensilla independent of odor identity or concentration.

Our results show that sensory signals from five odorants, including ethyl acetate, are transduced in part through G_o signaling. These findings support the possibility that a single odorant may activate multiple transduction pathways since previous studies showed that G_q is needed for optimal responses to isoamyl acetate, ethyl acetate and butanol (Kain et al., 2008; Kalidas and Smith, 2002) Activation of *Drosophila* OR cation channel function by multiple odorants implies that both OR channel function and G-protein signaling are required for optimal responses to a given odor (Sato et al., 2008). It is possible that odor bound ORs directly activate G_o and G_q, thus reinforcing and optimizing the ORN response by modulating cyclic nucleotide levels.

Conclusions

Our results demonstrate that G_o is required for maximal physiological responses to a diverse group of attractive and aversive odorants in *Drosophila*. Given that diminished physiological responses to odors persist in the absence of G_o signaling, it is likely that OR channel function, along with G-protein signaling, are required for optimal physiological responses to odors.

Methods

EAG and single-sensillum recording experiments were performed as previously described (Krishnan et al., 2008; Krishnan et al., 2005). Recordings were carried out during the middle of the day on 2-5 day old flies raised at 18^{o}C. Temperature sensitive *GAL80*^{ts20} was inactivated by placing flies at 32^{o}C for 18 hours. Heat-treated flies were

then kept at 18^{o}C for 24-48 hours for recovery. The Tet-On system was activated by feeding flies a 2% sucrose solution containing 2 mM doxycycline overnight. Dilutions of all odorants except CO_2 were made in mineral oil. Odors were delivered for approximately 500 ms. At least eight EAG or single unit recordings from at least four different flies were analyzed for each data point. To quantify spike frequency, recordings from 10 different ORNs from at least 4 different flies were analyzed. Spikes were manually sorted and spontaneous frequency was not subtracted from the odor-induced net response. Statistical significance with respect to pairwise comparison was calculated using Student's t-test, and multiple means were compared by one-way ANOVA. The Bonferroni test was used for post hoc analyses. The PTX-induced change in sensitivity to ethyl acetate was calculated using a fitted linear equation (EAG amplitude = -2.45 x negative log dilution of ethyl acetate + 22.3) derived from the dose response curve from PTX non-expressing flies. A response of 8.4 mV to a 10^{-3} dilution of ethyl acetate in PTX expressing flies equates to a $10^{-5.67}$ dilution of ethyl acetate in PTX non-expression flies, or a ~470-fold reduction in stimulus concentration to produce the same response.

CHAPTER III

CIRCADIAN RHYTHMS IN CHEMOSENSORY PHYSIOLOGY*

Background

Circadian changes in membrane potential and spontaneous firing frequency have been observed in microbial systems (Adamich et al., 1976), invertebrates (Barlow, 1983) and mammals (Yamazaki et al., 1998). Oscillators in olfactory sensory neurons (ORNs) from *Drosophila* are both necessary and sufficient to sustain rhythms in electroanntenogram (EAG) responses (Tanoue et al., 2004), suggesting that odorant receptors (ORs) and/or OR-dependent processes are under clock control. Since EAGs are of limited spatial resolution and do not necessarily reflect firing of action potentials, I measured single-unit responses in different antennal sensillae from wild-type, clock mutant, odorant-receptor mutant, and G protein-coupled receptor kinase 2 (Gprk2) mutant flies to examine the cellular and molecular mechanisms that drive rhythms in olfaction. Given the remarkable mechanistic and structural similarities between the *Drosophila* gustatory and olfactory systems and experiments demonstrating that the proboscis contains a self-sustaining oscillator (Plautz et al., 1997), I reasoned that the proboscis clock might control rhythms in gustatory physiology.

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Results

(a) Trichoid Sensillae Display Rhythms in Spike Amplitude

To determine whether other classes of sensillae exhibit rhythms in spike amplitude, I measured single-unit responses from trichoid sensillae, which are thought to mediate responses to pheromones (van der Goes van Naters and Carlson, 2007). When spike amplitude of the A neuron from T2 sensillae was quantified, I found an approximately 3-fold circadian change that peaked at ZT21, 4 hr later than the peak in ab1 and ab3 basiconics (Fig. 12A). This rhythm persisted in DD conditions and was absent in per^0 flies (Fig. 12B and 12C). The later peak phase of spike amplitude rhythms in trichoid sensillae could be due to differences in circadian oscillator phase between trichoid and basiconic sensillae, but the core circadian oscillator component TIMELESS (TIM) cycled in the same phase in trichoid and basiconic sensillae. As with the basiconic sensillae, no daily changes in spontaneous firing frequency were seen in T2 sensillae. Although odorants that produce robust responses in T2 sensillae have not been identified, trans-2-hexanal (Clyne et al., 1997) produces a modest yet reliable response. The frequency of trans-2-hexanal-evoked spikes in T2 sensillae remained constant at ZT5 and ZT21 (Fig. 12D), thus demonstrating that odor-evoked spike frequency in T2 sensillae doesn't vary over a diurnal cycle.

The T1 subset of trichoid sensillae is uniquely involved in the perception of the only volatile pheromone known in flies—11-cis vaccenyl acetate (cVA), a male-specific lipid that mediates aggregation behavior (Ha and Smith, 2006; Kurtovic et al., 2007). Spontaneous spike amplitude in T1 sensillae was rhythmic, with an approximately 2.5-fold higher amplitude at ZT21 than at ZT5, whereas spontaneous spike frequency in T1 sensillae was constant (Fig. 13). Unfortunately, I could not measure rhythms in cVA-evoked activity in T1 sensillae. These experiments show that the circadian clock controls spontaneous spike amplitude, but not spike frequency in T2, and T1 sensillae.

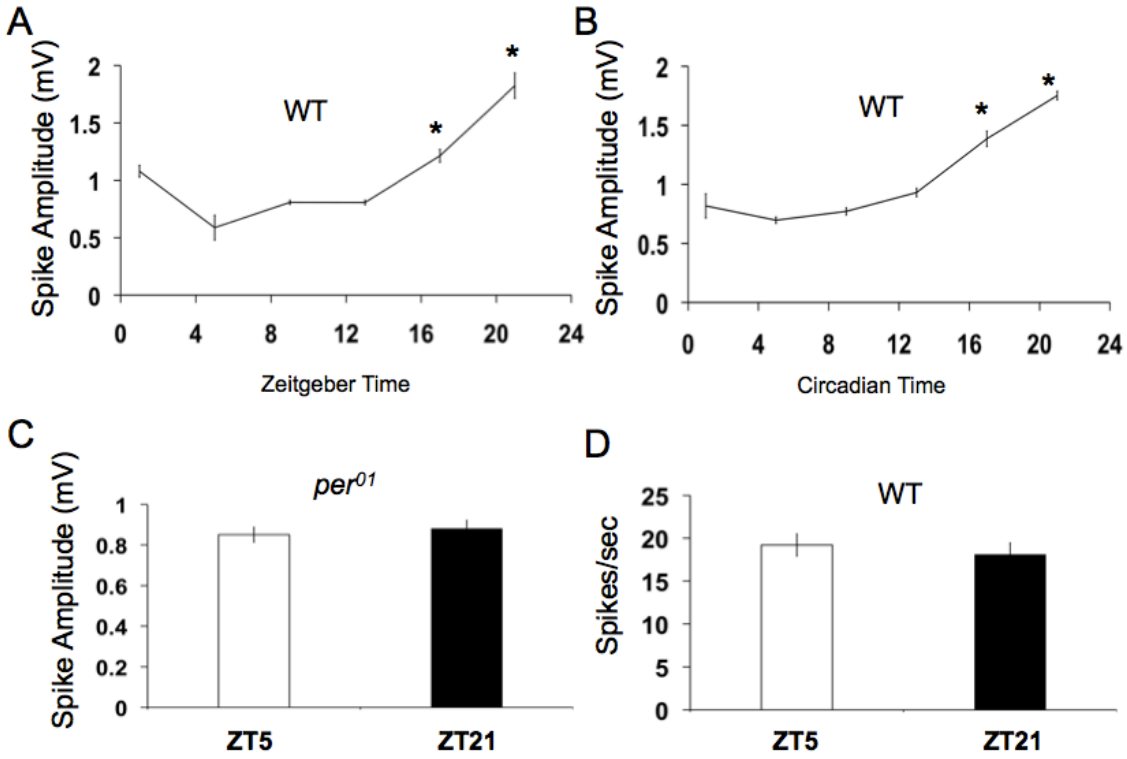

Fig. 12: Spontaneous spike amplitudes are under circadian-clock control in T2 sensillae. (A and B) Spontaneous T2A spike amplitude in WT flies during LD cycles (A) or DD (B) is rhythmic ($p < 0.001$). Asterisks indicate significant ($p < 0.05$) increase in amplitude at ZT17 and ZT21 (A) or CT17 and CT21 (B) compared with responses at all other times of day. (C) Spontaneous spike amplitudes are not rhythmic in *per*^{01} flies at ZT5 and ZT17 ($p > 0.5$). (D) Trans-2-hexanal-induced activity from a T2 sensillum in WT flies reveals that odor-induced firing frequency is not rhythmic ($p > 0.9$).

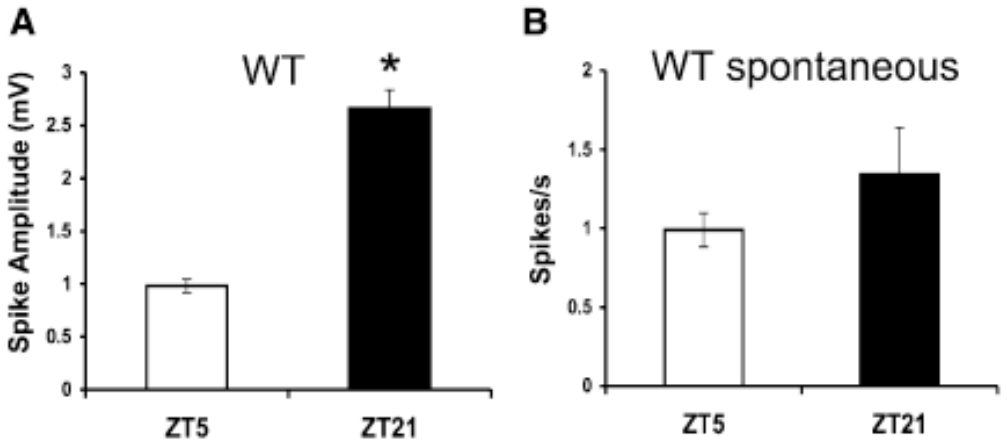

Fig. 13: Spontaneous spike amplitudes are under circadian-clock control in T1 sensillae. (A) Spontaneous spike amplitudes in wild-type flies during LD cycles. The asterisk indicates a significant ($p < 0.0001$) increase in response at ZT21 compared to ZT5. (B) Spontaneous firing frequency is not rhythmic in wild-type flies at ZT 5 and ZT21 ($p > 0.3$).

(b) Rhythms in Spike Amplitude Are Dependent on ORs and Gprk2

An Or83b deletion mutant that lacks Or83b messenger RNA (mRNA) and protein is anosmic because OR83b protein is necessary for the localization and function of odor-dependent cation channels in the ORN dendritic membrane in flies (Larsson et al., 2004). The Or83b deletion mutant shows spontaneous activity, albeit at lower levels, but no odor-induced responses (Larsson et al., 2004). Because I detect rhythms in spontaneous activity from T1, and T2 sensillae, I hypothesized that rhythms in spike amplitude will persist in the Or83b mutant. However, spontaneous spike amplitude did not show a significant rhythm in T2 sensillae from Or83b mutant flies recorded during LD cycles (Fig. 14). This result argues that ORs and/or OR-dependent processes are controlled by circadian clocks in ORNs.

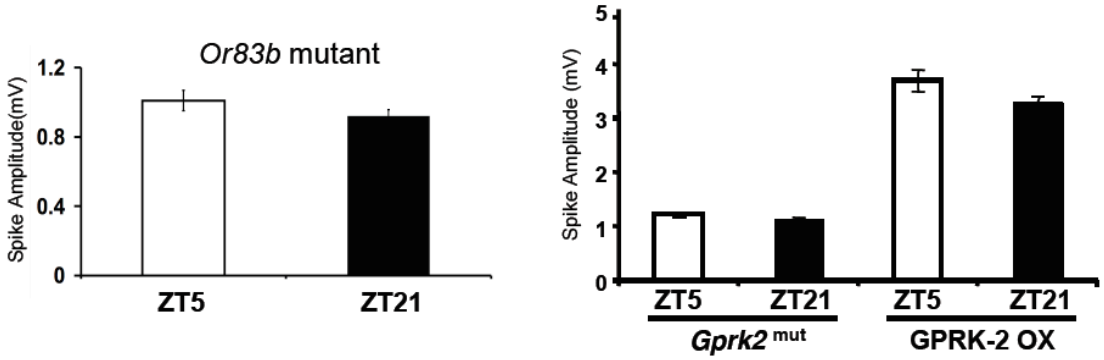

Fig. 14: *Or83b* and *Gprk2* mutants show no rhythm in spike amplitude. Left panel shows spontaneous spike amplitudes of T2 sensillae from Or83b null mutant flies at their respective peak and trough time points. No significant differences in the spike amplitudes of T2A neurons ($p > 0.2$) were seen in Or83b mutant flies. Spontaneous spike amplitudes of T2 sensillae from Gprk2 mutant and GPRK2 OX flies during LD cycles is shown on the right panel. Mean spike amplitudes at ZT5 and ZT21 for T2 sensillae were not significantly different in Gprk2 mutants and GPRK2 OE flies ($p > 0.1$). Mean spike amplitudes between Gprk2 mutants and GPRK2 OX flies at peak and trough time points was significant ($p < 0.0001$).

We recently showed that the abundance of *Drosophila* Gprk2 mRNA and protein cycle in antennae with a peak during the middle of the night and that GPRK2 levels determine the amplitude of EAG responses (Tanoue et al., 2008). Moreover, EAG amplitude and GPRK2 levels peak when ORs are localized predominantly in dendrites (*e.g.*, ZT17), and GPRK overexpression enhances OR localization to dendrites at times when ORs are normally at low levels in dendrites (*e.g.*, ZT5). These results are consistent with those showing that ORs must be present in the dendrite to produce rhythms in spike amplitude (Fig. 14) and suggest that GPRK2 levels may control the spike amplitude of single unit responses.

Single-unit responses were measured from Gprk2 mutants and flies that overexpress GPRK2 in ORNs to determine whether GPRK2 levels control spike amplitude. The amplitude of the spikes are constantly high when GPRK-2 is overexpressed (GPRK2

OX) in ORNs (Fig. 14). In contrast, spike amplitudes in the T2 trichoid sensillae were always close to the wild-type trough in *Gprk2*^{pj1} mutant flies, respectively (Fig. 14). This experiment, along with those of Tanoue et al. (Tanoue et al., 2008), demonstrate that the levels of GPRK2 regulate EAG and spike amplitude rhythms in different classes of sensillae.

One hypothesis to explain rhythms in the amplitude of spontaneous spikes and EAGs is that ion channel activity and/or composition is under circadian control. *Drosophila* ORs were recently found to form heteromeric odor-gated and cyclic-nucleotide-activated cation channels (Sato et al., 2008; Wicher et al., 2008). Tanoue et al. (2008) demonstrated that ORs accumulate in ORN dendrites in a circadian fashion, where OR abundance peaks near the middle of the night and is low during the day. These rhythms are dependent on the levels of GPRK2 and coincide with rhythms in the amplitude of both EAGs and spontaneous spikes. Taken together, these results suggest a model whereby GPRK2 controls the abundance and/or activity of OR-dependent odor-gated cation channels in ORN dendrites, which in turn alter membrane conductance to generate rhythms in the amplitude of spontaneous spikes and EAG responses. I can't exclude the possibility that the clock modulates other molecular or cellular targets to generate rhythms in EAG and spike amplitude such as other ion channels expressed in ORNs, the composition of sensillar lymph, and/or the size and shape of ORNs.

(c) GRN Spikes Are Controlled by the Circadian Clock

Recordings from single l-type sensillae were made in wild-type flies collected during 12 hr light:12 hr dark (LD) cycles. A different population of flies ($n \geq 6$) was recorded at each time point. The sweet-sensitive S neuron was stimulated by application of 100 mM sucrose. A 3.5-fold rhythm in S spike amplitude was detected with a peak at Zeitgeber time 1 (ZT1) and a trough at ZT17 (Fig. 15, Fig. 16A). The extent of diurnal influence

on spiking activity of S neurons was determined by recording the rate of firing in response to 100 mM sucrose.

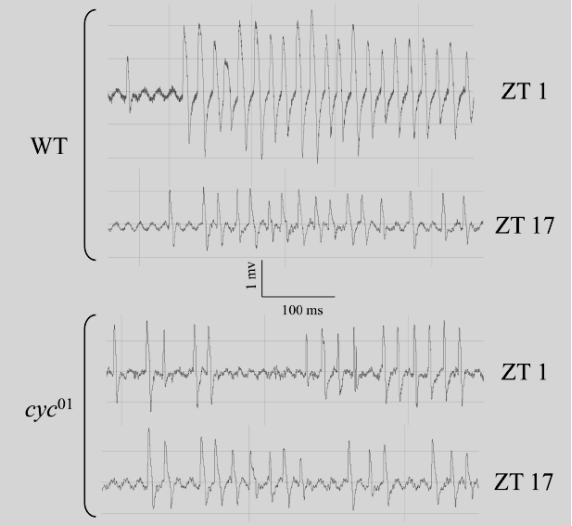

Fig. 15: Representative traces of sucrose-evoked single-unit activity recorded from S type GRNs under two different time points.

A 1.5-fold rhythm in spike frequency was detected, which showed a sharp trough at ZT17 (Fig. 16B). Because the waveforms of action potentials can encode biological information, I investigated changes in spike duration as a function of time of day. A 2-fold rhythm in S spike duration was found, with a peak at ZT1 and a trough at ZT17 (Fig. 16C). These rhythms in spike amplitude, frequency, and duration persisted in constant darkness (DD) (Fig. 16D–16F), thereby demonstrating that the rhythms are not a passive response to LD cycles but are driven by circadian clocks. These electrophysiological responses are constantly low in per^{01} and cyc^{01} null mutants, even in LD cycles (Fig. 16G–16L, Fig. 15), thus demonstrating that the clock is required for the daily increase in responses from S neurons.

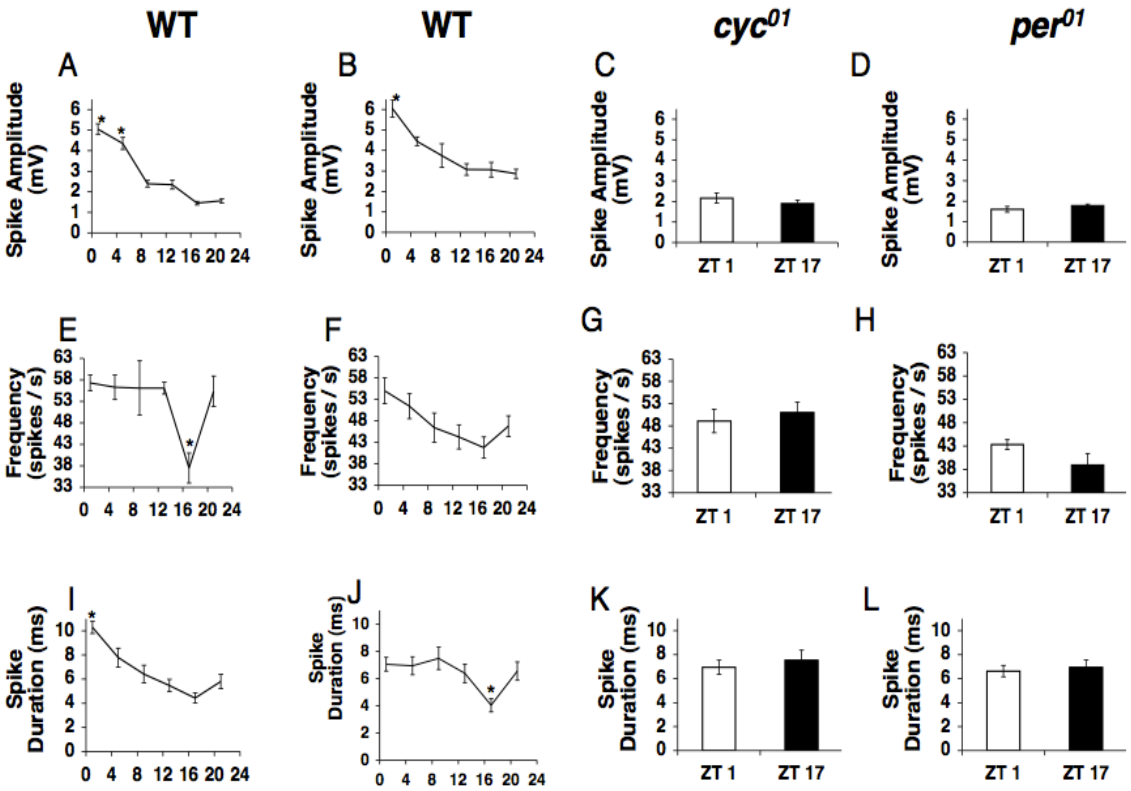

Fig. 16: S spikes are under circadian clock control in the L-type sensilla. (A–F) Spike amplitudes (A and D), frequencies (B and E), and durations (C and F) were rhythmic ($p \leq 0.02$) by one-way ANOVA in WT flies during LD cycles (A–C) or on the second day of DD (D–F). Asterisks indicate significant ($p < 0.05$) changes in spike parameters at a given time point compared to all other times of day. (G–L) Spike amplitudes (G and J), frequencies (H and K), and durations (I and L) in *cyc*^{01} (G–I) and *per*^{01} (J–L) flies are not rhythmic ($p > 0.18$). Each time point represents amplitudes calculated from a minimum of 30 individual spikes (in A, D, G, and J), frequencies calculated from a minimum of 10 individual gustatory receptor neurons (GRNs) (in B, E, H, and K), and spike durations calculated from a minimum of 20 individual spikes (in C, F, I, and L). All values are mean ± standard error of the mean (SEM).

To determine whether other classes of GRNs and other types of sensillae exhibit circadian rhythms in spike activity, I measured single-unit responses to the bitter compound caffeine (10 mM) in L2 neurons from s-type sensilla during DD. Rhythms in spike amplitude, frequency, and duration were detected that peaked at CT1 (Fig. 17A,

17C,and 17E). These rhythms were abolished in *cyc*^{01} mutants in DD (Fig. 17B, 17D, and 17F), in which spike amplitude and frequency were near the wild-type trough and spike duration was between the wild-type peak and trough values. These results demonstrate that circadian control of spike activity is broad, encompassing bitter-sensitive L2 neurons and sweet-sensitive S neurons in s-type and l-type sensillae, respectively.

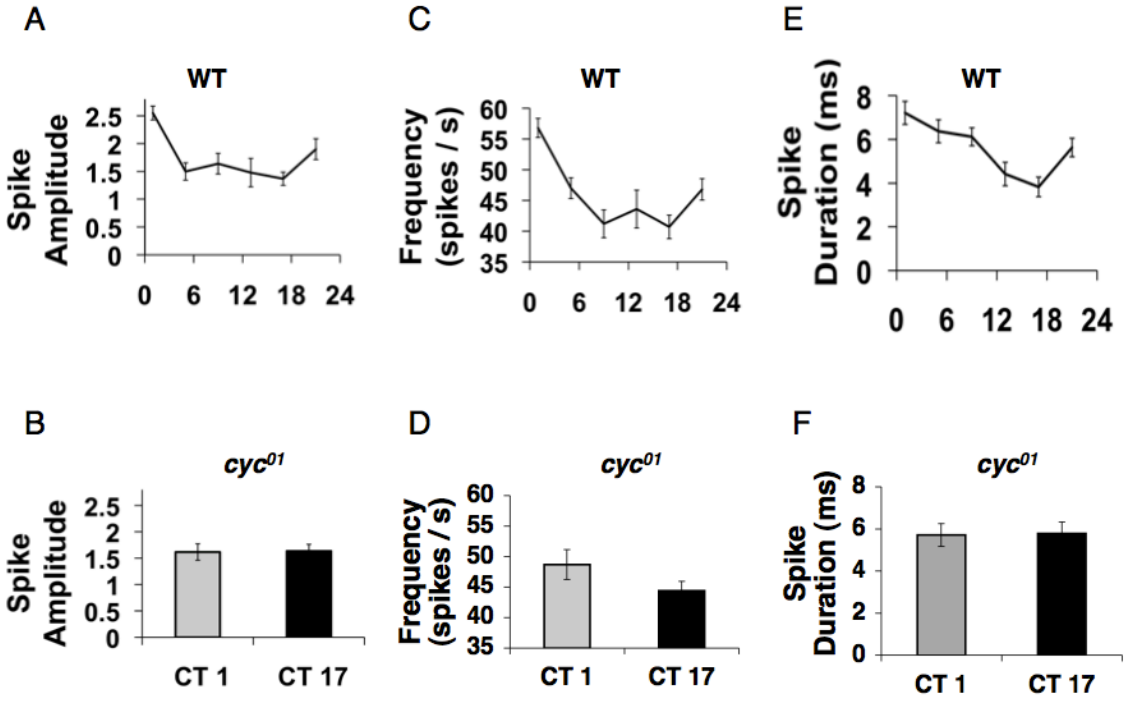

Fig. 17: L2 spikes are under circadian clock control in S-type sensilla. (A) The overall effect of time of day on spike amplitude is significant ($p < 0.005$) by one-way ANOVA. (B) Spike amplitudes in *cyc*^{01} flies at CT1 and CT17 are not significantly ($p > 0.92$) different. (C) The overall effect of time of day on spike frequency is significant ($p < 0.001$) by one-way ANOVA. Asterisk indicates significant ($p < 0.05$) changes in firing frequency at CT1 compared to all other times of day. (D) Spike frequencies in *cyc*^{01} flies at CT1 and CT17 are not significantly ($p > 0.13$) different. (E) The overall effect of time of day is significant ($p < 0.001$) on spike duration by one-way ANOVA. (F) Spike durations in *cyc*^{01} flies at CT1 and CT17 are not significantly ($p > 0.92$) different.

Rhythms in spike amplitude, frequency, and duration were all abolished in *Gprk2*^{06936} flies, in which spike frequency was close to the wild-type peak, but spike amplitude was midway between the wild-type peak and trough, and spike duration was only modestly higher than the wild-type trough. The effects were apparent even under LD cycles.

Conclusions

Our results demonstrate spike amplitude is controlled by circadian clocks trichoid sensillae. Rhythm in trichoid peaks ~ 4 hrs after the peak in basiconinc. The single-unit response rhythm requires GPRK2 expression and the presence of functional ORs in dendrites. These results argue that rhythms in GPRK2 levels control OR localization and OR-dependent ion channel activity and/or composition to mediate rhythms in spontaneous spike amplitude. Single-unit responses from labellar gustatory receptor neurons (GRNs) to attractive and aversive tastants also show diurnal and circadian rhythms in spike amplitude, frequency, and duration across different classes of gustatory sensilla. GRN sensitivity rhythm is dependent on Gprk2.

Methods

(a) Recording of Olfactory Single-Unit Responses

Flies (3–7 days old) were mounted in a specially designed apparatus, which was modified such that a fine glass capillary tube was used to both maneuver the antenna on the surface of the coverslip and hold the antenna in place. The antennal surface was observed under 1500x magnification that allowed individual sensillae to be resolved clearly using a BX-51W scope (Olympus). Recording in the dark was made possible with a filter with a cutoff of less than 600 nm (Leeds). Action potentials were recorded

with glass electrodes filled with 0.17 M NaCl with tip drawn to less than 1 mm diameter. The indifferent electrode was inserted into the eye of the fly and the recording electrode was inserted into the base of the sensillum so that the electrode is in contact with the sensillar lymph that bathes the dendrite. These electrodes were positioned with Huxley-style manual micromanipulators with fine controls (1 mm steps). Signals from the electrodes were fed into a differential amplifier (DP 301, Warner Instruments) and alternating current (AC) signals were recorded (300HZ–10KHz) and amplified1000x. Recordings were made from at least three different ORNs per fly. For all experiments described above, a minimum of four flies were measured. Single-unit recordings were stopped when signs of neuron damage characterized by a high frequency burst of firing were seen. Odorant stimulation was achieved by delivery of a quantifiable odor pulse, which interrupts a constant stream of air flowing over the preparation. The number of spikes initiated by the odor pulse was counted manually over 500 ms duration. Spike traces were analyzed with Axoscope (Axon) in offline mode, and peak-to-trough amplitudes of individual spikes were computed with software controls. Rate of spike firing was expressed as number of spikes/s. I could not measure rhythms in cVA-evoked activity in T1 sensillae because cVA-induced spikes could not be reliably distinguished from spontaneous non-T1 spikes having a similar frequency.

(b) Recording of Gustatory Single-Unit Responses

Male flies (3–10 days old) entrained to LD cycles for ≥3 days were collected during LD or the second day of DD and mounted, and the proboscis was immobilized. Individual labellar sensillae were observed under 1200× magnification. Recordings in the dark were made with a <600 nm filter. The indifferent electrode was inserted into the eye. The recording electrode contained tastant dissolved in 1 mM KCl and was used to stimulate a sensillum by physical contact with the tip of that sensillum. All recordings with a given genotype and tastant were performed at least six times per time point for ≥6 flies. A new

group of flies was recorded at each time point. Sucrose (100 mM) was used to stimulate S cells in accessible l-type sensilla, which respond to sugars in an identical manner (Hiroi et al., 2002). Caffeine (10 mM) was used to stimulate s6 and s2 sensilla, whose L2 neurons are responsive to bitter compounds (Hiroi et al., 2002). The number of spikes initiated by the tastant was counted manually over 500 ms duration beginning 50 ms after the onset of stimulation. Spike traces were analyzed with Axoscope (Axon) software in offline mode, in which the peak and trough values of individual spikes were used to compute amplitude. The time elapsed between the peak and trough values for an activity spike was used as a measure of spike duration (Gur et al., 1999).

CHAPTER IV

CIRCADIAN RHYTHMS IN APPETITIVE BEHAVIOR AND FEEDING*

Background

Circadian regulation of chemosensory processes is common in animals, but little is known about how circadian clocks control chemosensory systems or the consequences of rhythms in chemosensory system function. Taste is a major chemosensory gate used to decide whether or not an animal will eat, and the main taste organ in *Drosophila*, the proboscis, harbors autonomous circadian oscillators (Plautz et al., 1997). Oscillators in ORNs act upon OR trafficking to mediate olfaction rhythms (Tanoue et al., 2008). ORs and GRs belong to the same family of insect chemoreceptor proteins (Robertson et al., 2003), additionally, electrophysiological responses recorded from GRNs show rhythmic attributes (Chatterjee et al., 2010). Therefore, I hypothesized that appetitive behaviors may be under control of the clock. In response to contact chemoreception with a phagostimulatory chemical, flies elicit a reflex-like appetitive behavior wherein they extend the proboscis to attempt feeding (Dethier, 1976). To determine whether the circadian clock controls tastant-driven behavior, I measured proboscis extension reflex (PrER) responses at different times of day in wild-type and clock mutant flies during LD and DD conditions.

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Results

(a) Tastant-Induced Behavior Is Under Clock Control

PrER responses to 100 mM sucrose in wild-type flies showed a diurnal fluctuation that peaked at dawn (ZT1) and fell to trough levels by mid-night (ZT17) in LD (Fig. 18A). These trough-level PrER responses increased to near the peak level when stimulated with 500 mM sucrose (Fig. 18A), indicating a clock-modulated change in sensitivity to sucrose. PrER rhythms persisted in wild-type flies during DD, demonstrating that these rhythms are under circadian control (Fig. 18B). Rhythms in PrER responses remained at constant low levels in *per01* and *cyc01* mutants in LD (Fig. 18C and 18D), showing that the clock is necessary for increased PrER responses and that light does not have a strong masking effect on PrER rhythms. PrER responses to the sugar trehalose (100 mM), which also induces appetitive behavior, exhibited diurnal changes (Fig. 18E). Daily changes in responsiveness to a compound that deters appetitive behavior were measured by quantifying the reduction in PrER responses to a sucrose solution containing caffeine (Amrein and Thorne, 2005). The presence of caffeine decreased the probability of PrER strongly at ZT1 and only weakly at ZT17 (Fig. 18F). These results demonstrate that gustatory behavior to attractive and repulsive stimuli is under clock control.

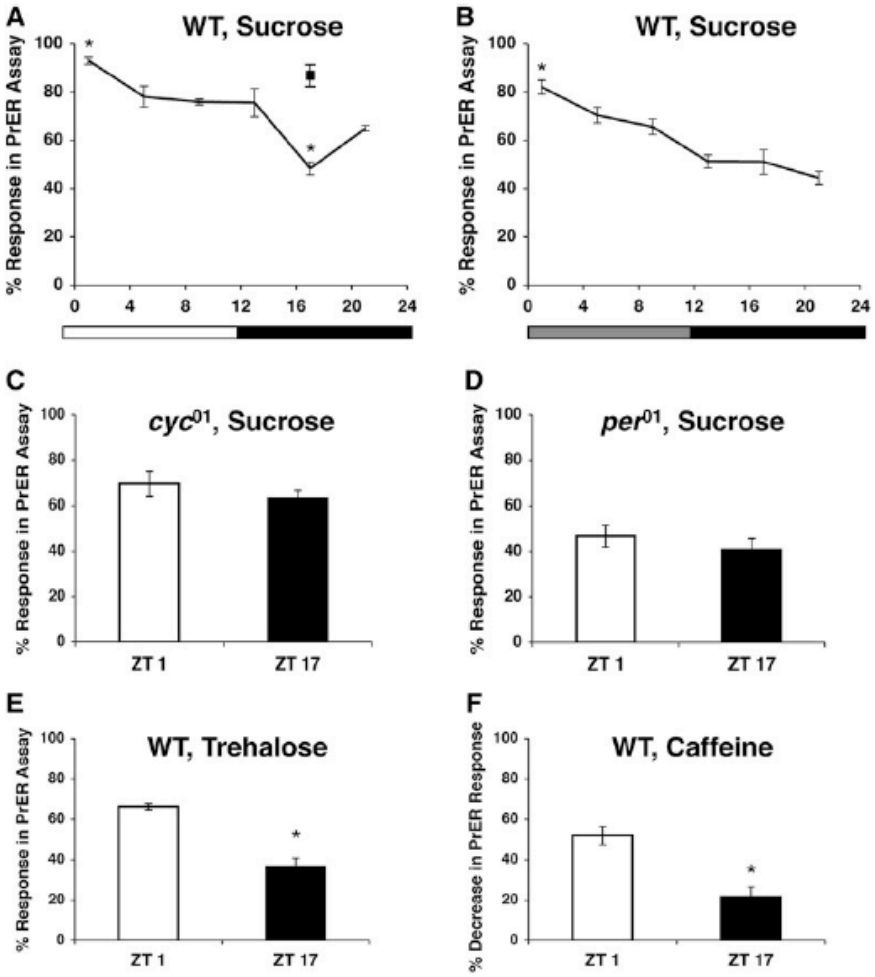

Fig. 18: *Drosophila* display circadian rhythms in gustatory behavioral responses. (A and B) PrER responses to 100 mM sucrose (black line) or 500 mM sucrose (filled square) under both LD and DD cycles are rhythmic ($p < 0.001$) by one-way ANOVA. Asterisks indicate significant ($p < 0.05$) changes in PrER behavior at ZT1 and ZT17 (A) or CT1 (B) compared to all other times of day. (C and D) PrER responses to 100 mM sucrose are arrhythmic ($p > 0.30$) in cyc^{01} and per^{01} flies at ZT1 and ZT17. (E) PrER responses to 100 mM trehalose were measured in WT flies at ZT1 and ZT17. Asterisks indicate a significant ($p < 0.001$) reduction in PrER responses at ZT17 compared to ZT1.
(F) Decrease in PrER responses to a 100 mM sucrose solution containing 10 mM caffeine versus 100 mM sucrose alone in WT flies at ZT1 and ZT17. Asterisk indicates significant ($p = 0.025$) decrease in PrER inhibition by caffeine at ZT17 compared to ZT1.

(b) GRN Clocks Are Necessary and Sufficient for PrER Rhythm

In the *Drosophila* olfactory system, peripheral clocks in ORNs drive rhythms in odor-induced physiological responses (Tanoue et al., 2004), which lead us to think that peripheral oscillators in GRNs drive PrER rhythms.

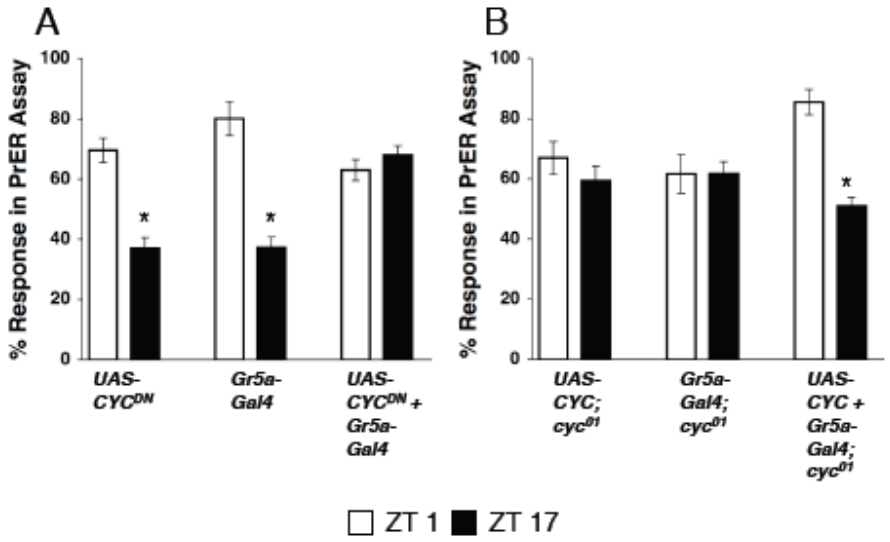

Fig. 19: Oscillators within GRNs are necessary and sufficient for PrER rhythms. (A) PrER responses were measured at ZT1 and ZT17 in wild-type flies bearing the Gr5a-Gal4, UAS-cycDN, or Gr5a-Gal4 + UAS-cycDN transgenes. The differences in mean PrER responses at ZT1 and ZT17 are significant ($p < 0.001$) in flies containing Gr5a-Gal4 or UAS-cycDN alone but are not significant ($p < 0.30$) in flies carrying Gr5a-Gal4 + UAS-cycDN. (B) PrER responses were measured at ZT1 and ZT17 in *cyc*^{01} flies carrying the Gr5a-Gal4, UAS-cyc, or UAS-cyc + Gr5a-Gal4 transgenes. There are no significant ($p > 0.30$) differences in PrER responses at ZT17 and ZT1 in *cyc*^{01} flies carrying either UAS-cyc or Gr5a-Gal4. The differences in mean PrER responses at ZT1 and ZT17 are significant ($p < 0.001$) in *cyc*^{01} flies carrying UAS-cyc + Gr5a-Gal4. Asterisks denote a significant ($p < 0.05$) change in PrER responses between ZT17 and ZT1.

The presence of peripheral clocks in GRNs on the proboscis was first confirmed via immunocytochemistry. Rhythmic PDP1 staining in ELAV-positive cells at the base of sensillae demonstrated (experiment done by Dr. Jerry Houl) that the GRNs within gustatory sensilla contain circadian oscillators.

To test the idea that local oscillators within GRNs are necessary for PrER rhythms, I expressed a dominant-negative form of CYC (CYC^{DN}) to abolish clock function in the sweet-sensitive S neurons that elicit PrER behavior in response to sucrose (Slone et al., 2007). Under LD conditions, PrER responses were abolished in flies containing both the Gr5a-Gal4 driver, which is expressed in S neurons (Wang et al., 2004), and UAS-cycDN responder, but not in control flies containing the Gr5a-Gal4 driver or UAS-cycDN responder alone (Fig. 19A). This result demonstrates that circadian oscillators in GRNs are required for PrER rhythms.

I then sought to determine whether local clocks in GRNs are sufficient for PrER rhythms by generating flies with circadian oscillators only in S neurons. For this, oscillator function was rescued exclusively in S neurons by using Gr5a-Gal4 to drive UAS-cyc expression in *cyc01* flies. PrER behavior in *cyc01* flies containing both Gr5a-Gal4 and UAS-cyc was rhythmic, whereas *cyc01* flies containing Gr5a-Gal4 or UAS-cyc alone were arrhythmic (Fig. 19B). These data demonstrate that clocks in GRNs are sufficient for PrER rhythms. Because clocks are not present elsewhere in *cyc01* flies containing Gr5a-Gal4 and UAS-cyc, these data also show that central clocks in the brain are not necessary for PrER rhythms. Taken together, these results demonstrate that GRN clocks are necessary and sufficient to control rhythms in gustatory behavior.

(c) Cycling Levels of GPRK2 Drive PrER Rhythm

Because circadian oscillators in GRNs are sufficient for PrER rhythms, the clock output pathway that controls this rhythm must also reside in GRNs. To identify a clock-controlled molecule involved in gustatory signal transduction, I focused my attention on GPRK2, which is required for rhythms in olfactory responses in *Drosophila* (Krishnan et al., 2008; Tanoue et al., 2008). Molecular analysis carried out by Dr. Shintaro Tanoue showed that the levels of a unique isoform of GPRK2 cycled 2-fold in wild-type proboscises with a peak at ZT17 and a trough at ZT1 in a clock-dependent manner. GPRK2 was detected in the cell body of GRNs, in the shaft of the sensillar hair that contains GRN dendritic projections and possibly support cells closely associated with GRNs.

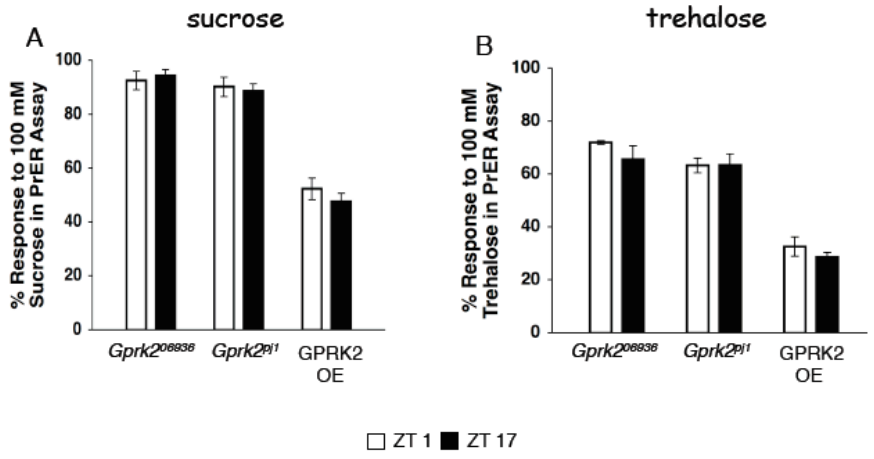

Fig. 20: G protein-coupled receptor kinase 2 levels control rhythms in PrER behavior. PrER responses to sucrose and trehalose were measured at ZT1 and ZT17 in WT flies carrying Gr5a-Gal4 and UAS-Gprk2, which overexpress GPRK2 in S neurons (GPRK2 OE), and in *Gprk2*^{06936} mutants (Gprk2 mutant). Mean PrER responses to sucrose (A) and trehalose (B) at ZT1 and ZT17 were not significant ($p > 0.16$) and remained at constant low levels in Gprk2 mutant flies and constant high levels in GPRK2 OE flies.

The levels of GPRK2 in the proboscis are lowest when PrER responses peak and peak when PrER responses are lowest. This antiphasic relationship suggests that GPRK2 levels may control rhythmic PrER behavior. Consistent with this possibility, PrER responses to sucrose and trehalose were constantly repressed when GPRK2 was overexpressed but were always high in the *Gprk2*^{06936} mutant (Fig. 20A-B). Thus, these experiments argue that cycling GPRK2 levels drive rhythms in PrER behavior.

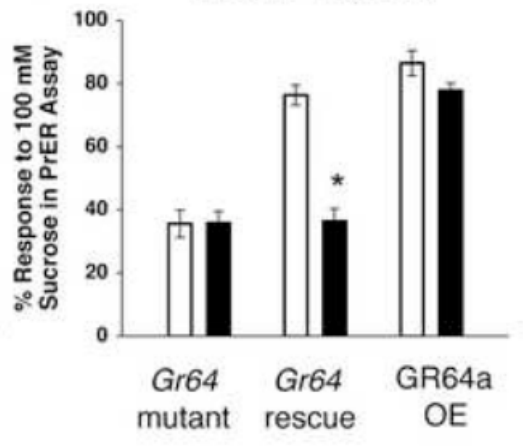

Fig. 21: Optimal GR level is required for PrER rhythm. PrER responses to 100 mM sucrose in Gr64 mutant (R1/+;R2/+;ΔGr64/ΔGr64), Gr64 rescue (R1/+;R2/+;ΔGr64/ΔGr64 carrying one copy of the UAS-Gr64abcd_GFP_f reporter), and GR64a-overexpressing flies at ZT1 and ZT17. The differences in mean responses at ZT1 and ZT17 are not significant in Gr64 mutants ($p > 0.90$) or GR64a-overexpressing flies ($p > 0.05$) but are significant ($p < 0.001$) in Gr64 rescue flies.

GPRK2 mediates circadian rhythms in the subcellular localization of *Drosophila* odorant receptors (ORs) (Tanoue et al., 2008). Because *Drosophila* ORs and GRs belong to the same family of insect chemoreceptor proteins, I wished to determine whether GPRK2-dependent regulation of rhythmic PrER responses relies on GRs. A mutant that removes all six *Drosophila* Gr64 genes (ΔGr64) shows drastically reduced PrER responses to most sugars (Slone et al., 2007). When ΔGr64 flies were stimulated with 100 mM sucrose at ZT1 and ZT17, their PrER responses were not rhythmic, but ΔGr64 mutants

rescued by a transgene containing the entire Gr64 gene cluster (Slone et al., 2007) recovered PrER rhythms (Fig. 21). Overexpression of the sucrose receptor Gr64a resulted in arrhythmic PrER responses that were near the circadian peak value (Fig. 21). Likewise, deletion of Gr5a, which is required for responses to trehalose (Dahanukar et al., 2001), resulted in constant low PrER responses to trehalose, whereas GR5a overexpression resulted in constant high responses to trehalose. These results imply that GRs are not only required to detect tastants but are also necessary for sustaining rhythms in tastant-evoked appetitive behavior.

(d) GRN Clocks Regulate Feeding

Both external sensory cues and internal metabolic state contribute to the regulation of feeding (Melcher et al., 2007). Recent work in *Drosophila* has shown that loss of clock function in fat body increases feeding by altering metabolic state (Xu et al., 2008). I sought to determine whether GRN oscillators also regulate feeding because they modulate taste sensitivity. Food ingestion was measured with a blue food dye that can be quantified spectrophotometrically and via the capillary feeder (CAFE) assay (Ja et al., 2007; Xu et al., 2008). Under LD conditions, flies that express CYC^{DN} in sweet-sensitive Gr5a neurons consumed significantly more food over 24 hr than controls carrying the driver or responder transgenes (Fig. 22A-B). Moreover, food intake was higher in the morning (ZT0–4) than in the evening (ZT12–16), demonstrating that increased consumption is not uniform during a diurnal cycle (Table1). This result shows that circadian clocks in a subset of GRNs act to limit the amount of food intake.

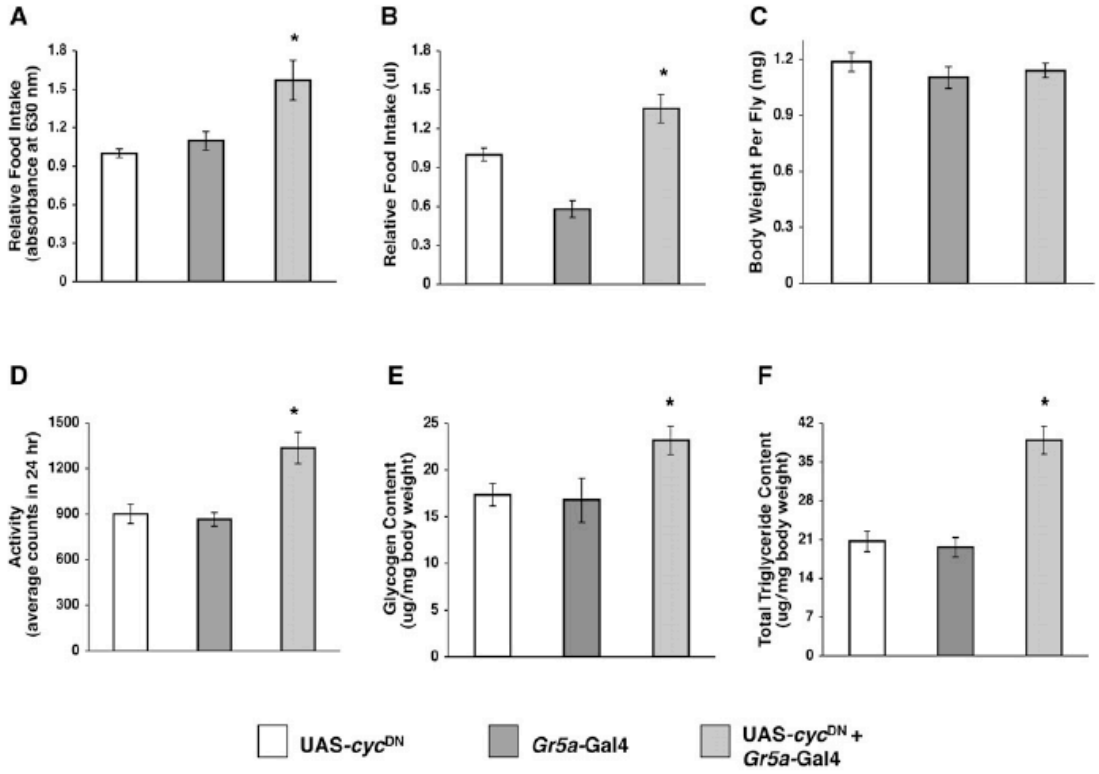

Fig. 22. Circadian clocks in Gr5a neurons regulate feeding, food storage, and activity. (A and B) Flies carrying both the Gr5a-Gal4 and UAS-cycDN transgenes show significantly ($p < 0.02$) increased feeding by (A) dyne-intake assay and (B) capillary feeding assay, compared to control flies containing either the Gr5a-Gal4 or the UAS-cycDN transgene. (C) The body weight of flies carrying both the Gr5a-Gal4 and UAS-cycDN transgenes was not different ($p > 0.50$) from control flies bearing the Gr5a-Gal4 or UAS-cycDN transgenes. (D) Overall activity measured as the number of times flies crossed an infrared light beam during a 24 hr period, was significantly ($p < 0.001$) increased in flies carrying both the Gr5a-Gal4 and UAS-cycDN transgenes. (E) Glycogen levels and (F) triglyceride levels are significantly ($p < 0.04$) higher in flies carrying both the Gr5a-Gal4 and UAS-cycDN transgenes than control flies bearing the Gr5a-Gal4 or UAS-cycDN transgenes.

Table 1: Eliminating circadian clock function in Gr5a neurons differentially alters PrER responses, food intake and activity level depending on time-of-day. PrER responses and food intake were measured at either ZT 0-4 or ZT 16-20 in flies of the indicated genotypes that had been starved for 24h and activity level was measured in *ad libitum* fed flies (see Experimental Procedures). Asterisks denote significantly ($p < 0.05$) different PrER responses, food intake and activity values in flies containing both the UAS-*cyc*^{DN} + *Gr5a*-Gal4 transgenes versus control flies containing UAS-*cyc*^{DN} or *Gr5a*-Gal4 alone. Values are shown as the mean ± S.E.M. The number in parentheses represents the number of independent repeats.

	ZT 0-4			**ZT 16-20**		
Genotype	**PrER response (%)**	**Food intake (ul)**	**Activity counts**	**PrER response (%)**	**Food intake (ul)**	**Activity counts**
UAS-*cyc*^{DN}	67.7 ± 2.87 (n=13)	0.572 ± 0.114 (n=6)	451 ± 32 (n=16)	43.4 ± 2.7 (n=11)	0.329 ± 0.026 (n=8)	69 ± 14 (n=16)
Gr5a-Gal4	76.1 ± 3.93 (n=12)	0.377± 0.097 (n=6)	433 ± 24 (n=16)	43.2 ± 2.7 (n=9)	0.342 ± 0.014 (n=7)	41 ± 15 (n=16)
UAS-*cyc*^{DN} + *Gr5a*-Gal4	58.8 ± 3.17 (n=13) *	0.873 ± 0.011 (n=6) *	676 ± 51 (n=16) *	54.7 ± 3.05 (n=22) *	0.208 ± 0.031 (n=8) *	34 ± 23 (n=16)

Although flies that lack clocks in Gr5a neurons eat more, they do not gain weight compared to controls carrying the driver or responder transgenes alone (Fig. 22C). Nevertheless, loss of clock function in Gr5a neurons led to a considerable increase in triglyceride and glycogen content (Fig. 22E-F). Increased triglyceride and glycogen content in flies lacking clocks in Gr5a neurons was associated with higher levels of

locomotor activity over a 24 hr period (Fig. 22D), in which increased activity levels coincided with increased feeding (Table 1). Thus, flies lacking clocks in Gr5a neurons eat more and store triglycerides and glycogen even though they expend more energy to fuel increased locomotor activity.

Conclusions

Circadian clocks in GRNs control neuronal output and drive behavioral rhythms in taste responses that peak at a time of day when feeding is maximal in flies. My results argue that oscillations in GPRK2 levels drive rhythms in gustatory physiology and behavior and that GRN clocks repress feeding. The similarity in gustatory system organization and feeding behavior in flies and mammals, as well as diurnal changes in taste sensitivity in humans, suggest that my results are relevant to the situation in humans.

Methods

(a) Proboscis Extension Reflex Assay

Three- to seven-day-old male flies that had been entrained to LD cycles for ≥3 days were starved for 24 hr, collected at different times during LD or the first day of DD, mounted on a slide, and allowed to recover for 30 min. Proboscis extension in response to 100 mM sucrose and 100 mM trehalose was recorded as described (Slone et al., 2007), with minor modifications. After the 30 minutes recovery time, flies were allowed to drink water until satiation. PrER responses to pure water were not rhythmic ($p > 0.39$) in WT flies. Each fly was given the same tastant (100 mM sucrose and 100 mM trehalose) three times with intervening water application and the number of extensions was recorded. To monitor responses to bitter compounds, compounds were added to 100 mM sucrose and

the effect on extension was examined (Wang, 2004). Recordings in the dark were made using a <600nm filter. At least three batches of ~10 flies each were tested for every substance at a given time point.

(b) Feeding Assays

Three- to ten-day-old male flies entrained for at least 3 LD cycles were given food containing 5% sucrose, 1% low-melting-point agarose, and 0.5% brilliant blue FCF (Wako) for 24 hr starting at ZT12. Flies were then collected and prepared for quantification of blue dye ingestion as described (Xu et al., 2008). For a given genotype, at least six independent experiments, each set consisting of 10 flies, were carried out. CAFE assays were used to measure feeding behavior of grouped fruit flies (Ja et al., 2007; Xu et al., 2008). For each genotype, CAFE assays were conducted as described (Xu et al., 2008), except that flies were habituated to feeding from glass capillaries for 24 hr and feeding was measured over 4 hr. CAFE assays were repeated at least five times for each data point. Levels of glycogen and triglycerides were measured as previously described (Xu et al., 2008).

(c) Activity Measurement

For each line, 7- to 10-day-old male flies were entrained for at least 3 days in LD cycles and placed in *Drosophila* activity monitors (Trikinetics). Activity was measured by counting the number of infrared beam breaks every 10 min and was analyzed with Clocklab software.

(d) Statistical Analysis

Statistical analysis was done with Statistica (Statsoft). Analysis of the effects of time of day was examined by one-way analysis of variance (ANOVA). Welch's ANOVA was used for heteroscedastic data set, provided Levene's test indicated unequal variances. Post hoc comparisons were done with Scheffe's test ($\alpha = 0.05$). Unpaired Student's t test (two-tailed) was used to compare values at peak and trough time points

CHAPTER V

DISCUSSION AND CONCLUSIONS

How *$G_{\alpha}o$* participates in olfactory signal transduction

Since *$G_{\alpha}o$* is expressed in *Drosophila* olfactory receptor neurons, I reasoned that *$G_{\alpha}o$* acts together with insect OR cation channels to mediate odor-induced physiological responses. To test whether *$G_{\alpha}o$* dependent signaling is involved in mediating olfactory responses in *Drosophila*, electroantennogram and single-sensillum recordings were analyzed from flies that conditionally express pertussis toxin, a specific inhibitor of *$G_{\alpha}o$* in *Drosophila*. PTX mediates the ADP-ribosylation of a unique cysteine reside present near the C terminus of *$G_{\alpha}o$*. Suppression of *$G_{\alpha}o$* signaling in olfactory receptor neurons reversibly reduced the amplitude and quickened the termination of EAG responses induced by ethyl acetate (Chatterjee et al., 2009). Expression of pertussis toxin reduced the frequency of odor-induced spike firing from individual sensory neurons (Chatterjee et al., 2009). These results demonstrated that *$G_{\alpha}o$* signaling is physiologically involved in increasing olfactory sensitivity in *Drosophila*. Independent of odorant identity and intensity, PTX dampened olfactory reception in a generalized manner (Chatterjee et al., 2009). My results demonstrated that *$G_{\alpha}o$* is required for maximal physiological responses to multiple odorants in *Drosophila*, and suggest that in addition to OR channel function; G-protein signaling is also required for optimal physiological responses to odors.

Immediate electrical signals (action potentials) generated by my chosen five odorants including ethyl acetate are produced at least partially through G_o signaling (Chatterjee et al., 2009). Because I tested only a restricted panel of odorants, I cannot completely rule out the possibility that activation of different, stimulus- and concentration-dependent signaling pathways in ORNs are mediated by other G proteins. In vivo modulation of no individual G protein could obliterate olfactory responses completely (Yao and Carlson,

2010). My findings also support the possibility that a single odorant may activate multiple transduction pathways: previously it was shown that G_q is needed for normal response to isoamyl acetate, ethyl acetate and butanol (Kain et al., 2008; Kalidas and Smith, 2002), I here show that G_o is also required to respond optimally to these odorants. Based on the quality and intensity of the odor stimulus it receives, each odorant receptor could stimulate different transduction pathways to varying extents (Kain et al., 2008). Interestingly, a single OR present within the same neuron can mediate both excitatory and inhibitory responses as a consequence of stimulation by two different odorants (Hallem et al., 2004). In light of recent results that demonstrate *Drosophila* ORs function as odor-gated and cyclic-nucleotide-activated cation channels, it is possible that ORs directly activate G_o (Sato et al., 2008; Wicher et al., 2008). Moreover, since cyclic nucleotides have been shown to activate cation channels formed by the OR-complexes (Wicher et al., 2008), it is tempting to hypothesize that G_o may directly modulate the levels of cyclic nucleotides in ORNs (Fig. 23). In a heterologous system, G_s seems to be the primary transducer of odor-activated metabotropic signaling (Wicher et al., 2008). The persistent activation of G_o may lead to the heterologous sensitization of adenylyl cyclases; increasing the activation of cyclases by $G_s\alpha$ (Watts et al., 1998). It is also possible that G_o may mediate olfactory reception by deactivating G protein regulated inward rectifying potassium (GIRK) current (Mark et al., 2000). In mammals, *$G_\alpha o$* has been shown to modulate the muscarinic regulation of L-type Ca^{2+} channels in heart (Valenzuela et al., 1997). Direct activation of the Rab5 GTPase by *$G_\alpha o$* is known to regulate endocytosis, which is involved in the internalization of cell surface receptors in fruit flies (Purvanov et al., 2010). It is therefore plausible that the role of *$G_\alpha o$* in changing the membrane density of ORs underlies its involvement in olfactory signal transduction. *$G_\alpha o$* is also involved in olfactory signaling in the nematode worm *C. elegans* (Matsuki et al., 2006). Acting upstream of *$G_\alpha q$* (EGL-30), *$G_\alpha o$* (GOA-1) antagonizes the DAG signaling cascade to mediate olfactory adaptation (Matsuki et al., 2006). In *Drosophila*, *$G_\alpha o$* is involved in other physiological functions with the ORNs. *Or83b*-expressing ORNs have $GABA_B$ receptors, which signal through PTX-sensitive

G$_{\alpha}$o at their axon terminals (Olsen and Wilson, 2008). GABA is involved in lateral presynaptic inhibition in the ORN~PN synapses (Olsen and Wilson, 2008). *G$_{\alpha}$o* in ORNs thus additionally mediates inhibitory modulation of synaptic transmission.

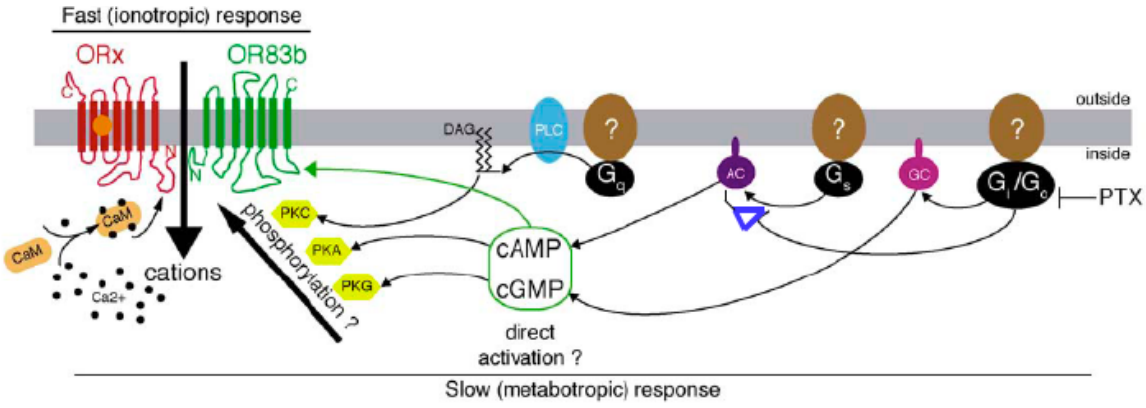

Fig. 23: Possible involvement of the PTX-sensitive *G$_{\alpha}$o* in the dual activation pathway of insect olfactory signal transduction (modified after, Nakagawa and Vosshall, 2009, *Current Opinion in Neurobiology*)

Whether PTX-mediated repression of G_o signaling is complete or not posits a caveat in my study. Because null allele of *G-oα47A* gene confers lethality, RNAi against G_o has been employed (Bredendiek et al., 2010). But RNAi also is unable to give rise to a protein-null molecular phenotype in most cases. Use of the MARCM system (mosaic analysis with a repressible cell marker) to generate G_o null clones in the antenna failed (Yao and Carlson, 2010), presumably due to the involvement of G_o in nervous system development. As a complementary approach to RNAi, competitive peptides have been used to knock down Gα protein activity in flies (Yao and Carlson, 2010). Peptides consisting of the 11 C-terminal amino acids of a Gα protein can not activate downstream signaling pathways but have been shown to decrease the receptor-mediated response to ligands by binding to GPCRs and competing with the endogenous Gα (Yao and Carlson, 2010). Use of this strategy failed to demonstrate any involvement of *G$_{\alpha}$o* in odor response (Yao and Carlson, 2010), but the efficacy of such a peptide against *G$_{\alpha}$o*

signaling have not been proved in vivo. In further analysis of $G_{\alpha}o$ function, a constitutively inactive GDP bound form of $G_{\alpha}o$ which is believed to have a dominant-negative effect may be used (Katanaev et al., 2005).

ORs are believed to represent a relatively recent branch of an ancient GR family (Robertson et al., 2003). A unifying model (Yao and Carlson, 2010) proposed that ORs and GRs are capable of acting via multiple mechanisms, one is ionotropic and one requiring G-proteins. There is no evidence in the literature of a G protein that is absolutely required for olfactory responses in flies. Perhaps ORs evolved to rely primarily on a ligand-gated ionotropic signaling mechanism, whereas GRs maintained the ability to signal via a G-protein-dependent mechanism. Indeed, mediators of G protein signaling, *e.g.*, G proteins ($G_{\alpha}s$, $G_{\alpha}o$, $G_{\alpha}q$ and $G_{\gamma}1$), and modulators of second messenger activity (adenylyl cyclase AC78C, IP3 receptor) are known to participate in sugar taste sensation in flies (Bredendiek et al., 2010; Ishimoto et al., 2005; Kain et al., 2010; Ueno and Kidokoro, 2008; Ueno et al., 2006; Usui-Aoki et al., 2005). GR-dependent response to CO_2 is also mediated in part by $G_{\alpha}q$ *and* $G_{\gamma}30a$ (Yao and Carlson, 2010). A limited ability of ORs and GRs to each signal through a secondary mechanism might reflect this evolutionary transition. My results indicate that to a partial but significant extent, odor responses are dependent on a G protein. We still need to know how and where G proteins act in insect olfactory signaling, and which G proteins are important. Modulation of the electrical signal, *e.g.*, termination and adaptation, has been reported in insect chemosensory systems (de Bruyne and Baker, 2008), indicating the involvement of secondary regulatory cascades, but their molecular basis is not well understood. Because properties of ion channels (open probability, inactivation kinetics etc.) are known to be regulated by a variety of second messengers—such as ions, cyclic nucleotides and lipids (Damann et al., 2008), a model of blended ionotropic/metabotropic modulation of odor-response in insects seems very probable. The following questions, nevertheless, still remain unanswered: is the channel pore formed by one or by both subunits? Does OR83b co-determine the ligand affinity and

selectivity of OR-X? What is the stoichiometry between OR-X and OR83b? How is the electrical response terminated? Does the receptor, similar to many neuronal ionotropic receptors, desensitize in the presence of the ligand? The cyclic nucleotide gated activation of the OR-OR83b channel may be potentiated by $G_{\alpha}o$. I demonstrated that G proteins are necessary for the correct functioning of the insect olfactory system. However, general neuronal sickness or the alteration of G protein-mediated signaling pathways downstream or independent of the olfactory receptors could be sufficient to explain the abnormal odor-evoked responses that I reported (Chatterjee et al., 2009).

Organization and outputs of the chemosensory clock

A wide variety of organisms display chemosensory rhythms (Table 2). The circadian circuit for chemosensory rhythm may be driven by clocks present in the – (a) association neurons in the chemosensory circuit, (b) both the central pacemaker and local oscillators which act either independently or cooperatively, (c) afferent sensory neurons, (d) inside motor neurons or effector tissues, or, (e) may be a direct consequence of general activity rhythm.

Chemosensory rhythms in mammals are brought about by clock controlled processing of neural activity in relay neurons of the CNS. In mouse, the olfactory bulb (OB) functions as circadian pacemaker because it drives a rhythm in the piriform cortex (Granados-Fuentes et al., 2006)). The SCN is dispensable for sustained neural activity rhythm in OB (Granados-Fuentes et al., 2006), but is needed for entrainment of the OB to environmental cycles (Granados-Fuentes et al., 2004a). It is well established that in mammals, the central clock plays important roles in synchronization between peripheral clocks under light-phase resetting conditions (Maywood et al., 2007). The OB clock interacts with the SCN clock or its output because OBX animals show a change in rhe free-running period and the pace of reentrainment (Granados-Fuentes et al., 2006).

Table 2: Widespread occurrence of the circadian rhythms in chemosensation in animals (individual sources of information have been cited in the main text).

Organism	Output	Peak	Trough	Amp. of rhythm in DD	Internally driven
Drosophila melanogaster (vinegar fly)	• Odor-evoked chemotactic behavior	CT 18	CT3	1.6	Yes
	• EAG	CT17	CT5	2	Yes
	• Spontaneous spike amplitude of ORNs	CT17	CT1	2	Yes
	• Proboscis extension reflex	CT1	CT17	2	Yes
	• Amplitude, frequency & duration of GRN spikes	CT1	CT17	2	Yes
Spodoptera littoralis (Egyptian Cotton Leafworm)	• EAG	CT6	CT21-3	1.4	Yes
	• Pheromone induced behavior	CT18-21	CT12-15	2.4	Yes
Leucophaea maderae (cockroach)	• EAG	CT0	CT12	1.3	Yes
	• ORN spike frequency	CT2	CT8	1.4	Yes
Manduca sexta (hawkmoth)	ORN spike frequency	ZT22-1	ZT8-11	1.7	Unknown
Protophormia terraenovae (blowfly)	• Proboscis extension reflex	CT5-9	CT1-4	2.7	Yes
	• ED_{50}	CT4		3.3	Yes
Glossina morsitans (tsetse fly)	• EAG	ZT8-12	ZT4-8	1.7	Unknown
	• ORN spike frequency	ZT8-12	ZT4-8	1.8	Unknown

Table 2: Continued

Organism	Output	Peak	Trough	Amp. of rhythm in DD	Internally driven
Triatoma infestans (vinchuca)	CO_2 directed orientation	CT14	CT8	6	Yes
rat	Spontaneous firing frequency of embryonic mitral cells from olfactory bulb	Dusk	Early morning	2	Yes
mouse	Odor evoked expression of immediate early genes in olfactory bulb and piriform cortex	CT16	CT8	4	Yes
human	Olfactory event-related potential (ERP)	4 pm	4 am		Unknown

Cockroach olfactory rhythms serve as example of multimodal regulation by independent clocks. Their master clock localized in the optic lobe dictates the EAG rhythm, while being redundant for the ORN frequency rhythm (Page and Koelling, 2003; Saifullah and Page, 2009). Why is neuronal impulse activity controlled by the peripheral clock, while the field potential generated at the level of the olfactory organ is controlled by the central clock is unclear as of now. However, these physiological rhythms are in antiphase to odor-evoked behavioral rhythms, implying that the behavioral rhythm is probably controlled at the level of central processing rather than sensory gating (Saifullah and Page, 2009).

Drosophila chemosensory rhythms are driven by autonomous oscillators present in afferent chemosensory neurons (Chatterjee et al., 2010; Tanoue et al., 2004). Oscillators in these sensory neurons are sufficient for the rhythmic output and disruption of the central protocerebral clockwork do not significantly influence the chemosensory rhythms (Chatterjee et al., 2010; Tanoue et al., 2004). It is not yet known whether all the

neurons that are involved in olfactory reception, *viz.*, ORNs, PNs, Kenyon cells *etc.*, are also mediators of olfactory behavior rhythm and whether additional neurons are involved in the circuitry for transferring and transforming the rhythm message. Although chemosensory rhythms seem to be conserved phenomena, the corresponding neural circuit organization is different across organisms.

Adaptive significance of chemosensory clocks

The widespread occurrence of chemosensory rhythm points toward its conservation across the animal kingdom. It has been proposed that the nervous system uses internal states set by mating and nutritional status to assign value to external sensory information from potential food sources, which is then used to guide food choice (Ribeiro and Dickson, 2010). Similarly, peripheral oscillators may influence sensory processing, such that the perceived meaning of a sensory input, in addition to depending on the nature and intensity of the stimulus, relies also on the circadian time when the signal is registered. Olfactory behavior in nocturnal insects, *e.g.*, moths and cockroaches, cycles in phase with wakefulness (Page and Koelling, 2003; Silvegren et al., 2005). Clock-mediated suppression of olfactory response occurs at times of relative inactivity, and thus should help the organism to save energy. Temporal coordination between behavioral response to pheromones in male moths, and biosynthesis and release of pheromones in females, both peaking in the latter part of the scotophase, may underlie the increased mating activity around late night (Silvegren et al., 2005). Intriguingly, physiological response to odorants at the level of peripheral sensory output, however, peaks at periods of quiescence in moths and cockroaches (Merlin et al., 2007; Page and Koelling, 2003; Saifullah and Page, 2009). The inverse phase relation of electrophysiological sensitivity rhythm and behavioral responsiveness rhythm suggests that the circadian system may independently act on the central processing centers in the olfactory pathway.

In *Drosophila*, physiological as well as behavioral response to odorants peak around midnight when individual flies are most inactive. This increased responsiveness may provide survival advantage by allowing escape from potential predators. By improving the gain (signal-to-noise ratio) of the olfactory system at a time when flies are sleeping or minimally active, the insects might reduce their vulnerability by having a stronger alarm system. This may also contribute to opportunistic feeding (Krishnan et al., 1999). It is also possible that at day, when temperature is high, odors are more volatile and they can travel longer distances. So there will be higher background odor (noise) during day. To filter this environmental noise, the fly may be downregulating the olfactory physiology at daytime. At colder nights the odor molecules traverse smaller distances and therefore a heightened sense of smell would be needed to detect the available odorants present at relatively lower concentrations. Electrophysiological response rhythms in the pheromone-responsive trichoid sensilla peaks at ZT21 (Krishnan et al., 2008) – time around which rhythms in male sex drive (Fujii et al., 2007) and the rhythms in mating activity (Sakai and Ishida, 2001) are at their respective peaks in *Drosophila*. Cutting off the antenna significantly compromises the rhythms in sex drive (Fujii et al., 2007). So it seems also possible that heightened olfactory response at night is needed for temporal gating of courtship. A number of GRNs in male's foreleg expressing Gr68a and Gr32a have been shown to be involved in courtship behavior (Bray and Amrein, 2003; Miyamoto and Amrein, 2008). Rhythmic neuronal activity of these neurons may contribute to male sex drive rhythm (Fig. 24).

My results indicate that a fly is most sensitive to tastants in the morning. This may allow the organism to temporally couple increased morning activity with food-detection machinery that works better at dawn, thereby leading to increased feeding. Taste sensitivity peaks around the same time of day as food consumption (Xu et al., 2008). Gustatory stimuli, along with olfactory cues, are major external signals, whereas feeding status and metabolic needs are key internal signals that regulate feeding (Melcher et al., 2007). The peak in feeding rhythms coincides with the early morning peak in gustatory

response rhythms suggesting a functional connection between feeding and the sensory input for feeding (Chatterjee et al., 2010). Rhythmic GRN activity also seemed to correlate with food intake in moths (Simmonds et al., 1991). Circadian rhythms in feeding-motivation provide a unique strategy to optimize food consumption. Thus, plasticity in gustatory physiology and behavior possibly provide the organism advantages in food acquisition (Fig. 24) at particular time of the day. This strategy can, on the other hand, minimize energy expenditure by shutting down the frequency of innate taste-evoked PrER behavior (behavioral noise) when flies are resting.

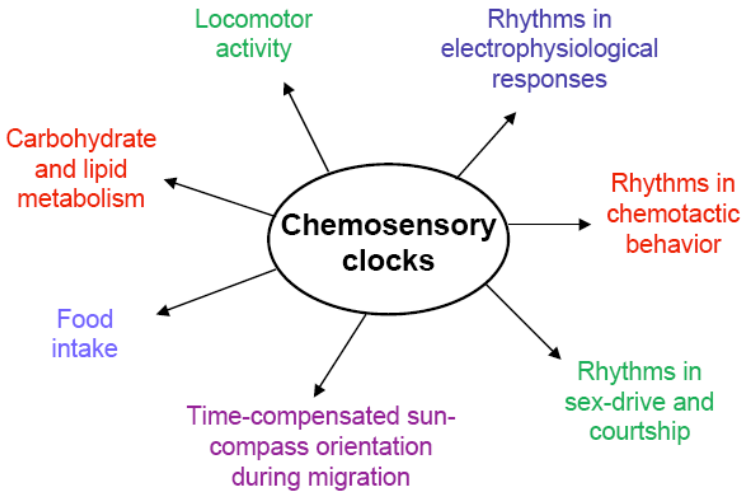

Fig. 24: Outputs of the circadian clocks in chemosensory organs.

Chemosensory cues received by male flies are required for the maintenance of courtship rhythm (Fujii et al., 2007). Local clocks in oenocytes temporally gate the production of male pheromones, and local clocks in GRNs and ORNs may temporally gate pheromone reception and signaling (Chatterjee et al., 2010; Krupp and Levine, 2010). Pheromonal cues underlie social entrainment of *Drosophila* clock (Levine et al., 2002b). A number of gustatory receptors are thought to be involved in pheromone sensing (Miyamoto and Amrein, 2008). The gustatory clock may impose temporal gating on chemosensory input

that may be transmitted to protocerebral neurons controlling locomotor activity and likelihood of mating. Such a two-way system of rhythmic pheromone production and rhythmic pheromone reception could function to define a time window for social interactions. The resulting social experience may in turn influence clocks to control pheromone production and/or chemosensory sensitivity, establishing a physiological feedback loop (Chatterjee et al., 2010; Krupp and Levine, 2010). Since the olfactory and the gustatory systems in fruit flies reach their highest acuity at different times in a day, it seems possible that the there may be phase-difference in the highest effectiveness of volatile and non-volatile pheromones as zeitgebers. Circadian clocks in chemosensory tissues are also involved in modulation of processes that are not directly mediated by altered sensitivity to odorants and tastants (Fig. 24). The antennal clock in the migratory monach butterfly is required for time compensated sun compass orientation (Merlin et al., 2009); the labellar clock in *Drosophila* restricts food-intake (Chatterjee et al., 2010). Sensory systems provide input to the clock. They are also subjected to the output action of clock. This can generate additional cellular feedback loops that increase the stability and robustness of the rhythms. The visual system must face a change in illumination of ~ 10 fold on a daily basis and still remain able to detect contrast. Vertebrate photoreceptors undergo circadian changes in retinomotor movement, outer segment membrane recycling, ion channel physiology, gene expression etc. helping the eyes to anticipate and adapt to sustained changes in illumination (Ko et al., 2009). The retina is more sensitive to light at night. High level of nocturnal melatonin drives dark-adaptive changes in the eye. The clock acting via melatonin regulates dopamine secretion, which peaks at day helping the retina to undergo light-adaptive changes. The Ras-MAPK-CaMKII output cascade in retina controls activity of CNG channels, expression and secretion of L-type VGCCs, and control of synaptic plasticity. These may lead to alteration of rod-cone dominance depending on whether the environment is scotopic or photopic. In flies an endogenous circadian rhythm causes daily oscillations in the volume of photoreceptor cell terminals (Pyza and Gorska-Andrzejak, 2008). The ensuing plasticity in photoreceptor anatomy and synaptic signal computation is sufficient to

control visual behavior. The strength of the optomotor turning response, a visually guided behavior, co-varies with synaptic-terminal volume oscillations of photoreceptor cells (Barth et al., 2010). Circadian changes in lamina volume in addition to mitigating cyclical changes in the visual coding efficiency at behavioral level, also seem to optimally adapt the visual system of flies to the ambient light environment thereby guaranteeing the best vision at dawn and dusk (Barth et al., 2010).

Gustation and olfaction in *Drosophila* has long been used as a model for sensory transduction, information coding and processing, neuronal development, cue-driven behavior, and associative learning. In light of my finding that physiological and behavioral responses to tastants and odorants are under circadian control, it will be important to consider time of day in the design and interpretation of experiments regarding taste and smell.

Molecular underpinnings of chemosensory rhythms

In *Drosophila*, main components of circadian time keeping are common between the central and the peripheral clocks. The operation of the chemosensory clock is dependent on the bHLH-PAS transcription factors CLOCK and CYCLE, and the transcriptional deactivators PER and TIM (Hardin, 2005). Chemosensory response rhythms are abolished in *Drosophila* core clock gene mutants (Chatterjee et al., 2010; Krishnan et al., 1999; Krishnan et al., 2008; Tanoue et al., 2004; Zhou et al., 2005). Expression of *per*, *cry1* and *cry2* mRNA is rhythmic in the antenna of *Spodoptera littoralis* under constant darkness (Merlin et al., 2007). But there seems to be one major mechanistic difference between the central clock and the chemosensory clock. *Drosophila* CRY, a flavin/pterin-containing protein, is a circadian photoreceptor not critical for oscillator function in central clock cells whereas CRY contributes to the operation of the core oscillator in sensory neurons possibly acting as a transcriptional repressor (Collins et al., 2006;

Krishnan et al., 2001). Molecular mechanisms of how the circadian clock regulates chemosensory responses are partly unveiled in *Drosophila*. DNA microarray analysis demonstrated that abundance of some of the *Or* (*Or46b*, *Or35a*) and *Obp* mRNAs are rhythmic (Ceriani et al., 2002; Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ueda et al., 2002).

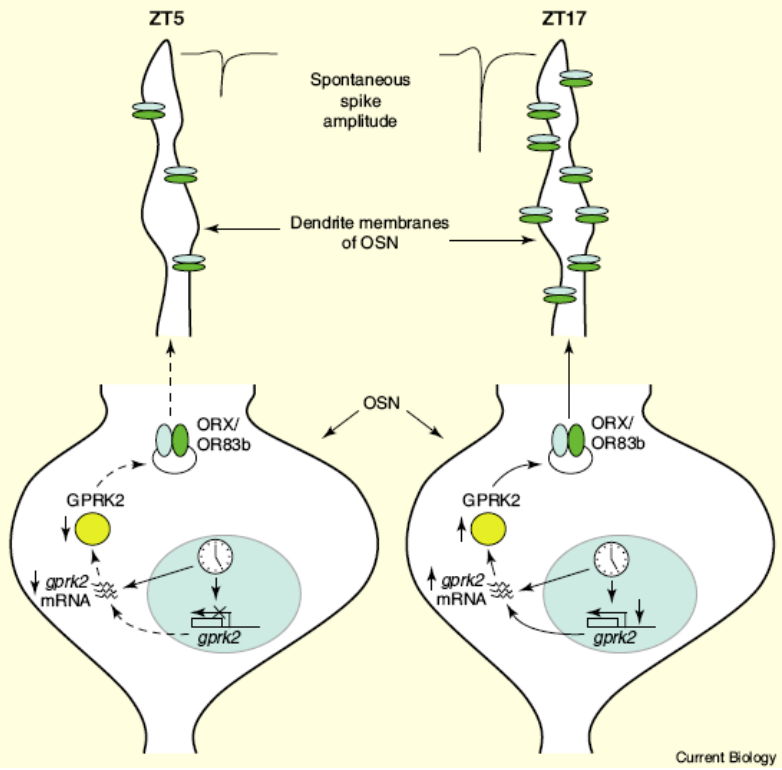

Fig. 25: Oscillations in *Gprk2* RNA level drives rhythm in olfaction. Increased titer of GPRK2 around midnight promotes translocation of OR/OR83b complexes into the dendrites of ORNs. (Emery and Francis, 2008).

In addition, *Gprk2* is required for both olfactory and gustatory response rhythms (Chatterjee et al., 2010; Tanoue et al., 2008). *Gprk2* mRNA and protein levels are high at mid-night and trough at early morning. However, *Gprk2* promoter does not contain any E-box and *Gprk2* mRNA levels, similar to another clock-regulated output gene *takeout* (So et al., 2000), are constitutively low not just in cyc^{01} flies but also in per^{01}

flies (Tanoue et al., 2008). High levels of GPRK2, by enhancing OR localization to dendrites of ORNs, create robust olfactory responses at mid-night (Fig. 25), and low levels of GPRK2 create weak olfactory responses at early morning (Tanoue et al., 2008). The simplest idea would posit that **[A]** GPRK2 phosphorylates the ubiquitous chaperone coreceptor OR83b which in turn modulates the shuttling of ligand-binding ORs. GPRK2 protein contains a kinase-like domain bearing sequence similarity to serine/threonine kinases, tyrosine kinases, protein kinase C, and GPCR kinase. On the other hand, bioinformatic analysis revealed the presence of a many consensus phosphorylation sites on the intracellular loops and N terminus regions of OR83b (Table 3). Ser/Thr phoshphorylation of a 7-TM receptor, *Smoothened*, by *Drosophila* GPRK2 has been recently shown to promote increased receptor activity (Chen et al., 2010). **[B]** GPRK2 may also act in a kinase-independent fashion. Binding of mutated GPRK2 lacking functional kinase domain to *Smoothened*, stabilizes the active conformation of this 7-TM receptor (Chen et al., 2010). In a similar manner, stability of functional OR/OR83b heterodimers deposited on dendritic membranes might be promoted by GPRK2. Whether phosphorylation by GPRK2 is required for olfaction rhythm can be determined by using (i) the kinase-dead $Gprk2^{KM}$ transgenic line, and (ii) overexpression of the GPCR-phosphatase gene Rdg^{C} *in ORNs*. **[C]** Additionally, GPRK2 may influence cytoskeletal reorganization or influence the properties of OR-interacting proteins to exert its effects. GPRK2-facilitated recruitment of arrestin to 7-TM receptors in mammalian cells allows anterograde ciliary transport of the receptors (Chen et al., 2004; Kovacs et al., 2008) lending support to the idea that fly GPRK2 may promote the shuttling of 7-TM ORs into the ciliated outer dendritic segments of ORNs.

Table 3: Possible phosphorylation sites on OR83b. The PHOSIDA motif matcher program was used to search for matching kinase motifs on OR83b sequence. Hits identified by the Support Vector Machine Predictor (SVM) are underlined – these are predicted phosphorylated serines (S) and threonines (T) recognized by sequence (surrounding +/- six residues) similarities with experimentally obtained human phosphosites. From left to right, the four columns show candidate phosphorylated residue in OR83b, kinases that can mediate phosphorylation of the given amino acid residue, consensus phosphorylation sequence for the kinase, topological location of the amino acid residue. Y=tyrosine, IC*x* =intracellular loop number *x*.

Amino acid	Kinase	Consesus phosphorylation sequence	Location
S4	GSK3	(S-X-X-X-S)	N terminus
S8	CK1	(S/T-X-X-X-S)	N terminus
Y10	ALK	(Y-X-X-I/L/V/M)	N terminus
T11	CK1	(S-X-X-S/T)	N terminus
Y26	ALK	(Y-X-X-I/L/V/M)	N terminus
Y102	ALK	(Y-X-X-I/L/V/M)	IC1
Y124	ALK	(Y-X-X-I/L/V/M)	IC1
S126	CAMK2	(R-X-X-S/T)	IC1
S247	NEK6	(L-X-X-S/T)	IC2
T250	CK1	(S-X-X-S/T)	IC2
S255	CAMK2	(R-X-X-S/T)	IC2
S261	NEK6	(L-X-X-S/T)	IC2
S263	CAMK2	(R-X-X-S/T)	IC2
	PKD	(L/V/I-X-R/K-X-X-S/T)	
	CHK1/2	(L-X-R-X-X-S/T)	
	CHK1	(M/I/L/V-X-R/K-X-X-S/T)	
S266	CK1	(S-X-X-S/T)	IC2
	CK2	(S/T-X-X-E)	
S268	?		IC2
S285	GSK3	(S-X-X-X-S)	IC2
S289	CK1	(S/T-X-X-X-S)	IC2
S290	?		IC2
S302	GSK3	(S-X-X-X-S)	IC2
	CAMK2	(R-X-X-S/T)	
S306	CK1	(S/T-X-X-X-S)	IC2
S421	CK2	(S/T-X-X-E)	IC3

Regarding the GRN clock in flies, GPRK2 seems to function like a traditional mammalian GPRK and the fly GPRK1 (Lee et al., 2004b), which are inhibitory components of sensory signaling cascade. The high levels of GPRK2 around midnight (Fig. 26) possibly suppress gustatory response by inactivating GRs via phosphorylation (Chatterjee et al., 2010). By means of a simple epistasis experiment I could provide genetic proof of an interaction between GRs and GPRK2. Recently antibodies against certain GRs (GR93a, GR33a) have become available (Moon et al., 2009) – permitting future immunohistochemical experiments to investigate the potential effect of GPRK2 on GR expression level/trafficking. It will be interesting to know whether GPRK2 is expressed cyclically in all classes of GRNs; and whether GPRK2 and GPRK1 has overlapping functions in GRNs. Taste-modalities which are not dependent on GRs, *e.g.*, *pickpocket* channel-mediated salt taste sensation, was also found to be rhythmic; indicating that the GRN clock does act on non-GR targets. I cannot exclude other possible mechanisms for clock-dependent taste modulation; the clock may in parallel control rhythmic oscillation of other molecules in the GRNs, such as *takeout*, a clock-controlled molecule expressed in the proboscis known to modulate GRN firing rate (Meunier et al., 2007). GPRK2 may be controlling the activity of other components of gustatory signal transduction machinery (Fig. 26), *e.g.* potassium channels (Ruiz-Gomez et al., 2007). Alternatively, non-neuronal accessory cells in gustatory sensilla may undergo GRN-clock driven oscillations to bring about perireceptor changes in the taste sensilla (Fig. 26).

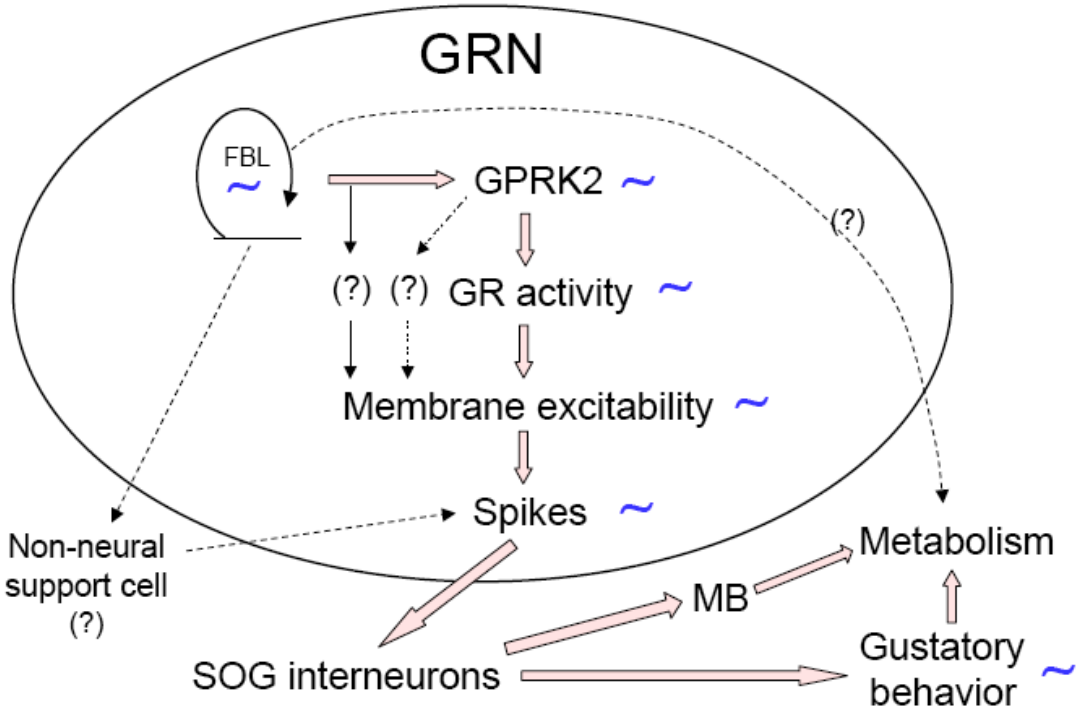

Fig. 26: Model of the clock output mechanism operating within GRNs.

Interestingly, a circadian rhythm in electroreception is also mediated by trafficking of voltage gated sodium channels into the membranes of electrocytes (Markham et al., 2009). Membrane deposition of sodium channels in electrocytes is controlled by a protein kinase (PKA) (Markham et al., 2009; Fortune and Chacron, 2009), as is the case in *Drosophila* ORNs. Current data supports a model in which cyclical changes in *Gprk2* levels drive rhythms in odorant receptor localization to dendrites that ultimately mediates rhythms in olfactory responses (Fig. 25). Surprisingly, high levels of GPRK2 give rise to weak gustatory responses, and low levels of GPRK2 cause strong gustatory responses, as measured by proboscis extension reflex behavior (Chatterjee et al., 2010). The phase of gustatory response rhythms is opposite to that of olfactory response rhythms. GPRK2 exhibits different isoforms in antenna and proboscis. Mammalian *Gprk2* homologue, *Gprk4*, exhibits 4 variant splicing forms having different functions

(Villar et al., 2009). It thus seems plausible that GPRK2 isoforms have divergent functions in different tissues.

Several future directions emerge from this discussion. *Gprk2* is one output gene known to regulate olfactory and gustatory responses. Understanding how *Gprk2* mRNA level is modulated will shed light on transcription regulatory networks that alter the expression of output genes. Characterizing the role of GPRK2 will provide important insights into the mechanisms altering the sensitivity of sensory neurons. Also, understanding the mechanisms by which GPRK2 influence 7TM receptor accumulation in dendrites will provide meaningful information for studies on protein trafficking, which is expected to ameliorate therapies for diseases like retinal dystrophy.

It is not clear whether GPRK2 underlies chemosensory rhythms in all insects. Clearly other components of chemosensory signal transduction pathway have been shown to undergo circadian modulations. Rhythmic RNA abundance of an odorant-degrading esterase enzyme was observed in the moth, *Spodoptera littoralis* (Merlin et al., 2007), but there was no definitive correlation between the phase of EAG rhythm and that of the enzyme expression. Coincidence between oscillating levels of the hormone leptin and sweet recognition threshold rhythms are reported in humans (Nakamura et al., 2008). *Drosophila* chemosensory rhythms undoubtedly provide the most complete picture of molecular mechanisms, and potential clock-controlled candidate molecules in other species need to be rigorously tested using molecular genetic and/or pharmacological assays.

Neurobiological correlates of chemosensory rhythms

As shown in Table 2, chemosensory rhythm in rodents is manifested as rhythmic changes in the frequency of action potentials of the association neurons, called mitral

cells, present in the olfactory bulb (Granados-Fuentes et al., 2004b). The behavioral outcomes of this physiological rhythm are currently unknown. The circadian connection from molecules to neural activity is clearer in insects. The phases of physiological and behavioral chemosensory rhythms in some moths and cockroaches are not consistent. This anomaly may arise because most of the physiological studies concerning olfaction rhythm in insects (Krishnan et al., 1999; Merlin et al., 2007; Page and Koelling, 2003) relied on EAG amplitude, a parameter that may not truly reflect changes in ORN activity, the biologically meaningful information transferred to second order neurons. Being a field potential, EAG sums up currents associated with receptor potential and action potential of ORNs, and the transepithelial potential that is generated as a result of uneven distribution of ions across the olfactory epithelium (Saifullah and Page, 2009). Documented instances exist where change in EAG amplitude is not correlated with a change in ORN frequency (Zhukovskaya and Kapitsky, 2006).

Perireceptor signaling events may underlie cyclical changes in chemosensory responses. The mRNA level of an odor-degrading enzyme is highest in the antenna a few hours prior to the peak in pheromone-evoked behavioral response rhythm in *Spodoptera* (Merlin et al., 2007). It is hypothesized that efficient OR deactivation at a particular time of the day should augment the signal resolution capacity (Merlin et al., 2007). Mathematical modeling on the sigmoid odor-response curve of tsetse flies at three different times of day revealed that a change in threshold sensitivity underlies the rhythm in ORN spike frequency, while neither spontaneous firing rate nor maximal firing rate is periodically modulated (Van der Goes van Naters et al., 1998). Since the spontaneous firing frequency that depends on the cell's resting membrane potential, and the steepness of the odor-response curve that depends on the multiformity of ORs, did not undergo cycling, the authors argued for diurnal invariance of intrinsic neuronal properties and opined that perireceptor events, *e.g.*, change in OBP may explain their findings (Van der Goes van Naters et al., 1998). Perireceptor changes may be induced by neurohormones,

e.g., tyramine and octopamine, whose abundance cycle, and which can modulate chemosensory physiology (Kutsukake et al., 2000; Pophof, 2002; Lehman, 1990).

Circadian rhythms were documented in amplitude of spikes from a multiple ORNs and GRNs belonging to different morphological classes of chemosensory sensilla of *Drosophila* (Krishnan et al., 2008). It would be interesting to determine whether spontaneous impulse activity is rhythmic in GRNs since such rhythms would demonstrate that the circadian oscillator does not act only on the tastant-evoked signaling cascade. With regard to ORN spikes, firing frequency was not rhythmic, in contrast to the situation with GRN spikes (Chatterjee et al., 2010; Krishnan et al., 2008). Technical difficulties precluded the authors from verifying whether the rhythm in sponateous spike amplitude of ORNs was reflected in the rhythm of odor-evoked spike amplitude, making any effort to extract a correlation between EAG and ORN response difficult. However, phase-correlation between the rhythms in spike amplitude, EAG and olfactory behavior suggests that spike amplitude may encode biologically relevant information that is somehow communicated between neurons in a given dedicated circuit to elicit behavioral modulation. Changes in GRN spike amplitude also successfully tracked daily changes in gustatory behavioral responses (Chatterjee and Hardin, 2010). The probability of PrER responses changed as a function of time of day in parallel to the phase of neurophysiological rhythms of GRNs, particularly the amplitude and frequency of GRN impulses (Fig. 27). This result suggests that amplitude, in addition to frequency of spikes, may code information translatable into behavior. Ca^{2+} imaging experiments/intracellular recording of action potentials from PNs should reveal whether change in spike height exert any influence on the strength of synaptic communication in the olfactory bulb. GPRK2-mediated dendritic translocation of channels should result in an increased receptor potential that may be reflected as heightened EAG amplitude (Tanoue et al., 2008). However, it is not clear how an enhanced receptor potential can lead to increased spike amplitude and invariant spike frequency. Precedence exists for a similar mechanism of amplitude control: increased membrane insertion of voltage gated

sodium channels has been shown to bring about an increase in the amplitude of action potentials in electric fish, ultimately resulting in a stronger display of signal (Markham et al., 2009). However, it is probable that change in spike height is not a result of change in the amplitude of action potentials in chemosensory neurons of fly. Structural changes such as widened dendritic arbor, increase in the diameter of dendrites, should heighten ORN spike amplitude (Hansson et al., 1994). Displacement of the spike initiation site toward the dendrite can also result in an increase of spike height (Guillet and Bernard, 1972). When OR conductance is elevated at night, a larger receptor potential ensues, so that the generator potential may be reached at a spatial location prior to the usual spike initiation zone of the receptor cell. This change will not affect the amplitude of true action potentials but an extracellular electrode will pick up larger single unit signals.

I demonstrated that clocks within the GRNs are both necessary and sufficient for PrER rhythms. Silencing and hyperexcitation of GRNs gave rise to arrhythmic phenotypes with constant low and constant high levels of PrER responses, respectively (Fig. 27). This reinforces the idea that the GRN clock changes taste-driven behavior by altering membrane excitability on a daily basis. The flow of rhythm-information from primary sensory neurons to downstream cells is required to generate rhythmic behavior, although clocks within these relay neurons, if present, are not necessary for PrER rhythms. It is also possible that the circuit for generating PrER behavior and the circuit for executing PrER rhythms are not completely identical. Both these circuits do share the GRNs, but neuroanatomical structures that are not required for PrER behavior *per se*, such as MB neurons (Malun et al., 2002), may form a functional part of the neural circuit for PrER rhythms.

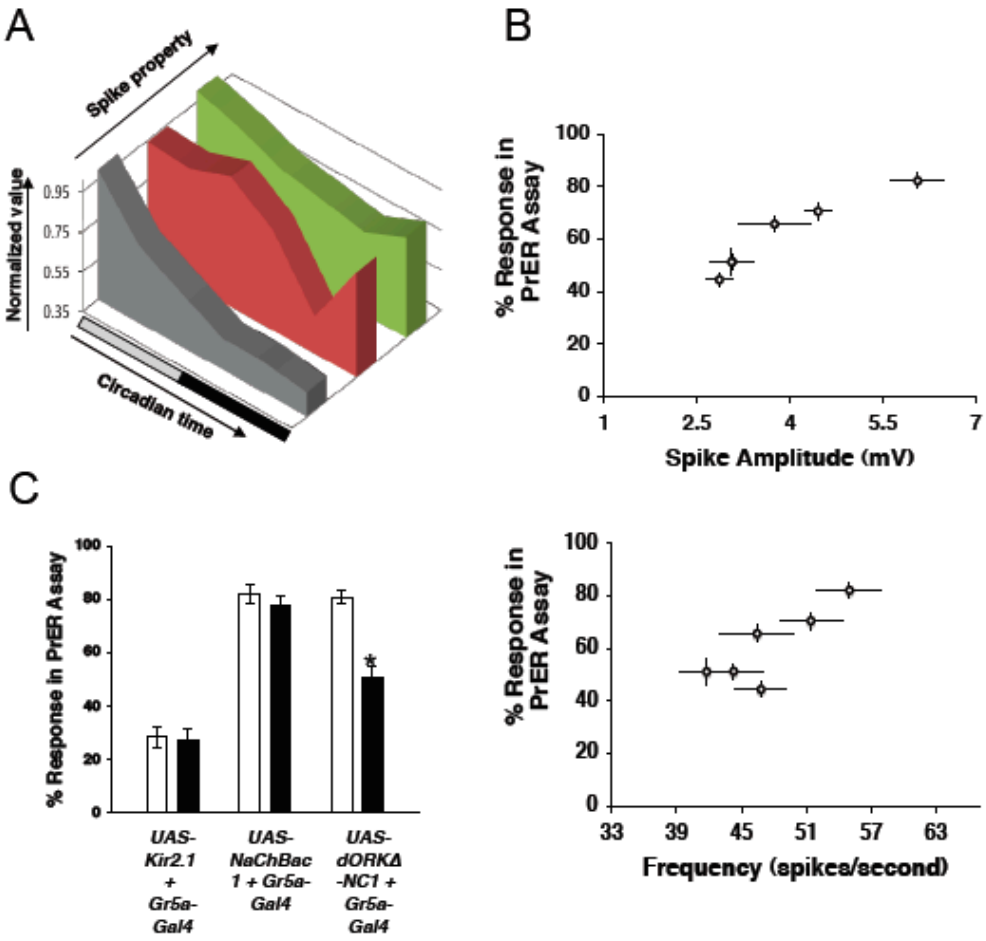

Fig. 27: Circadian regulation of gustatory physiology leads to PrER rhythm. A. The amplitude (gray), duration (red) and frequency (green) of S spikes exhibit synchronous circadian rhythms under DD. B. PrER response levels are positively correlated with the amplitude (upper panel) and frequency (lower panel) of GRN spikes. Spike amplitude and PrER response levels are strongly correlated (r^2=0.92) and highly significant ($p < 0.003$). C. Disruption of membrane excitability of GRNs abolishes PrER rhythms. PrER responses of *Gr5a-Gal4* flies carrying *UAS-Kir2.1*, *UAS-NaChBac1* or *UAS-dORK-ΔNC1* at ZT1 and ZT17. There are no significant ($p > 0.40$) differences in PrER responses at ZT17 compared with ZT1 in *Gr5a-Gal4* flies carrying either *UAS-Kir2.1* or *UAS-NaChBac1*. The differences in mean PrER responses at ZT1 and ZT17 are significant ($p < 0.001$) in *Gr5a-Gal4* flies carrying *UAS-dORK-ΔNC1*. White and black columns indicate mean PrER responses to 100 mM sucrose at respectively ZT1 and ZT17. (Chatterjee and Hardin, 2010).

A change in transepithelial potential may cause a change in spike height. V-ATPase powers the K^+/nH^+ antiporter to generate the transepthelial potential across an epithelium (Wieczorek et al., 2009). Accesory cells in insect chemosensory sensilla express the V-ATPase gene (Wieczorek et al., 2009), accessory cells also express core clock proteins (Chatterjee et al., 2010; Schuckel et al., 2007) and V-ATPase expression is rhythmic in flies (Bebas et al., 2002). But the clock in support cells is not required for EAG rhythm, and the clock in ORNs is sufficient for this rhythm (Tanoue et al., 2008), indicating that if accessory cells indeed contain a clock that modulate circadian olfactory response, that clock would be a slave oscillator under control of the ORN pacemaker.

Drosophila is an excellent model to probe the molecular bases of chemosensory rhythms whereas other insects seem to be better suited for cell-physiological studies of chemosensory rhythm, because larger insects offer technical ease for electrophysiological and pharmacological assays. Pheromone-evoked frequency of hawkmoth ORNs gradually decrease during the photophase reaching its trough around ZT 8-11 when the response becomes weaker and less phasic, signifying a compromise in the temporal resolution of the signal (Flecke et al., 2006; Flecke and Stengl, 2009). Octopamine (OA) and its precursor tyramine can occlude the midday decrease in ORN firing, but they also alter the receptor potential, which is typically not subjected to diurnal modulation in hawkmoth (Flecke and Stengl, 2009). OA titer in moth hemolyph is rhythmic (Lehman, 1990), and the moth olfactory hairs express the OA receptor (von Nickisch-Rosenegk et al., 1996). The current data indicate that midday suppression in olfactory responses may in part be mediated by biogenic amines, but the dawn enhancement of responses is controlled by an independent mechanism because the use of OA antagonist could not bring down the level of dawn responses significantly. While cGMP analogs could simulate the daytime dependent action of OA on ORNs, cAMP analogs failed to do so (Flecke et al., 2006; Flecke et al., 2010). OA receptors are GPCRs that, upon activation, increase intracellular cAMP concentration and also

independently elevate intracellular Ca^{2+} (Bischof and Enan, 2004). Accumulation of cGMP levels due to calcium elevation may lead to closure of medium-conductance delayed rectifier potassium channels (Dolzer et al., 2008). The resultant increase in membrane resistance and lengthening of repolarization phase may lead to a reduction in ORN frequency. A preliminary picture of second messenger action in diurnal control of *Manduca* ORN firing is emerging; identifying the OA-independent mechanism of nocturnal sensitivity and characterization of the components of OA signal transduction involved in this rhythm await.

Regulation of feeding and metabolism by GRN clock

Prandial behavior in flies and mammals is regulated by two major intermingled controls: the homeostatic and the hedonic systems. Homeostatic regulation of feeding ensures that circulating nutrient levels are sensed and maintained; lowering of which directly regulates feeding activity. The brain has an intrinsic circuitry that regulates the levels of various nutrients in the circulating fluid and in the body stores. Hedonic mechanisms make certain palatable foods intrinsically rewarding beyond their metabolic content. My results in flies strongly suggest that hedonic rewards are NOT the cause of increased appetite in flies having a compromised GRN clock (Chatterjee et al., 2010). The precise mechanism of energy homeostasis via regulation of feeding activity remains largely unknown in flies. In order to modulate their prandial behavior, adult flies may estimate their metabolic state by measuring (a) the levels of stored glycogen and/or triglyceride, (b) the rate of catabolic reactions such as glycogenolysis and/or lipolysis, (c) and the levels of amino acids and/or sugar and/or free fatty acids in hemolymph. Neurosecretory cells located in the brain and ring gland of *Drosophila* secrete insulin-like peptides (DILPs) and adipokinetic hormone (AKH, the insect glucagon) into the hemolymph. Their concerted action ensures sugar (fructose and trehalose in hemolymph) homeostasis. Targeted killing of insulin-producing cells leads to diabetic flies and,

conversely, having the AKH-producing cells ablated leads to low levels of circulating sugars (hypoglycemia) (Leopold and Perrimon, 2007). Excess sugar is stored as glycogen in muscles and fat body. On stimulation by AKH, the fat-body glycogen phosphorylase is activated to initiate glycogenolysis, and trehalose is released into the haemolymph (Leopold and Perrimon, 2007). The fat body acting like the mammalian adipocytes, also stores lipids. In food-deprived flies, the released fat from fat body is captured by the oenocytes (the fly 'hepatocytes') for energy production (Leopold and Perrimon, 2007). Information on energy shortage is relayed by lipolytic hormones, which activate a cyclic-AMP-dependent protein kinase cascade leading to the activation of specific lipases (*e.g.*, brummer) and lipid mobilization (Leopold and Perrimon, 2007).

Changing the nutrient compositions in the ingested food leads to alterations in feeding, fat metabolism and life span – *Drosophila melanogaster* makes adaptive food choices according to current nutritional requirements, sex, and mating status (Ribeiro and Dickson, 2010). It is possible that the clock is also used by the organism to select a certain kind of food (*e.g.* carbohydrates) at a certain time of day (*e.g.* morning, when calories need to quickly extracted from food in order to jump-start daily activity). If this is true, giving the fly a food at a time when metabolism of the food is suboptimal, may lead to metabolic problems, *e.g.*, hyperglycemia, obesity etc.

Clocks in *takeout*-expressing metabolic tissues were shown to restrict feeding, and neuronal clocks were found to promote feeding (Xu et al., 2008). In addition to fat bodies, antennal olfactory receptor neurons (ORNs) and labellar GRNs also express *takeout* (Dauwalder et al., 2002; Meunier et al., 2007). I disrupted the molecular clockwork within sugar-responsive GRNs and found those flies to be hyperphagic. Therefore, in contrast to other neuronal clocks, the gustatory clock helps the animal to restrict daily food consumption. Surprisingly, clocks in bitter-responsive neurons did not seem to influence feeding. I also did not observe any uniform correlation between PrER behavior and food intake, suggesting that the oscillators inside GRNs influence food

consumption possibly by altering the feedback of GRNs on tissues controlling the metabolic status of the fly. That altered sensitivity to tastants, brought about by elimination of the clock in GRNs is not the cause of my observed feeding phenotype, is supported by the fact that in spite of having a repressed and arrhythmic taste-response, *Gprk2* mutant flies do not show abnormal feeding behavior.

Neuronal circuits have been demonstrated to affect feeding in *Drosophila*. In adult flies, inhibiting hugin-expressing interneurons in the SOG (Fig. 26) causes rapid meal initiation and crop bloating, and ablating NPF neurons affects larval feeding, silencing *c673a-Gal4* leads to excess food consumption and fat storage in adults (Al-Anzi et al., 2009). *Gr28b.c* is coexpressed with *Gr5a*, and is present in pars intercerbralis (PI) of dorsal brain (Thorne and Amrein, 2008). Ablation of the DILP-secreting median-neurosecretory cells (m-NSCs) of PI triggers a starvation response (Ikeya et al., 2002), a behavior that I encountered on eliminating *Gr5a*-clock. It has been hypothesized that *Gr28b.c* defines higher-order neurons in the taste/feeding circuit and connects gustatory neurons with the endocrine signaling pathway controlling food intake (Thorne and Amrein, 2008). One possibility is that clocks in a subset of *Gr5a* neurons, which are connected with the m-NSCs, regulate the activity/release/synthesis of DILP. The GRN clock may also be involved in the secretion of neuropeptides from SOG and/or MB (Fig. 26), which can negatively modulate feeding behavior – for example sulfakinins and allatostatins suppresses feeding by inhibiting the contraction of insect visceral muscles, leucokinin (homolog of mammalian tachykinin) decreases meal size by stimulating foregut stretch signals that indicate satiety, and sNPF decreases appetite in adults (Al-Anzi et al., 2010; Nassel and Homberg, 2006). Because detection of neuropeptides in the small brain of a fly is technically challenging, as an alternative approach qRT-PCR may be used to verify (a) if any of these known neuropeptides that inhibit food intake are actually expressed in the GRNs, and (b) whether the clock genes have any role in controlling the expression levels of these peptides. A small number of candidate genes have been identified which are expressed in the nervous system, are clock regulated

(Table 4), and are also associated with processes that may potentially alter feeding. Neuronal clocks may regulate feeding by gating the activity/expression of these candidate molecules. A second point is notable – currently it is not clear whether my transgenic manipulation in GRNs led to hyperphagia by (a) eliminating potential non-circadian roles of *Clk* and *cyc* genes or (b) abolishing the bona fide circadian clock machinery. If feeding behavior is altered in flies expressing *per* and/or *tim* RNAi in *Gr5a* cells, this would argue for alternative (a).

Table 4: Clock-controlled candidate molecules that may exert neural control on feeding and metabolism.

Gene symbol	**Codes for**	**Biological processes involved**	**Peak under LD**	**Peak under DD**	**Level in *Clk^{Jrk}***
ple (pale)	Tyrosine-3-monooxygenase	light-induced masking, entrainment, obesity, (dopamine signaling)	2.4	1.6-2	high
CG1147 NPFR-1	Neuropeptide F receptor	insulin signaling, motivated feeding, (LN_ds and sLN_vs express its ligand)	?	1	low
slob	Slowpoke binding protein	locomotion, feeding, courtship, flight	15-16	8-13	medium
Eaat1	Excitatory amino acid transporter	glutamate signaling and synaptic transmission, (activated by feeding)	19	6	medium
5-HT1A	Serotonin receptor 1A	larval light response, sleep, (expressed in central clock neurons)	15	?	?
5-HT2	Serotonin receptor 2	anticipatory locomotor behavior, aggression	18	?	?

Flies with a compromised gustatory clock also showed gross metabolic defects in the form of significantly increased levels of stored fat and carbohydrate. Whether the hyperphagic phenotype leads to dyslipidemia, or metabolic dysregulation leads to both energy storage and feeding phenotypes - is currently unresolved. In which tissue does the fly store this extra load of fats and carbohydrates? I found that a part of the excess energy intake in my mutant flies was allocated to increased egg production in females. But these flies were no better at surviving the stressful challenge of starvation, suggesting that in spite of having higher lipid and carbohydrate levels in their bodies they were unable to use that stored food when needed. Deep sequencing techniques may be applied to compare the expression levels of genes involved in lipid metabolism, particularly those (*e.g.*, CG8093, a triacyl glycerol lipase) whose expression (Ceriani et al., 2002) is clock-regulated. Investigation of longevity and rate of normal metabolic activity in these flies may shed light on the fate of the excess food which they consumed.

Mammalian PER2 directly and specifically represses PPARγ, a nuclear receptor critical in adipogenesis and insulin sensitivity (Grimaldi et al., 2010). The circadian clock in flies may alter lipid accumulation in a similar manner. A clock-controlled protein CREB is involved in energy homeostasis of insects and mammals - blocking dCREB2 activity in the fat body increases food intake and lipid levels in flies (Iijima et al., 2009); clocks in GRNs may mediate metabolic balance by regulating CREB. Neuronal regulation of fat utilization has been documented before in flies – silencing of c673a-Gal4 and fru-Gal4 neurons causes obesity by changing the mRNA expression of key metabolic genes, *e.g.*, acetyl CoA-carboxylase (regulatory enzyme for de novo fatty acid biosynthesis), cytochrome P450 (involved in fat metabolism by oenocytes) (Al-Anzi et al., 2009). Microarray analysis has revealed a number of metabolic genes, which are under control of the clock in flies, including transaldolase, isocitrate dehydrogenase, long-chain fatty acid coA ligase etc. (Ueda et al., 2002).

GRNs are predicted to convey output to mushroom bodies (MB) and the corpora cardiaca (CC) via interneurons in the suboesophageal ganglion (SOG) (Melcher and Pankratz, 2005). Alteration of clock function in the olfactory receptor neurons, which ultimately send information to the MB and the lateral horn of protocerebrum, also influences feeding in flies (Xu et al., 2008). It is possible (Fig. 26) that clock-mediated changes in outputs from gustatory interneurons control the levels of food-intake regulating factors such as short neuropeptide F in MB (Lee et al., 2004a), and/or secretion of endocrine factors such as AKH from CC (Kim and Rulifson, 2004). The AKH receptor is actually expressed in the subset of GRNs where I abolished clock function, and AKHR mutant flies are obese (Bharucha et al., 2008). The fact that a small number of peripheral neurons known to be concerned with only taste reception may also control locomotor activity, feeding and metabolism of an organism indicate that homeostatic regulation of the 'milieu intérieur' is multilayered and complex.

Defects in the circadian clock cause metabolic disorders such as type II diabetes (Marcheva et al., 2010). As the circadian clock controls energy metabolism, running the clock itself is subjected to the metabolic state of the organism. Circadian gene expression rhythms in peripheral tissues, feeding rhythm, sleep-wake cycles and locomotor rhythms controlled by the brain are affected in mammalian models of obesity and diabetes (Kohsaka et al., 2007). Oxidative stress, which is often associated with aging, acting via FOXO signaling dampens the circadian clock in flies (Zheng et al., 2007). AKT and TOR-S6K pathways impact the brain circadian clock that drives locomotor rhythms. Increased activity of AKT or TOR lengthens circadian period, whereas reduced AKT signaling shortens it. Effects of TOR-S6K appear to be mediated by SGG/GSK3β, a known kinase involved in regulation of the core clock protein TIMELESS (Zheng and Sehgal, 2010). It has been proposed that a change in the timing of food availability alters metabolic cycles; but a concomitant change in behavioral cycles is needed to fine tune foraging time (Giebultowicz and Kapahi, 2010). In cases of food scarcity such as diapause in insects or torpor in mammals, TOR signaling may

sense the nutrient status and either alter the pace of the circadian clock or temporarily stop it altogether to achieve synchrony in behavior and physiology (Giebultowicz and Kapahi, 2010).

Function of odors in clock-entrainment

Emerging evidence supports the role of chemosensory system in non-photic and photic entrainment of the circadian clock. By mediating social interaction in animals, odorants may act as zeitgebers. The mammalian olfactory bulb serves as a relay center for sending olfactory information into higher brain centers. Ablation of the olfactory bulb (OBX) in the diurnal mammal degu (*Octodon degus*) blocks socially facilitated entrainment (Goel and Lee, 1997).

Surprisingly, photic entrainment is also delayed in OBX degus (Goel et al., 1998) supporting the idea of functional connectivity between the olfactory system and the central clock in the SCN of mammals. Odor enhances the amplitude of light-induced phase shift by about 50% (Amir et al., 1999). This behavioral change is mirrored by the increased number of Fos-immunoreactive cells in the SCN of degus subjected to a combined light and odor pulse (Amir et al., 1999). These experiments confirm that odors can modulate entrainment rate by reinforcing the action of photic zeitgeber.

Olfactory behavioral rhythm in males of the moth *Spodoptera littoralis* can be entrained by the odor from pheromone gland extract of conspecific females (Silvegren et al., 2005). The effect of odor is phase dependent – daily odor pulse around the middle of the subjective night is ineffective in imparting rhythmicity (Silvegren et al., 2005). It needs to be ascertained whether the ineffectiveness of odor pulse to reset the clock at subjective midnight is caused by the clock-controlled suppression of olfactory EAG responses to pheromones (Merlin et al., 2007) around this time. This provides an

interesting example in which the same tissue, *i.e.*, antenna that contains the oscillator (Merlin et al., 2007), also mediates local input for a particular rhythm.

Drosophila emits and receives olfactory cues that can reset the circadian clock in a social context (Levine et al., 2002). Fly odor alone effectively synchronizes the phase of circadian locomotor activity rhythm in wild-type flies maintained under constant darkness but has no effect on the phase coherence of rhythm in the olfactory mutant $para^{sbl}$ flies (Levine et al., 2002). Flies lacking oscillators in peripheral tissues fail to show sensitivity to the potentially resetting effect of fly odor on their activity rhythm (Levine et al., 2002). The social effect on circadian timing in *Drosophila* seems to, in part rely on the rhythmic emission of short-lasting fly-derived volatile cues, and also on the peripheral clock-controlled temporal modulation of olfactory reception (Levine et al., 2002). It would be interesting to know the identity of rhythmically transmitted pheromones, identify their cognate receptors, and verify if olfactory response to these cues is itself temporally gated.

Epilogue

A major objective of my research was to determine how temporal information from the circadian clock impinges on the nervous system to bring about physiological and behavioral plasticity in chemosensory responses. An organism must change its behaviors in an adaptive fashion based on its experience (*e.g.*, associative learning), metabolic status (*e.g.*, hunger), social interaction (*e.g.*, mating) etc. The phenomenon of clock-controlled plasticity in *Drosophila* chemosensory system function serves as an excellent model for neurogenetic investigations of how genetically pre-programmed innate behaviors could be internally modified. I challenged this overt rhythm with transgenic manipulations, and used electrophysiological, behavioral and biochemical tools of analysis to unravel the mechanistic links between the core oscillator and the

chemosensory responses.

I found that the circadian clock extensively remodeled action potentials of chemosensory neurons on a daily basis. ORN spikes elicited rhythm in amplitude, while GRN spikes showed rhythm in amplitude, frequency and duration of spikes (Chapter III). Although the amplitude of spikes is not typically regarded as encoder of biologically meaningful information, I found that changes in the amplitude of extracellularly recorded spikes were highly correlated with alterations in chemosensory cue-driven PrER behavior, which was also modulated by the circadian clock (Chapter IV). Oscillating protein levels of the kinase GPRK2 mediated the rhythms in smell and taste probably via its action on chemosensory receptor molecules (Chapter III and Chapter IV). Pacemakers within the GRNs not only regulated taste sensitivity, but also exerted significant control over food intake and metabolism. Elimination of the GRN clock triggered a starvation-response-like behavior rendering flies hyperactive, hyperphagic and obese (Chapter IV). GRN oscillators may influence feeding by providing feedbacks on the neuroendocrine signaling system. It seems possible that the abnormal feeding habit of human beings may result from aberrant running of the local clocks within chemosensory tissues.

I demonstrated that a clock-controlled molecule, GPRK2, which is known to act on GPCRs in worms, insects and mammals, also modulate chemosensory responses in *Drosophila*. This result opened the controversial question – whether insect chemosensory receptors act like GPCR proteins? I provided additional evidence (Chapter II) for the involvement of a G protein subtype, G_{o}*o* in chemosensory signal transduction. G_{o}*o* is required in the ORNs for optimal level of odor-evoked physiological responses. My results lend credence to the idea that second messengers generated via G protein-mediated signaling cascades can regulate the channel function of ORs.

Further work is needed to understand how does the clock regulate *gprk2* expression? How does GPRK2 serve opposing roles in antenna and proboscis? How is the rhythm in

spike amplitude translated into a rhythm in chemotactic behavior? What is the architecture of the neural circuit that dictates rhythmic chemosensory behavior? How does the oscillator in the GRN influence feeding? Does the chemosensory clock mediate social entrainment? Do rhythms in chemosensory cue-driven behaviors offer adaptive advantages for the fruit fly? Results obtained from these studies should stimulate further interdisciplinary research in the fields of chronobiology and sensory biology. The future is going to be an exciting and productive time as far as research is concerned.

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