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The *Drosophila* Circadian Pacemaker Circuit: Pas de Deux or Tarantella?

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Abstract

Molecular genetic analysis of the fruit fly *Drosophila melanogaster* has revolutionized our understanding of the transcription/translation loop mechanisms underlying the circadian molecular oscillator. More recently, *Drosophila* has been used to understand how different neuronal groups within the circadian pacemaker circuit interact to regulate the overall behavior of the fly in response to daily cyclic environmental cues as well as seasonal changes. Our present understanding of circadian timekeeping at the molecular and circuit level is discussed with a critical evaluation of the strengths and weaknesses of present models. Two models for circadian neural circuits are compared: one that posits that two anatomically distinct oscillators control the synchronization to the two major daily morning and evening transitions, *versus* a distributed network model that posits that many cell-autonomous oscillators are coordinated in a complex fashion and respond *via* plastic mechanisms to changes in environmental cues.

Keywords

oscillator; entrainment; photoperiod; morning-evening oscillator model

INTRODUCTION

All living organisms are faced with challenges associated with daily environmental changes. Furthermore, most organisms encounter varying degrees of seasonal changes. The question

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how organisms adjust their behavioral and physiological programs to such recurring environmental cycles has received considerable attention over the past four decades. It has now been established beyond doubt that biological systems use endogenous time-keeping systems called circadian clocks (from '*circa*' = approximate, '*dies*' = a day) that modulate a wide range of behavioral and metabolic processes. While we understand a great deal about the various components of the underlying molecular-genetic and neuronal machinery, it is still unclear how these components interact to regulate a wide range of precisely timed molecular and behavioral processes that can be synchronized to daily and annual environmental cycles and persist in the complete absence of environmental time-cues or zeitgebers (time-givers, from '*zeit*' = time, '*geber*' = giver).

In the fruit fly *Drosophila melanogaster*, circadian clocks regulate the timing of a wide range of behavioral and metabolic processes including adult emergence, activity/rest, egg-laying, olfaction, mating, larval photo-responses, axon-caliber of lamina neurons, bouton size of motor neuron terminals, and expression of numerous genes (Konopka and Benzer, 1971; Krishnan *et al.*, 1999; Sakai and Ishida, 2001; Mazzoni *et al.*, 2005; Howlader and Sharma, 2006; Taghert and Shafer, 2006; Mehnert *et al.*, 2007). It is conceivable that separate oscillators control different metabolic and behavioral phenomena and that these oscillators are coupled together such that they influence each other to make up a multi-oscillatory system. Although some of the seminal studies that laid down the canonical features of circadian pacemakers were based on behavioral studies using the fruit fly *Drosophila pseudoobscura* (Pittendrigh, 1954, 1960), the power of its close relative *Drosophila melanogaster* as a genetic tool transformed the field of circadian rhythms beginning with the identification of the various underlying molecular components of the circadian machinery, followed by the localization of cells that are the sites of pacemakers of the circadian oscillators. *Drosophila melanogaster* (henceforth referred to as *Drosophila*) continues to this day to be the model system of choice to investigate the details of molecular architecture, neural circuitry and interactions with other metabolic and homeostatic processes. Here we present a critical overview of recent developments and the current understanding in the field of *Drosophila* circadian rhythms and discuss some unanswered questions and theoretical inconsistencies of present empirically based models. Specifically, evidence will be discussed for two current models of how oscillators are coordinated in the *Drosophila* circadian circuit—a "morning and evening" oscillator model for which it has been posited that individual oscillators are anatomically restricted to two functional sets and "dance" a Pas de Deux, *versus* a distributed network model for which many cell-autonomous oscillators are coordinated in a more complex, but ultimately flexible fashion, akin to the southern Italian circle-dance, the Tarantella.

MOLECULAR BASIS FOR *DROSOPHILA* CIRCADIAN OSCILLATORS

A general consensus has emerged about the existence of feedback mechanisms (involving transcription and translation) with a remarkably similar architecture across multiple phylogenetic classes although its position as an essential feature of the circadian machinery has recently come into question (Nakajima *et al.*, 2005; Tomita *et al.*, 2005; Hardin, 2006; Fan *et al.*, 2007; Mori *et al.*, 2007; Rust *et al.*, 2007). In its simplest form, it is believed that the clock consists of at least one transcriptional-translational feedback loop (TTFL), while in more complex organisms such as *Drosophila*, there are predictions for at least two interlocked-TTFLs (Glossop *et al.*, 1999; Cyran *et al.*, 2003; Yu and Hardin, 2006). In *Drosophila*, the gene products of *period* (*per*) and *timeless* (*tim*) form the core of the *Drosophila* TTFL in pacemaker cells (Figure 1A). Levels of PERIOD (PER) and TIMELESS (TIM) proteins and their mRNAs exhibit cyclic expression in pacemaker cells, and PER and TIM translocate from cytoplasm to nucleus in a time-of-day-dependent manner (Siwicki *et al.*, 1988; Hardin *et al.*, 1990; Sehgal *et al.*, 1994). Starting at noon, the transcription of *per* and *tim* is activated by two proteins CLOCK and CYCLE (CLK and CYC) which heterodimerize and bind to E-box

sequences in the *per* and *tim* promoters. Thus, CLK and CYC form the positive limbs of one of the interlocked-TTFLs (Figure 1A). The rise in *per* and *tim* mRNA levels (peaking at dusk) is followed by an increase in PER/TIM protein heteromultimer in the cytoplasm. There is a delay of 6 hours between the rise in PER/TIM multimer formation and their mRNAs production (Hardin *et al.*, 1990; Hunter-Ensor *et al.*, 1996) partly mediated by DOUBLE-TIME (DBT kinase, a casein kinase Iεhomolog; Kloss *et al.*, 1998), which destabilizes PER by phosphorylating and thus facilitating its subsequent degradation. Previous versions of the TTFL models suggested that after PER and TIM have each been phosphorylated by Casein kinase 2 (CK2) and SHAGGY (SGG, a Glycogen Synthase Kinase 3 homolog) respectively the DBT-PER/TIM heteromultimer enters the nucleus around midnight (Martinek *et al.*, 2001; Lin *et al.*, 2002).

However, two independent studies using very different methods suggest that nuclear entry of PER and TIM could occur independently (Shafer *et al.*, 2002; Meyer *et al.*, 2006). Immunocytochemical assays of PER and TIM levels at high temporal resolution revealed that nuclear entry of PER precedes that of TIM in pacemaker neurons by at least 3 hours (Shafer *et al.*, 2002). *In vitro* studies using a FRET-based method in S2 cells detected cytoplasmic PER-TIM heteromer formation and disassociation prior to independent nuclear entry, leading the authors to propose that PER-TIM complex formation may act as an interval timer for an event that precedes nuclear entry (Meyer *et al.*, 2006).

If the translocation of PER-TIM-DBT complex is indeed required, it appears to be regulated by phosphorylation which may occur via PER phosphorylation by CK2 (Lin *et al.*, 2002), kinase activity of DBT (Bao *et al.*, 2001; Cyran *et al.*, 2005), concerted action of both DBT and CK2 (Nawathean and Rosbash, 2004; Nawathean *et al.*, 2007) or TIM phosphorylation by SGG (Martinek *et al.*, 2001), while cytoplasmic PER is stabilized by Protein Phosphatase 2A (PP2A) (Sathyanarayanan *et al.*, 2004), which dephosphorylates PER. A recent study (Fang *et al.*, 2007) suggests that another Protein Phosphatase (PP1) dephosphorylates and stabilizes TIM thus promoting PER accumulation and hetero-dimerization. Thus while independent methods confirm the formation of heteromers during the sequence of events that constitute the TTFLs, whether PER-TIM-DBT complex translocation is critical for circadian function remains unresolved. A mathematical model which assumes rapid and cytoplasm-limited PER-TIM heteromers posits that the dissociation of PER-TIM is a means of adjusting the period and phase of molecular oscillations by differential timing of nuclear entry of PER and TIM (Leise and Moin, 2007). This recent model suggests that heteromeric complex translocation is not required for circadian function (Leise and Moin, 2007).

The central feature of the updated TTFL model holds that DBT-PER and TIM complex represses *per* and *tim* transcription in the nucleus by binding to CLK and CYC transcription factors and releases CLK/CYC bound to the E-box sequences of the *per* and *tim* promoters (for most recent papers on this topic, see Kim and Edery, 2006). Thus, in direct contrast to CLK and CYC, TIM and PER form the negative limb of the TTFLs. TIM is eventually degraded by light through its interaction with the photosensitive CRYP-TOCHROME (CRY) and eventually targeted by proteosomal mechanisms (Naidoo *et al.*, 1999; Busza *et al.*, 2004; Dissel *et al.*, 2004). CRY has also been implicated recently as a potential transcriptional repressor of CLK in peripheral tissues (Collins *et al.*, 2006). Subsequently monomeric PER is phosphorylated and degraded until midnight because SLIMB an F-Box protein marks it for proteosomal degradation (Edery *et al.*, 1994; Zeng *et al.*, 1996; Grima *et al.*, 2002; Ko *et al.*, 2002). In addition to PER, DBT also hyperphosphorylates CLK (Kim and Edery, 2006; Yu *et al.*, 2006). Thus, DBT kinase modulates molecular clock function at multiple stages, components and cellular locations in the circadian cycle.

The loss of PER/TIM mediated repression frees CLK/CYC to begin a new cycle of the molecular clock. A second feedback loop for the *Drosophila* circadian molecular clock was proposed (Glossop *et al.*, 1999; Cyran *et al.*, 2003) interlocking with the first TTFL. The CLK/CYC complex in addition to transcriptional activation of *per* and *tim* also bind to E boxes of two other genes *vrille (vri*) and *par domain protein-1ε(pdp1ε*) (Glossop *et al.*, 1999; Cyran *et al.*, 2003). PDP-1 activates transcription of *Clk*, while VRI represses it by competitively binding to regulatory sequences called VRI/PDP1ε- boxes (V/P-boxes), upstream of *Clk* thus becoming the positive and negative elements in the second TTFL. VRI levels was shown to peak during the early part of the night, and was believed to repress *Clk* transcription in the late evening when PDP1εlevels rise to competitively bind V/P-boxes of *Clk* (shown by dotted and *hashed line* in Figure 1B) and allow its transcriptional activation (Cyran *et al.*, 2003; Glossop *et al.*, 2003). However, recent studies suggest that the role of VRI as a transcriptional repressor of *Clk* may not be an essential component of the circadian pacemaking machinery (Kim and Edery, 2006) and that *Clk* cycling is non-essential for clock function (Kim *et al.*, 2002). More recent work overturns yet another previous assumption of the second feedback loop oscillator model by revealing that PDP1εfunctions as an oscillator output component rather than a central oscillator component (Benito *et al.*, 2007). The authors also propose that an activator of *Clk* (indicated by a *blue bar* in Figure 1B) is responsible for the constant high levels of *Clk* seen in Clk^{Jrk} and cyc^{0} mutants and for rising levels of *Clk* in wild-type flies during the early day and late night phases when repression by VRI does not occur. In addition, CLK protein levels were revealed to be constant with circadian oscillation in its level of phosphorylation due to the action of the multifunctional DBT and the phosphatase PP2A (Kim and Edery, 2006; Yu *et al.*, 2006). In addition to the core-clock genes, CLK is thought to regulate mRNA levels of several output genes (Cyran *et al.*, 2003; Glossop *et al.*, 2003). This model assumes that post-translational modifications via DBT, CK2, SGG, PP2A, and perhaps yet unknown enzymes are determinants of the period length and the phase of the overt rhythms that the oscillator generates (Bae and Edery, 2006). The amplitude of the rhythm may be regulated by *Clk* mediated transcriptional activation of various genes (Kim *et al.*, 2002), including the most recent candidate, *clockwork orange* (*cwo*) (Kadener *et al.*, 2007; Lim *et al.*, 2007; Matsumoto *et al.*, 2007) which is proposed to contribute to robustness of the amplitude of mRNA oscillations of *vri*, *pdp-1ε*, *tim* and *per*.

The current TTFL model also does not explain the persistence of overt behavioral rhythms when *clk* mRNA is expressed using *per* or *tim* promoters such that its expression is no longer antiphasic to *per* and *tim* (Kim *et al.*, 2002) which according to the model is a critical factor for rhythm generation. Mathematical modeling data also support the hypothesis that robust oscillations can persist in the presence of constant levels of CLK, if total CLK levels remain below that of total PER levels (Leise and Moin, 2007). However, it is possible that the role of the second TTFL is to maintain the stability of the first TTFL (Harms *et al.*, 2004; Lakin-Thomas, 2006). Further, constitutive expression of either *tim* or *per* mRNA independently or together does not disrupt oscillations in PER and TIM protein and overt behavioral rhythms, which raises the question as to whether mRNA oscillation is a fundamental feature of the *Drosophila* circadian oscillator (Frisch *et al.*, 1994; Vosshall and Young, 1995; Cheng and Hardin, 1998; Yang and Sehgal, 2001; Harms *et al.*, 2004)—presenting a long-standing, but still not resolved challenge to the TTFL model. We note that thus far no study has simultaneously blocked mRNA oscillations of all known clock components, and therefore it is likely that there are yet- to be-identified, redundant components of the clock that continue to regulate rhythmicity observed in previous studies.

Can circadian oscillators unequivocally operate without TTFL features? They can. Over the past few years, *in vitro* studies have shown that a trio of purified cyanobacterial proteins Kai A, B, and C can interact in the absence of transcriptional machinery to function as a self-sustained biochemical oscillator under constant darkness (DD) with a period of ~ 24 hour and

can even exhibit a canonical feature of circadian oscillators which is temperature compensation (Nakajima *et al.*, 2005). Studies using mouse fibroblasts have led to the proposal that in fact post-translational modifications are the core oscillator, while transcriptional regulation merely enhances the amplitude and robustness of the oscillation (Kiyohara *et al.*, 2006). Recently, a combination of theoretical and empirical investigations has lead to the proposal that four phosphorylation states (phosphoforms) of the cyanobacterial Kai C protein are generated in an ordered pattern as a result of its intrinsic autokinase and autophosphatase activities and their modulation by Kai A (Rust *et al.*, 2007). Further this study proposes that the negative feedback is achieved by the inhibitory action of one of the Kai C phosphoforms together with Kai B upon Kai A activity. Another study conducted in mouse fibroblasts also challenges the current version of the TTFL model. This was achieved by administering exogenous cell-permeant mCRY1 and mCRY2 proteins modified to include the short hydrophobic sequence AAVLLPVL-LAAP which confers permeability across the plasma membrane, thus the levels of these exogenously administered proteins are not regulated by transcription. Exogenous cell-permeant mCRY1 and mCRY2 proteins can rescue circadian rhythmicity and can act as transcriptional repressors and cause phase shifts in the circadian oscillator (Fan *et al.*, 2007). These results suggest that previous assumption for cycling levels of CRY is non essential for a functional circadian oscillator in mammalian cells. Thus, the TTFL model, while appealing in many ways, has a number of unresolved inconsistencies.

CIRCADIAN CIRCUITS IN *DROSOPHILA* BRAIN

In the simplest model of organization, all circadian systems can be thought to be composed of input, core-pacemaker, and output components. This organization can be applied to our developing ideas of the *Drosophila* pacemaker circuit; although it is likely that a particular cell group in the pacemaker circuit has multiple overlapping functions (see below for specific examples).

Core Pacemaker Neurons

In *Drosophila*, cells that express known ‘clock genes’ occur all over the body. These cells can sustain molecular oscillations under constant conditions and can entrain to environmental zeitgebers such as light/dark and temperature cycles independent of the brain pacemakers (Plautz *et al.*, 1997; Glaser and Stanewsky, 2005). One working definition of a pacemaker is that it should be able to sustain rhythm under long-term DD (Kaneko *et al.*, 2006). In the brain numerous glial cells and approximately 150 neurons express clock genes rhythmically (Kaneko, 1998). Glia have previously been shown to contribute to the generation of activity/rest rhythm (Ewer *et al.*, 1992; Frisch *et al.*, 1994). Recent studies indicate that a biogenic amine synthase coded by the gene *ebony* is rhythmically expressed in glia with circadian period (Claridge-Chang *et al.*, 2001; Ueda *et al.*, 2002) regulates circadian locomotor activity/rest rhythm by coordinating the action of neurotransmitters such as dopamine and serotonin (Suh and Jackson, 2007). In this review we will focus on the clock pacemaker *neurons* and their circuits.

Even before the clock genes were discovered and their expression patterns identified, brain pacemakers were considered to be coupled bilaterally between two sides of the brain. This idea was developed from studies in other insects (Helfrich-Forster *et al.*, 1998). Advances in immunocytochemical reagents and reporter constructs allowed for the identification of the pacemaker neurons and their projection patterns and revealed that pacemaker neurons are interconnected both bilaterally and within a brain hemisphere (Helfrich-Forster and Homberg, 1993; Kaneko and Hall, 2000) (Figure 2). More recent studies demonstrate functional coupling among the different pacemaker neuronal subgroups (Peng *et al.*, 2003; Lin *et al.*, 2004; Stoleru *et al.*, 2005; Nitabach *et al.*, 2006).

Clock pacemaker neurons in adult *Drosophila* brain can be divided into two groups, lateral neurons (LN) whose cell bodies are located in the anterior lateral cortex of the central brain, and dorsal neurons (DN) with cell bodies in the dorsal cortex. LN are further divided into three groups 4–6 large and 4–6 small ventral LN (LN_v), and ~6 dorsal LN (LN_d). DN are also divided into at least three subgroups DN_1, DN_2, and DN_3 (Kaneko, 1998). Approximately 15 $DN_1$are located in the pars lateralis, a pair of DN_2 are located slightly ventral to DN_1 and are located just dorsal to the calyces of the mushroom body, and ~40 small DN_3neurons are located more dorsolaterally. In addition to these six clusters, there is another cluster of three *per*- and *tim*-expressing neurons in the posterior lateral cortex (LPN) (Kaneko and Hall, 2000; Shafer *et al.*, 2006) (Figure 2).

Projection patterns of most of the pacemaker neurons are shown in Figure 2, and extensively reviewed elsewhere (Helfrich-Forster, 2002,2003,2005), the only exception being LPN whose projection pattern is not yet known. The arborization patterns of LN_v were first identified by staining for antibody against the crustacean Pigment Dispersing Hormone (PDH) (Helfrich-Forster and Homberg, 1993). For the rest of the pacemaker neurons, *per*- and *tim-GAL4* driven marker gene expression have been used to reveal their morphology (Kaneko and Hall, 2000). Recent attempts from different research groups using various methods such as combination of *tim-GAL4* or anti-PDF staining along with anatomical mutations, combinations of *GAL4* drivers and *GAL80* constructs, new drivers, and cell-filling have revealed detailed and refined patterns of arborizations for DN_3 (Veleri *et al.*, 2003), DN_1 (Helfrich-Forster, 2003), small and large LN_v (Park and Griffith, 2006;Helfrich-Forster *et al.*, 2007), and LN_d (Helfrich-Forster *et al.*, 2007).

Most of the clock neurons (small LN_v, LN_d, DN_1, DN_2, and DN_3) send processes to the dorsal protocerebrum (Helfrich-Forster and Homberg, 1993; Kaneko and Hall, 2000) (Figure 2). The dorsal protocerebrum has connections to many areas of the brain and also contains many neurosecretory cells. Therefore this is a likely location where clock neurons are connected to various behavioral outputs. While processes from these five clusters of neurons extensively overlap in this region, there is no direct evidence of how putative functional coupling might occur—evidence for functional coupling of different neurons in the pacemaker circuit is largely inferential based on projection patterns, and more recently, mutant analysis (Peng *et al.*, 2003; Lin *et al.*, 2004) and genetic perturbation experiments (Nitabach *et al.*, 2006; de la Paz Fernandez *et al.*, 2007). Who directly talks to who in the pacemaker circuit is still an open question.

Where is information in the *Drosophila* pacemaker circuit integrated? Each large LN_v sends contralateral and ipsilateral projections that innervate the medulla and a small neuropil at the inner margin of the medulla called the accessory medulla (AMe) on both sides of the brain (Figure 2) (Park and Griffith, 2006;Helfrich-Forster *et al.*, 2007). This distinct anatomical feature suggests that the large LN_v mediate bilateral coupling of the two hemispheres of pacemaker circuit. The large LN_v unambiguously sends bilateral projections to the opposite hemisphere of the *Drosophila* brain (see Park and Griffith, 2006 for high resolution single cell fills). Besides large LN_v many other clock neurons (small LN_v, LN_d, DN_1, and DN_3) innervate the AMe (Helfrich-Forster, 2003;Veleri *et al.*, 2003;Shafer *et al.*, 2006;Helfrich-Forster *et al.*, 2007). This may be where oscillatory signals as well as light-input signals are integrated to form a coupled pacemaker circuit.

Inputs into Core Pacemaker Neurons

Light is a major Zeitgeber for the circadian clock, and *Drosophila* has many photoreceptors that participate in the entrainment of circadian rhythms. In addition to light-driven synaptic inputs, CRY a cell autonomous blue-light photoreceptor responsible for circadian entrainment is expressed in many if not all clock cells (Emery *et al.*, 2000; Klarsfeld *et al.*, 2004). Therefore,

molecular rhythms in clock cells can be entrained independent of the rest of the circuits through *cry*, and certainly that is the case for many peripheral oscillators (Stanewsky *et al.*, 1998). CRY interacts with TIM in a light-dependent manner and modifies it, enabling another protein JETLAG (JET) to ubiquitinate TIM thus facilitating its degradation via proteosomal pathways (Koh *et al.*, 2006; Peschel *et al.*, 2006; Van Gelder, 2006). However, locomotor activity rhythms of flies can be entrained without *cry* (Stanewsky *et al.*, 1998). The compound eyes, ocelli, a structure called the Hofbauer-Buchner eyelet (H-B eyelet), and CRY are all responsible for entraining activity rhythms, and flies lacking one or more of these are partially compromised in their entrainment (Stanewsky *et al.*, 1998; Helfrich-Forster *et al.*, 2001; Rieger *et al.*, 2003; Klarsfeld *et al.*, 2004). Among these photoreceptive structures, the H-B eyelet has a direct input into the AMe and possibly connects with large LN_v (Helfrich-Forster *et al.*, 2002a; Malpel *et al.*, 2002; Helfrich-Forster *et al.*, 2007). Exactly how the compound eyes and the ocelli connect to the clock neurons is not yet known. Besides these structures that are responsible for entraining locomotor activity rhythms, clock neurons such as DN_1 and DN_3 have been shown to be photoreceptive through unknown mechanisms (Veleri *et al.*, 2003; Klarsfeld *et al.*, 2004).

Temperature is another major Zeitgeber of the clock, and can be used to entrain clocks in DD as well as constant light (LL) where flies are normally arrhythmic (Wheeler *et al.*, 1993; Matsumoto *et al.*, 1998; Yoshii *et al.*, 2002; Glaser and Stanewsky, 2005; Yoshii *et al.*, 2005). Temperature-dependent entrainment of the clock seems to occur cell autonomously and temperature-sensing structures such as the antenna are dispensable (Glaser and Stanewsky, 2005). This process needs phospholipase C (Collins *et al.*, 2004; Majercak *et al.*, 2004) and a gene mutated in a novel mutant *nocte* (Glaser and Stanewsky, 2005). LN_v and LN_d are dispensable for locomotor activity rhythms under temperature cycles in LL (Yoshii *et al.*, 2005), suggesting that different pacemaker cells may be involved in temperature and light entrainment (Busza *et al.*, 2007; Miyasako *et al.*, 2007). Molecularly, interaction between CRY and TIM-PER complex is seen both after a heat pulse as well as a light pulse, suggesting that this may be a common mechanism for both light- and heat-mediated phase shifts (Kaushik *et al.*, 2007). However, the heat-mediated phase shifts of activity rhythms and accompanying CRY-TIM-PER interaction require rather high temperatures (~37°C) in wild-type flies (Kaushik *et al.*, 2007), whereas locomotor activity rhythms can be entrained by temperature cycles involving much lower temperatures (Wheeler *et al.*, 1993). Furthermore, molecular oscillations can be entrained by temperature cycles in the hypomorphic mutant cry^b (Stanewsky *et al.*, 1998; Glaser and Stanewsky, 2005). Therefore, CRY-TIM-PER interaction may not be responsible for entrainment involving moderate temperatures. Preliminary studies in our laboratory suggest that ion channels help modulate temperature sensitivity of the circadian pacemaker circuit within physiologically permissible range of temperatures (Sheeba V, Chou Y, Muirhead, KA, Sharma VK, Holmes TC, unpublished data).

Output from Core Pacemaker Neurons

Large LN_v have extensive arborizations with varicosities in the medulla of the optic lobe (Figure 2) (Helfrich-Forster and Homberg, 1993). Therefore, they may underlie communication with the visual system. The visual system also exhibits circadian rhythm in synaptic frequency of photoreceptor cells, screening pigment in photoreceptor terminals, and axon caliber, nuclear size, and dendritic spine of the lamina monopolar cells (Pyza and Meinertzhagen, 1993,1995,1997,1999;Gorska-Andrzejak *et al.*, 2005) suggesting that they are regulated by outputs from large LN_v. So what are the molecules that are involved in communication between pacemaker and output structures? Thus far three neuropeptides have been found to be expressed in subsets of clock neurons. PDF is expressed in large LN_v and all but one small LN_v (Helfrich-Forster, 1995,1997). Flies lacking either the neuropeptide or cells expressing it cannot sustain robust activity rhythms in constant darkness, and in light/dark

cycles (LD), have their evening peak of activity shifted earlier (Renn *et al.*, 1999,Figure 3). The main function of PDF seems to be coupling of molecular oscillations in different clock neurons, because genetic manipulation of PDF-expressing cells and the pdf^{01} mutation decreases synchrony of molecular oscillations within and among other clock neuronal subgroups (Peng *et al.*, 2003;Lin *et al.*, 2004;Stoleru *et al.*, 2005;Nitabach *et al.*, 2006). A similar role in synchrony of oscillators was previously proposed for a related peptide Pigment Dispersing Hormone (PDH), after injection of PDH into the cockroach brain (Petri and Stengl, 1997). While there is a growing body of functional evidence that PDF is an important neurotransmitter for circadian function, less is known about its receptor, PDFR (a.k.a. GOP, Han). The expression pattern of the receptor for PDF, the best known neuropeptide continues to remain unresolved. While one study, based on immuno-reactivity of a C-terminal sequence, suggests that the receptor is expressed on a pair of DN_1, and two- three DN_3 in addition to regions around the small LN_v, large LN_v, LN_d (Mertens *et al.*, 2005) another study also based on immuno-reactivity of N-terminus based antibody reveals a larger expression pattern and includes all the large LN_v, one of the LN_d and five to seven DN_1 and one DN_3 (PDFR designated as Han, Hyun *et al.*, 2005). Current understanding in the field is that neither of these antibodies accurately depicts the pattern of the receptor distribution. The third study used *in situ* hybridization and detected signals both in the dorsal as well as lateral brain regions that are known to be regions of pacemaker cells but cannot be confirmed due to lack of colabeling information (PDFR designated as GOP Lear *et al.*, 2005). Thus the final word on its localization awaits further studies.

Two-three of ~6 LN_d express the *Drosophila* neuropeptide F (NPF), which is a homologue of mammalian neuropeptide Y (NPY), a neuropeptide expressed in a subset of circadian suprachiasmatic nucleus (SCN) neurons (Lee *et al.*, 2006). Knockout $npy^{-/-}$mice exhibit defects in their ability to respond to non-photic time cues that ordinarily modulate clock period and these mice show weak entrainment to photoperiods (Harrington *et al.*, 2007). NPF expression is found mostly in males and is regulated by both *fruitless* and the clock genes *Clock* and *cycle*. Two of the DN_1 which have a slightly anterior location compared to the rest of this cluster (DN_{1a}) express a neuropeptide called IPNamide (Shafer *et al.*, 2006). These cells along with two other DN_1 (DN_{1p}) appear to project back to the AMe and LN_v, and thus could mediate a feedback signal within the pacemaker circuit.

FUNCTIONAL OUTPUTS OF THE PACEMAKER

The circadian clock has been shown to control a number of biological processes including cyclic expression of numerous genes. Unfortunately there is poor consensus among the five different studies in which DNA microarray techniques have been used to find cycling gene expressions in the *Drosophila* genome. The possible reasons behind the heterogeneity of results in the *Drosophila* circadian genomics studies has been discussed extensively elsewhere (reviewed in Etter and Ramaswami, 2002; Taghert and Shafer, 2006; Wijnen *et al.*, 2006). Briefly, use of different statistical tools, tissues, and experimental paradigms are thought to be the primary reasons behind the lack of consensus among fly micro-array data from different laboratories. In terms of the underlying output mechanism for a behavioral rhythm in *Drosophila*, the rhythms of adult emergence from the pupal case (eclosion) is perhaps the best understood thanks to endocrinological and developmental studies, and has been comprehensively reviewed elsewhere (Helfrich-Forster, 2005). One important aspect of this rhythm is that it is controlled by the clock in the ecdysteroid secreting prothoracic gland, which in turn appears to be controlled by the PDF-expressing LN_v (Myers *et al.*, 2003). This situation is very similar to many peripheral clocks in mammals, which are regulated by the central clock suprachiasmatic nucleus (SCN) (Yamazaki *et al.*, 2000).

Locomotor activity rhythm has been studied extensively as a behavioral readout of the circadian clock in *Drosophila* (Klarsfeld *et al.*, 2003). For robust free-running activity rhythms in DD, clocks in LN are necessary and sufficient (Ewer *et al.*, 1992; Frisch *et al.*, 1994). Among the LN, PDF-expressing small LN_v and their processes in the dorsal protocerebrum are especially important (Helfrich-Forster *et al.*, 1998; Renn *et al.*, 1999). As discussed in detail below several groups have shown data supporting the so-called 'morning and evening' oscillator model in which one group of clock neurons is responsible for the fly's morning peak of activity and another group for evening peak of activity under LD (Grima *et al.*, 2004; Stoleru *et al.*, 2004; Stoleru *et al.*, 2005; Rieger *et al.*, 2006; Stoleru *et al.*, 2007). The circuits downstream of the pacemaker neurons involved in generation of locomotor activity/rest rhythms are yet unknown. Presumably these circuits receive circadian signals from the pacemaker neurons in the dorsal protocerebrum and control arousal level as well as specific behaviors such as mating (Hendricks *et al.*, 2000; Shaw *et al.*, 2000; Sakai and Ishida, 2001). Sleep in flies is believed to be modulated by PKA-CREB pathway and serotonin receptor d5-HT1A in the mushroom body (Joiner *et al.*, 2006; Pitman *et al.*, 2006; Yuan *et al.*, 2006), but sleep regulation is complex and the circadian pacemaker circuit may not control these processes in the mushroom body directly, as activity rhythms are normal in mushroom body-ablated flies (Helfrich-Forster *et al.*, 2002b). Non-PDF-expressing cells regulate some rhythmic outputs such as olfaction and egg-laying rhythms (Krishnan *et al.*, 1999; Tanoue *et al.*, 2004; Howlader *et al.*, 2006).

DEVELOPMENT AND HETEROGENEITY OF THE PACEMAKER CIRCUIT

The expression of neuropeptides revealed heterogeneity among cells within the clusters small LN_v, LN_d, and DN_1. In the case of small LN_v, four PDF-expressing ones were the first to be discovered (Helfrich-Forster and Homberg, 1993). Later, it was found that there is a fifth PDF-negative PER- and TIM positive small LN_v (Helfrich-Forster, 1995; Kaneko *et al.*, 1997). Recent work suggests that the projection pattern of the fifth PDF-negative small LN_v is indistinguishable from those for PDF-expressing small LN_v (Helfrich-Forster *et al.*, 2007). Heterogeneity in cell body size and PER cycling amplitude among LN_d and $DN_1$was noted even before the neuropeptide expression was discovered (Rieger *et al.*, 2006). The relatively anterior position of two of the DN_1 neurons in pupae has been documented (Kaneko and Hall, 2000), and these DN_1 (DN_{1a}) have been distinguished as those not expressing GLASS protein and not eliminated by *glass*^{60j} mutation (Helfrich-Forster *et al.*, 2001; Klarsfeld *et al.*, 2004). In fact, DN_{1a} neurons are the cells that originate from larval DN_1 (Klarsfeld *et al.*, 2004), and send projections down to the AMe, while most other DN_1 neurons (except DN_{1p}) project proximally and make a commissure in the dorsal protocerebrum (Shafer *et al.*, 2006). In addition to these clusters, heterogeneity in cell body size and projection patterns have been found in DN_3 neurons, most of which project proximally to the pars intercerebralis while few project to the AMe (Veleri *et al.*, 2003).

Clock neurons differentiate at different developmental stages. Small LN_v are the first to differentiate and have PER and PDF expression from early first instar larval stage onward (Helfrich-Forster, 1997; Kaneko *et al.*, 1997). DN_{1a} and DN_2 start expressing PER in late first instar larvae, and these cells persist through metamorphosis (Kaneko *et al.*, 1997; Kaneko and Hall, 2000; Klarsfeld *et al.*, 2004). It has been suggested that LN_d and large LN_v are present from late third-instar larval stage, because weakly labeled neurons with characteristic projection patterns (POT-like processes for large LN_v and projections to the dorsal protocerebrum for LN_d) were found in late third instar larvae, and these cells could be observed in early pupae as well (Kaneko and Hall, 2000). These putative larval LN_d and large LN_v most likely correspond to weakly PER- and TIM-immunoreactive cells near LN in older larvae (Kaneko *et al.*, 1997). Later it was confirmed that these weakly labeled cells near the larval LN are indeed precursors of LN_d and large LN_v (Helfrich-Forster *et al.*, 2007). PER expression could be observed in majority of DN_1 and DN_3 from late pupal stage (Kaneko *et al.*, 1997).

SYNCHRONY OF MULTIPLE OSCILLATORS

Considering the fact that many different neuronal subgroups comprise the circadian pacemaker circuit the question arises as to how these neuronal subgroups are orchestrated to generate a single coherent rhythm in overt behavior. In terms of molecular oscillations, the cycling in levels of mRNA and protein for PER and TIM in the different subgroups of cells are synchronous (with one exception) under the influence of LD cycles. In larvae, precursors of the DN_2 cells oscillate in anti-phase with the rest of the pacemaker neurons both in LD and DD conditions (Kaneko *et al.*, 1997). In adults, the DN_2 oscillation is in-phase with the other pacemaker neurons in LD and for the first two days of DD (Blanchardon *et al.*, 2001) (Figure 4A, E), but on the fifth day of DD, they are out-of-phase leading to the proposition that under the influence of light the DN_2 cells are synchronized with the rest of the network (Veleri *et al.*, 2003) (Figure 4I); although its not clear as to what might mediate such synchrony since DN_2 are not known to express the photoreceptor CRY (Klarsfeld *et al.*, 2004).

The output molecule PDF is believed to be a synchronizing factor for oscillations among the multiple subgroups by two independent studies using the null mutant pdf^{01} (Peng *et al.*, 2003; Lin *et al.*, 2004). However these two studies disagree on one important point. Peng *et al.*, 2003 show dampening of *cry* and *tim* mRNA oscillations in DD and conclude that behavioral rhythms are generated by the action of PDF coordinating intra-cellular communication, while Lin *et al.*, 2004 show that the oscillations in PER levels in sLN_v are in fact not altered by the absence of functional PDF (also discussed in earlier section on molecular oscillators; Figure 4B, C). Instead, the level of synchrony—that is the cycling coherence between the individual cells within a subgroup was significantly reduced. The authors propose that PDF is responsible for maintaining tight synchrony in phase and amplitude of PER oscillation among the small LN_v and among the dorsal subgroup LN_d. There is good evidence for such synchronizing properties of a related peptide PDH in the cockroach, where injection of PDH in the AMe caused phase shifts in activity/rest behavior in a time dependent manner (Petri and Stengl, 1997).

Studies in our lab using transgenic expression of a voltage-gated Sodium channel NaChBac in the LN_v electrically perturbs a small subset of the pacemaker circuit—and these flies exhibit long-term complex locomotor activity/rest rhythms (Nitabach *et al.*, 2006). Further the molecular clock in the different neuronal subgroups in the circuit was desynchronized in flies that express NaChBac in the LN_v when assayed during the initial stages of exposure to DD (day 5)—this oscillator desynchrony corresponds with initial short-term arrhythmic locomotor activity (Figure 4L). But upon emergence of stable although complex rhythmicity after ~5–6 days, the molecular oscillations in the different subgroups assume a novel pattern of synchrony that is particularly striking in the dorsal pacemaker cell groups (Sheeba *et al.*, 2008, Figure 4N). We interpret these findings to imply that the pacemaker circuit responds to perturbation by compensatory homeostatic mechanisms that are not cell autonomous, but distributed throughout the neuronal subgroups as reflected by emergence of novel patterns of rhythmicity in behavior and synchrony in molecular oscillations from an initial arrhythmic behavior and asynchronous state of molecular oscillations. These results suggest that oscillator function is not strictly anatomically localized, but plastic throughout the circuit depending on input and cell-cell communication.

ELECTRICAL SIGNALING IN PACEMAKER NEURONS AND CIRCUITS

Before the molecular-genetic characterization of clock genes in *Drosophila*, circadian rhythms of spontaneous action potential firing was recognized as the key defining feature of pacemaker neurons in vertebrates and invertebrates (Inouye and Kawamura, 1979; Green and Gillette, 1982; Schwartz *et al.*, 1987; Michel *et al.*, 1993). Molecular circadian clocks show conservation

both at the operational level and in some cases, the molecular identity of clock components (*e.g.*, alleles of PERIOD are core clock components in both Drosophila and mammals, see Allada *et al.*, 2001). Correspondingly, it appears that there are conserved neurophysiological features of pacemaker neurons. Pacemaker neurons across different invertebrate and vertebrate species tend to fire spontaneous action potentials at high rates during the day and low rates at night (reviewed in Kuhlman and McMahon, 2006). Not surprisingly, neurophysiological recordings from pacemaker neurons in clock gene mutant rats and mice reveal corresponding changes in the phasing of action potential firing patterns (Liu *et al.*, 1997; Herzog *et al.*, 1998; Nakamura *et al.*, 2002). More recent work has shown circadian oscillation of ionic currents in mammalian pacemaker neurons (Colwell, 2000; Pennartz *et al.*, 2002; Ikeda *et al.*, 2003; Itri *et al.*, 2005). Ongoing work is identifying circadian regulated neurophysiological components in *Drosophila* pacemaker neurons (Rubovszky, Sheeba, Gu, Dahdal, O'Dowd, and Holmes, *Soc. Neuroscience Abs.* 459.16, 2006). Another recent neurophysiological study shows that *Drosophila* pacemaker neurons are light-sensitive, based on the finding that the threshold of current-evoked firing is modulated by light levels (Park and Griffith, 2006) although these authors did not report reliable spontaneous firing in these neurons.

Cell-Autonomous Electrical Signaling in Pacemaker Neurons

Isolated pacemaker neurons can retain circadian regulation of membrane electrical properties as shown by cell autonomous circadian rhythms in membrane potential and potassium conductance found in neurons of the mollusk *Bulla gouldiana* (Michel *et al.*, 1993) and spontaneous action potentials measured in isolated mammalian SCN neurons (Welsh *et al.*, 1995). So is the relationship between electrical signaling in pacemaker neurons and the circadian clock a "one-way street" for which information flows merely as an output function from the circadian clock to regulate electrical activity in the pacemaker neurons? Electrical signaling in pacemaker neurons has been proposed to be a core component of the circadian clock (Njus *et al.*, 1974, 1976; Nitabach *et al.*, 2005a). Experimental support for this idea came initially from the observation that modulation of membrane potential influences both input and output to the circadian clock in *Bulla* pacemaker neurons (McMahon and Block, 1987). Recent studies have revived the hypothesis that electrical signaling in pacemaker neurons is a core component of circadian clock. In *Drosophila*, electrical silencing of PDF-expressing LN_v by transgenic expression of mutant open rectifier or inward rectifier potassium channels causes run down of the free-running circadian clock in these neurons within few days in DD and abolishes rhythmic circadian locomotor behavior (Nitabach *et al.*, 2002; Nitabach *et al.*, 2005b). PER and TIM levels continue to oscillate in LN_v of flies with electrically silenced pacemaker neurons when they are kept in 12:12 hour LD cycles, thus electrical silencing specifically disrupts the free-running circadian clock in DD. Does expression of mutant potassium channels in LN_v actually cause electrical silencing? We have recently verified by whole cell patch clamp analysis that expression of the mutant high conductance open rectifier potassium (dORKΔ-C) channel in LN_v causes profound membrane hyperpolarization and abolishes spontaneous action potential firing that is normally observed in the LN_v (Gu, O'Dowd, and Holmes, unpublished results). While earlier patch clamp recordings of LN_v do not reveal spontaneous action potential firing (Park and Griffith, 2006), this study shows clearly that current-injection-evoked action potential firing of LN_v is attenuated by dORKΔ-C expression (Park and Griffith, 2006). Thus, all lines of evidence indicate that dORKΔ-C expression causes membrane hyperpolarization and abolishes action potential firing. Based on PER and TIM cycling results, we concluded that electrical silencing of the LN_v pacemaker neurons cell-autonomously disrupt circadian molecular oscillations (Nitabach *et al.*, 2002). However, as pointed out by Kuhlman and McMahon (2006), electrical silencing would also likely block output of the LN_v pacemaker neurons—and altered cell-cell communication within the pacemaker circuit could potentially feedback to the LN_v. We independently considered this as well, and attempted to address this possibility by testing the effects of expressing tetanus

toxin light chain throughout the *Drosophila* pacemaker circuit using the *tim-GAL4* driver line (UAS-TeTxLC/*tim-GAL4*). UAS-TeTxLC/*tim-GAL4* flies are behaviorally arrhythmic in constant darkness, but normally phased clock cycling persists in the LN_v pacemaker neurons after five days in DD (Nitabach *et al.*, 2005b). This suggests that the effect of electrical silencing on disruption of LN_v circadian oscillation is indeed a cell autonomous effect; although we cannot rule out the possibility tetanus toxin light chain expression does not completely block synaptic transmission in the pacemaker circuit. While this question has not been resolved, Nitabach *et al.* (2002) helped motivate a growing body of work examining circuit-level circadian function in *Drosophila*.

Circuit-Dependent Electrical Signaling between Components of the Pacemaker Neural Circuit

The circuit-wide effects of altering electrical excitability in a small subset of the *Drosophila* circadian pacemaker circuit have been tested. As described in a previous section on synchrony of multiple oscillators, targeted expression of the low-threshold voltage-gated sodium channel NaChBac in the LN_v causes short-term arrhythmicity, long-term changes in locomotor behavior and short-term molecular clock cycling desynchrony throughout the entire *Drosophila* pacemaker circuit, particularly in dorsal neurons (Nitabach *et al.*, 2006). Mutations in natively expressed *Drosophila* channels can also cause circuit-wide perturbation of circadian function as demonstrated by recent studies on SLOWPOKE (a large conductance calcium-activated potassium channel that is expressed widely throughout the *Drosophila* nervous system). Flies carrying severe mutations in the slowpoke channel gene exhibit weak circadian rhythms that selectively alter clock cycling in the *Drosophila* pacemaker circuit (de la Paz Fernandez *et al.*, 2007). Future neurophysiological and imaging studies will give us a clearer picture of the functional operation of the *Drosophila* pacemaker circuit. One note of caution that applies to most of the studies on *Drosophila* pacemaker circuit described thus far and to those discussed in the following sections is the fact that they do not account for the possibility of plasticity of the wild type neural circuit both during development and in response to environmental changes. Almost all genetic manipulations (and mutants) used in the study of the fly circuit have been chronic, thus pointing to the exciting possibility of unraveling the plasticity of the mature adult as well as the developing larval pacemaker circuit by designing circuit manipulations that are both temporally and spatially restricted.

THE “MORNING” AND “EVENING” OSCILLATOR MODEL

In 1976, Pittendrigh and Daan proposed that circadian clocks simultaneously “measure” daily and seasonal changes in day lengths using two mutually coupled oscillators: the ‘Morning’ (M) oscillator that tracks dawn and the ‘Evening’ (E) oscillator that tracks dusk (Pittendrigh and Daan, 1976). Although the model was originally conceived to explain the peculiar and rare phenomenon of “splitting” and “re-fusion” of morning and evening activity bouts in mammals, it was hoped that it would provide a basis for understanding other characteristic features of circadian pacemakers that could not be explained by other contemporary models based on a single oscillator (Pittendrigh, 1960). Previously a two-oscillator model had been developed to account for the bimodality of circadian rhythms often seen under LD cycles (Aschoff and Wever, 1966)

The M and E oscillator model assigns certain properties to the two oscillators such as (i) the free running period of M and E oscillators are differentially affected by light intensity; the period of morning oscillator is negatively correlated with light intensity and that of the evening oscillator is positively correlated with light intensity, (ii) when mutually coupled, the overall pacemaker period is different from the periods of the individual oscillators, (iii) the relative influence of the oscillators on each other depends upon their phase-relationship, and (iv) the coupling between the two oscillators depends upon the environmental conditions. In many

diurnal animals, during long summer days, morning activity occurs earlier and the evening activity occurs later compared to days with near 12:12 hour LD photoperiods, enabling them to avoid the midday heat (Majercak *et al.*, 1999). According to the M and E oscillator model, in the summer (long day conditions, for which LL may act as a proxy) the M oscillator would run with short period while the E oscillator would exhibit a long period. This could, in principle, decouple the two oscillators, resulting in behavioral "splitting." Indeed, under constant light conditions, behavioral activity components have been seen to split into multiple components in several diurnal and nocturnal rodents, in tree shrews, common marmosets, and in certain species of birds, reptiles, fishes and insects (Rosenwasser and Adler, 1986; Schardt *et al.*, 1989; Smietanko and Engelmann, 1989; Meijer *et al.*, 1990; Hong and Saunders, 1998). Multiple behavioral components that resemble "splitting" have also been observed in certain genetically modified *Drosophila* strains (Helfrich, 1986; Helfrich-Forster, 2000; Yoshii *et al.*, 2004; Nitabach *et al.*, 2006; Rieger *et al.*, 2006). An important point to note here is that such behavioral decoupling does not usually occur in the wild because constant bright light usually results in behavioral arrhythmicity (Aschoff, 1979; Konopka *et al.*, 1989) and when "splitting" occurs in experimentally manipulated environments such as dim LL (usually up to 100 lux), it is seen after prolonged exposure (as long as two months) to such conditions (Pickard *et al.*, 1993).

Although activity of some diurnal mammals, for example the northern tree shrew (*Tupaia belangeri*), undergoes splitting under DD, the split components are indistinguishable in terms of their response to light and carbachol perturbations (Meijer *et al.*, 1988; Meijer *et al.*, 1990). Similarly bilaterally distributed identical pacemakers in insects may temporally separate from each other (Koehler and Fleissner, 1978). These results suggest that the left and right SCN or bilaterally distributed pacemakers in insects, which are otherwise indistinguishable structurally, serve as M and E oscillators and show antiphasic coupling in "split" animals. Indeed, partial to complete lesions of one of the SCN lobes in hamsters leads to partial to complete elimination of one of the split activity components (Pickard and Turek, 1982; Davis and Gorski, 1984). Bimodality is also seen at the level of single unit electrical activity of coronal SCN slices of split hamsters (Mason, 1991; Zlomanczuk *et al.*, 1991). Although these studies suggest that the regulation of splitting occurs in the SCN, the left-right distribution of electrical activity is still unclear (Daan *et al.*, 2001). In a relatively recent study in rodents behavioral desynchronization was shown to be coupled with dissociation of clock gene expression in the ventrolateral and dorsomedial region of the SCN (de la Iglesia *et al.*, 2004).

From a purely functional perspective, the M and E oscillator model is appealing because it provides a relatively simple explanation for adjustments of circadian behavior—and thus by inference, circadian oscillator organization—in response to daily and seasonal changes. The M and E oscillator model is particularly attractive for *Drosophila melanogaster* as it exhibits two distinct bouts of locomotor activity under 12:12 hour LD cycles, one centered at dawn and the other around dusk suggesting that flies anticipate dawn and dusk transitions under natural cyclic environments.

While the M and E oscillator model was originally developed to explain "splitting" behavior in mammals in response to constant light, this model has been applied recently to *Drosophila melanogaster* using several genetic and behavioral approaches aimed at identifying the putative M and E oscillators in *Drosophila* circadian system. Helfrich-Forster (2000) suggested that the morning peak of activity is governed by a *per*-independent clock and is entrained by light signals via photoreceptors, while the evening peak is regulated by the TTFL involving *per* and is entrained by CRY. Studies by Yoshii and coworkers (2004) with the mutant cry^b showed that activity rhythm in these flies has a propensity to dissociate into two components with increasing light intensity, one with a short and the other with a long period (Yoshii *et al.*, 2004). Their investigations led them to the hypothesis that in these flies, the evening oscillator

is itself composed of two oscillators, both of which are PER dependent and receive information from photoreceptors (Yoshii *et al.*, 2004). Another approach has been to eliminate different subgroups of clock neurons or to restore clock gene expression in specific neurons in clock mutants (Grima *et al.*, 2004; Stoleru *et al.*, 2004), or to over express core clock genes in different neuronal subgroups to accelerate or alter the molecular oscillations in different neuronal subgroups in an attempt to change the timing of oscillator phase relationships between different oscillator subgroups (Stoleru *et al.*, 2005; Murad *et al.*, 2007; Stoleru *et al.*, 2007). The conclusion from these 2004 studies (Grima *et al.*, 2004; Stoleru *et al.*, 2004, Figure 5) was that the LN_v function as the M oscillator in the *Drosophila* circadian pacemaker circuit, while the LN_d functions as the E oscillator (while Stoleru and colleagues acknowledge that they cannot functionally distinguish between various CRY^+PDF^-cells, they favor the LN_d as the E oscillator because of the efferent connections from the LN_d to the LN_v region, pg. 868 in Stoleru *et al.*, 2004). One of these studies showed that transgenic flies lacking LN_v exhibit weak or no anticipation to lights-ON (morning) and altered lights-OFF (evening) anticipation (Figure 2 in Stoleru *et al.*, 2004). Flies that lacked most dorsal neurons exhibit disruptions in evening anticipatory activity. More recently another more detailed study of the behavioral polyrhythmicity and its underlying molecular basis in the cry^b mutant was conducted (Rieger *et al.*, 2006). The behavioral read-outs used to interpret the above studies included: 1) anticipatory activities during dawn and dusk and 2) "splitting" of morning and evening activity bouts and rhythmicity in LL (Grima *et al.*, 2004; Stoleru *et al.*, 2004; Yoshii *et al.*, 2004; Rieger *et al.*, 2006; Stoleru *et al.*, 2007).

Although, the claim has been made that the two oscillators are distinct but somehow coupled (Stoleru *et al.*, 2004), the results of this study along with numerous previous studies (Renn *et al.*, 1999; Blanchardon *et al.*, 2001; Nitabach *et al.*, 2002) clearly show a functional contribution by the so-called morning cells (LN_v) to the evening bout of activity. Further, cell-specific rescue experiments in circadian clock mutants indicate that absence of LN_v molecular oscillation does not modulate LD behavior (Stoleru *et al.*, 2004). While absence of *per* in the LN_v had no effect either on the morning or evening anticipatory activity, its absence in *cry* expressing cells or in all brain neurons abolished the morning peak and had marginal effect on the evening anticipatory activity (Fig. 4 in Stoleru *et al.*, 2004). Further, while absence of LN_v or its output (PDF) is known to abolish morning anticipatory activity it also modifies the evening anticipatory pattern (Figure 2 in Stoleru *et al.*, 2004; Figures 4 & 8 in Renn *et al.*, 1999; Figure 3). In both cases the evening anticipation and the evening peak are phase advanced. These results are consistent with those of previous studies that reported loss of morning anticipatory activity and phase advancement of evening activity peak in flies lacking LN_v function by ablation or carrying loss of function mutation for PDF or electrical silencing (Renn *et al.*, 1999; Blanchardon *et al.*, 2001; Nitabach *et al.*, 2002).

In the parallel study which tried to identify the M and E oscillators by restoring clock function in either the LN_v or in LN_v plus LN_d by transgenically expressing PER in *per* null (per^0) genetic background (Grima *et al.*, 2004), it was shown very convincingly that restoring PER expression in the LN_v reinstated the morning anticipatory activity alone, while restoring PER expression in both the LN_v plus LN_d restores both morning and evening anticipatory activity (albeit at weaker amplitudes than wild-type flies). This result is the most striking piece of evidence supporting the role of LN_v in regulating morning anticipatory activity via the action of PER protein. Although it was possible to restore the LD activity waveform by expressing PER in the LN_v and/or LN_d of per^0 flies, only 42% of flies showed weak rhythmic activity in DD and the activity profile showed a relatively broader peak in rescued flies compared to wild-type flies suggesting that additional dorsal neurons are important for the normal wild-type DD circadian behavioral pattern (Grima *et al.*, 2004). What is not clear is why disrupting LN_v function has such profound effect on the evening activity peak (Figure 3). One possibility is that LN_v inhibit the onset of evening activity bout—and that by ablation or electrical silencing

this putative inhibition is removed. Alternatively, since PER is a transcription factor that potentially influences the expression of hundreds of genes (Claridge-Chang *et al.*, 2001), the absence of PER could cause defects in receptors or other molecular machinery needed for cell-cell communication between different groups of pacemaker neurons.

In a follow-up to their previous study, Stoleru and coworkers (2005) using a slightly different approach speeded up molecular oscillations in different neuronal subgroups by ectopically expressing SGG (a circadian clock protein described in the section on molecular oscillations) in neuronal subsets of the pacemaker circuit, and examined its effect on activity/rest rhythm during the immediate three days following transfer to DD, and estimated the speed of the molecular oscillators on the fourth day in DD (measured in terms of mRNA levels) in the rest of the circuit (Stoleru *et al.*, 2005) (Figure 4F–H). The speed of the mRNA oscillations in the sLN_v was shown to significantly influence those in the LN_d, DN_1 and DN_3 cells, while the DN_2 cells remained unaffected. Surprisingly mRNA oscillation in lLN_v also remained unaltered despite the over expression of SGG in these cells. Further, when SGG expression is restricted to non-LN_v pacemakers, as expected, the molecular oscillation remained unaltered in sLN_v, but surprisingly the LN_d, DN_1 and DN_3 also remained in phase with sLN_v despite the presence of the accelerator molecule SGG in these cells (Figure 4H). The molecular oscillation was phase advanced in the DN_2 and lLN_v neurons (even though SGG is not expressed in lLN_v). Based on these results the authors proposed that there are two parallel circuits, one which comprises both the “morning” and “evening” oscillators and consists of the sLN_v (morning cells) controlling LN_d, DN_1, and DN_3 cells (evening cells) and the second circuit whose function is unknown, comprising cell autonomous DN_2 (dominant) oscillator and lLN_v. It should be noted that the *tim-GAL4* driver regulates expression in many more cells (including neurons and glia throughout the body) of the adult fly than those listed above (Kaneko and Hall, 2000). Further, the authors propose that sLN_v neurons provide a daily resetting signal that can function both as a delaying and advancing cue to other members (evening cells) within its circuit. The authors propose that PDF is likely to be the molecule that performs this function. These results are consistent with earlier reported findings that PDF regulates the overall synchrony of multiple oscillators distributed throughout the pacemaker circuit (Petri and Stengl, 1997; Lin *et al.*, 2004). Further studies will be necessary to unveil how both advances and delays in molecular oscillations are achieved by PDF. One unexplained inconsistency between this (Stoleru *et al.*, 2004) and other studies (Veleri *et al.*, 2003; Nitabach *et al.*, 2006) examining molecular oscillations in DD is that this study reports synchronous molecular oscillations on the fourth day in DD in all the pacemaker cell groups, while other studies have shown that DN_2 cells have anti-phase oscillations in PER protein by day 5 in DD (Veleri *et al.*, 2003, Nitabach *et al.*, 2006). This inconsistency may have arisen due to the fact that this study examined *tim* mRNA levels, while others assayed PER protein levels. If one compares activity profile over a 24-hour duration with *tim* mRNA levels in different neuronal subgroups on the fourth day in DD (Figure 4F–H), it appears that at this stage in DD phase of mRNA oscillation and activity peak has poor correlation with any of the neuronal subgroups. The reliability of mRNA levels as determinants of the state of circadian oscillator is potentially questionable based on the discussions in the earlier section herein on post-transcriptional regulation of molecular oscillator components.

More recent studies by Stoleru and coworkers (2007) and Murad *et al.* (2007) propose that dorsal neurons act as circadian pacemakers under LL. However, the LN_v may not be completely dispensable in LL because these studies also show that activity/rest behavior when SGG is over expressed in all known clock cells in a pdf^{01} genetic background does not phenocopy the behavior seen when SGG over expression was excluded from LN_vin *pdf*+ genetic background. In the former case, almost all flies were *arrhythmic* in contrast to the latter where 90% of flies were rhythmic (Stoleru *et al.*, 2007). Although it would be useful to know the phenotype of flies that express SGG in non-LN_v cells in a pdf^{01} genetic background, the results of this study

suggests that rhythmic locomotor activity in LL, driven by non-PDF cells is dependent upon the availability of PDF. These results are further complicated by the fact that overexpression of *morgue* (a gene likely to be involved in the circadian light input pathway) in pdf^{01} genetic background resulted in rhythmic behavior in about 60% of flies (Murad *et al.*, 2007), suggesting PDF independent mechanism in the generation of rhythmicity in LL.

In a separate study Rieger *et al.*, 2006 examined the "splitting" of behavioral activity/rest rhythm to refine the neural correlates of the M and E oscillators as defined by the studies of Yoshii and coworkers (Yoshii *et al.*, 2004). The activity/rest rhythm of cry^b mutant flies in LL predominantly splits into two bouts, each of which free-runs with either a faster or slower than 24-hour rhythm (short-period and long-period activity bouts, respectively) and respond in opposite ways to increase in light intensity, suggesting that they may be behavioral manifestations of M and E oscillators (Rieger *et al.*, 2006). They argue that the designation of LN_v and LN_d as M and E oscillators respectively is only partly justified. Their studies of cry^b mutants in LL examined the level of PER and TIM in different neuronal subgroups on day 1 or day 5 after flies were released into LL at different phases using level of activity as reference (Figure 4O, P). On day 1 of LL cry^b flies show only one distinct peak of activity while on day 5 they show two distinct peaks. The authors note that the two bouts do not appear to be derivatives of morning and evening activity bouts of the preceding LD regime, as the faster running bout invariably emerges from the evening activity bout. While all the neuronal subgroups, with oscillating levels of PER appeared to be in-phase on day 1 of LL and have high levels coinciding with the activity trough (Figure 4O), on day 5 sLN_v becomes antiphasic with LNd and fifth sLN_v and shows high PER levels coinciding with trough of short-period activity bout (Figure 4P). These results led to the conclusion by Rieger and colleagues (2003) that the four PDF expressing small LN_v function as both morning and evening oscillators ('M-E' oscillators, or 'Main' oscillator), while one PDF negative small LN_v (the fifth small LN_v) along with one of the LN_d cells forms the evening oscillator. The differential response of change in speed of the oscillator with changes in light levels supports the original M and E oscillator model, in LL (Wheeler *et al.*, 1993; Helfrich-Forster, 2000). Thus in contrast to the three preceding studies (Grima *et al.*, 2004; Stoleru *et al.*, 2004; Stoleru *et al.*, 2005) the cry^b results suggests that the PDF positive small LN_v neurons regulate not only the morning activity but also partly the evening activity, indicating that the circadian pacemaker circuit may be far more complex than envisaged in simple labeled-line models (Grima *et al.*, 2004; Stoleru *et al.*, 2004; Stoleru *et al.*, 2005).

The empirical evidence for anatomically restricted neural substrates for the oscillators that regulate the morning and evening anticipatory behavior in *Drosophila* is equivocal and it appears that many cell groups may jointly regulate activity/rest rhythm whether in LD, LL, or DD. If the LN_v and LN_d neurons are indeed the M and E oscillators of *Drosophila* respectively, they should respond differently to DD and LL. This was explored in a recent study (Stoleru *et al.*, 2007) by manipulating different subgroups of pacemaker neurons using ectopic expression of SGG. Over expression of SGG in all known clock cells using *tim-GAL4* driver makes the flies rhythmic in LL in contrast to wild-type control flies and those where SGG over expression is restricted to the LN_v; both of which are arrhythmic. Stoleru and colleagues (2007) conclude that the so called evening cells and DN_2 subgroups of neurons regulate the pacemaker speed in LL based on the interpretation that the DN_1 and DN_2 are the only cells that exhibit robust cycling in nuclear localization of PER with a pattern similar to that observed in DD in wild-type flies (Shafer *et al.*, 2002). Yet, the authors did not detect significant circadian oscillation in the levels of PER or TIM protein, which has been until now considered as a marker for circadian clock function (Stoleru *et al.*, 2007). It is worth noting in this study that by the use of the *tim-GAL4* driver in conjunction with the subtractive *pdf-GAL80* driver, the authors essentially extend the definition of "evening" cells to the entire set of clock cells that express TIM with the exception of PDF positive LN_v.

Another critical unexplained inconsistency in the assignment of distinct M and E oscillators to the LN_v and LN_d groups is that despite a near 12-hour phase difference between the observed morning and evening activity peaks, the molecular oscillations (both *tim* mRNA and PER protein) in the LN_v and LN_d neuronal groups remain nearly perfectly in phase in LD conditions, a true Pas de Deux (Stoleru *et al.*, 2004; Bachleitner *et al.*, 2007, Figure 4A, E). This raises the possibility that the neural circuits that control morning and evening activity bouts may be downstream from the LN_v and LN_d circadian pacemakers. The present M and E oscillator model for *Drosophila* has no explanation for how two sets of oscillators that operate in-phase can control temporally distinct bouts of behavior that are almost completely antiphasic. A comparison of activity levels with the oscillations in the known pacemaker cells (irrespective of whether mRNA or protein levels are examined), suggests that it is highly unlikely that the phase of molecular oscillations and phase of activity have a simple one-to-one correlation (Figure 4).

One possible explanation for the conundrum noted above is that different oscillators can control different outputs at a downstream circuit level. Previous studies in mammals have shown that nocturnal and diurnal rodents that show similar phase of PER oscillation have differences in the brain regions outside the pacemaker center (Smale *et al.*, 2003; Nixon and Smale, 2004; Schwartz *et al.*, 2004; Saper *et al.*, 2005; Schwartz and Smale, 2005). This suggests that molecular and behavioral oscillations may not be linearly correlated and that unimodal rhythm in clock protein cycling might be transformed into a bimodal output. The other possibility is that circadian pacemaker neurons may segregate in a spatio-temporal manner, wherein the same neural subgroup may function as both morning and evening oscillators at different times of the day. Indeed, some of the results of studies described above show that there may be a complex hierarchy among different neuronal subgroups, most of which is still largely unclear, and that circadian outputs are regulated efforts of the entire pacemaker circuit. However, such inferences have not been taken into account, instead it was first proposed that LN_v act as the M oscillator and the LN_d act as the primary E oscillator (Grima *et al.*, 2004; Stoleru *et al.*, 2004; Stoleru *et al.*, 2005; Stoleru *et al.*, 2007), and later the cell groups LN_d, the DNs (with the exception of the DN_2, Stoleru *et al.*, 2005) were extended as E oscillators. In the most recent study *tim-GAL4/pdf-GAL80* driven SGG expression (which essentially targets ~ 90% LN_d; 55% DN_1; 75% DN_2 and 40% DN_3) was implied to target E cells (Stoleru *et al.*, 2007). Thus we have seen a rather flexible assignment of functional identity (morning or evening cells) partly due to constraints due to the lack of availability of more restricted driver lines and the urge to define simple distinct anatomical neural correlates to the two-oscillator model.

Does the M and E Oscillator Model Explain How Circadian Circuits Adapt to Seasonal Change?

One of the original motivations for the development of the M and E model was to explain how the circadian circuit tracks daily and seasonal changes in day and night length. Seasons are marked by gradual changes in day length, spectral properties of light throughout the day, temperature, food availability, and often profound long-term alterations in physical environment. The studies above have led the proponents of the *Drosophila* M and E oscillator model to test whether pacemaker neuronal subgroups respond differently to environmental manipulations that are proxies for seasonal change—and if so, whether M and E oscillator organization accounts for entrainment to varying photoperiods encountered due to changing seasons. Stoleru and colleagues (2007) show that flies with SGG over expression in all known clock neurons (with the exception of PDF-positive LN_v) show diminished responses to light pulses during the early part of the night and conclude that “evening” cells govern entrainability at these phases. In DD, LN_v-restricted over-expression of SGG causes shortening of period (τ_{DD} = 21.7 hours), close to the shortening achieved when SGG is over expressed in all known pacemakers τ_{DD} = 20.8 hours), whereas SGG over expression in the dorsal neurons alone has

no observable effect on period (τ_{DD} = 23.7 hours) (Stoleru *et al.*, 2005). Previous studies have shown that the LN_v are responsible for entraining circadian rhythms especially during the late subjective night (Emery *et al.*, 2000). Since the LN_v rely more on the changing levels of clock proteins during the night, it is hypothesized that the active phases during long winter nights would be locked to dusk (Stoleru *et al.*, 2007). In other words, by virtue of having greater sensitivity to light during the early subjective night, the dorsal neurons would play a greater role during the long summer days, while the LN_v neurons that serve as the key oscillators in DD, are expected to play a prominent role in winter conditions. The authors tested this hypothesis by comparing the anticipatory activity of flies under different photoperiods which may be comparable to those that flies experience if they live in temperate latitudes during spring/fall (12:12 hours LD), summer (14:10 hours LD) and winter (10:14 hours LD). The phase of anticipatory activity to both the ON and OFF transitions of flies where molecular oscillations in LN_v or dorsal neurons were speeded-up was compared to control phase of anticipatory activity. Under spring/fall-like conditions both types of genetic manipulations resulted in phase advances in both transitions, although the magnitude of transition was always greater when dorsal neurons were manipulated. Under “summer-like” photoperiod, control flies show almost no anticipation to lights ON. Under the same light regimen there is a dramatic phase advance in evening anticipatory activity when LN_v are speeded up, this advance is even greater with speeding-up of dorsal neurons. But both manipulations barely evoke phase advances for morning anticipation. The authors interpret their results to imply that dorsal neurons by virtue of their more dominant clock under long days have the ability to phase shift both morning and evening anticipatory activity.

Murad *et al.* (2007) also hypothesize that DN_1 are likely to possess a dominant function during “summer-like” long day photoperiods due to the persistence of molecular oscillations in these cells under LL as opposed to the dominance of LN_v during “winter-like” conditions due to their role in the persistence of rhythms in DD. Rieger *et al.* (2006), propose that the extended period of activity seen under long day conditions are in fact due to the period-shortening of the PDF positive sLN_v (main oscillators) and the period-lengthening of the fifth sLN_v evening oscillator in combination with one of the LN_d which is effected by input from the compound eye photoreceptors. They maintain that CRY on the other hand, causes period lengthening in all pacemaker neurons in which it is present (which include the so called morning and evening cells). Thus the above studies suggest a complex interaction among pacemaker neurons rather than a simple anatomically distinct two-oscillator mechanism enabling entrainment to diverse photoperiods in *Drosophila*. Further, the use of the *tim-GAL4* driver, which is known to have a wide pattern of expression including the eyes and other tissues, limits the interpretive power of the results (in terms of oscillator identity) in some of the above studies (Stoleru *et al.*, 2004, 2005, 2007; Murad *et al.*, 2007). A more refined separation of neuronal subgroups along with an experimental paradigm that mimics other environmental factors that are dramatically altered in the different seasons (such as temperature) will probably give us a better understanding of how the neural circuit adjusts to changing seasons.

How faithful are the experimental conditions used in the above studies representing seasonal change? Specifically, can altered day length effectively act as a signifier to the animal for seasonal state? Yes, indeed many studies using mainly multivoltine insect species have shown that they exhibit over-wintering diapause in response to short photoperiods (reviewed in Saunders, 2005). *D. melanogaster* enters ovarian diapause upon exposure to low temperature and short day lengths characterized by block of haemolymph yolk protein uptake by developing oocytes (Saunders, 1990). One possible mechanism for the induction of diapause has been thought to occur via circadian oscillator whose amplitude dampens with increasing night length (first proposed by Bunning, 1969). Is there a role for any of the known circadian molecular components in entrainment to seasonal changes? Under a range of different photoperiods in combination with different temperatures, the observed alterations in locomotor activity profiles

has been demonstrated to be due to alternate splice forms of a regulatory region of the *per* gene as well as gene phospolipase C (Majercak *et al.*, 1999; Collins *et al.*, 2004; Majercak *et al.*, 2004). A recent collaborative effort by several research groups revealed two alleles of *tim* gene among populations collected from different latitudes in Europe: one that codes for an ancestral short form and a more recent derivative that codes for both a long and short form (Sandrelli *et al.*, 2007; Tauber *et al.*, 2007). Based on molecular, biochemical and behavioral analysis of seasonal ovarian diapause the authors conclude that light signals are perceived by both the seasonal and circadian timer via TIM degradation pathways (Bradshaw and Holzapfel, 2007; Sandrelli *et al.*, 2007; Tauber *et al.*, 2007). In contrast, an independence of circadian molecule *per* and the photoperiodic timer was previously proposed by Saunders (1990) based upon studies of ovarian diapause in *per* mutant flies (Saunders, 1990).

Distributed Oscillator Model: an Alternative to the M and E Oscillator Model

Empirical evidence suggests that circadian pacemaker circuits in both invertebrates and vertebrates function in a distributed spatio-temporal manner. Taken as a whole it appears that different genetic manipulations push the organism's genetic architecture to different equilibrium steady states, each of which is capable of utilizing the perturbed pacemaker circuit to generate a unique behavioral pattern. While it was quite obvious from the beginning that results of empirical studies testing the M and E oscillator model by eliminating one or other components may not represent how oscillators function in wild-type animals, they have provided us with interesting insights as to how these neuronal subgroups interact to influence behavior when genetically per-turbed.

What is the oscillator organization in mammalian circadian circuits? One of the limitations of studying the circadian circuit in flies is the lack of a highly resolved continuous record of individual oscillators cycling in the large ensemble context of pacemaker circuit. Ideally, we would like to see the precise phase of oscillation between individual cells throughout an entire cycle. Immunocytochemical measurements of clock proteins in fly pacemaker neurons provide only snapshots of dynamic oscillator ensemble activity. While we lack the means to monitor multiple oscillators continuously in *Drosophila* at present, there is a growing body of such highly resolved data for the mammalian circuit. As noted above, cell autonomous firing of spontaneous action potentials occurs in isolated SCN neurons. Remarkably, spontaneous action potential firing in dispersed SCN neurons occurs with markedly different phases and periods (Welsh *et al.*, 1995). This phase and period dispersal of spontaneous firing does not appear to be an artifact of the isolated culture system, as highly variable circadian phase and period relationships between individual pacemaker neurons are found also in multiunit recordings of SCN *in vivo* and slice preparations (Meijer *et al.*, 1997; Schaap *et al.*, 2003). Direct comparison of the phase relationship of SCN multiunit firing *in vivo* and *in vitro* show that the phase of *in vivo* firing is actually more variable than *in vitro* (Meijer *et al.*, 1997). This suggests that greater phase variation between individual oscillators carries more information such as encoding of ultradian components (Meijer *et al.*, 1997).

Since circadian rhythmicity of molecular components of the clock is considered as *conditio sine qua non* by many investigators for circadian function, what do measurements of the clock itself say about the provocative findings above? Direct support for the electrophysiological findings comes from imaging studies of clock cycling readout in SCN slice cultures. The McMahon group imaged clock cycling in SCN slice cultures prepared from transgenic mice carrying the m*Per1* promoter-driven short-lived GFP reporter gene and find that highly variable phased clock cycling in individual neurons occurs across the SCN (Quintero *et al.*, 2003). The Okamura group came to fundamentally the same conclusion by imaging SCN slices prepared from mice carrying the m*Per1* promoter-driven luciferase reporter gene (Yamaguchi *et al.*, 2003). They find also that blockage of spontaneous action potentials by bath application of the

voltage-gated sodium channel blocker tetrodotoxin (TTX) further disrupts the synchrony and amplitude of individual oscillators. Thus, there is considerable physiological evidence to suggest that the SCN oscillator organization is dispersed as multiple oscillators with widely differing phases and periods. This suggests a more complex organization for the mammalian SCN, akin to the Tarantella (the pell-mell circle dance enjoyed at old-fashioned Southern Italian weddings—as depicted in the film *The Godfather*). The above results collectively raise questions of whether simple two component models such as the M and E oscillator model, while appealing, can fully account for the functional operations of circadian neural circuits (see also Rohling *et al.*, 2006 for a recent insightful discussion on the SCN). In the case of *Drosophila*, the limited temporal resolution of current methods precludes assignment of phase relationship between molecular oscillations in the various oscillator subgroups and behavior. The complexity of different oscillator phases in relation to behavioral activity summarized in Figure 4 suggests that a Tarantella model may apply to the *Drosophila* circadian circuit.

Drosophila has been a fruitful test ground for understanding circadian biology from molecular to behavioral levels. While much of our discussion on the *Drosophila* TTFL clock model and M and E circuit model is critical, these studies have collectively provided what is arguably our best understanding of circadian mechanisms for *any* model organism. The noted inconsistencies in present models raise questions for future tests. To what extent are transcriptional loops required for self-sustained clocks? How does clock cycling couple to pacemaker neuronal activity? How does phase distribution of oscillation among different neuronal groups in the pacemaker circuit code for circadian physiological and behavioral output? How do different oscillator groups communicate synaptically? Are oscillator subpopulations anatomically fixed to two functional morning and evening groups, or are they plastically distributed throughout the circuit by recent responses to environmental changes? Can we detect changes in clock and pacemaker circuit function in response to realistic tests of gradual seasonal change? And finally, how does the circadian circuit output couple to circuits that mediate activity versus sleep? Much of what we have learned about circadian biology in flies has informed our understanding of circadian mechanisms in mammals. There is a remarkable conservation of function at the level of the molecules that constitute the circadian clock. The field of *Drosophila* circadian biology is currently enjoying a shift from inquiry focused on the molecular components of the circadian clock to understanding how circadian information is encoded at the systems level. The advent of more powerful physiological and imaging tools combined with genetics promises to soon reveal whether there are similar levels of conservation for the cellular and circuit physiology between flies and mammals.

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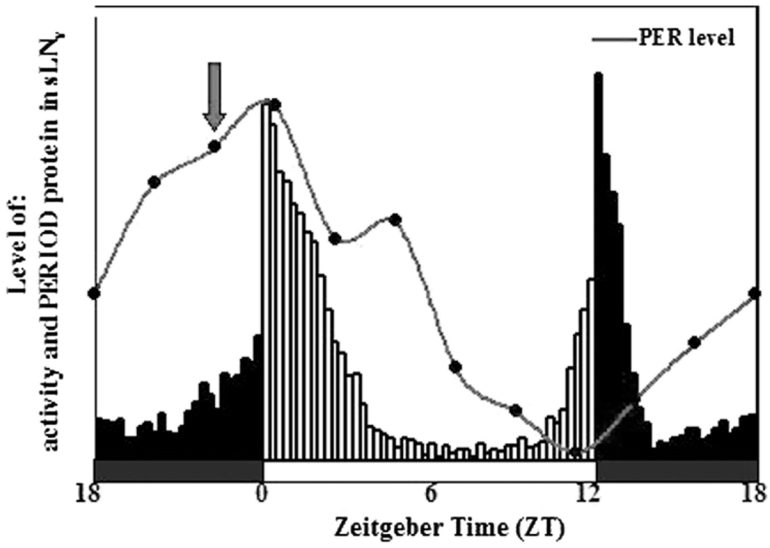
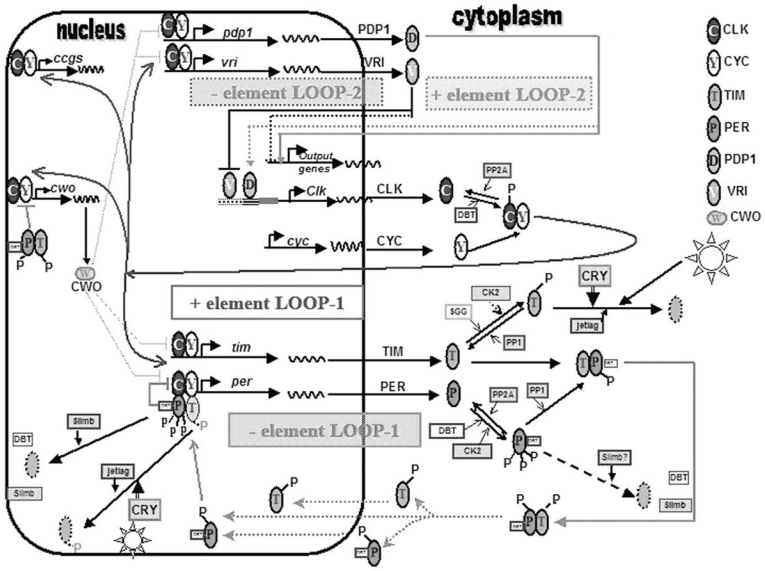


(a)



(b)

FIGURE 1.

FIGURE 1A The *Drosophila* molecular circadian clock in pacemaker cells: the essence. The rhythm in activity levels of adult flies shows two peaks, one around dawn and the other around dusk. The pattern of activity rest rhythms is greatly influenced by level and sub cellular localization of PERIOD protein in a group of ventral lateral pacemaker neurons (small LN_v and similarly in other pacemaker neurons). Highest levels of PER is seen around dawn (defined as Zeitgeber Time 0, ZT0) and peak nuclear localization occurs shortly before dawn (around ZT 22, Shafer *et al.*, 2002; *red arrow*). The levels of *per* mRNA follow a similar pattern with an approximately 6-hour phase advance. Oscillations in mRNA, protein levels and post-translationally modified states of several other genes have been implicated in the generation of rhythmic behavioral and metabolic processes, the intricacies of which are described in Figure1B.

FIGURE 1B The *Drosophila* molecular circadian clock in pacemaker cells: the gory details. The *Drosophila* circadian clock consists of interlocked sets of transcription/translation

feedback loops (TTFL), which have mRNA and protein components that cycle in abundance and subcellular localization with a near 24-hour period. Dotted or dashed arrows indicate pathways that are not completely resolved or not considered essential for the self-sustained biochemical oscillator function. Lines ending in arrows indicate activation while those ending in a hatched bar indicate repression or inhibition. Degraded proteins are indicated by ovals with dotted walls. Light ordinarily recalibrates the clock at the onset of each day. During the previous night, PER and TIM proteins have accumulated at their highest levels in the nucleus, acting as transcriptional repressors of their own mRNA expression by binding to the transcriptional activators (only *per* repression is shown for simplicity) CLK-CYC, thus forming the negative limb of one of the TTFLs (loop1). Starting at dawn, light degrades TIM via a CRYPTOCHROME (CRY) mediated pathway, subsequently monomeric PER is phosphorylated and degraded after SLIMB an F-Box protein marks it for proteosomal degradation. By noon, PER and TIM's degradation releases transcriptional repression and the transcription of *period* (*per*) and *timeless* (*tim*) genes is activated by CLK-CYC which bind to E-box sequences in *per* and *tim* promoters forming the positive limb of loop1 and attaining peak levels of *per* and *tim* transcripts at dusk. In the cytoplasm, DOUBLE-TIME (DBT) kinase complexes with and destabilizes PER by phosphorylating and thus facilitating its subsequent degradation. PER and TIM are each also phosphorylated by Casein kinase 2 (CK2) and SHAGGY (SGG), respectively. Since TIM is light sensitive, TIM levels can begin to rise in the cytoplasm only after dusk, after which it complexes with the DBT-PER heteromers. Levels of PER and TIM are maximal by mid-night. The entry of DBT-PER/TIM heteromultimer into the nucleus around midnight is controversial, it is possible that they dissociate such that PER (along with DBT) enters the nucleus at least 3 hours before TIM does. Cytoplasmic PER is stabilized by Protein Phosphatase 2A (PP2A), which dephosphorylates PER while Protein Phosphatase1 (PP1) dephosphorylates and stabilizes TIM thus promoting PER accumulation and hetero-dimerization. Total CLK levels remain constant with circadian oscillation in its phosphorylation state due to the action of the multifunctional DBT and perhaps other kinases and phosphatases including PP2A. CLK heterodimerizes with the constitutively present CYC and this CLK-CYC complex in addition to activation of *per* and *tim* transcription also binds to E boxes of at least two other genes *vrille (vri*) and *par domain protein-1ε(pdp-1ε*) to activate their transcription. PDP-1 in turn activates transcription of *Clk*, while VRI represses it by competitively binding to regulatory sequences called VRI/PDP1ε- boxes (V/P-boxes, shown by *dotted and hashed line*), upstream of *Clk*, forming a second feedback loop that interlocks with the first via CLK/CYC. Recent studies indicate that the second loop may not be an essential component of the circadian pacemaking machinery as *Clk* cycling is nonessential for clock function. Instead, the cycling in phosphorylation state of CLK is thought to contribute towards maintaining a robust period. In addition to core-clock genes, CLK regulates mRNA levels of several output genes (and *cry* via VRI and possibly PDP1ε- not shown). PER-TIM complex is also thought to repress CLK/CYC transcription of *vri* and *pdp-1ε*(not shown). PDP1εis now believed to function as an oscillator output component rather than a central oscillator component. The amplitude of the circadian biochemical oscillator may be regulated by *Clk*-mediated transcriptional activation of various genes including *clockwork orange* (*cwo*) which is proposed to contribute to robustness of the amplitude of mRNA oscillations of *vri*, *pdp-1ε*, *tim*, and *per*. For a depiction of how the amounts and location of these clock molecules vary over time (see Yu and Hardin 2006).

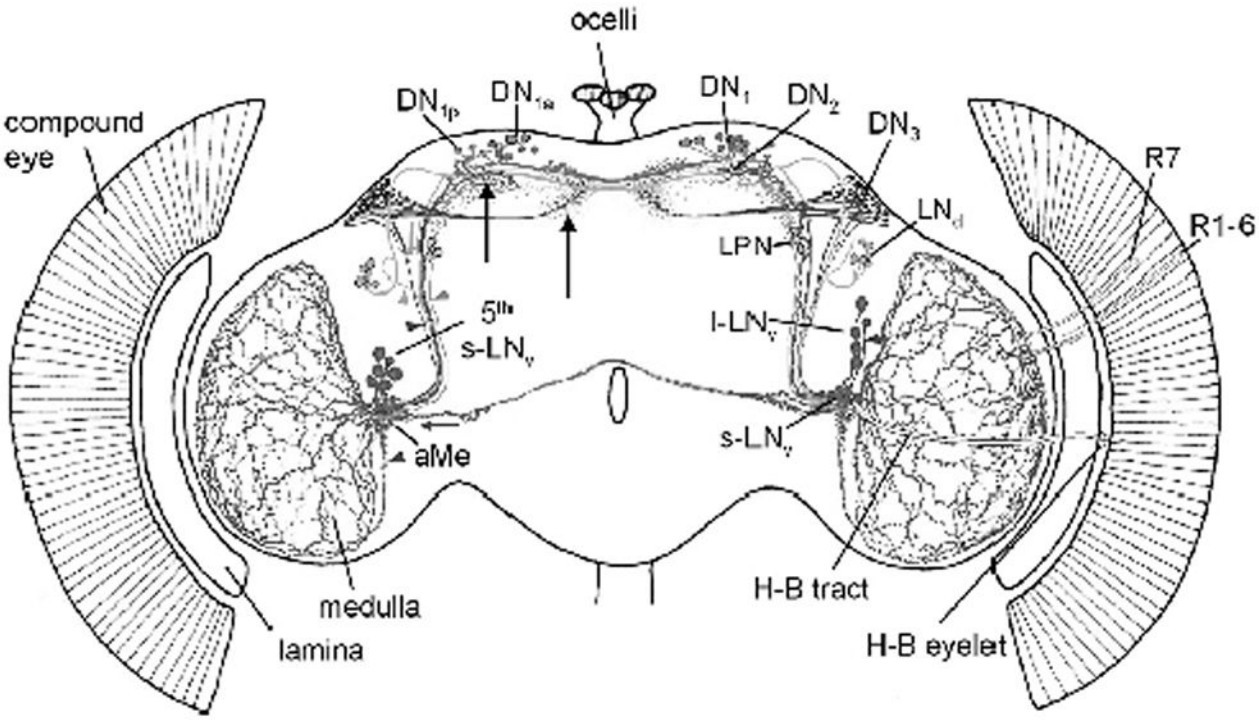


FIGURE 2.

Neuronal network that regulates circadian rhythmicity in the adult *Drosophila* brain. Locations and putative arborization patterns of *Drosophila* clock neurons as originally illustrated by Helfrich-Forster *et al.* (2007). Each neuronal cluster is depicted in distinct color for clarification of neuronal morphology in a frontal view of a brain: Large LN_v, *brown*; PDF-positive small LN_v, *red*; PDF-negative fifth small LN_v, *dark violet*; LN_d, *orange*; $DN_{1a,p}$, lilac; DN_1, *blue*; DN_2, *light blue*; DN_3, *navy*; LPN cell bodies, *green*; photoreceptors including H-B eyelet, *yellow*. Bedsides PDF-positive LN_v, fifth small LN_v (*dark violet arrowhead*), $DN_{1a,p}$ (*lilac arrowhead*), LN_d (*orange arrowhead*), and DN_3 (navy arrowhead) invade the AMe ipsilaterally. Large LN_v (*brown arrow*) and LN_d (*orange arrow*) send contralateral projections to the AMe. *Brown arrowhead* points to the ventral elongation of the AMe which receives innervations from large LN_v. (Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

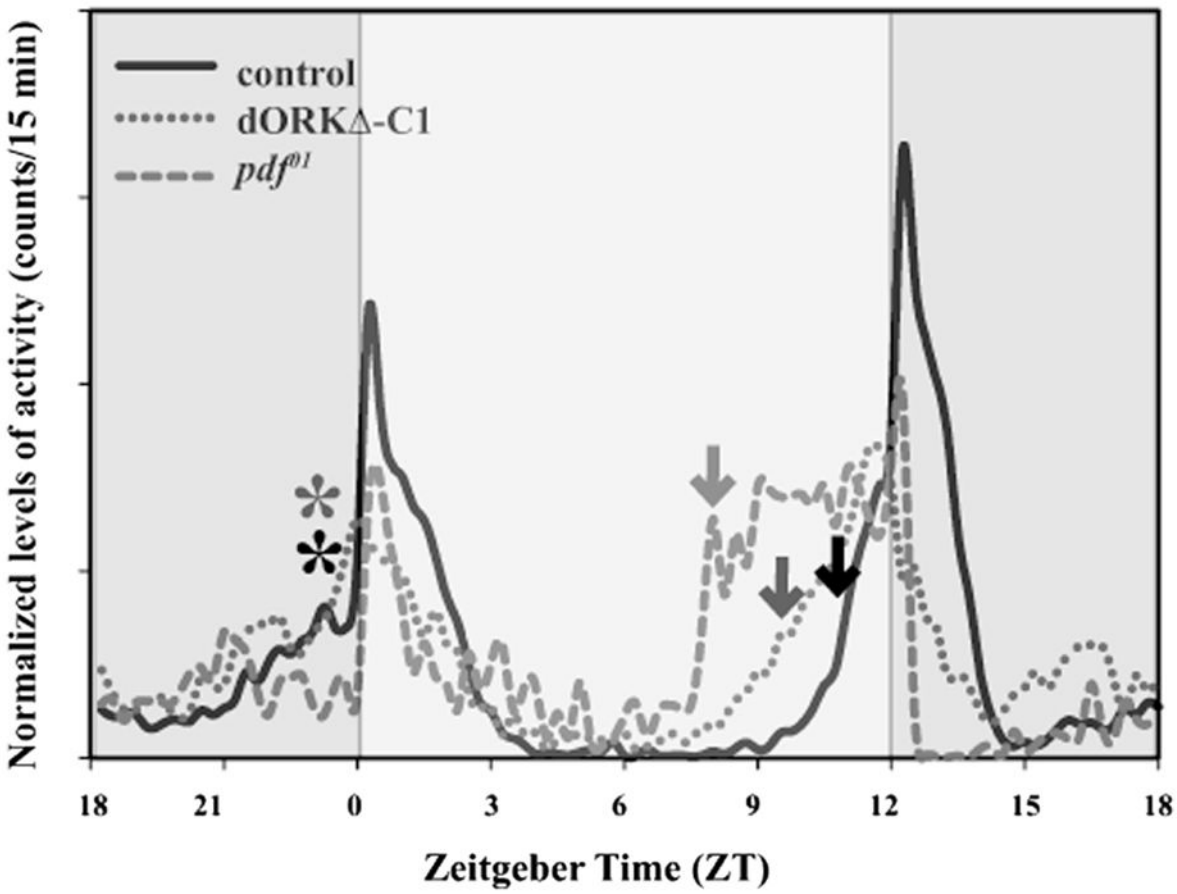


FIGURE 3.

LN_v regulation of morning and evening locomotor activity. Representative activity profiles of three genotypes under 12:12 hour light/dark cycles, *blue shaded areas* represent darkness, and the *yellow shaded area* represents light. All three genotypes show enhanced activity around dawn and dusk. The controls (*solid black curve*) show an increase in activity both in anticipation of dawn and dusk as indicated by the *black asterisk* (morning anticipation) and *arrow* (evening anticipation). When LNv neurons are electrically silenced by targeted expression of the dORKΔ-C1 channel (*red dotted curve*) evening anticipation is phase advanced as indicated by the *red arrow* and occurs around 3 hour before lights OFF while morning anticipation is not significantly altered (*red asterisk*). A more severe phenotype is obtained with expression of Kir2.1 channel with both a loss of morning anticipation and a shift in evening anticipation (data not shown). The null mutant pdf^{01} (*green dashed curve*) shows complete loss of morning anticipation as well as a large phase advance in the evening anticipation. These results indicate a role of the LN_v cells in mediating both peaks of the activity rest cycle in *Drosophila*.

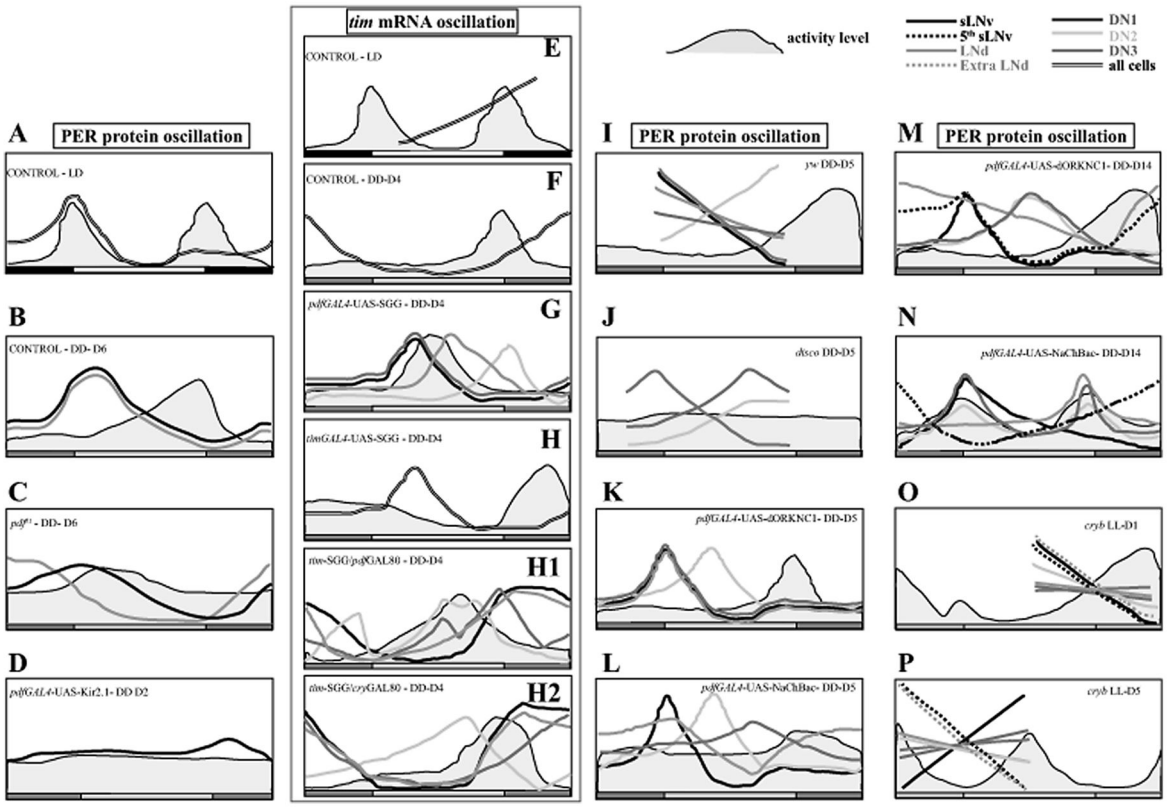


FIGURE 4.

Schematic summary of locomotor activity level and its relationship with molecular oscillation in *Drosophila* brain circadian pacemaker neuronal subgroups. *Horizontal bars* at the bottom of each panel depict the light regime. *White* and *black bars* indicate the light and dark duration under light/dark (LD) cycles. Constant darkness (DD) is denoted by *pale* and *dark grey bars* corresponding to the light and dark durations of previous entrainment regime and similarly constant light (LL) is denoted by white and pale grey bars. Activity level is indicated by the *grey filled wave* above the LD bars. The neuronal subgroups are color coded in most panels except in cases where the oscillation is synchronous in all the cells assayed, in which case a *double lined curve* is used. (A) The adult locomotor activity shows two clear peaks in activity under LD 12:12 hr and the level of PER in all cells occur at approximately the same phase and coincides with the morning peak in activity (Bachleitner *et al.*, 2007). Upon transfer to DD, locomotor activity of wild-type flies usually shows only one peak; very often this peak appears to be derived from the evening peak of prior entrainment. (B-C) Lin *et al.*, (2004), showed that molecular oscillations in sLN_v and LN_d stay tightly synchronized in case of wild-type controls (*yw*) for up to 6 days in DD, and that in pdf^{01} flies the dampening in activity rhythm is accompanied by a dampening in molecular oscillations among the sLN_v probably due to loss in intercellular communication among sLN_v and a phase advance as well as dampening in oscillations among LN_d, suggesting that PDF is the agent of synchrony both within and between sLN_v and LN_d subgroups. (D) Such a dampening and ultimate stop in molecular oscillations was also seen when LN_v are electrically silenced using Kir2.1 channels along with arrhythmic locomotor behavior in DD (Nitabach *et al.*, 2002). (E-H) mRNA levels have been used as an indicator of the state of the clock in some studies (Stoleru *et al.*, 2004, 2005, 2007). (E) Under LD 12:12 hr, high level of *per* mRNA is seen soon after lights OFF and the level is lowest around early morning. (F) Stoleru *et al.* (2005) report that mRNA level oscillates with a single peak on the fourth day of DD in all the neuronal groups examined and the phase of oscillation

remains in close synchrony among the different neuronal subgroups. (G) When SGG is expressed in LN_v, the "morning" cells, activity is phase advanced and so is the phase of mRNA oscillation in all cells; with greatest advance in sLN_v and DN_1 cells, followed by LN_d and lastly DN_2. (H) Such a phase advance is seen in all the neuronal subgroups to a similar degree when SGG is expressed using the *tim-GAL4* driver. (H1) When SGG expression is restricted to non-LN_v cells, only DN_2 cells (and lLN_v, not shown) are phase advanced and other cells are similarly phased as sLN_v indicating a dominance of sLN_v. (H2) Alternatively when DN_2 cells alone express SGG, the advance in molecular oscillation does not alter any of the other cells except lLN_v (not shown) (I-J) Veleri *et al.* (2003) assayed molecular oscillations in control (*yw*) and *disco* mutant flies after 5 days in DD and report that oscillation in DN_2 cells are now in anti-phase with the rest of the neuronal groups. *disco* mutants which lack almost all the LN neurons exhibit anti-phase oscillations in both DN_1 and DN_2 cells compared to DN_3 oscillations, which have similar phase as controls. (K) Out-of-phase oscillations in DN_2 were also detected in rhythmic control flies expressing dORK-NC1 channel after five days in DD (Nitabach *et al.*, 2006). (L) In contrast, flies expressing NaChBac channel in LN_v exhibit arrhythmic behavior and asynchrony in molecular oscillations among the different neuronal subgroups when assayed after 5 days in DD. (M) After 14 days in DD, control flies continue to exhibit robust rhythmic activity with a single peak in activity level. The peak in PER levels in the sLN_v occurs at the trough of activity level. The oscillation in DN_1 and DN_2 are delayed with respect to the sLN_v, and LN_d shows a dampened oscillation with a clear trough just before activity onset. (N) NaChBac flies show two clear bouts of activity at day 14 DD, one bout exhibits a shorter than 24-hour free running period and the other a longer than 24-hour free running period. The sLN_v show peak PER levels coinciding with the long-period activity bout. The DN_1 show two peaks each coinciding with one peak of activity. DN_2 cells also show higher PER levels coinciding with activity peaks, although they are not significantly different from the other two time points. Under LL, while most wild-type flies are arrhythmic, cry^b mutants exhibit two periodicities. This is evident after at least 5 days of LL (Rieger *et al.*, 2006). (O) On the first day of LL only one peak in activity is seen and at that time point, the sLN_v, DN_2 and a subset of LN_d express low levels of PER. At the trough of activity profile, the levels of PER is high in these cells. Other cells do not exhibit significant oscillation. On the fifth day of LL, the activity pattern shows two distinct bouts. DN_2, fifth sLN_v and one large LN_d (extra LN_d, *dotted orange line*) appear synchronous and express high PER coinciding with the activity peak of the short-period bout, and low PER levels corresponding to the time of the long-period bout. PER levels in other four sLN_v are in anti-phase with the above cells and have high PER coinciding with the long-period bout. The oscillations in other cell groups as determined by sampling at these two active phases are not statistically significant.