PHOTIC AND HORMONAL ZEITGEBERS TO A PHOTOSENSITIVE PROTHORACIC GLAND CLOCK THAT REGULATES STEROID SYNTHESIS IN THE INSECT *RHODNIUS PROLIXUS* (HEMIPTERA)

NICHOLAS RAPP

A Thesis submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of
Master of Science

Graduate Programme in Biology
York University
Toronto, Ontario

December 2013

GENERAL ABSTRACT

The prothoracic glands (PGs) in the insect *Rhodnius prolixus* contain a photosensitive circadian clock controlling synthesis of the steroid molting hormones. We examined the response of the PG clock to internal hormonal Zeitgebers. Immunohistochemistry revealed the depletion of nuclear PER from PG cells in both continuous light (LL) and continuous dark (DD). LL and DD PGs were incubated *in vitro* and exposed to a 1h pulse of brain neuropeptides. In LL PGs, but not in DD PGs, expression and circadian cycling of PER were reinitiated by both prothoracicotropic hormone (PTTH) and insulin-like peptide(s) (ILPs), and both peptides were capable of reinitiating rhythmic ecdysteroid synthesis. ILPs and PTTH both have known stimulatory effects on steroidogenesis by PGs. We infer at least two brain neuropeptide Zeitgebers are capable of contributing to entrainment of the PG clocks by acting as signals of darkness to the PGs and stimulating a rhythmic clock output (ecdysteroids).

ACKNOWLEDGEMENTS

Thank you to Dr. Colin Steel for introducing me to the world of scientific research and allowing me to participate in this project; it was truly an unforgettable experience.

Thank you to Dr. Xanthe Vafopoulou for showing me the techniques necessary for me to be successful, I will always remember the "lab parties".

Thank you to my lab mates Michael Cardinal-Aucoin, Martha Hindley-Smith, Tudor Saroiu and Samantha Leung; you were all there for the good times and the bad, offering never ending support and always able to put a smile on my face.

Thank you to my Mom and Dad for always believing in me and supporting me in everything I have taken on.

TABLE OF CONTENTS

List of Figures

Abbreviations

ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
CNS	Central nervous system
CRY	Cryptochrome; clock protein
CYC	CYCLE; clock protein
DABCO	1,4-diazabicyclo[2.2.2]octane; antioxidant used in mounting medium
DBT	Double-time, kinase of the PER protein
dCLK	*Drosophila* CLOCK; clock protein
DD	Continuous dark environment
DNs	Dorsal clock neurons
EcR	Ecdysteroid receptor
FITC	Fluorescein isothiocyanate
FRQ	Frequency, Clock gene/protein in fungi
IHC	Immunohistochemistry
ILP	Insulin-like peptide
LL	Continuous light environment
LNs	Lateral clock neurons
MO	Molecular oscillator
PDF	Pigment-dispersing factor
PER	PERIOD; clock protein
PFA	Paraformaldehyde; fixative
PGs	Prothoracic glands
PP2A	Protein phosphotase-2-alpha
PTTH	Prothoracicotropic hormone
RG	Ring Gland
RIA	Radioimmunoasssay
RFI	Relative immunofluorescence intensity
SCN	Suprachiasmatic nucleus
TIM	TIMELESS; clock protein
TOR	Target of rapamycin
TTFL	Transcription/translation feedback loop
TTX	Tetrodotoxin
WTC	White-collar, clock gene/protein in fungi

CHAPTER I:

GENERAL INTRODUCTION

1.1. Development of Circadian Rhythms

All organisms on Earth live in a rhythmic environment, containing daily cycles of environmental phenomena such as light/dark and temperature. This rhythmic environment is produced by the Earth spinning on its axis producing day lengths of approximately 24 hours. Circadian rhythms have been found in all groups of organisms existing within the 24 hour rhythmic environment of Earth, except viruses. The term circadian itself derives from the latin "circa diem" meaning "about a day". The key component of circadian rhythms is that they are endogenously controlled, independent of the external world. Thus, these endogenous circadian rhythms were first identified by placing organisms in aperiodic environments, those lacking external environmental cycles (e.g. continuous darkness). As these endogenous circadian rhythms were further elucidated specific criteria were set in order to indentify a rhythm as circadian. In order for a rhythm to be considered circadian it must: 1) persist in aperiodic conditions, in the absence of environmental cues, 2) the free-running period length of the rhythm must be approximately, but not exactly, 24 hours, and 3) The rhythm must be temperature compensated, meaning unlike most biological processes the timing is unaffected by changes in temperature within a physiological range (reviewed by Pittendrigh, 1993).

It is believed circadian clocks first evolved on primitive Earth in order to confine the process of DNA replication to the night to avoid the harmful cosmic radiation during

the day (Edmunds, 1988). These circadian clocks controlling DNA replication then spread to regulate the timing of other biological processes. The development of daily rhythmic biological processes (e.g. sleep/wake cycles, hormone rhythms, metabolism etc.) allowed organisms to be able to function within this rhythmic environment, as they are able to anticipate daily events (Pittendrigh, 1954; Henley et al., 2009). Circadian rhythms have been determined to be generated by specialized clock cells. Clock cells are located throughout multicellular organisms and contain specific molecular mechanisms used to produce rhythmic "clock gene" expression (see Section 1.2). Endogenous rhythmic expression of clock genes and their protein products leads to the production of rhythmic outputs to act on their target tissues. As mentioned above, the endogenous free-running period length of these rhythms, generated by circadian clocks, is not exactly 24 hours; therefore, circadian clocks synchronize with external timing cues. These external timing cues are known as Zeitgebers, German for "time-giver", and are generally environmental timing signals which occur with 24 hour periodicity. Some examples of Zeitgebers would be the daily light/dark cycle and daily temperature cycles (Pittendrigh, 1954); however, Zeitgebers will be more fully discussed in Section 1.4. Circadian clocks will synchronize to these daily Zeitgebers in order to maintain a precise 24 hour periodicity via a process known as entrainment (Nishiitsutuji-Uwo and Pittendrigh, 1968). Synchronization of circadian clocks to the daily 24h cycle allows organisms to function within the 24 hour cycle of their environments and produces biological phenomena at specific time points during a day. This synchronization is required because if the rhythms were allowed to free-run (i.e. occur in the absence of entraining

Zeitgebers) the biological processes they control would occur at slightly different times each day.

1.1.1. Importance of Studying Circadian Rhythms

Circadian rhythms have been found to govern the timing of essential biological processes in every organism in which they have been investigated. The ubiquity and evolutionary conservation of these endogenous biological rhythms speaks to the important role they play in allowing organisms to survive on Earth. All levels of biological functions have been found to be driven to some extent by circadian timekeeping. At the molecular level, rhythmic gene expression has been identified (Balsalobre et al., 1998); cellular processes such as liver metabolism (Lavery and Schibler, 1993) and nitrogen fixation are under circadian control, as are homeostatic mechanisms such as body temperature (Buhr et al., 2010), and whole animal rhythms such as the sleep/wake cycle (Shaw et al., 2002). Further supporting the importance of circadian rhythmicity, beyond their essentially ubiquitous presence, is the fact that disruption of these endogenous rhythms in all organisms studied has led to serious adverse effects (Arthur et al., 1930; Went, 1960; Pittendrigh and Minis, 1972; Boden and Kennaway, 2006).

The generation of internal desynchronization of circadian rhythms in animals has been demonstrated to be detrimental to the organism. Internal desynchronization is when endogenous biological rhythms are occurring out of phase with one another, due to rapid phase shifts or prolonged exposure to aperiodic conditions. Internal desynchronization is

the cause of a common phenomenon experienced by humans: jet-lag. The adverse effects felt by individuals travelling rapidly across time-zones, thus forcing a rapid phase shift in the circadian clockwork, are attributed to the internal desynchrony of the individuals' endogenous biological rhythms (Rudiger, 2004). Disruptions in the circadian timekeeping system due to internal desynchronization have been linked to serious health problems in mammals such as decreased lifespan, increased risk of depression (Turek, 2007; Lall et al., 2012), obesity, diabetes (Shi et al., 2013) and cancer (Bernstein, 2002; Savvidis and Koutsilieris, 2012). Shift workers experience these rapid phase shifts in their circadian clockwork due to their ever changing schedules and have been found to exhibit all of the above health issues with greater frequency than the rest of the population (Knutsson, 2003). Therefore, the importance of studying circadian rhythms to human health cannot be denied.

The benefits of the study of circadian rhythms are already being felt in human medicine. The use of chronotherapeutics/chronopharmacology is a rapidly expanding area of research producing positive results. Chronopharmacology involves administering drugs at specific times of day, which has been found to maximize the benefits/effectiveness of the drugs while at the same time minimizing any potential adverse side-effects (e.g. toxicity/ill-effects related to chemotherapy) (Lemmer and Labrecque, 1987). Additionally, hospitals are now beginning to recognize the negative effects of non-circadian lighting regimes. Therefore, the amount of ambient light and noise during the night is now being reduced and blue-light filters are being employed during the night. Light waves occurring in the blue spectrum are responsible for

entrainment of the molecular oscillator involved in circadian clocks (see Section 1.2). Blue light filters are being investigated as a potential method to reduce circadian disruption from improper lighting regimes but allow hospital staff to continue their work without hindrance (Udell, Jacob A., personal communication).

The importance of studying circadian rhythms is clear; however, trying to understand the underlying mechanisms of these systems in mammals/humans can be very difficult. The clock cells in the mammalian brain are located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is an extremely small structure composed of a heterogeneous population of cells (i.e. glial cells, neuroendocrine cells, clock neurons); therefore, studying circadian mechanisms directly in the SCN is a major challenge (Welsh et al., 1995). The molecular and cellular mechanisms composing the circadian clockwork have been highly conserved throughout the animal kingdom (Herzog, 2007). In fact, the original canonical clock genes, later found to have mammalian homologues, were discovered in the insect *Drosophila melanogaster* (Kanopka and Benzer, 1979; Smith and Konopka, 1981, 1982). The conservation of circadian clock machinery allows for the use of insect model organisms to elucidate complex underlying principles and later apply this knowledge to the more complex mammalian system.

1.2. The Molecular Oscillator-Transcription/Translation Feedback Loops

1.2.1. Molecular Machinery Behind the Clock

The existence of endogenous circadian rhythms had been well established in a wide variety of organisms for many years before molecular mechanisms controlling these rhythms were determined. There have now been as many as three distinct molecular mechanisms found to be involved in governing circadian rhythmicity. In the cyanobacterium *Synechococcus elongates,* cyclic expression of the Kai proteins acts as the molecular mechanism driving circadian rhythmicity (Dong and Golden, 2008). In the fungus *Neurospora crassa,* the clock genes *frequency (frq)* and *white collar (wtc)* are involved in a transcription/translation feedback loop (TTFL) that generate a circadian rhythm of conidiation (Dunlap et al., 2007). The molecular clock machinery discovered in animals additionally involves a series TTFLs to compose the molecular oscillator (MO), with the first clock gene discovered being the *period (per)* gene (Hardin et al., 1990). The *per* gene was originally discovered in *Drosophila* but was eventually found to have closely related homologues in mice/mammals (m*per* 1, 2, and 3). The evolution of these TTFL mechanisms and their subsequent conservation demonstrates their importance in circadian regulation of gene expression and ultimately circadian timekeeping.

It should be noted that there is an ever expanding literature regarding circadian rhythmicity in the absence of MOs (reviewed in Lakin-Thomas, 2006). Prokaryotes which lack true nuclei are able to maintain circadian rhythmicity in the absence of such a mechanism and an example in mammals can be found in red blood cells which have also

been shown to contain circadian rhythms in the absence of a nucleus (O'Neill and Reddy, 2011). Therefore, to say the MO is entirely responsible for generating circadian rhythmicity is a gross overstatement. It is a component of the clock, not the clock as a whole. The scientific community has only begun to scratch the surface of how circadian rhythms are truly generated by clock cells and what in fact composes the "clock".

1.2.2. Insect Molecular Oscillator

As mentioned above, the insect MO responsible for producing circadian rhythms of clock gene expression in clock cells is governed by a complex interaction between specific clock genes and their protein products. The canonical clock genes involved in this MO within clock cells are *period (per)* and *timeless (tim),* which produce their protein products PERIOD (PER) and TIMELESS (TIM). PER and TIM were determined to form a heterodimer that indirectly inhibits further transcription of the *per* and *tim* genes (Vosshall et al., 1994; Gekakis et al., 1995). Two additional clock proteins, CLOCK (dCLK) and CYCLE (CYC), were then discovered. These proteins also form a heterodimer which acts as a promoter of *per* and *tim* gene transcription (Hao et al., 1997; Allada et al., 1998; Rutila et al., 1998; Darlington et al., 1998). PER and TIM dimerize, enter the nucleus, and bind dCLK to prevent its dimerization with CYC (Lee et al., 1999; Yu et al., 2006). Preventing this dimerization inhibits the promoting effects on the *per/tim* genes. While this new aspect of the MO shed new insight into the molecular clockwork it could still not account for the photic entrainment and 24 hour periodicity of PER and TIM expression in clock cells. The discovery of the involvement of the

cryptochrome protein (CRY) helped to explain how entrainment to light:dark cycles is achieved (Emery et al., 1998; Ishikawa et al., 1999; Emery et al., 2000). CRY is a blue-light sensitive molecule which, when excited by light, will actively bind to and cause degradation of TIM (Ceriani et al., 1999; Naidoo et al., 1999). Degradation of TIM prevents the formation of the PER/TIM heterodimer (PER becomes unstable and degrades as well) (Lee et al., 1996). Degradation of the PER/TIM dimer prevents translocation of this complex to the nucleus, thus preventing binding to dCLK. dCLK can once again dimerize with CYC and promote the transcription of *per* and *tim*. The discovery of CRY explains the photosensitivity of clock cells within the MO as during the day light activates CRY to degrade TIM, only when light is no longer present can the PER/TIM heterodimer form. This allows for rhythmic expression of these clock proteins with peak expression during the night and low levels of expression during the day. The 24 hour periodicity of PER/TIM expression and the MO as a whole are controlled by post-translational modifications of the clock proteins. Phosphorylation and dephosphorylation of the PER protein by the kinases Doubletime (DBT) and casein kinase 2 (Kloss et al., 2001; Lin et al., 2002) and the phosphotase PP2A (Sathyanarayanan et al., 2004) respectively regulate the stability of PER and produce the daily 24 hour rhythm of expression of PER in the MO. Additional aspects of this MO have since been discovered that account for the rhythmic regulation of the dCLK protein (Cyran et al., 2003; Lim et al., 2007; reviewed in Allada and Chung, 2010); however, to avoid complication these aspects will not be covered here. Discovery of the canonical clock genes and proteins created a convenient tool for identifying clock cells within an

organism. Expression of these essential clock proteins is specific to clock cells; therefore, observation of rhythmic expression of these genes/proteins allows for the easy identification of clock cells in insects.

1.2.3. Mammalian Molecular Oscillator

Upon discovery of the *period* gene in insects, a set of homologous genes was discovered in mice and given the same name (m*per* 1, 2, and 3). The discovery of clock gene homologues in mammals continued over the years until eventually a MO similar to the one described in insects was identified, demonstrating the conservation of the MO mechanism across phyla (Allada, 2003; Stanewsky, 2003). The precise details of the mammalian MO will not be addressed here except to note the high level of conservation of this mechanism indicates the use of model organisms such as insects is extremely useful and highly applicable to mammalian clock systems.

1.3. Organization of Circadian Clocks

1.3.1. The Insect Brain Clock

The classical view of circadian clocks was of specialized clock cells located strictly in the brain acting as the master circadian clock. From the master brain clock, timing information would be passed, via nervous and/or hormonal outputs, to peripheral tissues containing "slave" oscillators. These slave oscillators were thought to be incapable of maintaining rhythmicity without receiving input from the master brain clock. The original view has now been modified with the discovery of a wide array of peripheral

tissue clocks able to maintain rhythmicity independent of brain inputs. The current widely accepted view is of the brain clock acting as the synchronizer of several clocks throughout an organism, ensuring all tissue clocks work together to provide timing information to the organism and coordinate the timing of essential biological processes (reviewed by Tomioka et al., 2012).

The discovery of essential canonical clock genes has allowed for the identification of clock cells within the insect brain, with clock cells generally being defined as those possessing cyclic expression of the clock proteins PER and TIM. Clock cells possessing cyclic expression of both PER and TIM have been identified in the brains of only two insects: *Drosophila melanogaster* and *Rhodnius prolixus* (Siwicki et al., 1988; Helfrich-Förster, 1995; Hunter-Ensor et al., 1996; Kaneko et al., 1997; Vafopoulou et al., 2010). Using these clock genes and cyclic clock protein expression as markers, the neuroarchitecture of the insect brain clock has been elucidated. Clock cells in the brain of both *Drosophila* and *Rhodnius* can be found in two distinct clusters per brain hemisphere. The first cluster of cells is located at the base of the optic lobe and is known as the lateral neurons (LNs); the second cluster is found in the posterior region of the brain and is known as the dorsal neurons (DNs). Both the LNs and DNs have been shown to contain cyclic expression of both PER and TIM (Helfrich-Förster, 1995; Vafopoulou et al., 2010), identifying them as clock cells. Axonal connections exist between the LNs and DNs in the same hemisphere (Kaneko and Hall, 2000); additionally, connections exist between LNs in contralateral hemispheres allowing for communication between both sides of the brain. The LNs are able to receive light input through axonal

connections extending towards the compound eye, using these inputs to entrain to the external 24 hour light/dark cycle.

1.3.2. The Mammalian Brain Clock

It has been determined that, like insects, mammals contain a central brain clock. The centralized brain clock in mammals, consisting of approximately 10,000 cells, is in the SCN of the hypothalamus (Moore, 1991). The SCN was determined to be the central mammalian brain clock through a series of *in vivo* and *in vitro* experiments. When the SCN of the hypothalamus is ablated circadian rhythmicity is lost, thus arrhythmic animals are produced. Rhythmicity can be restored through transplantation of a donor SCN from an entrained animal into the hypothalamus of the arrhythmic SCN-ablated animals (Ralph et al., 1990). Furthermore, isolated SCNs *in vivo* and *in vitro* maintain a circadian rhythm of spontaneous neuronal firing (Inouye and Kawamura, 1979; Green and Gillette, 1982). Isolated SCN clock cells appear to have individual differences in period lengths of neuronal firing (Welsh et al., 1995; Liu et al., 1997; Herzog et al., 2004); therefore, it was determined that SCN clock cells communicate via gap junctions in order to synchronize the rhythmic phases of all the clock neurons (Colwell, 2000; Long et al., 2005; Liu et al., 2007). This synchronization allows for the production of a single circadian period by the SCN to transmit timing information to target tissues. Clock cells located in the SCN are able to receive external timing information (i.e. light/dark cues) directly via a specialized nervous connection with melanopsin-containing photosensitive retinal ganglion cells in the eye known as the retinohypothalamic tract

(Moore et al., 1995; Provencio et al., 1998; Gooley et al., 2001; Berson et al., 2002). Clock cells in the SCN are the only clock cells in mammals capable of directly receiving entraining photic cues. Thus, like in insects, the central brain clock in the SCN is responsible for synchronizing clock cells located in a variety of tissues throughout the organism.

1.3.3. Peripheral Tissue Clocks in Insects

Circadian rhythms were originally attributed to clocks exclusively in the brain because the initial observations only examined overt behavioural rhythms. These rhythms were linked to the brain due to observed loss of rhythmicity caused by removal of brain clock function (Roberts, 1974; Sokolove, 1975; Page, 1978). However, as mentioned previously, all levels of biological processes are governed by circadian clocks and, as this became more evident, the concept of a strictly brain-centred circadian system became less likely. The discovery of clock genes and proteins allowed for identification of clock cells in tissues outside of the brain (Plautz et al., 1997). These peripheral tissue clocks in insects have proven to be more than simple slave oscillators but rather autonomous clock cells. These cells are capable of maintaining rhythmicity in the absence of cues from the brain. In addition, many of these peripheral tissue clocks have been found to be themselves photosensitive (Plautz et al., 1997). An autonomous photosensitive peripheral clock has been studied extensively in the prothoracic glands (PGs) of the insect *R. prolixus* (see Section 1.5.3). These peripheral clocks are not exclusive to the PGs in insects but have been found to be wide spread in tissues such as

Malpighian tubules (Hege et al., 1997), antennae (Merlin et al., 2006; Schuckel et al., 2007), epidermis (Wiedenmann et al., 1986; Ito et al., 2008), and testes (Gvakharia et al., 2000). The discovery of these peripheral tissue clocks has changed the focus of circadian organization within an organism. The brain clock is no longer considered the master clock driving rhythmicity in all other tissues; instead there are several clocks located throughout the organism working together to coordinate the timing of biological processes. These clocks are able to use hormones and nervous signals as means of communicating and synchronizing their functions. The brain clock generally provides synchronizing information to allow each individual clock to 'tick' with the same phase.

Entrainment mechanisms governing insect peripheral tissue clocks still remain unclear. It has been demonstrated in insects such as *Drosophila* and *Rhodnius* that light can pass through the cuticle and provide entraining input to peripheral tissue clocks. Furthermore, tissues such as the Malpighian tubules (Giebultowicz et al., 2000) and the PGs (Vafopoulou and Steel, 1998, 2001) have been shown to be directly photosensitive and entrainable by photic cues *in vitro*. Therefore, evidence exists for direct photic entrainment of peripheral tissue clocks in insects. The use of internal factors in the entrainment of peripheral tissue clocks in insects remains unclear. Evidence exists which implicates neuropeptides rhythmically release from the brain that modulate the phase of circadian clocks in peripheral tissues (Pelc and Steel, 1997). Thus, the possibility remains that entrainment of these peripheral clocks *in vivo* is under the control of the central brain clock. The brain clock receives photic input from the eyes and, through the use of rhythmic release of neuropeptides, entrains clocks in the periphery. However, it is

not yet clear if rhythmic neuropeptide inputs from the brain override photic inputs and entraining these clocks in insects.

1.3.4. Peripheral Tissue Clocks in Mammals

In keeping with the analogous nature of circadian clocks between insects and mammals, it is no surprise that rhythmic clock gene expression was found in many mammalian tissues as well. Much like insects, these tissues are able to maintain these oscillations in the absence of inputs from the brain (SCN). A wide variety of clock cells have been discovered in mammalian tissues; the retina (Tosini and Menaker, 1996) liver, kidneys, lungs, adrenal glands, and testes (Yamamoto et al., 2004; Yoo et al., 2004; Dibner et al., 2010) have all been implicated as peripheral tissue clocks. As with insects, these clocks regulate the timing of critical biological functions relevant to their tissue of origin. An important distinction exists between mammalian and insect peripheral clocks; mammalian peripheral clocks are not directly photosensitive, they cannot be entrained directly by light cues (Yamazaki et al., 2000). In mammals, the clock in the SCN is the only clock which receives photic information from the eyes; therefore, peripheral clocks in mammals must receive entraining information via humoral and/or nervous factors. It is clear that, like the insect system, the mammalian system is composed of a complex network of clocks throughout the organism, communicating and working together to coordinate the timing of biological processes in the whole organism.

1.4. Entrainment of Clock Cells by Photic and Non-photic Zeitgebers

1.4.1. Photic Zeitgebers

Entrainment of circadian clocks by external timing cues is vital to keep organisms synchronized with the external world. As mentioned in Section 1, timing signals used to entrain circadian clocks are known as Zeitgebers. The most common and well-known Zeitgeber used by circadian clocks is the daily 24 hour light/dark cycle. The rotation of the Earth on its axis produces a consistent daily light/dark cycle and is therefore an ever present source of timing information to be used by circadian clocks. Specifically, circadian clocks use the transitions from light to dark and vice versa to set the phase of rhythmic biological processes. In other words, lights-off and lights-on are the two major Zeitgebers used by circadian clocks (Pittendrigh and Minis, 1964). In insects, light information is received by the compound eyes; however, this is not necessarily the main route by which light/dark information is processed by the brain clock. In *Drosophila,* removal of the compound eyes does not produce arrhythmic flies, nor does removal of rhodopsin-containing cells known as the H-B eyelets (Helfrich-Förster et al., 2001; reviewed by Helfrich-Förster, 2002). Therefore, it appears that the clock cells in the brain are in fact directly photosensitive, employing the above elements and the photopigment CRY(described in Section 1.2.2), to entrain the core MO. In addition, direct photoentrainment of insect peripheral clocks has been demonstrated *in vitro* (Emery et al., 1997; Vafopoulou and Steel, 1998). In mammals the clock cells in the SCN and peripheral tissues are not directly photosensitive; therefore, direct photic cues are not the only Zeitgebers used to entrain circadian clocks.

1.4.2. Non-photic Zeitgebers

While light has been shown to be the most prominent Zeitgeber and most powerful, there are many additional non-photic Zeitgebers used by circadian clocks to entrain to a daily 24 hour rhythm. A common feature amongst all Zeitgebers is they all contain daily fluctuations with a period of 24 hours. Daily cycles in temperature was one of the first non-photic Zeitgebers found to affect the clock system (Zimmerman et al., 1968). Temperature levels generally follow a predictable pattern, with lows occurring at night and in the early morning and peaks during the day. A critical component of circadian rhythms is that they are temperature-compensated (Bruce and Pittendrigh, 1956; Hastings and Sweeney, 1957); however, they are clearly capable of detecting changes in temperature and responding to these changes as a method of entrainment. Temperature appears to be an important Zeitgeber to the circadian clocks of organisms that survive in dark places such as underground burrows. The mechanism by which circadian clocks entrain to temperature changes is still poorly understood; however, it has been noted that in *Drosophila*, increases temperature in constant light environments lead to an up-regulation of *clk* expression (and vice versa) (Yoshii et al., 2002, 2007). Furthermore, in *clk*-null flies, temperature-based entrainment is lost (Yoshii et al., 2002, 2007), linking *clk* as a major component in temperature response by the clock system.

Another prevalent form of entrainment of circadian clocks is the use of hormonal and nervous signals. The majority of Zeitgebers used to entrain circadian clocks are derived from the external environment. Hormonal and nervous entrainment occurs internally thus they will be referred to as internal Zeitgebers. Entrainment of circadian

clocks by internal Zeitgebers in mammals occurs in both brain and peripheral tissue clocks. Clocks in the brain receive external photic timing cues via nervous connection to the eyes and transmit this information to peripheral clocks via the rhythmic release of neuropeptides. As previously mentioned, the peripheral clocks studied in mammals are not photosensitive; therefore, these clocks are entrained by hormonal/nervous signals from the brain and through changes in body temperature. One of the most well-studied hormonal Zeitgebers in mammals is corticosteroid release from the adrenals (Balsalobre et al., 2000; Torra et al., 2000). The exact method of entrainment of the clock cells in the adrenal cortex is not yet fully understood; however, it appears to be partly through regulation by the autonomic nervous system (ANS) (Ottenweller and Meier, 1982; Jasper and Engeland, 1994; Urlich-Lai et al., 2006). Regulation of the adrenal clock by the ANS gates the responsiveness of the adrenals to adrenicorticotropic hormone (ACTH) with a circadian rhythm, and thus sets the phase of the circadian rhythm of corticosteroid production (Oster et al., 2006). It has been demonstrated *in vitro* and *in vivo* that glucocorticoids are capable of stimulating clock gene expression in other peripheral tissues such as the liver, kidneys, and lungs; thus implicating these steroids as hormonal Zeitgebers to these tissues (Balsalobre et al., 2000; Yamamoto et al., 2005). Internal Zeitgebers are an essential component of the mammalian clockwork; however, due to its complexity, peripheral clocks in mammals remain difficult to study, they receive a wide variety of inputs, both nervous and hormonal, making it difficult to determine which factors are key in circadian regulation.

Due to the apparent similarities and conservation of key mechanisms governing circadian timekeeping between insects and mammals, insects are an extremely useful model system for the investigation of these core concepts but lack the complexity found in the mammalian system. There is evidence that suggests that inputs from the brain are important in maintaining proper functioning of peripheral clock cells. Pigment-dispersing factor (PDF) is a neurochemical output known to be associated with the LN clock cells in insects. Genetic ablation of the PDF-producing LNs in *Drosophila* results in a disruption in rhythmic clock gene expression in the PG-equivalent ring gland (RG) cells and rhythmic eclosion (Myers et al., 2003), thus implicating the central brain clock in the regulation of the PG clock. In addition, contrary to a report by Emery et al. (1997), disconnection of the PGs from the CNS in the RG of *Drosophila* has recently been shown to result in a loss of rhythmic PER immunoreactivity in the PGs (Morioka et al., 2012). Furthermore, in the insect *R. prolixus,* it has been demonstrated that rhythmic release of the ecdysteroidogenic neuropeptide prothoracicotropic hormone (PTTH) is required *in vivo* to set the phase of the rhythmic ecdysteroid production by the PGs (Pelc and Steel, 1997; Vafopoulou and Steel, 2001). There is clear evidence for the importance of internal (hormonal and nervous) factors in the regulation of peripheral clocks in insects. The interaction of both internal and external Zeitgebers is vital to the coordination of the circadian system within both insects and mammals.

1.5. Circadian Orchestration of Development in Rhodnius prolixus

1.5.1. Rhodnius as a Model Organism

Rhodnius is a classic model organism of insect physiology, beginning with the pioneering work by Wigglesworth possessing a strong foundation for physiological studies. Furthermore, the circadian regulation of development in *Rhodnius* has been studied extensively by our lab (explained in greater detail in the following sections), allowing for more complex studies of the circadian system in this insect. *Rhodnius* is an extremely useful model organism for studying circadian regulation of physiological processes. *Rhodnius* exist in a state of complete developmental arrest between receiving blood meals. Upon engorging on a blood meal, a moulting cycle is initiated in the larval stages and a reproductive cycle in the adults. All major developmental processes begin following the blood meal; therefore, the timing of development of an entire experimental population can be synchronized with a single blood meal (Buxton, 1930; Wigglesworth, 1933). This synchronization is extremely useful when investigating circadian regulation of biological processes (e.g. rhythmic release of ecdysteroids, PTTH etc.). While *D. melanogaster* is a commonly used insect model for molecular and genetic studies of circadian rhythms, its small size makes physiological investigations (such as measurement of hemolymph ecdysteroid titers) difficult if not impossible. In addition, the peripheral clock cells in the RG (equivalent to the PGs) are innervated and integrated into a conglomerate endocrine structure in *Drosophila* while in *Rhodnius* the PGs are discrete structures that lack innervation (Wigglesworth, 1952). The simpler organization

and larger size of *Rhodnius'* organs make them ideal for physiological studies of the circadian system.

1.5.2. Circadian Rhythmicity of Ecdysteroid Production

The PGs in *Rhodnius* have been well established as the site of ecdysteroid synthesis/release in the larval insect. During the approximately 21-day moulting cycle that occurs after feeding the 5^{th} instar, there is a smooth increase to peak levels, followed by a smooth decrease in the hemolymph ecdysteroid titre (Steel et al., 1982). It was later determined that throughout the developmental profile of ecdysteroid production massive changes of the hemolymph ecdysteroid titre were occurring each day (Ampleford and Steel, 1985). Ecdysteroids are the major moulting hormone in larval *Rhodnius* as well as many other insects, and evidence of rhythmicity in ecdysteroid production has been found in several other species including *Galleria mellonella, Bombyx mori, Periplaneta americana* (Cymborowski et al., 1991; Sakurai et al., 1998; Richter, 2001). Furthermore, it was determined that these daily rhythms are, in fact, under circadian control since they were shown to persist in aperiodic conditions with a free-running period around 24 hours that is temperature-compensated (Ampleford and Steel, 1985). The circadian rhythms in the ecdysteroid titer demonstrated peaks in the scotophase (dark period) and troughs in the photophase (light period). Synthesis of ecdysteroids by the PGs has been shown to account for the daily oscillations and developmental profile of the hemolymph ecdysteroid titre (Vafopoulou and Steel, 1989). Circadian rhythms of ecdysteroid production by the PGs *in vivo* and *in vitro* have since been confirmed, with rhythms of ecdytseroid synthesis found to persist in continuous darkness (DD) with peaks in the

subjective scotophase (Vafopoulou and Steel, 1991). The circadian rhythm of ecdysteroid synthesis by the PGs is used to coordinate the timing of development in *Rhodnius,* ecdysteroid target tissues are widespread and thus these hormones are acting as messengers of timing information to their target tissues.

It has been demonstrated in *Rhodnius* that the ecdysteroid receptor (EcR) is expressed in a wide range of target tissues, with a major target being the epidermis (Vafopoulou et al., 2005). Furthermore, expression of EcR in these target tissues cycles rhythmically in conjunction with the daily changes in the hemolymph ecdysteroid titre (Vafopoulou et al., 2005). The cycle of EcR expression in *Rhodnius* epidermis was determined to free-run in DD, indicating it is under circadian control (Vafopoulou and Steel, 2006). The free-running rhythm of EcR expression in epidermal cells occurred in concert with the free-running ecdysteroid rhythm. Therefore, ecdysteroids in the hemolymph are providing temporal information to epidermal cell and other target tissues in *Rhodnius*.

1.5.3. The PGs Contain an Autonomous Circadian Clock

Prior to the discovery of peripheral clocks, the prevailing understanding was that hormonal rhythms under circadian control were strictly driven by the "master" clock in the brain. However, the discovery of clock gene expression in peripheral tissues challenges this view and suggests new possibilities for circadian regulation of development in insects. *In vitro* approaches led to the discovery that the PGs in *Rhodnius* were, in fact, photosensitive and implicate the PGs as containing their own circadian

oscillator (Vafopoulou and Steel, 1991, 1992). Animals kept long-term in continuous light (LL) are made arrhythmic, as long-term exposure to LL has been well established to disrupt circadian clock function (Ampleford and Steel, 1986; Vafopoulou and Steel, 1991). PGs from LL animals continue to produce ecdysteroids; however, rhythmicity is lost. Transfer of PGs from LL animals to DD *in vitro* followed by long-term incubation produces an immediate increase in ecdysteroid production, and a restoration of rhythmicity (Vafopoulou and Steel, 1998). Thus, the PGs responded to direct photic input in the absence of influences from the central clock in the brain. The PGs have been implicated as containing circadian clocks in several other insect species such as *Drosophila* (Emery et al., 1997), *Samia cynthia* (Mizoguchi and Ishizaki, 1982), and *Bombyx mori* (Cymborowski et al., 1991). Additionally, cockroaches have been shown to contain rhythmic release of ecdysteroids indicating clock control of the PGs (Richter, 2001). It was concluded that the PGs in *Rhodnius* must contain an autonomous photosensitive circadian clock responsible for regulating the circadian rhythm of ecdysteroid synthesis (Vafopoulou and Steel, 1998). However, new aspects of the interaction between the PG clock and the clock in the brain continue to be uncovered.

1.5.4. Circadian Regulation of Prothoracicotropic Hormone (PTTH)

PTTH is a classic insect hormone known to stimulate ecdysteroid synthesis by the PGs in larval insects. Within larval insects the PGs are the only known target tissue of PTTH. PTTH was previously thought to be strictly a larval hormone; however, our lab has recently demonstrated rhythmic release of this hormone within the adult insect

(Vafopoulou et al., 2012). For the purposes of this report only the larval aspects of PTTH will be discussed. Our lab demonstrated that, contrary to the previous view of a single release of PTTH at the beginning of development, PTTH is released with a daily rhythm throughout development (Vafopoulou et al., 1996; Vafopoulou and Steel, 1996a). Rhythmic release of PTTH peaked during the scotophase; furthermore, this release produced a rhythmic PTTH titre in the hemolymph. The rhythm of brain content of PTTH was found to be out of phase with the release and hemolymph rhythms; suggesting PTTH is synthesized and stored during the photophase and released during the scotophase. The timing of peak PTTH release from the brain coincides with peak levels of ecdysteroid synthesis by the PGs *in vivo*. The daily rhythm of PTTH release was found to be under circadian control, persisting in aperiodic conditions for several cycles Vafopoulou and Steel, 1996b). These results demonstrate that production and release of both PTTH and ecdysteroids is under circadian control; thus linking a classical developmental hormonal axis to the circadian clock system.

1.5.5. Close Association of PTTH Producing Cells and Clock Cell Axons

Circadian control of PTTH release suggests association with clock cells in the brain. Furthermore, transfer of LL animals to DD reinitiated rhythmic PTTH release from the brain, indicating a close association with a photosensitive circadian clock (Vafopoulou and Steel, 2001). Insect brains were double-labelled using immunohistochemistry (IHC) for PDF (known brain clock output) and PTTH in an attempt to associate clock cell axons with those of PTTH producing cells (Vafopoulou et

al., 2007). It was demonstrated that PTTH-producing cells and their axons are very closely associated with PDF-containing axons originating from the LNs (Vafopoulou et al., 2007). The close association of these axons and the presence of varicosities at these sites indicates communication between an output of the clock system and PTTH cells (Vafopoulou et al., 2007). PTTH release is known to be under circadian control; therefore, it was concluded that rhythmic PTTH release is regulated by the LNs in the brain of *Rhodnius*. The results further solidified circadian clock control of the timing of development through the regulation of major developmental hormones.

1.5.6. Role of Insulin-like Peptides in Endocrine Axis of Development

There is growing evidence showing that the family of small neuropeptides known as the insulin-like peptides (ILPs) are involved in the regulation of insect development. Duve et al. (1979) were the first to demonstrate immunological and physiological insulin activity in flies, *Calliphora vomitoria*, and the first of these peptides was isolated in the silkmoth *Bombyx mori*. Originally deemed "small PTTH" due to the ecdysteroidogenic affects it elicited in *S. cynthia* PGs, this peptide was later renamed bombyxin (Mizoguchi et al., 1987). This turned out to be the first of many ILPs discovered, ultimately forming a large family of related peptides that occur in many insect species and are now known to be the ILPs. These ILPs have been shown to act as growth factors and effectors of development, and have roles in a wide variety of processes including metabolism, reproduction and the stimulation of ecdysteroidogenesis. It has been demonstrated that bombyxin has ecdysteroidogenic effects on the *Rhodnius* PGs (Vafopoulou and Steel,

1997). The stimulation of ecdysteroid synthesis generated by bombyxin was found to be much weaker than that elicited by either *Bombyx* PTTH or *Rhodnius* PTTH (Vafopoulou and Steel, 1997). In addition to the ecdysteroidogenic effects of bombyxin, the peptide was found to exist in *Rhodnius* (Vafopoulou and Steel, 2002) and be rhythmically released by *Rhodnius* brain neurons (Vafopoulou and Steel, 2012). Rhythmic release and ecdysteroidogenic effects of this peptide suggest it may play a role in the endocrine axis of development previously thought to be exclusive to PTTH and ecdysteroids. Using the same method employed for PTTH, potential interactions between ILP-producing cells and clock cells in the brain were investigated. ILP production and release was determined to be rhythmic within the ILP-producing cells themselves, with rhythmic release occurring in the scotophase (Vafopoulou and Steel, 2012), matching the results found in Vafopoulou and Steel (2002). Furthermore, like PTTH-producing cells, the ILP-containing cells demonstrated intimate associations with clock cell axons stained with PDF (Vafopoulou and Steel, 2012). These close associations with the PDF-containing axons indicate communication between the ILP-producing cells and the LNs. It was inferred that the LNs are involved in the regulation of rhythmic release of neuropeptides from the brain which are involved in regulation of development. Clock control of rhythmic ILP release further indicates a potential role as a neuropeptide factor acting on the PG clock in *Rhodnius*.

1.5.7. Coordination Between Clock Cells in the Brain and PGs

The main hormones involved in regulating *Rhodnius* development are associated with circadian clocks in both the brain and PGs. The former view was of the

neuropeptide PTTH driving ecdysteroid synthesis in the PGs; however, the PGs have been clearly shown to be capable of retaining rhythmic synthesis of ecdysteroids in the absence of PTTH input. In addition, clock controlled rhythmic release of ILPs from the brain have ecdysteroidogenic effects on the PGs. However, removal of circadian brain clock inputs through decapitation or flaccid paralysis by TTX injection provided great insight into the coordination and communication between these two clocks (Pelc and Steel, 1997). When neuropeptide inputs from the brain are removed using the above methods the rhythm of ecdysteroid synthesis by the PGs remains but its phase is reversed (Pelc and Steel, 1997). The peak ecdysteroid production in these animals occurred during the photophase as opposed to the scotophase peak observed in control animals. Therefore, rhythmic neuropeptide inputs from the brain, such as PTTH, are required to set the phase of the PG clock/ecdysteroid rhythm *in vivo* (Pelc and Steel, 1997; Vafopoulou and Steel, 2001). There is clear communication between the two clocks through the use of internal hormonal entraining signals to coordinate development. This was a major discovery regarding the presence and role of internal Zeitgebers regulating peripheral tissue clocks in insects, thus offering an important parallel to the mammalian system. However, it remains unclear whether these inputs can directly affect the molecular clockwork in *Rhodnius* PGs and whether or not they are capable of exerting their effects in the absence of light cues.

1.6. Objectives

There is still a gap in our understanding of how internal factors such as hormones and nervous control regulate/entrain peripheral tissue clocks. The mammalian system is

highly complex and difficult to use to investigate this aspect of clock control. As demonstrated above, there is a high level of conservation between the insect and mammalian clock systems making insects ideal model organisms for the study of fundamental circadian processes. *Rhodnius* in particular is well suited for this kind of investigation as a great deal is already known about the circadian control of development and there is already an established axis of entrainment by internal hormonal Zeitgebers on the PG clock. Furthermore, the PTTH/ecdysteroid axis is highly analogous to the hypothamlamic-pituitary-adrenal (HPA) axis found in mammals; therefore, it can offer important comparative physiological insights into mammalian clock control of peripheral clocks.

The purpose of my investigation is to further elucidate the relationship of circadian regulation of the PGs by internal and external Zeitgebers. Using *in vitro* incubation techniques in conjunction with IHC and confocal laser scanning microscopy, I studied the ability of photic and hormonal cues to re-start the PG clock. The PG clock was deemed functional through the observation of rhythmic expression of the PER protein *in vivo* and *in vitro*. The PTTH-like and ILP-like neuropeptides were determined to be able to re-start an arrhythmic PG clock and appear to be acting as signals of darkness to the PG clock. Furthermore, re-starting the PG clock using these hormonal factors re-initiates rhythmic ecdysteroid synthesis by the PGs. My aim is to better understand the relationship of two separate methods of entrainment used by the PG clock and how these cues regulate rhythmic ecdysteroid synthesis.

GENERAL INTRODUCTION REFERENCES

Allada R. 2003. Circadian clocks. A tale of two feedback loops. *Cell* **112:**284–286.

Allada R, Chung BY. 2010. Circadian organization of behaviour and physiology in *Drosophila. Annu Rev Physiol* **77:**605-624.

Allada R, White NE, So WV, Hall JC, Rosbash M. 1998. A mutant Drosophila homolog of mammalian clock disrupts circadian rhythms and transcription of period and timeless. *Cell* **93:**791–804

Ampleford, E.J., and Steel, C.G.H. 1985. Circadian control of a daily rhythm in hemolymph ecdysteroid titre in the insect *Rhodnius prolixus* (Hemiptera) *Gen Comp Endo* **59:** 453-59.

Ampleford, E.J., and Steel, C.G.H. 1986. Induction of rhythmic modulation of hemolymph ecdysteroids in the insect *Rhodnius prolixus* by treatments which elicit rhythmic ecdysis. *Gen Comp Endo* **63:** 353-361.

Arthur JM, Guthrie JD, Newell JM. 1930. Some effects of artificial climates on the growth and chemical composition of plants. *Am J Bot* **17(5):**416-482.

Balsalobre A, Damiola F, Schibler U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93:**929-937.

Balsalobre, A., Brown, S.A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H.M., Schutz, G., Schibler, U. 2000. Resetting of circadian time in peripheral tissues by glucocorticoid signalling. *Science* **289:**2344-2347.

Bernstein L. 2002. Epidemiology of endocrine-related risk factors for breast cancer. *J Mammary Gland Biol Neoplasia* **7(1):**3-15.

Berson DM, Dunn FA, Takao M. 2002. Phototransduction by retinal ganglion cells that set the circadian clock. *Science* **295:**1070–1073.

Boden MJ, Kennaway DJ. 2006. Circadian rhythms and reproduction. *Reproduction* **132:**379-392.

Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N, Schibler U. 2002. Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr Biol* **12:**1574–1583.

Bruce, V., Pittendrigh, C.S. 1956. Temperature independence in a unicellular "clock". *Proc Natl Acad Sci USA* **42:**676-682.

Ceriani MF, Darlington TK, Staknis D, Mas P, Petti AA, Weitz CJ, Kay SA. 1999. Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science* **285:**553–556.

Colwell CS. 2000. Rhythmic coupling among cells in the suprachiasmatic nucleus. *J Neurobiol* **43(4):**379-388.

Cymborowski, B., Muszynska-Pytel, M., Porcheron, P., Cassier, P., 1991. Hemolymph ecdysteroid titers controlled by a circadian clock mechanism in larvae of the wax moth, *Galleria mellonella. J. Insect Physiol.* **37:** 35–40.

Cyran SA, Buchsbaum AM, Reddy KL, Lin MC, Glossop NR, et al. 2003. *vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. *Cell* **112**:329–341.

Darlington TK, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N, Steeves TDL, Weitz CJ, Takahashi JS, Kay SA. 1998. Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. *Science* **280:**1599–1603.

Dibner, C., Schibler, U., Albrecht, U. 2010. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu. Rev. Phys.* **75:**517-549.

Dong G, Golden SS. 2008. How a cyanobacterium tells time. *Curr Opin Microbiol* **11:**541-546.

Edmunds, L.N. 1988. *Cellular and Molecular Bases of Biological Clocks.* New York: Springer-Verlag. pp. 1-497.

Emery, I.F., Noveral, J.M., Jamison, C.F., Siwicki, K.K. 1997. Rhythms of *Drosophila period* gene expression in culture. *Proc Natl Acad Sci USA* **94**: 4092-4096.

Emery P, So WV, Kaneko M, Hall JC, Rosbash M. 1998. CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* **95:**669–679.

Emery P, Stanewsky R, Helfrich-Forster C, Emery-Le M, Hall JC, Rosbash M. 2000. *Drosophila* CRY is a deep-brain circadian photoreceptor. *Neuron* **26:**493–504.

Gekakis N, Saez L, Delahaye-Brown A-M, Myers MP, Sehgal A, Young MW, Weitz CJ. 1995. Isolation of timeless by PER protein interactions: defective interaction between timeless protein and long-period mutant PERL. *Science* **270:**811–815.

Giebultowicz JW, Stanewsky R, Hall JC, Hege DM. 2000. Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. *Curr Biol* **10:**107–110.

Gooley JJ, Lu J, Chou TC, Scammell TE, Saper CB. 2001. Melanopsin in cells of origin of the retinohypothalamic tract. *Nat. Neurosci.* **4**:1165.

Green DJ, Gillette R. 1982. Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice. *Brain Res* **245:**198-200.

Gvakharia BO, Kilgore JA, Bebas P, Giebultowicz JM. 2000. Temporal and spatial expression of the period gene in the reproductive system of the codling moth. *J Biol Rhythms* **15:**27–35.

Hao H, Allen DL, Hardin PE. 1997. A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila melanogaster. Mol Cell Biol* **17**:3687–3693.

Hastings, J.W., Sweeney, B.M. 1957. On the mechanism of temperature independence in a biological clock. *Proc Natl Acad Sci USA* **43:**804-811.

Helfrich-Förster C. 1995. The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster. Proc Natl Acad Sci USA* **92:**612-616.

Helfrich-Förster C. 2002. The circadian system of *Drosophila melanogaster* and its light input pathways. *Zoology* **105:**297-312.

Helfrich-Förster C. 2004. The circadian clock in the brain: a structural and functional comparison between mammals and insects. *J Comp Physiol A* **190:**601-613.

Helfrich-Förster C, Stengl M, Homberg U. 1998. Organization of the circadian system in insects. *Chronobiol Int* **15(6):**567-594.

Helfrich-Förster, C., Winter, C., Hofbauer, A., Hall, J.C., Stanewsky, R. 2001. The circadian clock of fruit flies is blind after elimination of all known photoreceptors. *Neuron* **30:**249-261.

Henley, D.E., Leendertz, J.A., Russell, G.M., Wood, S.A., Taheri, S., Woltersdorf, W.W., Lightman, S.L. 2009. Development of an automated blood sampling system for use in humans, *J Med Eng Tech* **33:**199-208.

Herzog ED, Aton SJ, Numano R, Skaki Y, Tei H. 2004. Temporal precision in the mammalian circadian system: a reliable clock from less reliable neurons. *J Biol Rhythm* **19(1):**35-46.

Herzog ED. 2007. Neurons and networks in daily rhythms. *Nature* **8:**790-802.

Hunter-Ensor M, Ousley A, Sehgal A. 1996. Regulation of the *Drosophila* protein timeless suggests a mechanism for resetting the circadian clock by light. *Cell* **94:**677-685.

Inouye ST, Kawamura H. 1979. Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proc Natl Acad Sci USA* **76(11):**5962-5966.

Ishikawa T,Matsumoto A, Kato T Jr, Togashi S, Ryo H, IkeganaM, Todo T, Ueda R, Tanimura T. 1999. DCRY is a *Drosophila* photoreceptor protein implicated in light entrainment of circadian rhythms. *Genes to Cells* **4:**57–65.

Ito C, Goto SG, Shiga S, Tomioka K, Numata H. 2008. Peripheral circadian clock for the cuticle deposition rhythm in *Drosophila melanogaster. Proc Natl Acad Sci USA* **105:**8446–8451.

Jasper, M.S., Engeland, W.C. 1994. Splanchnic neural activity modulates ultradian and circadian rhythms in adrenocortical secretion in awake rats. *Neuroendocrinology* **59:**97-109.

Kaneko M, Helfrich-Förster C, Hall JC. 1997. Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*: newly identified pacemaker candidates and novel features of clock gene product cycling. *J Neurosci* **17(17):**6745-6760.

Kloss B, Rothenfluh A, Young MW, Saez L. 2001. Phosphorylation of period is influenced by cycling physical associations of doubletime, period, and timeless in the *Drosophila* clock. *Neuron* **30:**699–706.

Konopka, R.J. and S. Benzer. 1971. Clock mutants of *Drosophila melanogaster. Proc. Natl. Acad. Sci USA* **68:** 2112–2116.

Knutsson A. 2003. Health disorders of shift workers. *Occup Med* **53:**103-108

Lakin-Thomas P. 2006. Transcriptional feedback oscillators: maybe, maybe not... *J Biol Rhythm* **21(2):**83-92.

Lall GS, Atkinson LA, Corlett SA, Broadbridge PJ, Bonsall DR. 2012. Circadian entrainment and its role in depression: a mechanistic review. *J Neural Transm* **119:**1085-1096.

Lee C, Parikh V, Itsukaichi T, Bae K, Edery I (1996) Resetting the *Drosophila* clock by photic regulation of PER and a PER–TIM complex. *Science* **271:**1740–1744.

Lee C, Bae K, Edery I .1999. PER and TIM inhibit the DNA binding activity of a Drosophila CLOCK–CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription. *Proc Natl Acad Sci USA* **19:** 5316–5325.

Lemmer B, Labrecque G. 1987. Chronopharmacology and chronotherapeutics: definitions and concepts. *Chronobiol Int* **4(3):**319-329.

Lim C, Chung BY, Pitman JL, McGill JJ, Pradhan S, et al. 2007. Clockwork orange encodes a transcriptional repressor important for circadian-clock amplitude in *Drosophila. Curr. Biol.* **17:**1082–1089.

Lin JM, Kilman VL, Keegan K, Paddock B, Emery-Le M, Rosbash M, Allada R. 2002. A role for casein kinase 2alpha in the *Drosophila* circadian clock. *Nature* **420:**816–820.

Liu C, Weaver DR, Strogatz SH, Reppert SM. 1997. Cellular construction of a circadian clock: period determination in the suprachiasmatic nuclei. *Cell* **91:**855-860.

Liu AC, Welsh DK, Ko CH, Tran HG, Zhang EE, Priest AA, Buhr ED, Singer O, Meeker K, Verma IM, Doyle III FJ, Takahashi JS, Kay SA. 2007. Intercellular coupling confers robustness against mutations in the SCN circadian clock network. *Cell* **129:**605-616.

Long MA, Jutras MJ, Connors BW, Burwell RD. 2005. Electrical synapses coordinate activity in the suprachiasmatic nucleus. *Nat Neurosci* **8(1):**61-66.

Merlin C, Francois M.C, Queguiner I, Mai¨be`che-Coisne´ M, Jacquin-Joly E. 2006. Evidence for a putative antennal clock in *Mamestra brassicae*: Molecular cloning and characterization of two clock genes—*period* and *cryptochrome*—in antennae. *Insect Mol Biol* **15:**137–145.

Mizoguchi, A., Ishizaki, H., 1982. Prothoracic glands of the saturniid moth *Samia cynthia* ricini possess a circadian clock controlling gut purge timing. *Proc. Natl. Acad. Sci. U S A.* **79:** 2726–2730.

Moore RY. 1991. The suprachiasmatic nucleus and the circadian timing system. In: Klein DC, Moore RY, Reppert SM (eds) *Suprachiasmatic Nucleus: The Mind's Clock.* New York: Oxford University Press, pp 13-15.

Moore RY, Speh JC, Card JP. 1995. The retinohypothalamic tract originates from a distinct subset of retinal ganglion cells. *J. Comp. Neurol.* **352**:351–66.

Morioka, E., Matsumoto, A., Ikeda, M. 2012. Neuronal influence on peripheral circadian oscillators in pupal *Drosophila* prothoracic glands. *Nature Comm.* **3 (909):** 1-11.

Myers, E.M., Yu, J., Sehgal, A. 2003. Circadian control of eclosion: interaction between a central and peripheral clock in *Drosophila melanogaster. Curr Biol* **13**: 526-533.

Naidoo N, Song W, Hunter-Ensor M, Sehgal A. 1999. A role for the proteasome in the light response of the timeless clock protein. *Science* **285:**1737–1741.

Nishiitsutsuji-Uwo J, Pittendrigh CS. 1968. Central nervous system control of circadian rhythmicity in the cockroach: ii. the pathway of light signals that entrain the rhythm. *Zeit ver Physiol* **58:**1-13.

O'Neill, J.S., Reddy, A.B. 2011. Circadian clocks in human red blood cells. *Nature* **469:** 498-505.

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffman, M.W., Eichele, G. 2006. The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metab* **4:** 163-173.

Ottenweller, J.E. Meier, A.H. 1982. Adrenal innervation may be an extrapituitary mechanism able to regulate adrenocortical rhythmicity in rats. *Endocrinology* **111:**1334-1338.

Page TL, Caldarola PC, Pittendrigh CS. 1977. Mutual entrainment of bilaterally distributed circadian pacemakers. *Proc Natl Acad Sci USA* **74(3):**1277-1281.

Page TL. 1978. Interactions between bilaterally paired components of the cockroach circadian system. *J Comp Physiol* **124:**225-236.

Pelc, D., Steel, C.G.H., 1997. Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotropic hormone. *Gen. Comp. Endocrinol.* **108:** 358–365.

Pittendrigh CS. 1954. On temperature independence in the clock system controlling emergence time in *Drosophila*. *Proc Natl Acad Sci USA* **40(10):**1018-1029.

Pittendrigh CS. 1993. Temporal organization: reflections of a darwinian clock-watcher. *Annu Rev Physiol* **55:**17-54.

Pittendrigh, C.S., Minis, D.H. 1964. The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *Am Nat* **98:**261-294.

Pittendrigh CS, Minis DH. 1972. Circadian systems: longevity as a function of circadian resonance in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **69(6):**1537-1539.

Provencio I, Jiang G, De Grip WJ, Hayes WP, Rollag MD. 1998. Melanopsin: an opsin in melanophores, brain, and eye. *Proc. Natl. Acad. Sci. USA* **95**:340–45.

Ralph MR, Foster RG, Davis FC, Menaker M. 1990. Transplanted suprachiasmatic nucleus determines circadian period. *Science* **247(4945):**975-978.

Richter, K., 2001. Daily changes in neuroendocrine control of molting hormone secretion in the prothoracic gland of the cockroach, *Periplaneta americana*. *J. Insect Physiol.* **47:** 333–338.

Roberts SK. 1974. Circadian rhythms in cockroaches: effects of optic lobe lesions. *J Comp Physiol A* **88:**21-30.

Reppert SM, Weaver DR. 2001. Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* **63:**647-676.

Rudiger HW. 2004. Health problems due to night shift work and jetlag. *Der Internist* **45(9):**1021-1025.

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC. 1998. CYCLE is a second bHLH-PAS protein essential for circadian transcription of Drosophila period and timeless. *Cell* **93:**805–814.

Sakurai, S., Kaya, M., Satake, S., 1998. Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the larval–pupal stadium of the silkworm, *Bombyx mori*: role of low ecdysteroid titer in larval–pupal metamorphosis and a reappraisal of the head critical period. *J. Insect Physiol.* **44:** 867–881.

Sathyanarayanan S, Zheng X, Xiao R, Sehgal A. 2004. Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. *Cell* **116:**603–615.

Savvidis C, Koutsilieris M. 2012. Circadian rhythm disruption in cancer biology. *Mol Med* **18:**1249-1260.

Schuckel J, Siwicki KK, Stengl M. 2007. Putative circadian pacemaker cells in the antenna of the hawkmoth *Manduca sexta*. *Cell Tissue Res* **330:**271–278.

Siwicki KK, Eastman C, Petersen G, Rosbash M, Hall JC. 1988. Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* **1:**141-150.

Smith, RF., Konopka, RJ. 1981. Circadian clock phenotypes of chromosome-aberrations with a breakpoint at the per locus. *Mol Gen Genetics* **183(2):**243-251.

Smith, RF., Konopka, RJ. 1982. Effects of dosage alterations at the per locus on the period of the circadian clock of *Drosophila*. *Mol Gen Genetics* **185(1):**30-36.

Sokolove PG. 1975. Localization of the cockroach optic lobe circadian pacemaker with microlesions. *Brain Res* **87:**13-21.

Stanewsky R. 2003. Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. *J Neurobiol* **54:**111–147.

Steel, C.G.H., Bollenbacher, W.E., Smith, S.L., Gilbert, L.I. 1982. Haemolymph ecdysteroid titres during larval-adult development in *Rhodnius prolixus:* correlations with moulting hormone action and brain neurosecretory cell activity. *J Insect Physiol.* **28 (6):** 519-25.

Tomioka K, Uryu O, Kamae Y, Umezaki Y, Yoshii T. 2012. Peripheral circadian rhythms and their regulatory mechanism in insects and some other arthropods: a review. *J Comp Physiol B* **182:**729-740.

Torra, I.P., Tsibulsky, V., Delaunay, F., Saladin, R., Laudet, V., Fruchart, J.C., Kosykh, V., Staels, B. 2000. Circadian and glucocorticoid regulation of Rev-erb alpha expression in liver. *Endocrinology* **141:**3799-3806.

Tosini G, Menaker M. 1996. Circadian rhythms in cultured mammalian retina. *Science* **272:**419–421.

Ulrich-Lai, Y.M., Arnhold, M.M., Engeland, W.C. 2006. Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH. *Am J. Primatol* **290:**1128-1135.

Vafopoulou, X., Steel, C.G.H., 1989. Developmental and diurnal changes in ecdysteroid biosynthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera) in vitro during the last larval instar. *Gen. Comp. Endocrinol.* **74:** 484–493.

Vafopoulou, X., Steel, C.G.H., 1991. Circadian regulation of synthesis of ecdysteroids by prothoracic glands of the insect *Rhodnius prolixus*: evidence of a dual oscillator system. *Gen. Comp. Endo.* **83:** 27–34.

Vafopoulou, X., Steel, C.G.H., 1992. In vitro photosensitivity of ecdysteroid synthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera). *Gen.Comp. Endocrinol.* **86:** 1–9.

Vafopoulou, X. and Steel, C.G.H. 1996a. The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. *Pro. Natl Acad Sci* **93 (8):** 3368-3372.

Vafopoulou, X. and Steel, C.G.H. 1996b. Prothoracicotropic hormone from the brain-retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval-adult development. *Gen Comp Endocrinol.* **102 (1):** 123-129.

Vafopoulou, X. and Steel, C.G.H. 1997. Ecdysteroidogenic action of *Bombyx* prothoracicotropic hormone and bombyxin on the prothoracic glands of *Rhodnius prolixus in vitro*. *J Insect Physiol* **43 (7):** 651-656.

Vafopoulou, X. and Steel, C.G.H. 1998. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis in vitro. *J Comp Physiol A* **182 (3):** 343-349.

Vafopoulou, X. and Steel, C.G.H. 2001. Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine zeitgebers. *J Insect Phys* **47 (8):** 935-941.

Vafopoulou, X. and Steel, C.G.H. 2002. Prothoracicotropic hormone of *Rhodnius prolixus*: partial characterization and rhythmic release of neuropeptides related to *Bombyx* PTTH and bombyxin. *In Repro Devel.* **42 (2-3):** 111-120.

Vafopoulou, X., Steel, C.G.H., 2006. Ecdysteroid hormone nuclear receptor (EcR) exhibits circadian cycling in certain tissues, but not others, during development in Rhodnius prolixus (Hemiptera). *Cell Tissue Res.* **323:** 443–455.

Vafopoulou, X. and Steel, C.G.H. 2012. Insulin-like and testis ecdysiotropin neuropeptide are regulated by the cicadian timing-system in the brain during larval-adult development in the insect *Rhodnius prolixus* (Hemiptera). *Gen Comp Endocrinol.* **179:** 277-288.

Vafopoulou, X, Sim, C-H, Steel, C.G.H. 1996. Prothoracicotropic hormone in *Rhodnius prolixus: in vitro* analysis and changes in amounts in the brain- retrocerebral complex during larval-adult development. *J Insect Physiol* **42 (4):** 407-415.

Vafopoulou, X., Steel, C.G.H., Terry, K., 2005. Ecdysteroid receptor (EcR) shows marked differences in temporal patterns between tissues during larval–adult development in Rhodnius prolixus: correlations with haemolymph ecdysteroid titres. *J. Insect Physiol.* **51:** 27–38.

Vafopoulou, X., Steel, C.G.H., and Terry, K.L. 2007. Neuroanatomical relations of prothoracicotropic hormone neurons with the circadian timekeeping system in the brain of larval and adult Rhodnius prolixus (Hemiptera). *J Comp Neurol* **503 (4):** 511-524.

Vafopoulou, X., Terry, K.L., and Steel, C.G.H. 2010. The circadian timing system in the brain of the fifth larval instar of *Rhodnius prolixus* (Hemiptera). *J Comp Neurol* **518 (8):** 1264-1282.

Vafopoulou, X., Cardinal-Aucoin, M., Steel, C.G.H. 2012. Rhythmic release of prothoracicotropic hormone from the brain of an adult insect during egg development. *Comp Biochem Physiol A* **161 (2)**: 193-200.

Vosshall LB, Price JL, Sehgal A, Saez L, Young MW. 1994. Specific block in nuclear localization of period protein by a second clock mutation, timeless. *Science* **263:**1606–1609.

Welsh DK, Logothetis DE, Meister M, Reppert SM. 1995. Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron* **14:**697-706.

Went FW. 1960. Photo- and thermoperiodic effects in plant growth. *Cold Spring Harbor Symp Quant Biol* **25:**221-230.

Wiedenmann G, Lukat R, Weber F. 1986. Cyclic layer deposition in the cockroach endocuticle: a circadian rhythm? *J Insect Physiol* **32**:1019–1027.

Wigglesworth, V.B., 1952. The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in moulting. *J. Exp. Biol.* **29:** 561–570.

Yamamoto T, Nakahata Y, Soma H, Akashi M, Mamine T, Takumi T. 2004. Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. *BMC Mol. Biol.* **5**:18.

Yamamoto, T., Nakahata, Y., Tanaka, M., Yoshida, M., Soma, H., Shinohara, K., Yasuda, A., Mamine, T., Takumi, T. 2005. Acute physical stress elevates mPeriod1 mRNA expression in mouse peripheral tissues via a glucocorticoid responsive element. *J Biol Chem* **280**:42036-42043.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda, M., Block, G.D., Sakaki, Y., Menaker, M., Tei, H. 2000. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288**:682–685.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr, E.D., Siepka, S.M., Hong, HK., Oh, W.J., Yoo, O.J., Menaker, M., Takahashi, J.S. 2004. PERIOD2::LUCIFERASE realtime reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci USA* **101**:5339–5346.

Yoshii, T., Sakamoto, M., Tomioka, K. 2002. A temperature-dependent timing mechanism is involved in the circadian system that drives locomotor rhythms in the fruit fly *Drosophila melanogaster. Zoo. Sci.* **19(8)**:841-850.

Yoshii T, Fujii K, Tomioka K. 2007. Induction of *Drosophila* behavioral and molecular circadian rhythms by temperature steps in constant light. *J Biol Rhythms* **22**:103–114.

Yu W, Zheng H, Houl JH, Dauwalder B, Hardin PE. 2006. PER dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes Dev* **20**:723–733.

Zimmerman WF, Pittendrigh CS, Pavlidis T. 1968. Temperature compensation of the circadian oscillation in Drosophila pseudoobscura and its entrainment by temperature cycles. *J Insect Physiol.* **14**:669–684.

CHAPTER II:

MANUSCRIPT

Photic and hormonal Zeitgebers to a photosensitive prothoracic gland clock that regulates steroid synthesis in the insect *Rhodnius prolixus* (Hemiptera)

Authors:
Nicholas Rapp
Department of Biology, York University, 4700 Keele Street, Toronto, ON, M3J 1P3, Canada
E-mail: nickr0o2@yorku.ca

Colin G.H. Steel
Department of Biology, York University, 4700 Keele Street, Toronto, ON, M3J 1P3, Canada
E-mail: csteel@yorku.ca
Phone: (416) 736-2100 x33437

Running Title:
Photic and hormonal Zeitgebers to the PG clock in *Rhodnius*

Correspondence to:
Colin G.H. Steel
Department of Biology, York University, 4700 Keele Street, Toronto, ON, M3J 1P3, Canada
E-mail: csteel@yorku.ca
Phone: (416) 736-2100 x33437

Keywords:
Ecdysteroids, prothoracic gland, immunohistochemistry, period, prothoracicotropic hormone (PTTH), insulin-like peptides (ILPs), bombyxin, peripheral and brain clocks.

ABSTRACT

The paired prothoracic glands (PGs) of larval *Rhodnius prolixus* each contain a photosensitive circadian clock that controls synthesis of the steroid molting hormones at night. Using immunohistochemistry and confocal laser scanning microscopy, we examined whether the PG clock is also responsive to internal hormonal Zeitgebers, by observing the relative fluorescence intensity of the canonical clock protein PERIOD (PER) in PG cells exposed *in vitro* to brain neuropeptides. Nuclear PER is depleted from PG cells in prolonged continuous light (LL). These LL PGs were incubated *in vitro* and exposed to a 1h pulse of brain neuropeptide fractions of either whole brain extract, <10kDa fraction or >10kDa fraction; both PER cycling and rhythmic steroidogenesis were reinitiated by all brain extracts tested. PER is also depleted from PG cells in prolonged continuous darkness, these PGs were unresponsive to pulses of neuropeptide extracts, showing these neuropeptides are specifically acting as signals of darkness to the PGs. The <10kDa fraction contains insulin-like peptides and the >10kDa fraction contains prothoracicotropic hormone (PTTH). Removal of these peptides from their respective fractions via double immunoprecipitation resulted in each fraction losing the ability to re-initiate PER expression in the PGs. Both PTTH and ILPs affect steroidogenesis by PGs in *Rhodnius* and *in vivo* release of both of these neuropeptides from the brain occurs during the scotophase, in synchrony with PG secretion of molting hormones. We infer there are at least two brain neuropeptide Zeitgebers (PTTH and ILPs) capable of contributing to entrainment of the PG clocks and stimulation of rhythmic ecdysteroid synthesis. Further, both PTTH and ILPs act as signals of darkness, not light.

INTRODUCTION

Circadian rhythms are essential to the optimal functioning of an organism living within the 24h cyclic environment on Earth. Endogenous circadian clocks maintain internal temporal order by organizing behavioural, physiological, and molecular biology to allow organisms to adapt and anticipate daily external events. These circadian rhythms are generated with approximately 24h periodicity by specialized circadian clock cells. Circadian clocks are entrained by external timing cues, known as Zeitgebers, and generate rhythmic nervous or endocrine outputs that convey timing information to target tissues throughout an organism that otherwise lack access to such information. These clock cells were initially associated only with the nervous system; however, there is a widely expanding literature on 'clocks' located in peripheral tissues (Dibner et al., 2010; Tomioka et al., 2012). The specific roles of these peripheral tissue clocks within the organs in which they reside remain unclear, as does the relationship between central and peripheral clocks.

The circadian system has now been studied extensively in mammals; however, much of the pioneering research in the field was performed in insects. The molecular clockwork used to drive circadian rhythmicity in clock cells was first discovered in *Drosophila melanogaster* (Konopka and Benzer, 1971; Hardin et al., 1990) and later found to be conserved in mammals (Allada, 2003). Circadian oscillations are governed by a set of interlocking transcription/translation feedback loops which have been dubbed the molecular oscillator (MO). The *period (per)* gene is a vital component of the MO, rhythmically producing the canonical clock protein PERIOD (PER). The PER protein

dimerizes with the clock protein TIMELESS (TIM) and translocates into the nucleus to indirectly inhibit transcription of the *per* and *tim* genes; thus completing the negative feedback loop (Vosshall et al., 1994; Gekakis et al., 1995). Many other genes and their protein products are involved in proper functioning of the MO and a complete review of its function can be found in Allada and Chung (2010). The elucidation of the molecular mechanisms involved in generating circadian periodicity within cells has provided useful tools to the circadian physiologist. Cycling clock genes and proteins, such as *per* and PER, respectively, can be used as markers to study the organization of circadian clock cells in the brain and throughout the organism (Siwicki et al., 1988; Helfrich-Förster, 1995; Kaneko et al., 1997; Vafopoulou et al., 2010).

The insect *Rhodnius prolixus* is a classical model organism used in insect physiology and is ideally suited for circadian studies (Wigglesworth, 1934). *Rhodnius* exist in a state of developmental arrest between blood meals until administration of a large blood meal initiates development to the next stage. Therefore, the timing of development can be precisely synchronized across an entire experimental population (Buxton, 1930; Wigglesworth, 1933). The circadian organization and orchestration of larval-adult development in *Rhodnius* have been studied extensively by this lab (reviewed in Steel and Vafopoulou, 2006; Vafopoulou and Steel, 2007, 2010, 2012b). In insects, ecdysteroids, the insect moulting hormones, are critical regulators of larval development that act on multiple target tissues to induce developmental gene expression (Vafopoulou et al., 2005) and are synthesized by the prothoracic glands (PGs). In *Rhodnius*, the PGs produce ecdysteroids with a circadian rhythm, ultimately generating rhythmic peaks in

ecdysteroid levels in the hemolymph throughout larval development (Ampleford and Steel, 1985; Vafopoulou and Steel, 1991). The PGs have been shown to possess an autonomous clock capable of functioning independent of clock cells in the brain (i.e. the PGs represent a peripheral clock) (Vafopoulou and Steel, 1998). The PGs are directly photosensitive and respond to light cues both *in vivo* and *in vitro* (Vafopoulou and Steel, 1992; Vafopoulou and Steel, 1998; Vafopoulou and Steel, 2001). In addition to photic cues, the PG ecdysteroid rhythm is affected by hormonal cues. The developmental hormone prothoracicotropic hormone (PTTH) is released from the brain and is known to set the phase of ecdysteroid synthesis *in vivo* (Pelc and Steel, 1997). Little is known regarding the entrainment of circadian clocks in insects by internal factors such as hormones or nervous inputs. *Rhodnius* is an ideal model for such studies, as they possess a known pathway of hormonal entrainment which can be investigated in greater detail.

The picture of entrainment of mammalian peripheral tissue clocks remains unclear. A plethora of examples of clock cells have been found to reside in nearly every tissue studied. None of the mammalian peripheral tissue clocks discovered are directly photosensitive; therefore, entrainment of these clocks must rely on internal factors (Yamazaki et al., 2000). However, the complexity of mammalian organization makes studying entrainment by internal Zeitgebers extremely difficult. Therefore, the simpler organization found in insects makes them ideal models to better our understanding of the principles governing circadian timekeeping.

The present paper examines the role of photic (external) and hormonal (internal) inputs to the PG clock system. Using immunohistochemistry and confocal laser scanning

microscopy, PER rhythmicity in the PGs was used as a marker for a functional clock system. Animals maintained in long-term continuous light (LL) lose the intrinsic rhythmicity of ecdysteroid synthesis by the PGs (Vafopoulou and Steel, 1991; Vafopoulou and Steel, 1998). In this study, we found that PER cycling ceased in these arrhythmic LL animals and that PER remained at low levels, indicating PG clock function has stopped. We report that both photic (transfer to constant dark) and hormonal (brief exposure to brain neuropeptide extract) cues successfully induced PER expression and cycling in LL PGs, thus restarting the stopped clock. Synchronous rhythmic ecdysteroid synthesis was also re-initiated in every case. Furthermore, entraining neuropeptides act specifically as signals of darkness, rather than light, to the PG clock. These results provide insights into the entraining mechanisms that govern the PG clock and illuminate the communication between central and peripheral clocks.

MATERIALS AND METHODS

2.1. Animals and prothoracic gland dissection:

Rhodnius were reared at 28°C ± 0.5 °C in a cycle of 12h light and 12h dark (12L: 12D). A blood meal was provided to a population of fifth instar larvae, synchronizing their development to the adult stage; the day of feeding is considered day 0. Ecdysis to the adult is gated by the circadian system (Ampleford and Steel, 1982) and occurs around day 21 after a blood meal. PGs were removed from male fifth instars on day 12 after a blood meal. *Rhodnius* PG cells are arranged on the surface of paired inner lobes of the thoracic fat body; therefore, the whole lobe was dissected (includes the PG cells). Arrthythmic animals were produced through transfer from a 12L:12D cycle to either continuous light (LL) or continuous dark (DD) (28°C ± 0.5 °C) for a minimum of 4 weeks prior to feeding. Rhythmicity is known to damp out after approximately 1 week in LL (Ampleford and Steel, 1986) and prolonged maintenance in LL is known to abolish the intrinsic rhythmicity of ecdysteroid synthesis by PGs (Vafopoulou and Steel 1991, 1992, 1993).

For *in vivo* experiments, PGs were dissected from LD animals to observe the PER rhythm in the intact animal. To assess the affect of light, LL animals were transferred to DD and PGs were dissected at 1h after lights-off and then at 6h intervals. For *in vitro* experiments, arrhythmic LL insects were used throughout.

2.2. Antibodies:

The PER antibody used was a custom made (Genscript, Piscataway, NJ) Protein A purified polyclonal antibody produced in guinea pig. This antibody was raised against a 14-amino acid synthetic peptide corresponding to a highly conserved region of the *Drosophila* PER protein, known as the PAS region (residues 605–618; KSSTETPPSYNQLN; known as peptide PER-S). The PAS region is essential for protein dimerization, a vital component for proper functioning of the MO, thus conservation of this region is essential (Siwicki et al., 1988). This antibody was used at a dilution of 1:500 for both immunohistochemistry and western/immunoblot procedures. A BLAST search revealed an extremely high sequence identity of PER-S with PER sequences of other insects (Vafopoulou et al., 2010). For example, 100% sequence identity is seen between *Drosophila melanogaster* and other drosophilids, and other insects including *Blatella germanica, Ceratitis capitata, Sarcophaga bullata, Anastrepha suspensa*, and *Apis mellifera* insects (Vafopoulou et al., 2010). For these reasons native *Rhodnius* PER is the probable target of the antibody employed in the following study, which is further confirmed by SDS-PAGE and western blot analysis as well as immunohistochemistry presented here (see Results).

Protein A purified guinea pig polyclonal antibody (Genscript, Piscataway, NJ) raised against a synthetic peptide corresponding to the C-terminus of *Bombyx* bombyxin (GIVDECCLRP; known as bombyxin A-10 A chain) was used for double immunoprecipitation of bombyxin/ILPs. A custom made Protein A purified polyclonal antibody (Genscript, Piscataway, NJ) made in guinea pig against a

synthetic peptide corresponding to the N-terminus of *Bombyx* PTTH (known as PTTH[1–15]; GNIQVENQAIPDPPC) was used for PTTH double immunoprecipitation.

2.3. SDS-PAGE and Western blot

PGs were homogenized in lysis buffer and processed as described in Vafopoulou and Steel (2012). For SDS-PAGE and western blotting were performed using standard protocols as in Vafopoulou and Steel (2012). 10 PG pairs were used per well. Proteins were separated using 10% SDS PAGE and transferred electrophoretically onto PVDF membranes for 1h at 100V. PVDF membranes were processed according to protocol set out in Vafopoulou and Steel, 2002. Secondary antibody was an anti-guinea pig IgG raised in rabbits that was conjugated to horseradish peroxidise and used at a dilution of 1:200. Immunoreactive material was visualized with 3,3-diaminobenzidine hydrochloride and hydrogen peroxide as a peroxidase substrate.

2.4. Imunohistochemistry:

PGs were dissected in *Rhodnius* saline (pH 7.2; Lane et al., 1975) and immediately fixed in freshly prepared 4% paraformaldehyde for 2h at room temperature as outlined in an established protocol (Vafopoulou, 2009). PGs were incubated in primary antibody solution (1:500 dilution) for 24h at 4°C, followed by 3 15 minute washes with phosphate buffered saline (PBS). PGs were then incubated in secondary antibody for 2h at room temperature. Secondary antibody was anti-guinea pig IgG conjugated to fluorescein isothiocyanate (FITC), used at a dilution of 1:200. PGs were

mounted in 90% glycerol in PBS containing 1% 1,4-diazabicyclo[2.2.2]octane (DABCO).

For controls, primary antibody was replaced with primary buffer and secondary antibody was replaced with secondary buffer. Fluorescence levels observed in the controls proved to be indistinguishable from the background and no autofluorescence was observed.

Digital optical sections at 1.0μm distances were viewed with an Olympus FV300 confocal laser scanning microscope. Microscope parameters were kept constant. Fluorescence levels were quantified by measuring mean pixel intensity in the nuclei and cytoplasm of PG cells displaying PER fluorescence (Table 1). Observed images were processed using Image J with an established protocol (Vafopoulou et al., 2010). The line tool was employed to take measurements of mean pixel intensity in the subcellular location of interest. Use of the line tool allows for discrimination between cytoplasmic and nuclear fluorescence and avoidance of confounding dark structures such as the nucleoi. Five measuresments per cell were taken in a minimum of 5 cells per time point. Statistical comparisons of mean pixel intensities between time points were performed using the Kruskall-Wallis test.

2.5. In vitro incubations:

PGs were dissected from arrhythmic LL animals and incubated for 40h (maximum time for maintainence of viable tissue) at 28°C ± 0.5 °C in 50μl of medium containing 4 parts *Rhodnius* saline (Lane et al., 1975) enriched with antibiotics and 1 part

Rhodnius hemolymph (Vafopoulou and Steel, 1998). This hemolymph was collected from day 2 larvae, which have been shown to contain only trace levels of ecdysteroids (Vafopoulou and Steel, 1989) and no detectable levels of PTTH (Vafopoulou and Steel, 1996a).

To assess the effect of light on the PER rhythmicity *in vitro,* arrhythmic LL glands from day 12 animals were incubated *in vitro* as above in DD for 40h. Glands were fixed for immunohistochemistry at set intervals beginning at 1h after the start of the incubation and then at 6h intervals. Fresh incubation media was provided for non-fixed glands at each time point.

In order to investigate the potential involvement of neuropeptides on the PG clock, arrhythmic LL glands were incubated in LL for 1h in media containing 1 brain equivalent extract (either whole brain, >10kDa fraction or <10kDa fraction; see section 2.6) at which point incubation medium was removed and replaced with fresh medium lacking brain extract, glands were rinsed thoroughly before fresh medium was added. Glands were fixed and subjected to immunohistochemical procedures beginning at 1h after the pulse of brain extract, and then every 6h for a total of 40h. Fresh incubation medium was provided for non-fixed glands at each time point.

Ecdysteroid release by the PGs *in vitro* was also examined. Incubation media were collected beginning 1h after exposure to pulse of brain extract and subsequently at 6h intervals, with fresh medium restored at each interval. Collected medium was stored in methanol at -20 °C. One gland from each animal was placed in the experimental regime *in vitro* while the contralateral gland (control) was incubated in LL.

2.6. Removal of PTTH and ILP activity with double immunoprecipitation

Brain extract from day 12 photophase fifth instar *Rhodnius* were prepared as in Vafopoulou and Steel (1996) and then partially purified by ultrafiltration using Amicon Ultra-0.5 ml (Ultracel-PL membrane) centrifugal tubes (Millipore, Billerica, MA, USA). Extract from 10 brains was centrifuged using a 10kDa filter, separating the upper and lower molecular weight fractions. The >10kDa and <10kDa fractions used in re-initiation experiments were in 100µl of *Rhodnius* saline.

Incubation media used for double immunoprecipitation contained either the >10kDa or <10kDa fractions re-constituted in 100µl of PBS, the >10kDa fraction and <10kDa fractions were allowed to react with anti-PTTH and anti-bombyxin respectively, for 24h at 4 °C. Antibodies were used at a dilution of 1:100. A secondary antibody, anti-guinea pig IgG was added at a dilution of 1:200 and allowed to react with both fractions for 2h at room temperature. The >10kDa fraction was centrifuged with a 100kDa filter to remove the antibodies and any bound protein, the filtrate was then centrifuged using a 10kDa filter and the subsequent concentrate was re-constituted in 100µl of *Rhodnius* saline. The <10kDa fraction was centrifuged using a 10kDa filter removing all bound protein and was reconstituted in 100ul of *Rhodnius* saline.

2.7. Ecdysteroid radioimmunoassay (RIA):

The ecdysteroid RIA was performed as described by Steel et al. (1982) using the H-21B antiserum (produced by Horn et al. 1976 and received from Dr. E. Chang, U.C.

Davis) and α-[23,24-3H(N)]ecdysone (sp. act. 83.2 Ci/mmol) (PerkinElmer, Billerica, MA) as the labeled ligand. 20E (Sigma, Oakville, ON) was used as the standard and the results are therefore expressed as 20-hydroxyecdysone (20E) equivalents.

2.8. *Stimulation index*

Synthesis of ecdysteroids by PGs *in vitro* declines progressively with time (described in Vafopoulou and Steel, 1998), thus it was necessary to correct for this decline in order to assess rhythmicity of ecdysteroid synthesis *in vitro*. In experiments involving pulses of neuropeptides, one gland was used in the experimental regime while the contralateral gland was left in LL as a control. The differences of the test and control glands were determined at each time point, mean differences were calculated for 5 pairs and the significance of these differences was determined using a paired-sample *t-test*. Ecdysteroid release by the PGs *in vitro* was then plotted as both 1) the differences of the means between control and experimental glands at each time point and 2) the calculated *t* values, producing a stimulation index representing the significance of the differences plotted in 1) (Vafopoulou et al., 1996).

RESULTS

3.1. SDS-PAGE and western blot with anti-PER

The specificity of the anti-PER was confirmed using western blot analysis. *Rhodnius* PGs were extracted at 7h after lights-off (mid-scotophase) and 7h after lights-on (mid-photophase), in order to investigate daily differences in protein levels in PGs. SDS-PAGE western blots with anti-PER revealed an immunoreactive protein band at 115kDa corresponding to the expected molecular weight of the PER protein on SDS-PAGE (Figure 1), as previously confirmed using 3 separate anti-PERs (Vafopoulou et al., 2010). A minor peptide band seen at 65kDa, is thought to represent a breakdown product or subunits of the PER peptide (Vafopoulou et al., 2010). PER staining was more intense using PGs from scotophase animals, as opposed to photophase animals (Fig. 1). These results suggest daily cycling of the PER protein in the PGs and correspond to the pattern observed for PER expression from clock cells in the *Rhodnius* brain (Vafopoulou et al., 2010). Additionally, an immunopositive protein band from scotophase wild-type *Drosophila* head extracts co-migrated with those from *Rhodnius* PGs (Fig. 1C). These findings together support the conclusion that the anti-PER used here specifically binds native *Rhodnius* PER.

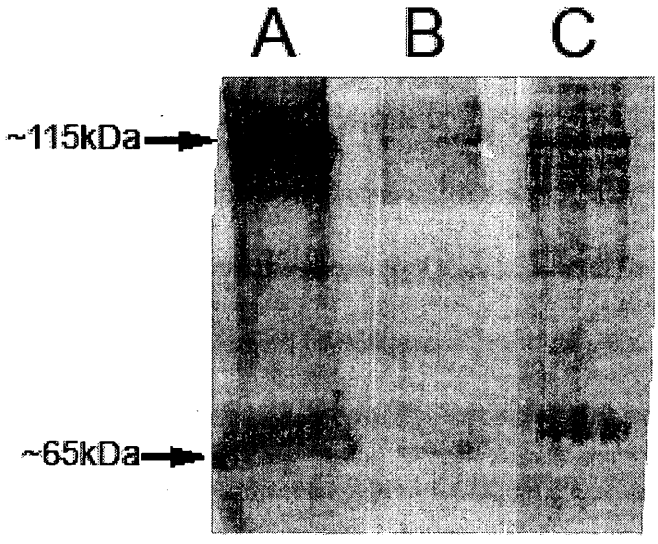
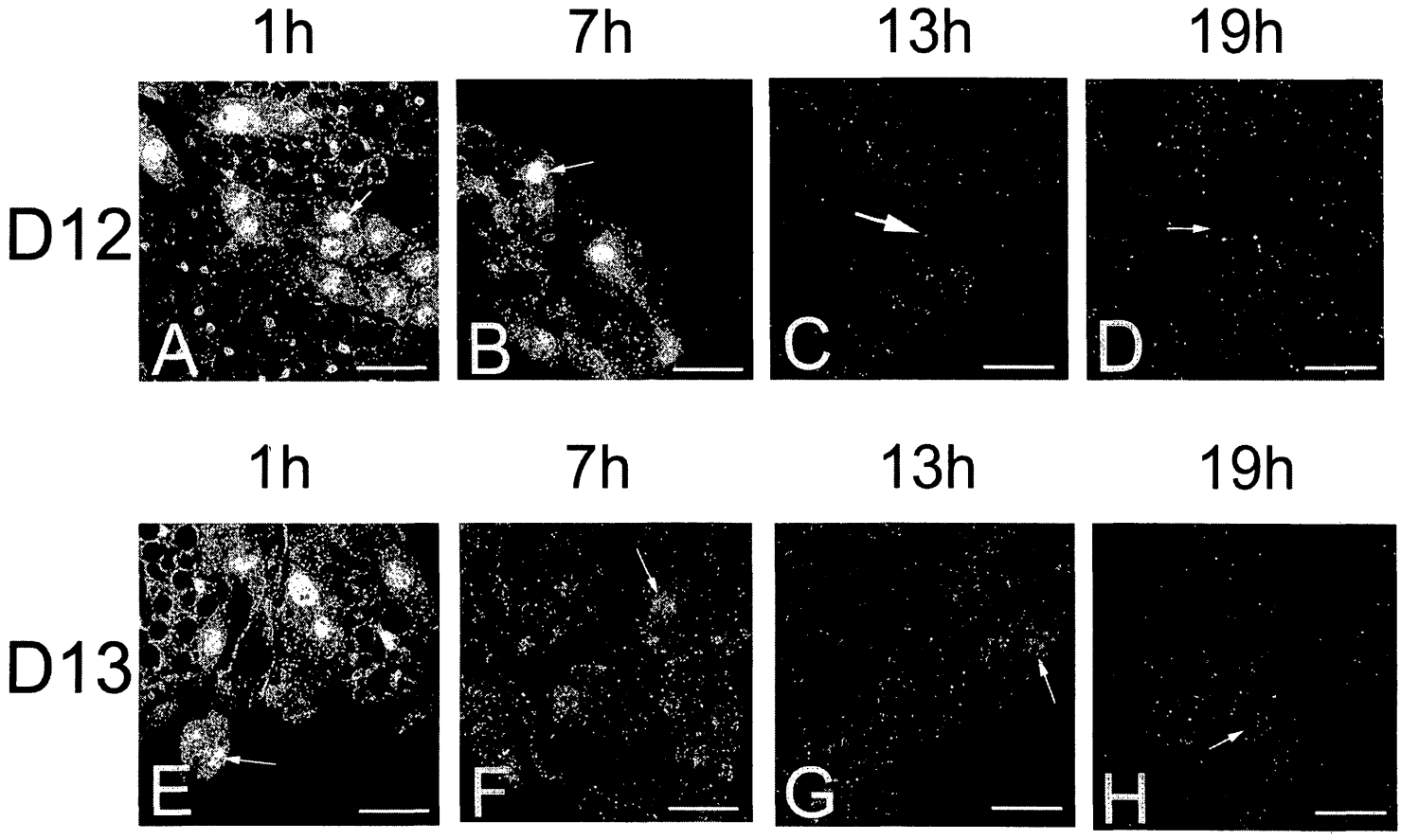


Figure 1: Western blot probed with anti-PER, comparing *Rhodnius* PGs with *Drosophila* head extract. Lanes A and B contain *Rhodnius* PGs (10 PGs per well) from mid-scotophase and mid-photophase respectively. Lane C contains *Drosophila* head extracts (5 heads). Immunoreactive protein band found in all lanes, with greater expression during the scotophase, occurs at ~115kDa the expected molecular weight of the PER protein.

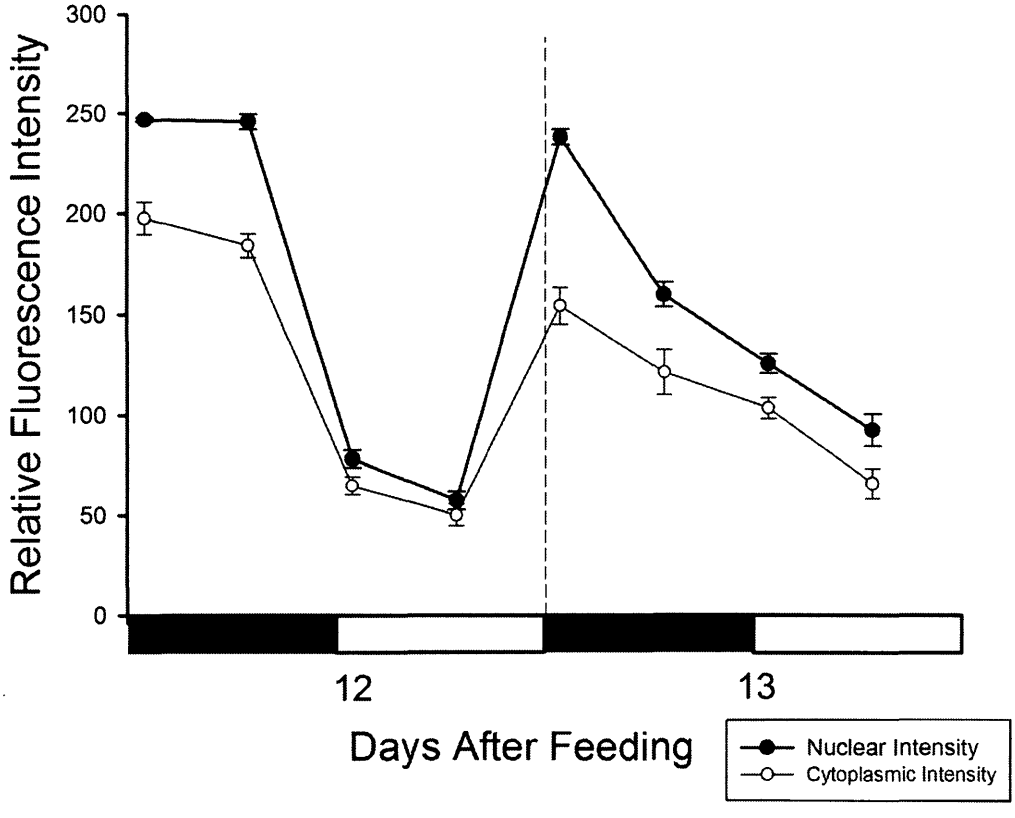
3.2. Daily Rhythm of PER *Cycling in 12L:12D Animals*

Rhythmic PER expression in the PGs *in vivo* was investigated in animals entrained to a 12L:12D photoperiod. PGs were dissected at 6h intervals for 2 consecutive days. PER immunofluorescence was evident at every time point examined (Figure 2). Furthermore, PER fluorescence was found to cycle rhythmically within the 24h cycle with peak PER fluorescence during the scotophase and reduced fluorescence during the photophase. In addition, cyclic sub-cellular localization of PER fluorescence can be seen in the PGs displaying bright nuclear localization during the scotophase (Fig. 2A-H). Relative immunofluorescence intensity (RFI) demonstrates a clear quantifiable rhythm of PER fluorescence across 2 consecutive days (Fig. 2I), comparison of daily peak and trough values were determined to be statistically significant ($p<0.05$) using a Kruskall-Wallis test with scotophase values in the nucleus being roughly 4 times those of the photophase. These results indicate a pattern of rhythmic PER expression in the PGs of *Rhodnius,* with peak expression and enhanced nuclear localization in the scotophase and are consistent with previous findings suggesting *Rhodnius* PGs possess a circadian clock (Vafopoulou and Steel, 1991, 1992, 1998).

Figure 2: Confocal laser scanning images of immunofluorescence of PER in PG cells. Days 12 and 13 after feeding are shown displaying a clear daily rhythm of PER expression in the PG cells. Images presented as time points occurring at 1h-19h after lights off on day 12 after feeding (A-D) and 1h-19h after lights off on day 13 (E-H). Strong nuclear immunofluorescence during the scotophase was observed, with low PER expression seen in the photophase. Pattern of rhythmicity was observed over both days. Scale bar= 50μm. (I) Mean relative fluorescence intensity of above images in Figure 3. Points represent mean ± SEM of intensity of a minimum of 5 PG cells per time point. Clear daily rhythmicity of immunofluorescence observed on days 12 and 13. Differences between daily peak intensity and daily low were shown to be statistically significant using a Kruskal-Wallis test ($P<0.05$), indicating the rhythm is significant.


1h
7h
13h
19h
D12
A
B
C
D
1h
7h
13h
19h
D13
E
F
G
H

I


Relative Fluorescence Intensity
300
250
200
150
100
50
0
12
13
Days After Feeding
Nuclear Intensity
Cytoplasmic Intensity

3.3. Photic Induction of PER Rhythmicity in Arrhythmic LL Animals in vivo

Prolonged exposure to LL produces completely arrhythmic animals (Ampleford and Steel, 1986; Vafopoulou and Steel, 1991, 1998). Animals which have been made arrhythmic were shown to have low arrhythmic levels of PER in the PGs (Figure 3). It was investigated whether a lights-off photic cue would reinitiate rhythmic PER expression in arrhythmic LL animals. Transfer of animals from LL to DD induced PER expression as determined by bright PER fluorescence beginning as early as 1h after transfer to DD (Figure 4A). In addition, rhythmicity of PER fluorescence was reinitiated, with two clear rhythmic peaks across two consecutive days roughly 24h apart (Fig.4A, E). The peak of PER fluorescence was noted during the subjective scotophase following exposure to an entraining lights-off cue (Fig. 4I). Daily differences in peak and trough levels of RIF were found to be statistically significant ($p<0.05$). Furthermore, cyclic nuclear localization of PER in the PG cells was noted with bright nuclear fluorescence existing in the subjective scotophase (Fig. 4A, B, E, F). These results agree with those observed in entrained animals and indicate that transfer to DD reinitiated rhythmic PER expression in previously arrhythmic PGs. Therefore, *in vivo,* the PGs are receiving and responding to photic cues (see Discussion).

Figure 3: Confocal laser scanning images of immunofluorescence of PER in LL PG cells. Representative images of arrhythmic low levels of PER expression in LL PGs. LL control images taken every 6 hours, arrhythmicity in PER expression (A-D). LL controls were taken at all time points of all subsequent experiments and demonstrated the same/similar arrhythmic levels of PER throughout.

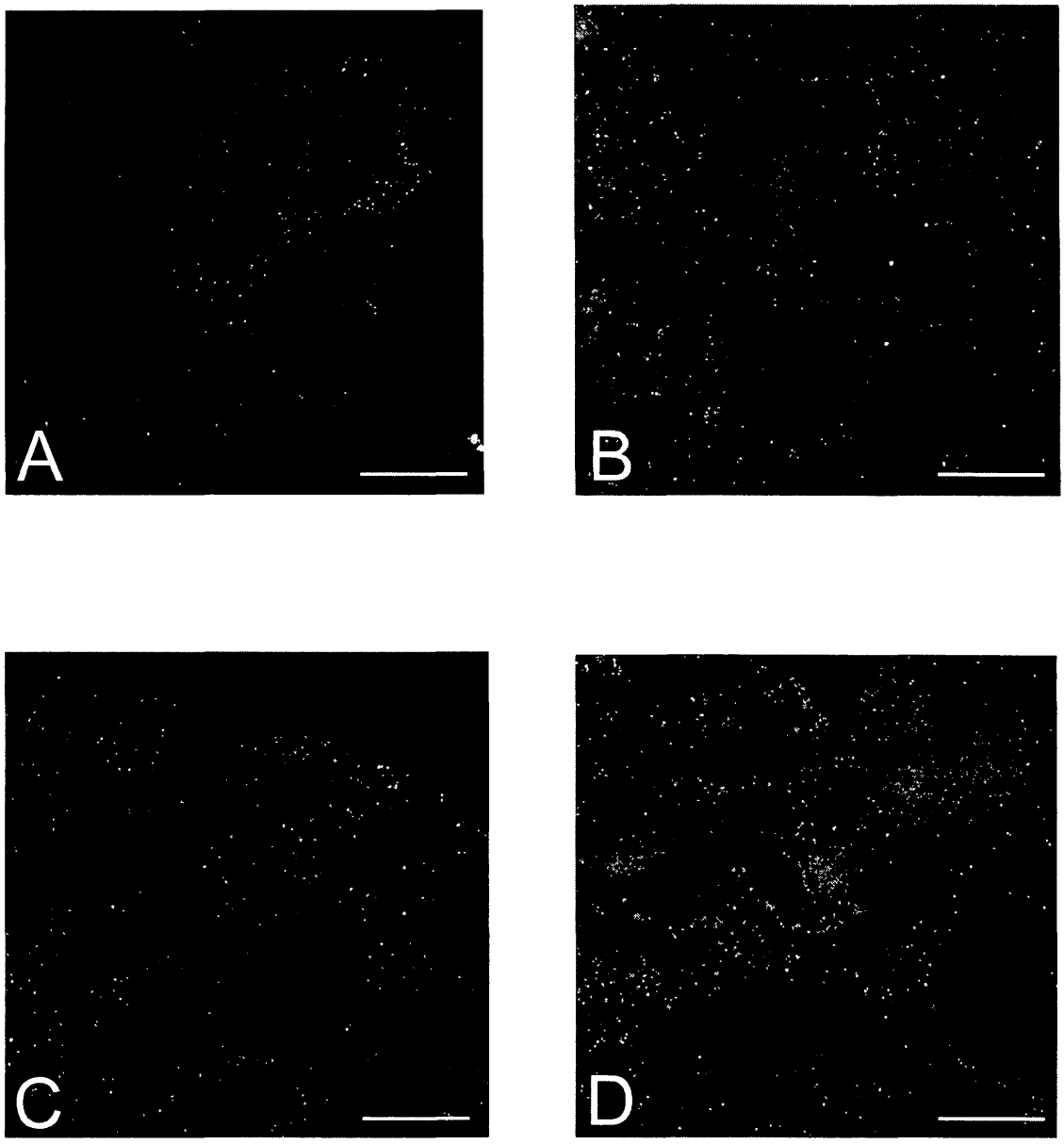
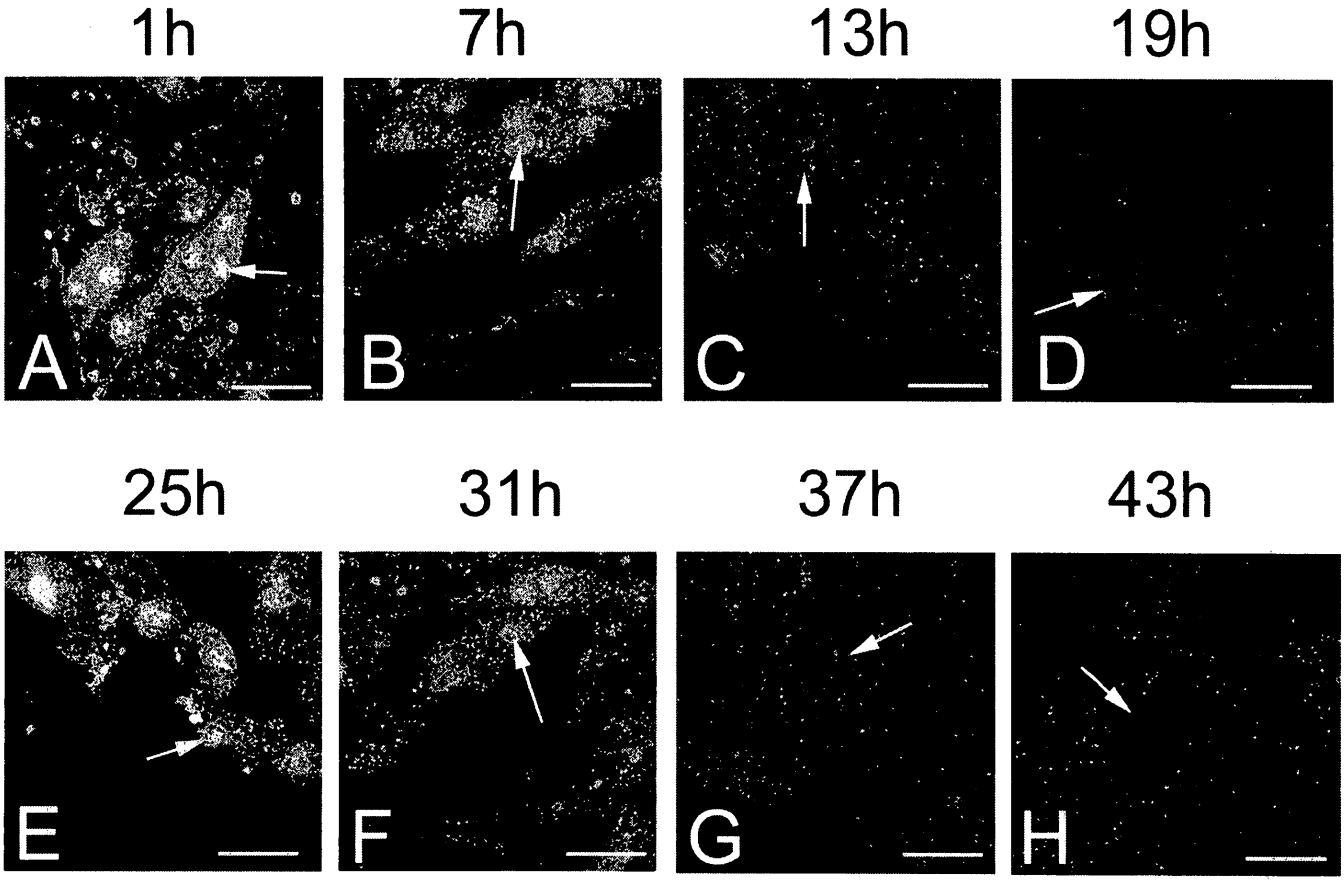
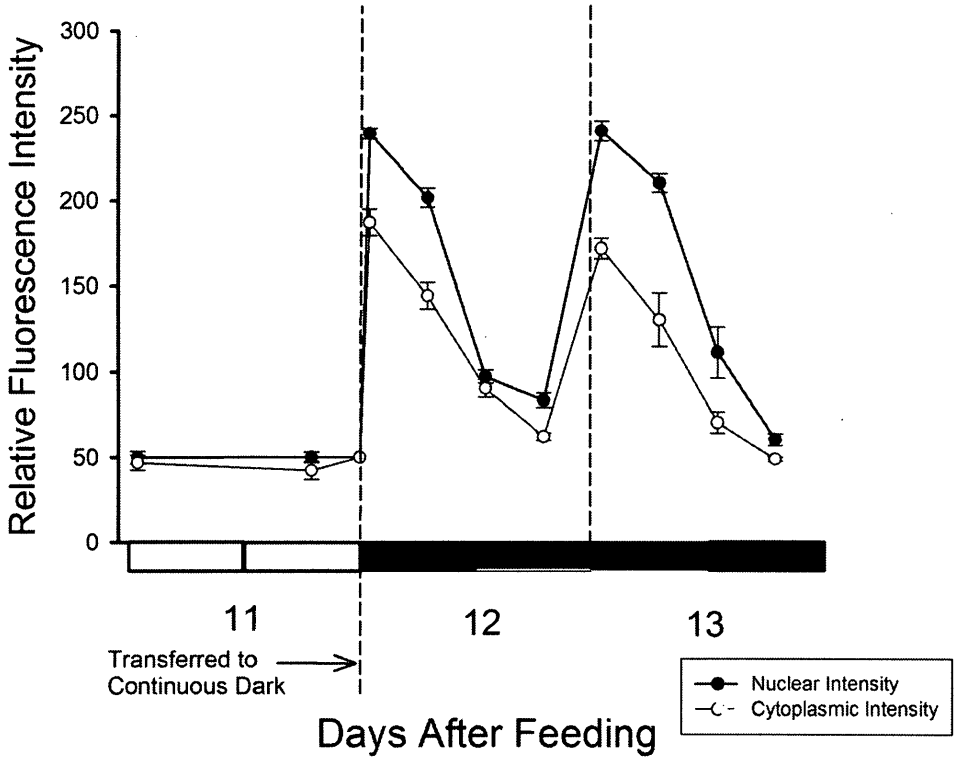

A
B
C
D

Figure 4: Confocal laser scanning images of immunofluorescence of PER in PG cells. Demonstrates reinitiation of rhythmicity of PER expression in PG cells of arrhythmic LL animals following transfer to DD. Each image represents a specific time point indicated above spanning from 1h-43h after lights-off (A-H). Rhythmicity is initiated and maintained across days 12 and 13. Scale bar = 50μm. (I) Mean relative fluorescence intensity of images. Points represent mean ± SEM of intensity of a minimum of 5 PG cells per time point (Nuclear and cytoplasmic measurements included). Differences between daily peak intensity and daily low were shown to be statistically significant using Kruskal-Wallis test ($P<0.05$), indicating the rhythm is significant.


1h
7h
13h
19h
A
B
C
D
25h
31h
37h
43h
E
F
G
H

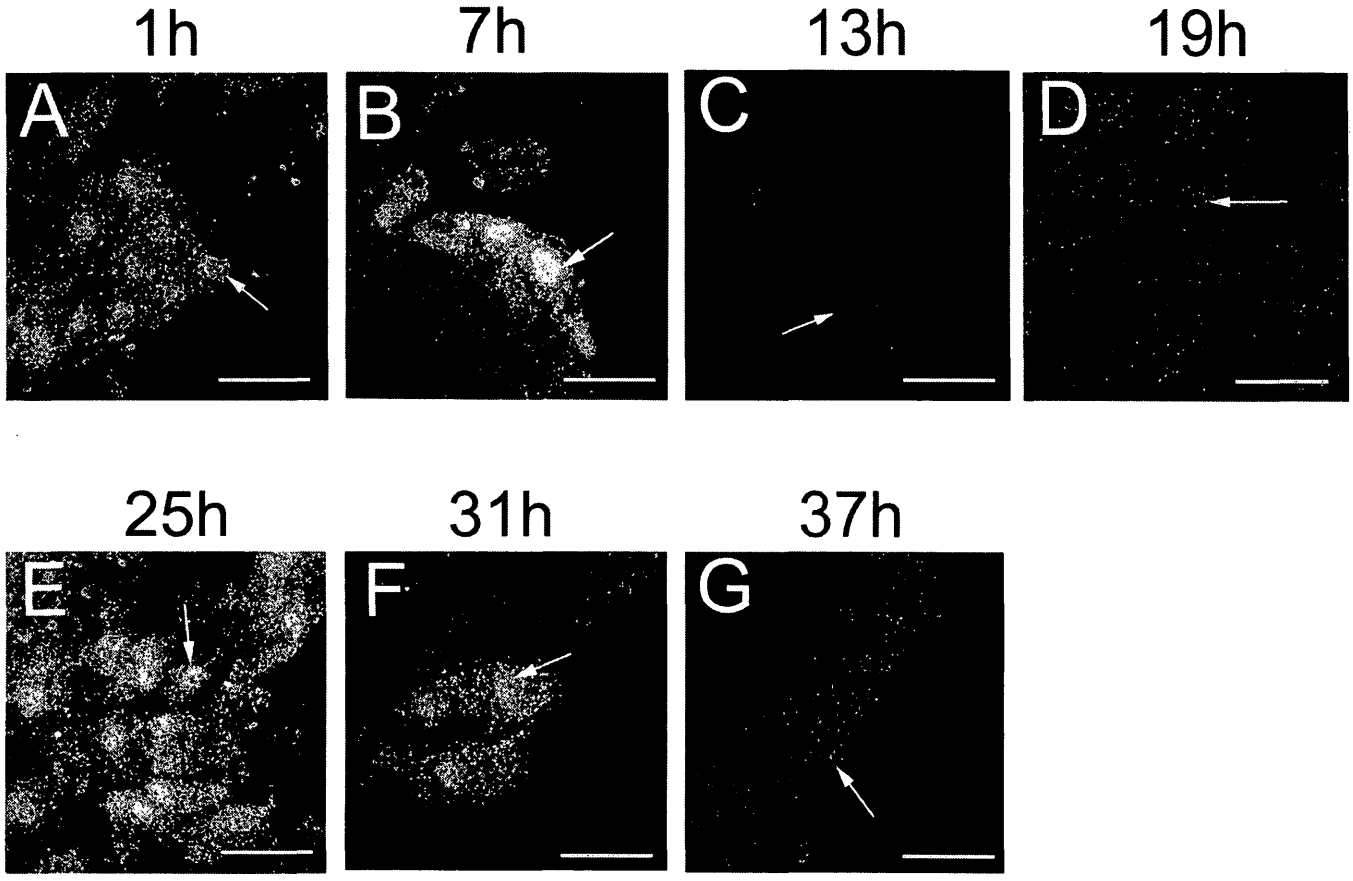
I


Relative Fluorescence Intensity
300
250
200
150
100
50
0
11
12
13
Transferred to
Continuous Dark
Days After Feeding
Nuclear Intensity
Cytoplasmic Intensity

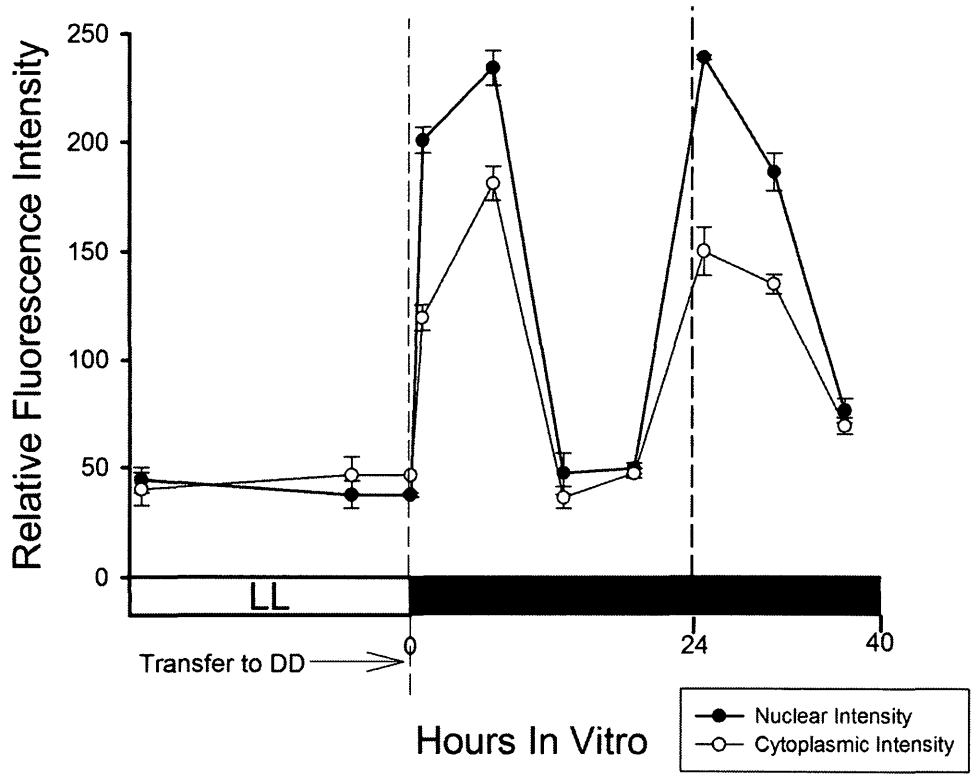
3.4. Photic Induction of PER Rhythmicity in Arrhythmic LL PGs in vitro

In vitro incubation of arrhythmic LL PGs in DD has been shown to reinitiate rhythmic ecdysteroid synthesis, further supporting that PGs are themselves directly photosensitive (Vafopoulou and Steel, 1992; 1998; 2001). It has not been shown whether the reinitiation of steroid synthesis results from reinitiation of cycling of the MO. In order to examine this, the reinitiation of PG clock function was investigated by observing PER expression in LL PGs incubated *in vitro* in DD (Figure 5). Bright PER fluorescence was observed as early as 1h after the start of the incubation (Fig.5A), indicating reinitiation of the MO and direct photosensitivity of the PG clock. Rhythmic PER fluorescence was reinitiated across a 40h long-term *in vitro* incubation, with levels comparable to those seen *in vivo* (Fig.5H). Two rhythmic peaks of PER fluorescence were observed with a period length around 24h. Cyclic nuclear localization of PER fluorescence was once again observed in PGs, corresponding to time points containing peak PER fluorescence (Fig.5). Reinitiation of rhythmic PER fluorescence corresponds to the rhythms of PER expression observed *in vivo* (Fig. 4). Furthermore, the rhythm of PER expression is synchronous with the previously reported rhythm of ecdysteroid synthesis *in vitro* (Vafopoulou and Steel, 1998). These results show that reinitiation of PER rhythmicity is linked to rhythmic ecdysteroid production and the PG clock is directly photosensitive (see Discussion).

Figure 5: Confocal laser scanning images of immunofluorescence of PER in PG cells. Demonstrates reinitiation of rhythmicity of PER expression in PG cells of arrhythmic LL animals following transfer to DD *in vitro*. Each image represents a specific time point indicated above spanning from 1h-43h after lights-off (A-G). Rhythmicity is initiated and maintained across a two day span. Scale bar = 50μm. (H) Mean relative fluorescence intensity of images. Points represent mean ± SEM of intensity of a minimum of 5 PG cells per time point (Nuclear and cytoplasmic measurements included). Differences between daily peak intensity and daily low were shown to be statistically significant using Kruskal-Wallis test ($P<0.05$), indicating the rhythm is significant.


1h
7h
13h
19h
A
B
C
D
25h
31h
37h
E
F
G

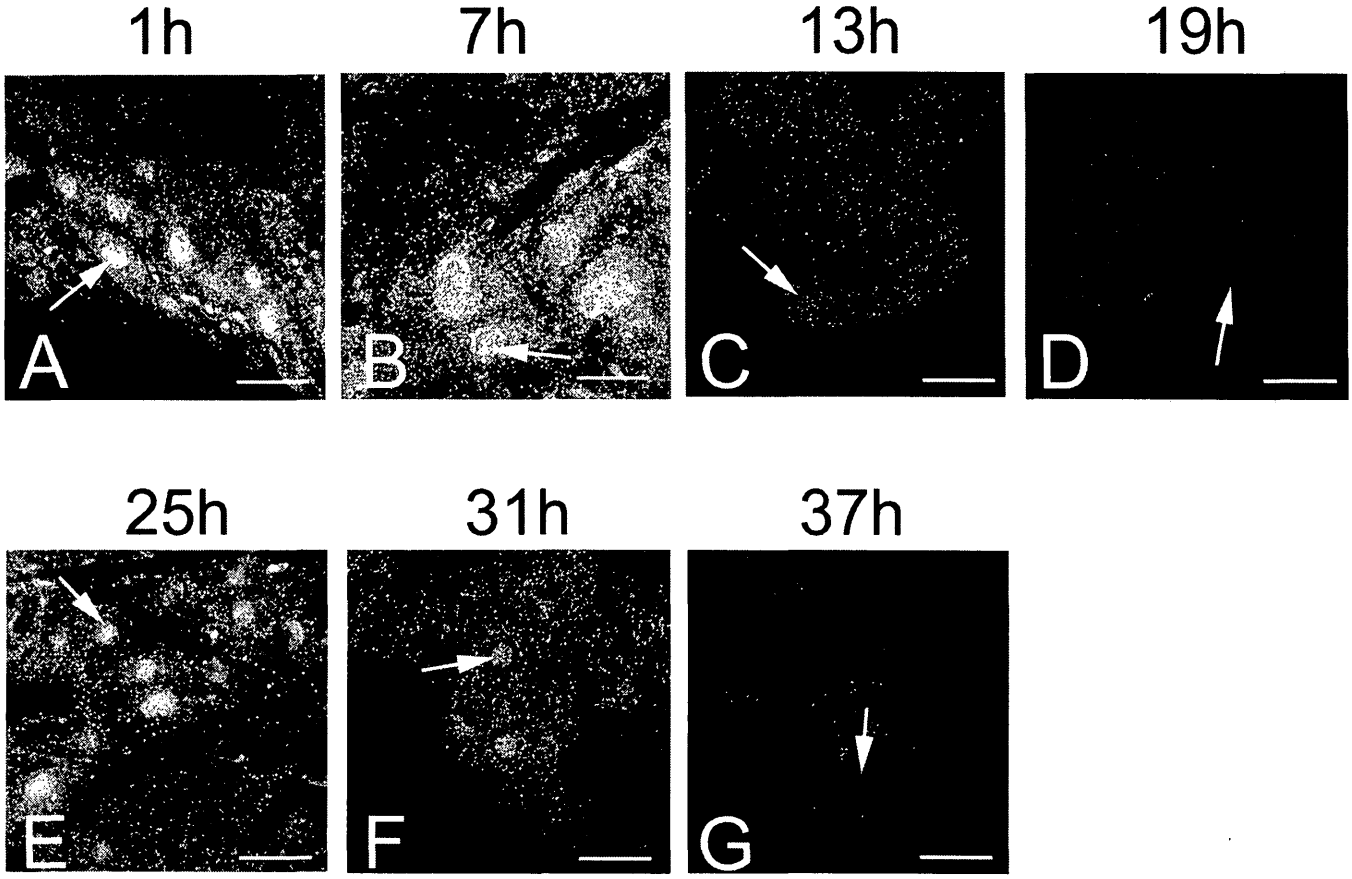
H


Relative Fluorescence Intensity
250
200
150
100
50
0
LL
Transfer to DD
0
24
40
Hours In Vitro
Nuclear Intensity
Cytoplasmic Intensity

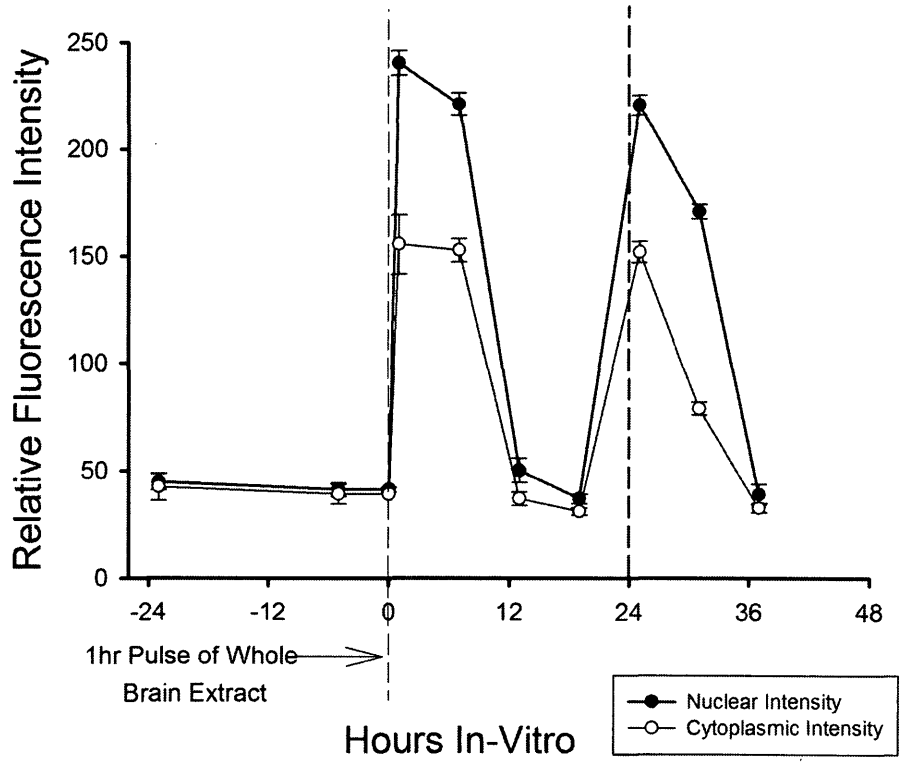
3.5. Neuropeptide Induction of PER Rhythmicity in LL PGs in vitro

The neuropeptide PTTH has previously been shown to modulate the phase of rhythmic ecdysteroid production by the PGs, suggesting that the peptide acts on the PG clock (Pelc and Steel, 1997). However, it is unknown whether peptides such as PTTH affect the clock machinery directly. The ability of neuropeptides to reinitiate rhythmic PER expression and rhythmic ecdysteroid production in arrhythmic LL PGs was investigated to determine whether these neuropeptides directly affect the PG clock. Whole brain extract was provided, immediately following dissection, for 1h to arrhythmic LL PGs incubated in LL *in vitro* (Figure 6). The pulse of brain extract reinitiated PER fluorescence in arrhythmic LL PGs (Fig.6A); furthermore, rhythmicity of PER fluorescence was observed over the 40h long-term incubation. Bright PER fluorescence was observed beginning at 1h after the pulse of brain neuropeptide extract, with 2 clear rhythmic peaks of fluorescence with a period of roughly 24h (Fig.6H). Differences in daily peak and trough values were determined to be statistically significant ($p<0.01$). Cyclic nuclear localization was once again observed in the PGs, with enhanced nuclear localization during peak PER fluorescence (1h, 7h, 25h and 31h hours after the pulse) (Fig.6A,B,E,F). PER fluorescence levels were comparable to those observed *in vivo*. This finding demonstrates that a 1h pulse of brain extract was sufficient, in the absence of light cues, to reinitiate rhythmic PER expression. Furthermore, the rhythm of PER expression in the PGs is identical to that induced through transfer to DD (Fig.5), suggesting that the brain extract neuropeptides may be acting as a signal of darkness.

Figure 6: Confocal laser scanning images of immunofluorescence of PER in PG cells. Demonstrates reinitiation of rhythmicity of PER fluorescence in arrhythmic PG cells after exposure to a 1h pulse of *Rhodnius* whole brain extract *in vitro*. Each image represents a specific time point indicated above spanning from 1h-43h after lights-off (A-G). Rhythmicity is initiated and maintained across a two day span. Scale bar = 50μm. (H) Mean relative fluorescence intensity of images. Points represent mean ± SEM of intensity of a minimum of 5 PG cells per time point (Nuclear and cytoplasmic measurements included). Differences between daily peak intensity and daily low were shown to be statistically significant using Kruskal-Wallis test ($P<0.05$), indicating the rhythm is significant.


1h
7h
13h
19h
A
B
C
D
25h
31h
37h
E
F
G

H


Relative Fluorescence Intensity
250
200
150
100
50
0
-24
-12
0
12
24
36
48
1hr Pulse of Whole Brain Extract
Hours In-Vitro
Nuclear Intensity
Cytoplasmic Intensity

Ecdysteroid production by the PGs following a 1h pulse of whole brain extract was also investigated. Pairs of PGs were dissected and incubated *in* vitro in LL for 40h with one gland from each pair being incubated in modified *Rhodnius* saline (see Materials and Methods) alone (control) while the contralateral gland was provided with the 1h pulse of brain extract (experimental) (Figure 7). Comparisons were made between LL control glands and contralateral experimental glands to account for the gradual decline in ecdysteroid production observed in a long-term incubation such as this (Vafopoulou and Steel, 1998). Points on the left side of the vertical dashed line indicate differences between glands both left in LL conditions, differences of means were minimal and thus stimulation indices are approximately zero (Fig.7B). When comparing LL control glands to those provided with the pulse of brain extract, there is a large increase in ecdysteroid production by the PGs provided with the pulse of brain extract (Fig.7B). This large and significant increase is noted as early as 1h after the pulse and ecdysteroid production remained significantly greater in experimental glands at 7h after the pulse. Ecdysteroid levels drop to below those produced by the LL control glands at 13h and 19h after the pulse (Fig.7B). This is followed by a second statistically significant peak at 25h after the pulse of brain extract. The two distinct statistically significant peaks of ecdysteroid synthesis by the experimental glands indicate the pulse of whole brain extract induced a reinitiation of rhythmic ecdysteroid production by the PGs, with an approximately 24h period length. Therefore, a 1h pulse of brain extract reinitiated rhythmic ecdysteroid synthesis in conjunction with re-starting the PG clock.

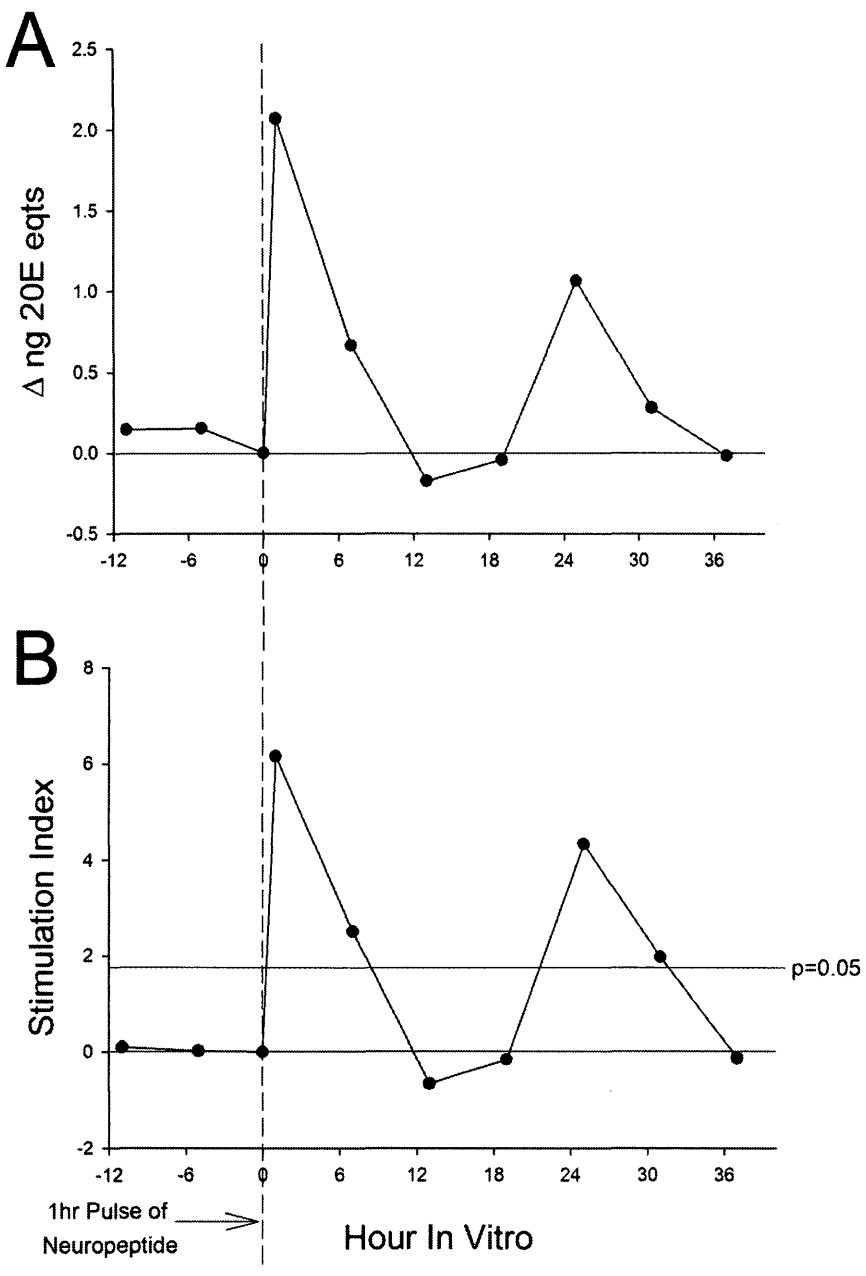


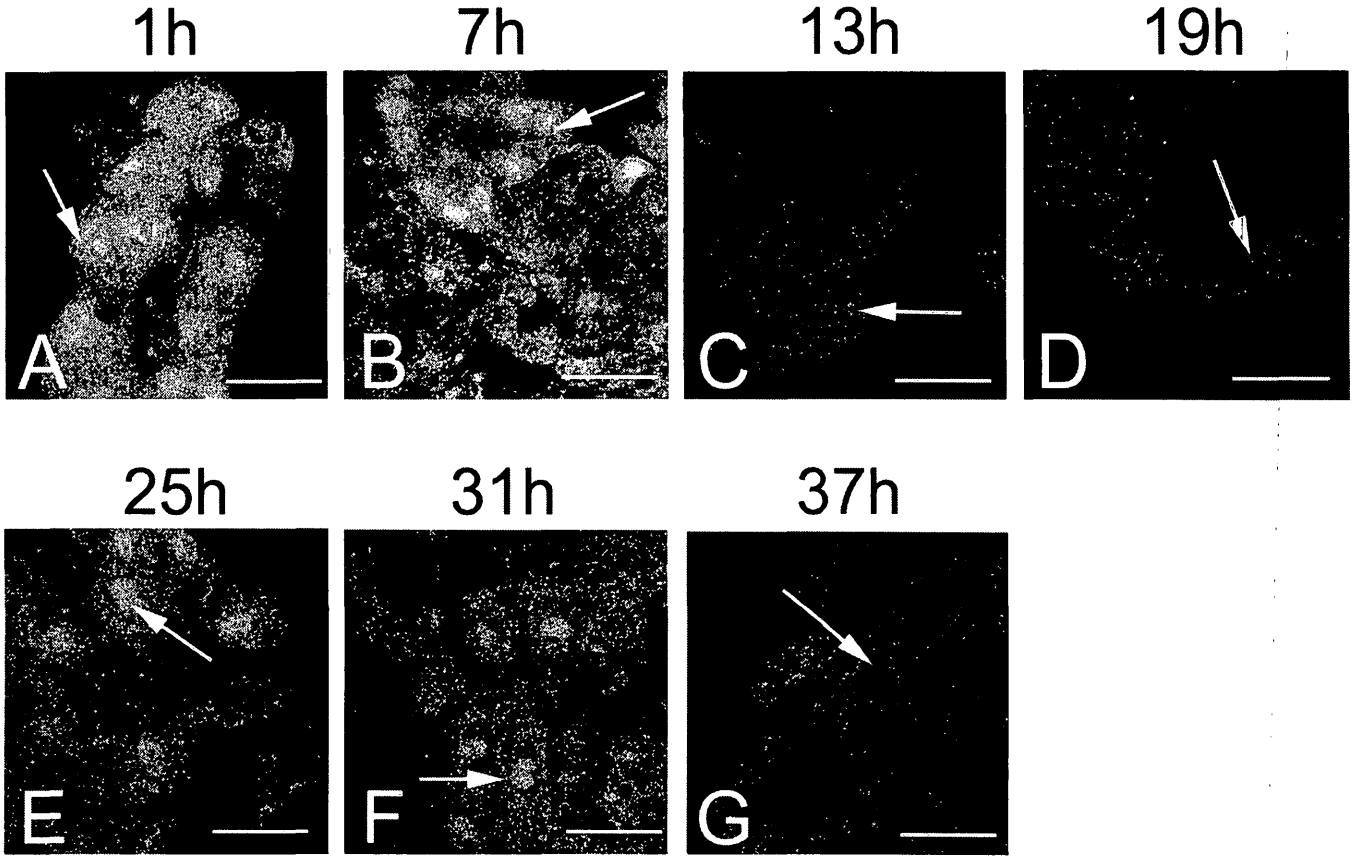
Figure 7: Rhythm of ecdysteroid synthesis induced in vitro by 1h incubation of arrhythmic LL glands in whole brain extract. One gland of each pair from each animal was incubated with brain extract, the other remained in LL control. Data are expressed as (A) the mean numerical differences between synthesis by test glands and that of control glands (n=10 replicants of paired glands), and (B) significance of the differences calculated a paired sample t-test. Solid line at p=0.05 show levels at which synthesis by experimental glands is above synthesis by LL control glands.

In order to more specifically characterize the neuropeptide(s) responsible for reinitiaiting rhythmicity of PER expression and ecdysteroid synthesis by the PGs, brain extracts were fractionated using size exclusion centrifugation into >10kDa and <10kDa fractions. Molecular size groups were chosen to separate known ecdysteroidogenic peptides, PTTH (>10kDa fraction) and ILPs (<10kDa fraction). Both peptides are rhythmically released and closely associated with clock cell axons in the brain (Vafopoulou and Steel, 2007, 2012). Providing a 1h pulse of either the >10kDa (Figure 8) or <10kDa (Figure 9) fraction resulted in rhythmic PER fluorescence in LL PGs. There were two clear statistically significant rhythmic peaks of PER fluorescence over the time course of the *in vitro* incubation (Fig.8H and Fig.9H). Peak fluorescence was noted at 1h and 25h after the pulse of either >10kDa or <10kDa fractionated brain extract. Nuclear localization of PER was found to be cyclic, with enhanced nuclear expression noted at 1h, 7h, 25h and 31h after the pulse of brain extract. The results indicate that the >10kDa fraction and/or the <10kDa fraction of brain extract is sufficient to reinitiate rhythmic PER fluorescence in arrhythmic LL PGs; therefore, the known ecdysteroidogenic peptides (reviewed in Marchal et al., 2010), PTTH and ILPs, both appear as likely candidates to act on the MO (see Discussion).

The ability of the >10kDa and the <10kDa brain neuropeptide fractions to reinitiate rhythmic ecdysteroid synthesis by the PGs was investigated (as described above for whole brain extract). Following the pulse of >10kDa or <10kDa peptide, there was a statistically significant increase in ecdysteroid production when compared to LL control glands (Figure 10B,D). This significant increase is noted as early as 1h after the pulse,

ecdysteroid production remained significantly greater in experimental glands at 7h after the pulse, after which point it began to decline. A second statistically significant increase/peak was noted at 25h after the pulse of brain extract. The two statistically significant peaks of ecdysteroid production by experimental glands indicates the rhythmicity of ecdysteroid synthesis can be restored by a 1h pulse of >10kDa peptide and/or <10kDa peptide (Fig.10). PGs provided with a pulse of <10kDa brain extract showed the lowest levels of stimulation of ecdysteroid synthesis (Fig.10D). The greatest stimulation was observed in glands provided with a pulse of whole brain extract, suggesting a potential synergistic effect of the 2 fractions.

Figure 8: Confocal laser scanning images of immunofluorescence of PER in PG cells. Demonstrates reinitiation of rhythmicity of PER fluorescence in arrhythmic PG cells after exposure to a 1h pulse of *Rhodnius* brain extract, >10kDa fraction. Each image represents a specific time point indicated above spanning the period from 1h-43h after lights-off (A-G). Rhythmicity is initiated and maintained across the two days of study. Scale bar = 50μm. (H) Mean relative fluorescence intensity of images. Points represent mean ± SEM of intensity of a minimum of 5 PG cells per time point (Nuclear and cytoplasmic measurements included). Differences between daily peak intensity and daily low were shown to be statistically significant using Kruskal-Wallis test ($P<0.05$), indicating the rhythm is significant.


1h
7h
13h
19h
A
B
C
D
25h
31h
37h
E
F
G

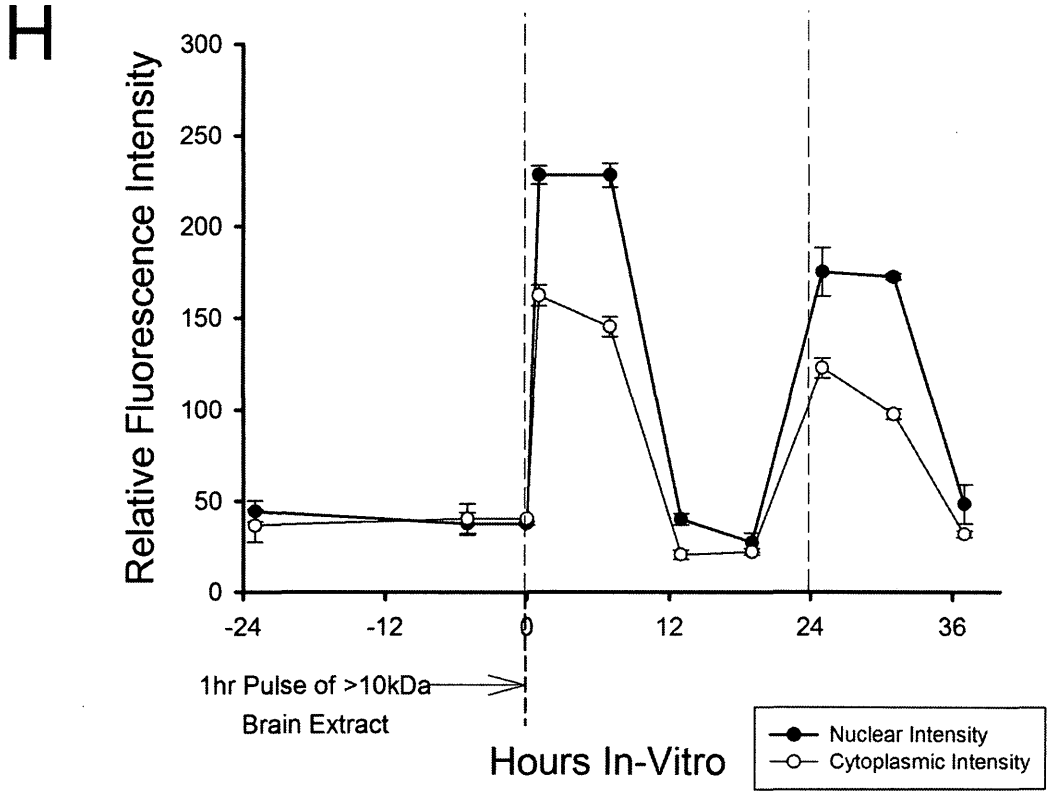
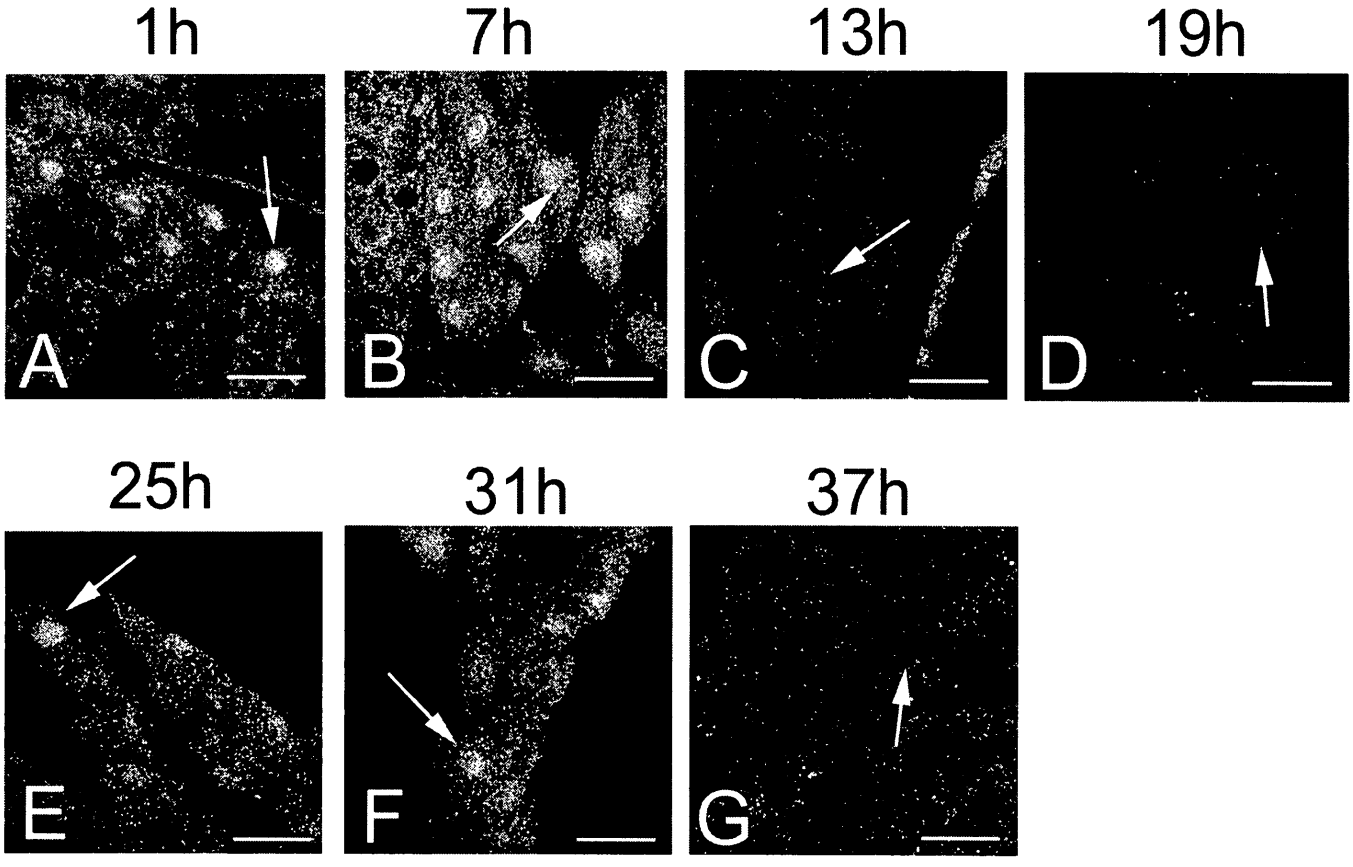

H
Relative Fluorescence Intensity
300
250
200
150
100
50
0
-24
-12
0
12
24
36
1hr Pulse of >10kDa
Brain Extract
Hours In-Vitro
Nuclear Intensity
Cytoplasmic Intensity

Figure 9: Confocal laser scanning images of immunofluorescence of PER in PG cells. Demonstrates reinitiation of rhythmicity of PER fluorescence in arrhythmic PG cells after exposure to a 1h pulse of *Rhodnius* brain extract, <10kDa fraction. Each image represents a specific time point indicated above spanning from 1h-43h after lights-off (A-G). Rhythmicity is initiated and maintained across a two day span. Scale bar = 50μm. (H) Mean relative fluorescence intensity of images. Points represent mean ± SEM of intensity of a minimum of 5 PG cells per time point (Nuclear and cytoplasmic measurements included). Differences between daily peak intensity and daily low were shown to be statistically significant using Kruskal-Wallis test ($P<0.05$), indicating the rhythm is significant.


1h
7h
13h
19h
A
B
C
D
25h
31h
37h
E
F
G

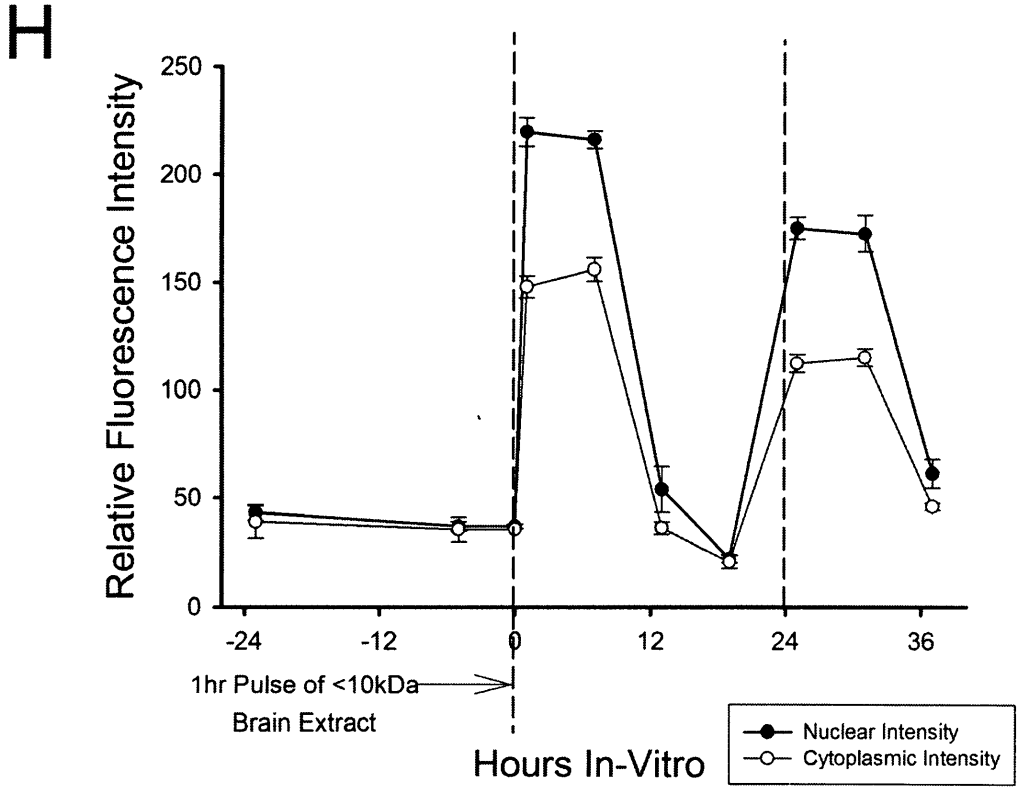
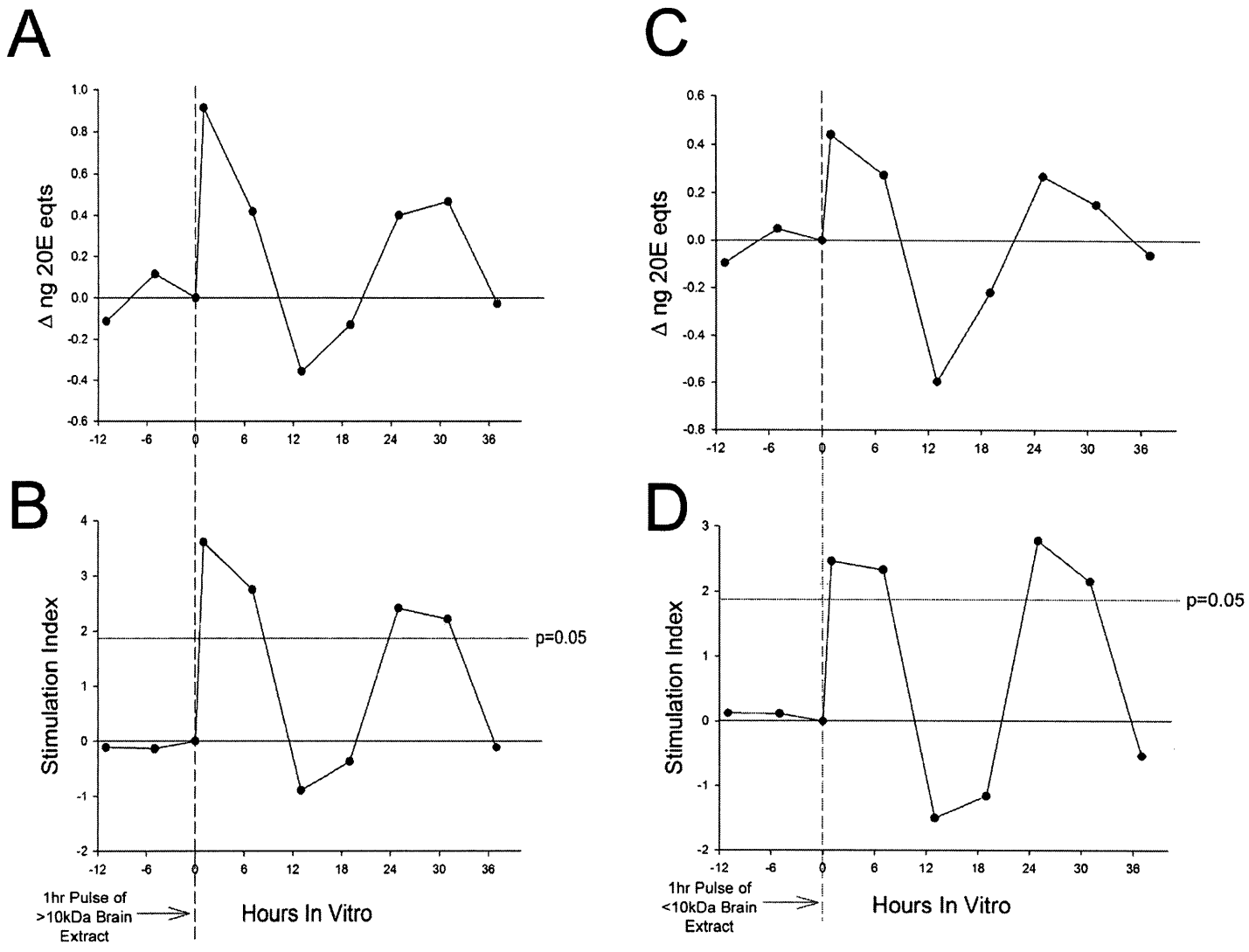

H
Relative Fluorescence Intensity
250
200
150
100
50
0
-24
-12
0
12
24
36
1hr Pulse of <10kDa
Brain Extract
Hours In-Vitro
Nuclear Intensity
Cytoplasmic Intensity

Figure 10: Rhythm of ecdysteroid synthesis induced in vitro by 1h incubation of arrhythmic LL glands in brain extract, >10kDa brain extract (A,B) and <10kDa brain extract (C, D). One gland of each pair from each animal was incubated with brain extract, the other remained in LL control. Data are expressed as (A,C) the mean numerical differences between synthesis by test glands and that of control glands (n=10 replicants of paired glands), and (B,D) significance of the differences calculated a paired sample t-test. Solid lines at p=0.05 show levels at which synthesis by experimental glands is above synthesis by LL control glands.


A
B
C
D
Δ ng 20E eqts
Stimulation Index
p=0.05
1hr Pulse of
>10kDa Brain
Extract
1hr Pulse of
<10kDa Brain
Extract
Hours In Vitro

3.6. Removal of PTTH and ILP Activity via Double Immunoprecipitation

Since both PTTH (Vafopoulou et al., 1996; Vafopoulou and Steel, 1996a, 1996b) and ILPs (Vafopoulou and Steel, 1997) are known to stimulate ecdysteroidogenesis by *Rhodnius* PGs and to be under control of the brain circadian clock (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012) the possibility that these neuropeptides are responsible for the effects on PER expression and ecdysteroid synthesis by PGs reported here was investigated (Figure 11). Thus, removal of PTTH-like and ILP-like activity from the fractionated brain extracts (>10kDa and <10kDa respectively) via double immunoprecipitation was performed (see Materials and Methods). Arrhythmic LL PGs were provided with a 1h pulse of either >10kDa extract with PTTH activity removed (Fig.11A) or <10kDa extract with ILP activity removed (Fig.11B) immediately following dissection. The pulses of either double-immunoprecipitated fraction were unable to elicit any change in PER fluorescence when compared to LL controls. The rapid increase in PER fluorescence initiated by both brain extract fractions (see above) was not evident when PTTH and ILP activity were removed from the >10kDa and <10kDa fractions respectively. These results implicate both PTTH and ILPs as modulators of the *Rhodnius* PG clock and suggest these neuropeptides are acting as hormonal Zeitgebers to the PG clock in *Rhodnius* (see Discussion).

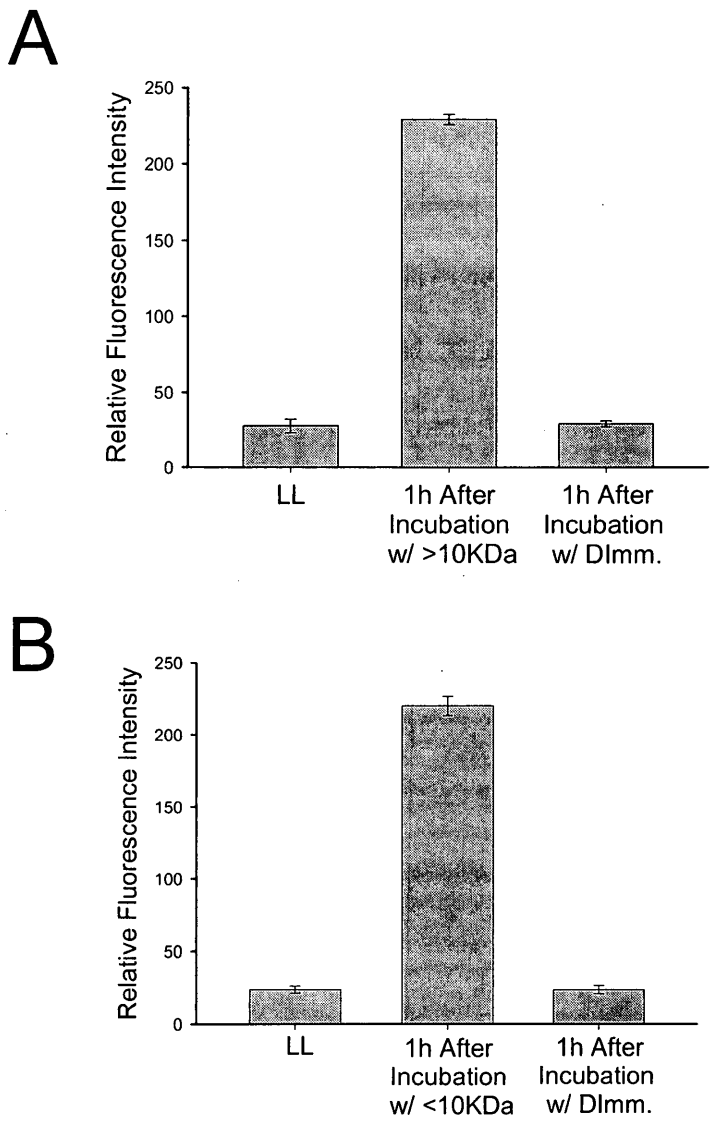
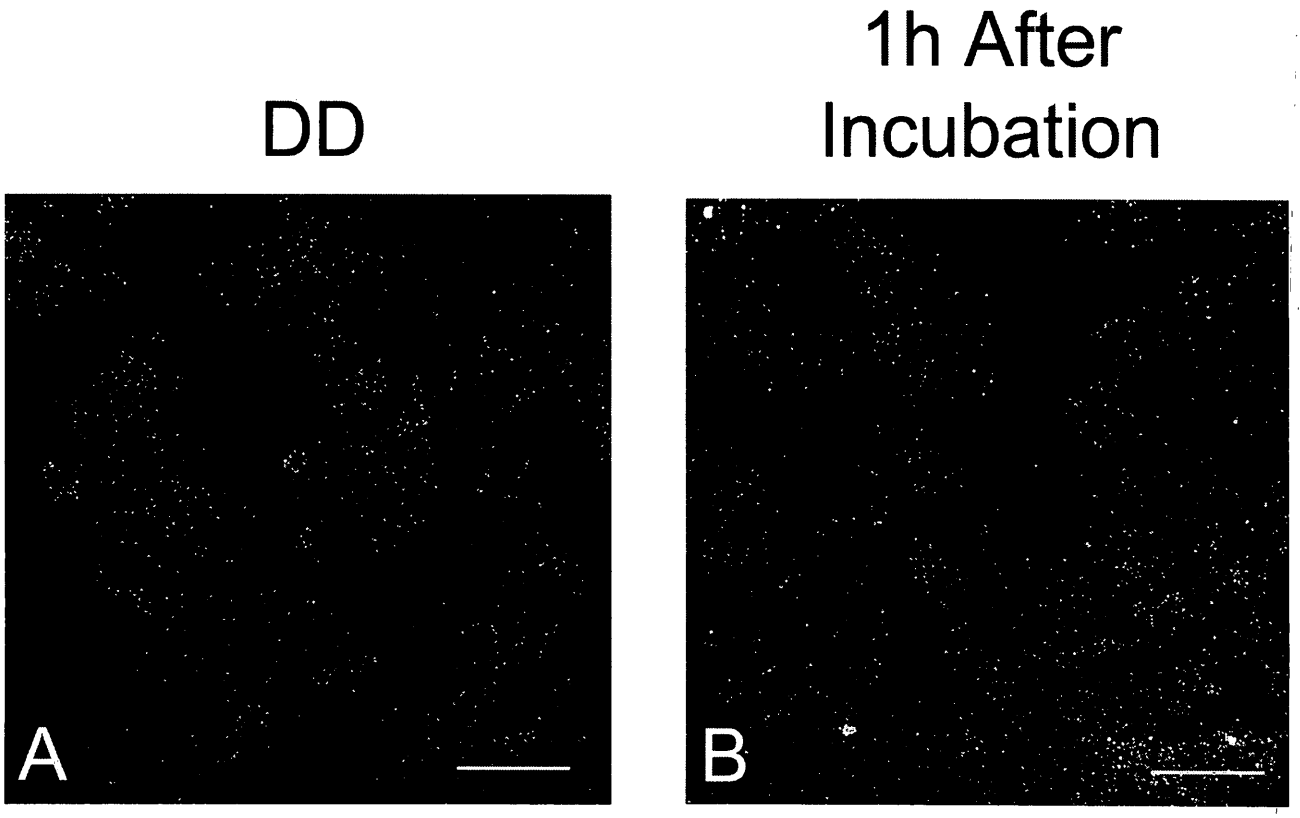


Figure 11: Quantification of immunofluorescence in LL PGs provided with 1h pulse of brain extract (A) LL PGs provided with 1h pulse of >10kDa brain extract with PTTH-like activity removed, no significant difference exists between LL control PGs and those in which PTTH activity has been removed from the >10kDa fraction. (B) LL PGs provided with 1h pulse of <10kDa brain extract with ILP-like activity removed, no significant difference exists between LL control PGs and those in which ILP activity has been removed from the <10kDa fraction.

3.7. Neuropeptide Induction of PER Expression in Arrhythmic DD PGs in vitro

The 1h pulse of brain extract (whole brain, >10kDa and <10kDa) produced the same results as providing LL PGs with a lights-off cue, suggesting these neuropeptide inputs to the PGs act like signals of darkness. To investigate if the previously described neuropeptides can also act like a pulse of light to the PG clock, animals were made arrhythmic through long-term exposure to DD. After 4 weeks in DD, PGs were found to contain minimal PER fluorescence similar to the case for arrhythmic LL glands. Arrhythmic DD animals were dissected in the dark with the assistance of non-photoreceptive red light. PGs were incubated *in vitro* in DD and provided with a 1h pulse of brain extract (as described above). Following incubation PER fluorescence in experimental glands was found not to be significantly different (data not shown) from DD control glands (Figure 12). The lack of response by DD PGs to a pulse of neuropeptides indicates these peptides are not seen as light by the MO in the PGs, only as darkness.

Figure 12: Confocal laser scanning images of immunofluorescence of PER in PG cells. Animals were made arrhythmic through long-term exposure to DD, PGs were excised from DD animals and provided with a 1h pulse of whole brain extract (all steps were performed in DD). Incubation of arrhythmic DD PGs did not reinitiate PER expression as there was no significant difference (data not shown) in immunofluorescence between DD controls and experimental glands. Results confirm brain extract neuropeptides acting as pulse of darkness for LL PGs; therefore, unable to provide an entraining signal to arrhythmic DD PGs.


DD
1h After Incubation
A
B

DISCUSSION

The PG clock of *R. prolixus* was one of the first definitively demonstrated peripheral clocks due to the simplicity of its structure, its ease of maintenance *in vitro,* and its production of a measurable rhythmic hormonal output (ecdysteroids). Peripheral tissue clocks have now been well established in a variety of insect tissues (Plautz et al., 1997; Tomioka et al., 2012). The PGs specifically have been a source of investigation due to their role in regulating larval and pupal development. The present study investigated regulation of the PG clock in *Rhodnius* by both internal and external Zeitegbers. Reinitiation of rhythmic expression of the canonical clock protein PER was observed in the PGs through the use of both photic and hormonal Zeitgebers, supported by previous findings that the PGs possess an autonomous clock susceptible to regulation by both influences (Pelc and Steel, 1997; Vafopoulou and Steel, 1998, 2001).

Chronic exposure to LL is known to disrupt the circadian system and in insects this is believed to be the result of the arrest of the clock itself (Pittendrigh, 1981); this view is supported by our present findings. In *Rhodnius*, prolonged maintenance in LL is known to abolish the intrinsic circadian rhythm of ecdysteroid synthesis by the PGs (Vafopoulou and Steel, 1991, 1998). We infer that the loss of rhythmic ecdysteroid synthesis is due to the reported arrest of the MO in PGs of LL insects.

However, stopping the MO in the PGs is not permanent. Rhythmic PER expression in the PGs was rapidly re-initiated, therefore, the clock was re-started, by transfer of LL animals to DD. The observed rhythmicity corresponds to the PER rhythm observed in entrained animals with peak PER expression and strong nuclear localization

occurring in the subjective scotophase. Furthermore, PER rhythmicity continued for at least 2 cycles with a free-running period around 24h and is therefore endogenously generated within the PG cells. At least 2 different mechanisms may be responsible for re-starting the PG clock: 1) a direct response to light cues, since PGs are known to be directly photosensitive (Vafopoulou and Steel, 1998; Vafopoulou and Steel, 2001) or 2) a response to hormonal cues, such as PTTH, controlled by the brain clock. The PGs in *Rhodnius* are not innervated (Wigglesworth, 1952); therefore, internal influences to the PG clock system must be hormonal as opposed to nervous.

The direct effect of light on the PG clock was investigated by incubating arrhythmic LL PGs in DD *in vitro*. This has been previously shown to restore rhythmic ecdysteroid production *in vitro* (Vafopoulou and Steel, 1998). PER expression in the PGs was rapidly induced following exposure of PGs *in vitro* to lights-off and PER cycling was re-initiated (rhythmic PER expression continued for at least 2 cycles in DD). Nuclear localization of PER during peak expression supports its role as a vital transcription factor and key component to the molecular clockwork. These results demonstrate that the PER-based clock in the PGs rapidly responds to external photic cues (Vafopoulou and Steel, 1998, 2001) and that light cues are sufficient, in the absence of other timing information, to restart the PG clock.

The rapid re-initiation of PER expression corresponds with the re-initiation of rhythmic ecdysteroid synthesis by the PGs and PER was found to cycle in synchrony with ecdysteroid production by PGs *in vitro* (Vafopoulou and Steel, 1998). Therefore, it is inferred that the rhythm of ecdysteroid synthesis by the PGs is regulated by an

autonomous photosensitive PER-based clock located within the PGs themselves. Results from *Drosophila* suggest that a similar photosensitive PER-based clock resides in the ring gland and can be entrained by light in the absence of other temporal cues (Emery et al, 1997). This ring gland clock has not been associated with a rhythmic output as has been shown for *Rhodnius* PGs and rhythmic ecdysteroid synthesis. However, it has recently been suggested that clocks in the PGs of *Drosophila* ring glands do, in fact, require nervous input to retain rhythmicity of nuclear PER expression (Morioka et al., 2012). *Rhodnius* is an ideal organism to illuminate this discrepancy, as *Rhodnius* PGs are not innervated; therefore, it is ideally suited to investigate the relationship between external and internal Zeitgebers.

Non-photic hormonal cues also successfully re-initiated PER cycling in arrhythmic PGs. Brain neuropeptide extracts were prepared whole or were separated into >10 kDa and <10 kDa fractions, which contain PTTH and ILPs, respectively. A 1h pulse of such brain neuropeptide(s) extracts (whole and fractionated) induced rhythmic PER expression in arrhythmic LL PGs *in vitro*. Increased PER expression was observed immediately following the 1h pulse of neuropeptide and cyclic nuclear localization of PER was also restored. Therefore, the 1h pulse of neuropeptides was sufficient to re-start the PG clock.

PTTH and ILPs were selected as the major candidate peptides affecting the clock system. PTTH is a classical neuropeptide in larval insects, with the PGs being its only known target tissue (reviewed by Bollenbacher and Granger, 1985). The main role of PTTH is promotion of ecdysteroid synthesis by the PGs (Warren et al., 1988). In

addition, PTTH-producing cells are closely associated with the axons of clock cells in the brain (Vafopoulou et al., 2007), resulting in rhythmic scotophase release of this peptide. Bombyxin was originally known as "small PTTH" due to its observed ecdysteroidogeneic properties in PGs of *Samia cynthia ricini* (reviewed by Ishizaki, 2004). Further study identified bombyxin to be part of a large family of peptides known as the insulin-like peptides (ILPs) (reviewed by Wu and Brown, 2006). ILP-producing cells have rhythmic scotophase release as well, and demonstrate a similar close association with the clock system in the brain (Vafopoulou and Steel, 2002; Vafopoulou and Steel, 2012). The role of these peptides in promotion of ecdysteroidogenesis by the PGs in *Rhodnius* and their relationship with the clock system explains their candidacy as the neuropeptides affecting the PG clock.

PTTH-like and ILP-like peptides were removed from their respective fractions by double-immunoprecipitation. These double-immunoprecipitated fractions failed to induce PER expression in arrhythmic LL PGs. These results implicate both PTTH and ILPs as modulators of the PG clock. The rhythm of PER expression induced by brain extract parallels the pattern seen in PGs provided with a lights-off cue. Furthermore, both PTTH and ILPs are known to be released during the scotophase (Vafopoulou and Steel, 1996a, 1996b; Vafopoulou et al., 2007; Vafopoulou and Steel, 2002, 2012). Therefore, we infer these neuropeptides may be acting like signals of darkness to the PG clock. We conclude internal hormonal influences play a key role in the regulation of the PG clock and can act on the clock in PG cells independently of external light cues.

The neuropeptide influences investigated here clearly have the same affect as a pulse of darkness on the LL PGs. Thus, these internal factors have been implicated as hormonal Zeitgebers to the PG clock. A pulse of neuropeptides failed to induce PER expression in arrhythmic DD PGs; therefore, it was inferred these neuropeptide influences are selectively acting like signals of darkness to the PG clock. This selective entrainment by brain neuropeptides/hormones offers clues to their effects on the MO. A likely method of action would be through the inhibition of the clock protein CRY (Emery et al., 1998; Ishikawa et al., 1999). When provided with light cues, CRY actively inhibits the clock protein TIM (Ceriani et al., 1999; Naidoo et al., 1999). Inhibition of CRY would allow for the formation of the PER/TIM heterodimer required for the completion of the transcription/translation feedback loop comprising the insect MO (Lee et al., 1996). However, further investigation is required regarding the formation of arrhythmic animals through long-term exposure to DD and the methods of action of the examined neuropeptides on the PG clock system.

There is a wealth of literature studying hormonal factors responsible for regulating ecdysteroid synthesis by the PGs, such as PTTH and ILPs (reviewed by Marchal et al., 2010). Both peptides are known to stimulate ecdysteroidogenesis in *Rhodnius* PGs (Vafopoulou and Steel, 1997). PTTH is the major ecdysteroidogenic neuropeptide known to act on PGs, and was found to modulate the phase of ecdysteroid synthesis by the PGs (Pelc and Steel, 1997); therefore, it is a hormonal input to the *Rhodnius* PG clock system capable of overriding external (photic) timing cues (Pelc and Steel, 1997; Vafopoulou and Steel, 2001) and setting the phase of rhythmic ecdysteroid

synthesis *in vivo*. Thus, rhythmic release of neurohormones, such as PTTH and ILPs, regulated by the brain clock allows for communication and coordination between the clock in the brain and the autonomous PG clock.

PGs provided with a pulse of >10 kDa brain extract fraction produced a significantly greater increase in ecdysteroid production than those provided with a pulse of <10 kDa fraction. PTTH, present in the >10 kDa fraction, has typically been shown to exert greater ecdysteroidogenic effects than ILPs (Vafopoulou and Steel, 1997). In *Rhodnius*, it has previously been demonstrated that PGs subjected to *Bombyx* PTTH show greater levels of stimulation than when exposed to *Bombyx* bombyxin (Vafopoulou and Steel, 1997). Therefore, the greater level of stimulation produced by the >10 kDa fraction, which contains PTTH not ILPs, is consistent with previous findings (Vafopoulou and Steel, 1997). It should be noted that PGs exposed to a pulse of whole brain homogenate (i.e. containing both PTTH and ILPs) produced the greatest levels of stimulation of ecdysteroid synthesis. It is inferred that these peptides stimulate ecdysteroidogenesis by the PGs in an either synergistic or additive manner. A similar type of cooperative effect of PTTH and ILPs has previously been reported in regulation of timing of development and body size in *Drosophila*, removal of PTTH activity does not completely compromise an insect's ability to produce ecdysteroids or to moult (McBrayer et al., 2007). The lower level of stimulation of ecdysteroid synthesis by ILPs suggests that this may not be their primary function in *Rhodnius*. ILPs have been widely accepted as growth factors in insects; therefore, their role may lie in promoting growth of the PG cells themselves. The results presented here connect rhythmic hormonal inputs,

PTTH and ILPs, to the molecular clockwork of the PGs and to its rhythmic output (ecdysteroids). This system provides a clear demonstration of the interaction between central and peripheral clocks since rhythmic PTTH and ILP release are controlled by the brain clock (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012) and these neuropeptides provide inputs to the autonomous peripheral clock in the PGs. These hormonal cues appear to be able to override direct external photic cues to the PGs *in vivo* (Pelc and Steel, 1997), emphasizing the importance of coordination and synchronization between central and peripheral clocks.

There is an apparent redundancy between PTTH and ILPs in this system. This highlights the importance of the proper functioning of this system in the tightly regulated process of *Rhodnius* development. Therefore, if one or more of these Zeitgebers is not functioning properly another is in place to regulate/stimulate the rhythmic synthesis of ecdysteroids in the larval insect to coordinate the upcoming moult. Together these influences increase the robustness of this system. There is evidence of PTTH setting the phase of the ecdysteroid rhythm (Pelc and Steel, 1997); therefore, the main function in clock regulation is to entrain the rhythm to ensure peak expression during the scotophase as opposed to strictly ecdysteroidogenesis. The direct affects of ILPs on the PG clock, reported here, implicate it as another internal factor in the entrainment of the PG clock *in vivo*. The results presented here provide insights into the role of external (photic) and internal (hormonal) factors in controlling a peripheral clock (PGs) and how they precisely regulate the timing of development. Furthermore, this is the first report of neuropeptides directly initiating rhythmic clock protein expression in any tissue.

This study provides new insights into the entrainment of peripheral tissue clocks in the mammalian system. Circadian regulation of the mammalian HPA axis (reviewed in Kalsbeek et al., 2012) closely parallels the axis observed here in *Rhodnius*. Rhythmically released neuropeptides (PTTH and/or ILPs) from the brain act on the PGs to promote rhythmic steroid hormone (ecdysteroid) synthesis. The axis in *Rhodnius* is analogous to the promotion of glucocorticoid synthesis by clock cells in the adrenal glands of mammals (Andrews and Folk, 1964; Andrews, 1971; Oster et al., 2006). Furthermore, glucocorticoids (much like ecdysteroids) have been indentified as messengers of timing information to surrounding tissues (Balsalobre et al., 2000; Torra et al., 2000). While light can pass through the cuticle in *Rhodnius* to entrain the PGs (Vafopoulou and Steel, 2001), mammalian peripheral tissues are unable to receive direct light cues. Therefore, rhythmic release of neuropeptides from the brain to act on these peripheral tissue clocks is likely the primary form of entrainment. External light information is processed in the SCN in the hypothalamus (Wright and Czeisler, 2002) and transmitted from the brain in the form of rhythmic neuropeptide release to entrain and set the phase of peripheral clocks (Moore and Eichler, 1972; Ottenweller and Meier, 1982; Jasper and Engeland, 1994; Ishida et al., 2005). The methods of entrainment by internal Zeitgebers are more complex in the mammalian system as most tissues contain both hormonal and nervous inputs; therefore, studying the basic underlying principles in an insect such as *Rhodnius* is exceedingly beneficial. The remarkable similarities between these two systems (insect and mammalian) cannot be overlooked; therefore, we

conclude the apparent conserved evolution of circadian organizational principles in insects and mammals emphasizes its importance to survival.

REFERENCES

Allada R. 2003. Circadian clocks. A tale of two feedback loops. *Cell* **112:**284–286.

Allada R, Chung BY. 2010. Circadian organization of behaviour and physiology in *Drosophila. Annu Rev Physiol* **77:**605-624.

Ampleford, E.J., Steel, C.G.H. 1982. Circadian control of ecdysis in *Rhodnius prolixus* (Hemiptera). *J Comp Physiol.* **147:**281-286.

Ampleford, E.J., Steel, C.G.H. 1985. Circadian control of a daily rhythm in hemolymph ecdysteroid titre in the insect *Rhodnius prolixus* (Hemiptera) *Gen Comp Endocrinol.* **59:** 453-59.

Ampleford, E.J., Steel, C.G.H. 1986. Induction of rhythmic modulation of hemolymph ecdysteroids in the insect *Rhodnius prolixus* by treatments which elicit rhythmic ecdysis. *Gen Comp Endocrinol.* **63:** 353-361.

Andrews, R.V. Folk, G.E. 1964. Circadian metabolic patterns in cultured hamster adrenal glands. *Comp Biochem Physiol* **11:**393-409.

Andrews RV (1971) Circadian rhythms in adrenal organ cultures. *Gegenbaurs Morphol Jahrb* **117:** 89-98.

Balsalobre, A., Brown, S.A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H.M., Schutz, G., Schibler, U. 2000. Resetting of circadian time in peripheral tissues by glucocorticoid signalling. *Science* **289:**2344-2347.

Bollenbacher, W.E., Granger, A.A., 1985. Endocrinology of the prothoracicotropic hormone. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon Press, Oxford, pp. 109–151.

Buxton PA. 1930. The biology of the blood-sucking bug, *Rhodnius prolixus. Trans Entomol Soc Lond* **128**:227–234.

Ceriani MF, Darlington TK, Staknis D, Mas P, Petti AA, Weitz CJ, Kay SA. 1999. Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science* **285:**553–556.

Dibner, C., Schibler, U., Albrecht, U. 2010. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu. Rev. Physiol.* **75:**517-549.

Emery, I.F., Noveral, J.M., Jamison, C.F., Siwicki, K.K. 1997. Rhythms of *Drosophila period* gene expression in culture. *Proc Natl Acad Sci USA* **94**: 4092-4096.

Emery P, So WV, Kaneko M, Hall JC, Rosbash M. 1998. CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* **95**:669–679.

Gekakis N, Saez L, Delahaye-Brown A-M, Myers MP, Sehgal A, Young MW, Weitz CJ. 1995. Isolation of timeless by PER protein interactions: defective interaction between timeless protein and long-period mutant PERL. *Science* **270**:811–815.

Helfrich-Förster C. 1995. The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster. Proc Natl Acad Sci USA* **92**:612-616.

Horn, D.H.S., Wilkie, J.S., Sage, B.A., O'Connor, J.D. 1976. A high affinity antiserum specific for the ecdysone nucleus. *J Insect Physiol* **22:** 901-905.

Ishida, A., Mutoh, T., Ueyama, T., Bando, H., Masubuchi, S., Nakahara, D., Tsujimoto, G., Okamura, H. 2005. Light activates the adrenal gland: timing of gene expression and glucocorticoid release. *Cell Metab* **2**:297-307.

Ishikawa T,Matsumoto A, Kato T Jr, Togashi S, Ryo H, IkeganaM, Todo T, Ueda R, Tanimura T. 1999. DCRY is a *Drosophila* photoreceptor protein implicated in light entrainment of circadian rhythms. *Genes to Cells* **4**:57–65.

Ishizaki, H. 2004. Molecular characterization of the brain secretory peptides, prothoracicotropic hormone (PTTH) and bombyxin, of the silkmoth *Bombyx mori. Proc Jpn Acad, Ser. B* **80:** 195-203.

Jasper, M.S., Engeland, W.C. 1994. Splanchnic neural activity modulates ultradian and circadian rhythms in adrenocortical secretion in awake rats. *Neuroendocrinology* **59**:97-109.

Kalsbeek, A., van der Spek, R., Lei, J., Endert, E., Buijs, R.M., Fliers, E. 2012. Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. *Mol Cell Endocrinol* **349**:20-29.

Lane NJ, Leslie RA, Swales LS. 1975. Insect peripheral nerves: accessibility of neurohaemal regions to lanthanum. *J Cell Sci* **18:** 179-197.

Lee C, Parikh V, Itsukaichi T, Bae K, Edery I (1996) Resetting the *Drosophila* clock by photic regulation of PER and a PER–TIM complex. *Science* **271**:1740–1744.

Marchal, E., Vandersmissen, H.P., Badisco, L., Van de Valde, S., Verlinden, H., Iga, M., Van Weilendaele, P., Huybrechts, R., Simonet, G., Smagghe, G., Broeck, J.V. 2010. Control of ecdysteroidogenesis in prothoracic glands of insects: a review. *Peptides* **31:**506-519.

McBrayer, Z., Ono, H., Shimell, M.J., Parvy, JP., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-Villemant, C., Gilbert, L.I. 2007. Prothoracicotropic hormone regulates development timing and body size in *Drosophila. Develop Cell* **13:**857-871.

Moore, R.Y., Eichler, V.B. 1972. Loss of circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res* **42:**201-206.

Morioka, E., Matsumoto, A., Ikeda, M. 2012. Neuronal influence on peripheral circadian oscillators in pupal *Drosophila* prothoracic glands. *Nature Comm.* **3 (909):** 1-11.

Naidoo N, Song W, Hunter-Ensor M, Sehgal A. 1999. A role for the proteasome in the light response of the timeless clock protein. *Science* **285:**1737–1741.

Ottenweller, J.E. Meier, A.H. 1982. Adrenal innervation may be an extrapituitary mechanism able to regulate adrenaocortical rhythmicity in rats. *Endocrinology* **111:**1334-1338.

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffman, M.W., Eichele, G. 2006. The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metab* **4:** 163-173.

Pittendrigh, C.S. 1981. Circadian Systems: Entrainment. In. Aschoff, J. (Eds), Handbook of Behavioural Neurobiology, Vol. 4, *Biological Rhythms*. Plenum, New York, pp. 95-123.

Plautz, J.D., Kaneko, M., Hall, J.C., Kay, S.A. 1997. Independent photoreceptive circadian clocks throughout *Drosophila. Science* **278:**1632-1635.

Siwicki KK, Eastman C, Petersen G, Rosbash M, Hall JC. 1988. Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* **1:**141-150.

Steel, C.G.H., Bollenbacher, W.E., Smith, S.L., Gilbert, L.I. 1982. Haemolymph ecdysteroid titres during larval-adult development in *Rhodnius prolixus:* correlations with moulting hormone action and brain neurosecretory cell activity. *J Insect Physiol* **28 (6):** 519-25.

Tomioka K, Uryu O, Kamae Y, Umezaki Y, Yoshii T. 2012. Peripheral circadian rhythms and their regulatory mechanism in insects and some other arthropods: a review. *J Comp Physiol B* **182:**729-740.

Torra, I.P., Tsibulsky, V., Delaunay, F., Saladin, R., Laudet, V., Fruchart, J.C., Kosykh, V., Staels, B. 2000. Circadian and glucocorticoid regulation of Rev-erb alpha expression in liver. *Endocrinology* **141:**3799-3806.

Vafopoulou, X. 2009. Ecdysteroid receptor (EcR) is associated with microtubules and mitochondria in the cytoplasm of prothoracic gland cells of *Rhodnius prolixus*. *Arc Insect Biochem Physiol.* **72 (4):** 249-262.

Vafopoulou, X., Steel, C.G.H., 1989. Developmental and diurnal changes in ecdysteroid biosynthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera) in vitro during the last larval instar. *Gen. Comp. Endocrinol.* **74:** 484–493.

Vafopoulou, X., Steel, C.G.H., 1991. Circadian regulation of synthesis of ecdysteroids by prothoracic glands of the insect *Rhodnius prolixus*: evidence of a dual oscillator system. *Gen. Comp. Endocrinol.* **83:** 27–34.

Vafopoulou, X., Steel, C.G.H., 1992. In vitro photosensitivity of ecdysteroid synthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera). *Gen.Comp. Endocrinol.* **86:** 1–9.

Vafopoulou, X., Steel, C.G.H. 1996a. The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. *Pro. Natl Acad Sci* **93 (8):** 3368-3372.

Vafopoulou, X., Steel, C.G.H. 1996b. Prothoracicotropic hormone from the brain-retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval-adult development. *Gen Comp Endocrinol.* **102 (1):** 123-129.

Vafopoulou, X., Steel, C.G.H. 1997. Ecdysteroidogenic action of *Bombyx* prothoracicotropic hormone and bombyxin on the prothoracic glands of *Rhodnius prolixus in vitro*. *J Insect Physiol.* **43 (7):** 651-656.

Vafopoulou, X., Steel, C.G.H. 1998. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis in vitro. *J Comp Physiol A* **182 (3):** 343-349.

Vafopoulou, X., Steel, C.G.H. 2001. Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine zeitgebers. *J Insect Physiol* **47 (8):** 935-941.

Vafopoulou, X., Steel, C.G.H. 2002. Prothoracicotropic hormone of *Rhodnius prolixus*: partial characterization and rhythmic release of neuropeptides related to *Bombyx* PTTH and bombyxin. *In Repro Devel* **42 (2-3):** 111-120.

Vafopoulou, X., Steel, C.G.H. 2012a. Cytoplasmic travels of the ecdysteroid receptor in target cells: pathways for both genomic and non-genomic actions. *Frontiers Endocrinol.* **3 (43):** 1-16.

Vafopoulou, X., Steel, C.G.H. 2012b. Insulin-like and testis ecdysiotropin neuropeptide are regulated by the cicadian timing-system in the brain during larval-adult development in the insect *Rhodnius prolixus* (Hemiptera). *Gen Comp Endocrinol.* **179:** 277-288.

Vafopoulou, X, Sim, C-H, Steel, C.G.H. 1996. Prothoracicotropic hormone in *Rhodnius prolixus: in vitro* analysis and changes in amounts in the brain- retrocerebral complex during larval-adult development. *J Insect Physiol.* **42 (4):** 407-415.

Vafopoulou, X., Steel, C.G.H., Terry, K., 2005. Ecdysteroid receptor (EcR) shows marked differences in temporal patterns between tissues during larval–adult development in Rhodnius prolixus: correlations with haemolymph ecdysteroid titres. *J. Insect Physiol.* **51:** 27–38.

Vafopoulou, X., Steel, C.G.H., Terry, K.L. 2007. Neuroanatomical relations of prothoracicotropic hormone neurons with the circadian timekeeping system in the brain of larval and adult Rhodnius prolixus (Hemiptera). *J Comp Neurol* 503 **(4):** 511-524.

Vafopoulou, X., Terry, K.L., Steel, C.G.H. 2010. The circadian timing system in the brain of the fifth larval instar of *Rhodnius prolixus* (Hemiptera). *J Comp Neurol* **518 (8):** 1264-1282.

Vosshall LB, Price JL, Sehgal A, Saez L, Young MW. 1994. Specific block in nuclear localization of period protein by a second clock mutation, timeless. *Science* **263:**1606–1609.

Warren, J.T., Sakurai, S., Rountree, D.G., Gilbert, L.I., Lee, S.-S., Nakanishi, K., 1988. Regulation of the ecdysteroid titer of Manduca sexta: reappraisal of the role of the prothoracic glands. *Proc Natl Acad Sci U S A.* **85:** 958–962.

Wigglesworth VB. 1933. The physiology of the cuticle and of ecdysis in *Rhodnius prolixus* (Triatomidae, Hemiptera); with special reference to the function of the oenocytes and of the dermal glands. *Q J Microsc Sci* **76:**269–318.

Wigglesworth VB. 1934. Insect physiology. *London: Methuen.*

Wigglesworth, V.B., 1952. The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in moulting. *J. Exp. Biol.* **29:** 561–570.

Wright Jr, K.P., Czeisler, C.A. 2002. Absence of circadian phase resetting in response to bright light behind the knees. *Science* **297:** 571.

Wu, Q., Brown, M.R. 2006. Signaling and function of insulin-like peptides in insects. *Annu Rev Entomol* **51:** 1-24.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda, M., Block, G.D., Sakaki, Y., Menaker, M., Tei, H. 2000. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288**:682–685.

CHAPTER III:

GENERAL DISCUSSION

3.1. New Insights into Rhodnius Development

The present thesis demonstrated the ability of both photic and neuropeptide inputs from the brain to re-start an autonomous peripheral clock in the PGs of *R. prolixus*. Lights-off cues and neuropeptide pulses not only induced expression of PER in the PGs, these timing cues also produced cyclic PER expression, indicating the PG clock had been re-started. Furthermore, it was shown that these neuropeptide inputs re-initiate production of a rhythmic output of ecdysteroids by the PG clock. The findings presented here demonstrate the PG clock machinery (molecular oscillator/MO) responds directly to photic (external) and hormonal (internal) inputs; thus, providing new insights into the regulation of this clock system and circadian clocks as a whole.

The circadian control of development in *Rhodnius* has been studied extensively by this lab; however, the results presented in this thesis expand our understanding of this process. Previous work by this lab has identified hormonal Zeitgeber influences on the PGs, demonstrating that *in vivo* PTTH overrides the entraining influences of light on the PGs to set the phase of rhythmic ecdysteroid synthesis (Pelc and Steel, 1997; Vafopoulou and Steel, 2001). However, it had not been previously demonstrated that the action of PTTH in rhythmic ecdysteroid synthesis involved specific action of PTTH on the MO in PGs. The results demonstrated here provide evidence for this association, as a pulse of PTTH-like peptide re-started the PG clock *in vitro,* as shown through the re-initiation of rhythmic PER expression. Furthermore, ILP-like peptides were capable of eliciting the

same effect as PTTH, demonstrating multiple neuropeptide influences acting on the PG clock itself, as opposed to strictly on mechanisms involved in ecdysteroid synthesis. As has been previously mentioned, both PTTH (Vafopoulou and Steel, 1996a, 2007) and ILPs (Vafopoulou and Steel, 2012) have been shown to be released by the brain rhythmically during the scotophase in *Rhodnius*. Furthermore, PTTH has been shown to be released with a circadian rhythm (Vafopoulou and Steel, 1996b). The neurosecretory cells responsible for the production of the above neuropeptides are closely associated with clock cell axons in the brain of *Rhodnius*; thus communication between the brain clock and these cells has been reported (Vafopoulou and Steel, 2007, 2012). The observed ability of these peptides to re-start the PG clock when presented as a pulse, that mimics their release *in vivo* further strengthens their link to the clock in the brain.

Direct photosensitivity of the PG clock has been previously inferred by our lab (Vafopoulou and Steel, 1998); however, this is the first demonstration of light directly regulating the expression of a clock protein in *Rhodnius* PGs. Furthermore, this is one of the only known examples demonstrating both direct photosensitivity and the action of internal Zeitgebers on a single peripheral clock system. Therefore, these results offer new insights into the regulation of circadian clocks in *Rhodnius* and insects as a whole. A paradox does appear to exist, of conflict between the internal and external Zeitgebers to the PG clock in *Rhodnius*. In the absence of internal influences PGs entrained to light contain a rhythm of ecdysteroid synthesis in reverse phase when compared to the normal *in vivo* rhythm (Pelc and Steel, 1997). The results presented here help to reconcile this paradox as it was demonstrated that PTTH and ILPs reinforce the signal of darkness to

the PGs to produce the observed *in vivo* rhythm of ecdysteroid synthesis with a scotophase peak.

Both PTTH and ILPs have been previously shown to stimulate ecdysteroidogenesis in *Rhodnius* PGs (Vafopoulou and Steel, 1997) and, as demonstrated here, stimulate ecdysteroid production and re-initiate rhythmic synthesis in arrhythmic PGs. PTTH and ILPs have a potential synergistic effect to produce ecdysteroids in *Rhodnius* PGs. Evidence of the cooperation and synergy between these hormones has previously been demonstrated with respect to regulating the timing of moults, and viability and body size of *Drosophila* during larval-adult development (McBrayer et al., 2007). Therefore, rhythmic release of PTTH and ILPs, are acting together directly on the PG clock (see above), to stimulate ecdysteroid production and control the phase of the ecdysteroid rhythm *in vivo*. There is some evidence that PTTH is rhythmically released not only in *Rhodnius* but in the insects *Bombyx mori* (Mizoguchi et al., 2001, 2002) and *Periplaneta americana* (Richter, 2001); therefore, PTTH is likely an important developmental hormone involved in circadian regulation of development in insect systems as a whole. There is a clear association of PTTH and ILPs in the regulation of the PG clock in *Rhodnius*; thus, these peptides are key players in the circadian orchestration of development.

3.2. Expanding Role of PTTH as an Insect Hormone

PTTH has long been known as a key developmental hormone in insects (reviewed by Bollenbacher and Granger, 1985). This neuropeptide is produced by neurosecretory

cells in the brain of insects and, depending on the insect, it is released either through nervous pathways or hormonally into the hemolymph. The peptide was originally purified from the silkmoth *B. mori* approximately 20 years ago (Kawakami et al., 1990; Kataoka et al., 1991); however, new insights regarding this hormone and its role in development continue to be discovered. PTTH was previously thought to be a strictly larval hormone in insects known to stimulate ecdysteroid production by the PGs (Warren et al., 1988). The original thinking had been for PTTH to be released only twice (or once depending on the insect) during insect development, to cue the commencement of development at the head critical period (Kopec, 1922; Wigglesworth, 1934). This line of thinking was challenged by this lab as it was demonstrated not only that PTTH present throughout development in *Rhodnius* (Vafopoulou and Steel, 1996a), but it is additionally synthesized and released into the hemolymph with a circadian rhythm (Vafopoulou and Steel, 1996b). The discovery of circadian control of PTTH presented a new aspect of its involvement in regulation of development; however, PTTH remains the primary ecdysteroidogenic factor across several different insect species. In addition to PTTH's expanding role as a hormonal factor involved in circadian control of development, it has also been found to be present in adult *Rhodnius*; thus, challenging the view that it is a strictly larval hormone. In the adult, PTTH is also rhythmically released by the brain, likely functioning in the regulation of reproductive processes in the adult insect (Vafopoulou et al., 2012). While PTTH has been a known ecdysteroidogenic hormone in insects for decades, new and exciting roles are still being discovered.

The ability of the PGs in *Rhodnius* to respond directly to photic cues and produce ecdysteroids with a circadian rhythm both *in vitro* and *in vivo* would appear to indicate that PTTH is not necessary (Vafopoulou and Steel 1992, 1998, 2001). However, it is clear that PTTH plays a vital role providing stimulation of ecdysteroid production by the PGs and as an overriding internal Zeitgeber used to set the phase of ecdysteroid production *in vivo* (Pelc and Steel, 1997). The present thesis has demonstrated PTTH is responsible for re-starting the clock in arrhythmic LL PGs in *Rhodnius*, thus expanding the role of PTTH to a direct regulator of the molecular clockwork in the PG clock system.

These results inspire the question of how PTTH regulates the clock and whether or not it also directly affects the enzymes responsible for ecdysteroid synthesis. Rhythmic release of PTTH by the brain results in rhythmic binding to its receptor, recently identified as the receptor tyrosine kinase Torso (Rewitz et al., 2009). Binding of the Torso receptor by PTTH elicits the activation of second messenger molecules such as calcium-calmodulin and cyclic AMP (cAMP) (Gilbert et al., 2002). Both calcium (Harrisingh et al., 2007) and cAMP (Dodd et al., 2007; O'Neill et al., 2009) have been implicated in the regulation of circadian rhythms in clock cells; therefore, they remain likely candidates for PTTH's communication with the MO in the PGs. Furthermore, in *Drosophila* it has been shown that removal of brain inputs to the RG results in a disruption in calcium signalling in the PG cells, along with disruption of the PG clock (Morioka et al., 2012) potentially linking these two systems through a neuropeptide signal such as PTTH.

It appears evident that PTTH induces the expression of the clock protein PER in the PGs; however, it remains unclear how this interaction with the clock system results in rhythmic stimulation of ecdysteroid synthesis. The biosynthesis of ecdysteroids requires a set of genes known as the Halloween genes (*spook, phantom, disembodied, shadow and shade*) (reviewed by Rewitz et al., 2007); therefore, these genes are the likely targets for regulation of rhythmic synthesis by the PGs. It remains unclear how PTTH is regulating these biosynthetic genes either via the MAPK signal transduction pathway (which has not yet been definitively demonstrated) or via clock-related transcription factors induced by the pulse of PTTH. Further investigation is required to determine the mechanism of regulation. Rhythmic ecdysteroid production may be due to the circadian control of a rate-limiting step in the ecdysteroid biosynthetic pathway, as has been shown for example in the pineal-melatonin system in vertebrates via rhythmic expression of N-acetyltransferase. Control of rhythmic steroid hormone synthesis in the mammalian adrenal glands is apparently the result of rhythmic expression of the protein StAR (Stocco, 1999), a key component in steroid hormone synthesis that controls the transport of cholesterol across the mitochondrial membrane. Therefore, a similar mechanism may exist in the PGs to regulate ecdysteroid synthesis which is stimulated either directly or indirectly by PTTH.

3.3. Role of Insulin-like Peptides in Insect Development

Insulin-like peptides (ILPs) in insects comprise a very large family of peptides closely related in sequence and structure to the well known vertebrate family of peptides

(insulin-like peptides/insulin-like growth factors). Insect ILPs all contain 2 interchain disulfide bonds between the A and B peptides and a single intrachain disulfide bond in the A peptide to comprise the mature hormone. This structure is conserved between insects and mammals, specifically through the conserved positioning of 6 cysteine residues within the A and B peptides. Since the discovery of this family of peptides in the silkmoth *B. mori,* 38 distinct bombyxin genes have been characterized. These genes encode the propeptides used to form the mature proteins. The identification of the bombyxin genes in *B. mori* eventually led to the identification of ILPs in other lepidopterans such as *S. cynthia* (Nagata et al., 1999) and *Agrius convolvuli* (Iwami et al., 1996a). Evidence of the existence of ILPs has now been demonstrated in several other insect species spanning across several orders such as: *Locusta migratoria* (Lagueux et al., 1990; Hetru et al., 1991), *P. americana* (Raabe, 1986; Verhaert et al., 1989), *D. melanogaster* (Van den Broeck, 2001, Brogiolo et al., 2001) , *R. prolixus* (Vafopoulou and Steel, 2012). Immunostaining of brain sections with anti-insulin in a variety of insect species has identified neurosecretory cells in the brain which project axons into the corpus allatum and corpus cardiacum suggesting their release into the blood as neurohormones (Thorpe and Duve, 1984; Mizoguchi et al., 1987; Goltzene et al., 1992; Cao and Brown, 2001). Additionally, ILPs have been localized to areas such as the gut, Malpighian tubules, epidermis, PGs, and fat body (Verhaert et al., 1989; Iwami et al., 1996b). indicating a wide range of target tissues and functions for this family of neuropeptides.

The functional role of ILPs is as varied as the identified target tissues for this vast group of peptides. ILPs have been shown to play important roles in the regulation of reproduction in insects as well as life span of insects; however, both factors apply mainly to adults insects and will not be covered here in any detail (reviewed in Wu and Brown, 2006). Vertebrate insulin has been well documented as an essential hormone involved in the metabolism of carbohydrates with loss of function of insulin resulting in diabetes. Regulation of metabolism is a conserved function of ILPs found in insects. However, as opposed to promoting the accumulation of carbohydrate stores, injection of bombyxin (an ILP) results in lowered glycogen content in the fat body in the insect *B. mori* (Satake et al., 1997). Furthermore, introduction of insulin to the fly *D. melanogaster* resulted in an increase in glucose oxidation and lactate production (Ceddia et al., 2003). Therefore, ILPs in insects function to make energy stores available to the surrounding tissues during larval development in insects.

The apparent liberation of energy stores by ILPs in insects closely parallels an additional function of this family of peptides which is the regulation of cell size and overall growth of the insect. The majority of insulin-mediated cell and tissue growth studies have been done in *D. melanogaster.* Overexpression of *Drosophila* ILP genes results in the production of larger flies through an increase in cell proliferation and cell growth (Brogiolo et al., 2001). The PI3K/Akt signal cascade has been well documented as a major signal transduction pathway induced by the binding of insulin to its receptor in mammals. Targeted mutation of PI3K, as well as other members of this pathway in insects, suppresses cell growth, cell proliferation, and overall development in a manner

similar to that observed in starved larvae (Weinkove et al., 1999; Huang et al., 1999; Scanga et al., 2000; Rulifson et al., 2002). There appears to be tight communication and coordination between the PI3K pathway initiated by the binding of ILPs to their receptor and to the signalling pathway mediated by the protein TOR (target of rapamycin). The TOR pathway responds to nutrient availability to the tissue in which it is expressed. TOR null mutant flies demonstrate similar phenotypes (reduced cell size, reduced cell number, growth defects) to those lacking expression of ILPs, thus there is a tight interplay between ILP mediated growth and the availability of nutrients essential for metamorphosis (Oldham et al., 2000; Zhang et al., 2000). Therefore, a key role of insect ILPs is in ensuring the necessary nutrients are available through metabolism of energy stores (as described above) and then using these nutrients to promote cell and tissue growth during larval development.

The PGs have been found to be a key tissue involved in coordinating this interplay of nutrient availability and development. The PGs of *D. melanogaster* have been suggested as sensors of metabolic status of the insect. As discussed above, ILPs function to increase cell size and promote cell proliferation during developmental cycles in insects. ILP-mediated growth of the PG equivalent cells in *Drosophila* is used to determine critical weight and thus mediate a moult to the next stage via release of ecdysteroids (Mirth et al., 2005; Mirth and Riddiford, 2007). As was presented here, the PG clock in *Rhodnius* is being regulated by ILPs, which ultimately promotes ecdysteroid synthesis. Therefore, a potential function of the ILPs in *Rhodnius* would be to regulate the growth of the PG cells, which increase drastically in size following a blood meal

during a developmental cycle. In addition to stimulating PG cell growth, ILPs may be providing nutritional information via the TOR signaling pathway (Layalle et al., 2008) to the PG clock which, in turn, leads to stimulation of ecdysteroid synthesis by the PGs to work in conjunction with the stimulatory effects of PTTH, thus, producing the developmental profile of ecdysteroid production found in *Rhodnius* during a larval-adult moult. The roles of ILPs in *Rhodnius* still remain unclear; however, the findings presented here demonstrating that ILPs provide input to the PG clock system and stimulate rhythmic ecdysteroid production suggest that these hormones function as an important component of the circadian regulation of development.

3.4. Regulation of Insect Clocks by Internal Zeitgebers

Peripheral clocks have been identified in a wide variety of insect peripheral tissues. These peripheral tissue clocks have generally been found to be directly photosensitive (Plautz et al., 1997); therefore, they would not require inputs from the brain clock to entrain to the 24h light/dark cycle. The results presented here, as well as previous evidence in *Rhodnius* regarding hormonal entrainment of rhythmic ecdysteroid synthesis (Pelc and Steel, 1997), demonstrate the importance of internal Zeitgebers in regulating insect peripheral clock systems. It remains unclear whether entrainment of peripheral tissue clocks in other insects occurs completely independent of each other through direct response to light cues or if hormonal/nervous inputs play a larger role than previously supposed, as was determined to be the case in *Rhodnius*. Recent evidence is beginning to suggest that internal factors may have significant influences in entraining

clock systems. Removal of nervous inputs to the *Drosophila* PGs found in the RG have been shown to disrupt rhythmic expression of *per* as well as preventing rhythmic nuclear localization of PER in these cells (Morioka et al., 2012). However, these results directly conflict with a previous study under similar conditions (Emery et al., 1997); therefore, the situation in *Drosophila* is unclear. Additionally, indirect evidence has linked ILPs to regulation of clock protein expression in the clock cells in the brain. Overexpression of Akt and/or TOR resulted in a lengthening of circadian activity rhythms (Zheng and Sehgal, 2010). Furthermore, elevated TOR expression resulted in a delay in nuclear localization of TIM in the observed clock cells (Zheng and Sehgal, 2010). Both Akt and TOR have been shown to be linked to the signal cascade initiated by ILPs; therefore indicating ILPs can affect the molecular oscillator in *Drosophila*. Moreover, this is the only other evidence, outside of the results in the present thesis, of a canonical clock protein being regulated by a hormone. This is one of the only other examples outside of the present study in *Rhodnius* linking an affect on the circadian MO to an observable circadian output.

The lack of knowledge regarding hormonal/nervous regulation of circadian clocks in insects is due to the limitations of other insect model systems. *Drosophila* is extremely useful as a molecular and genetic model organism; however, due to its small size direct physiological analysis of circadian clocks is exceedingly difficult. *Rhodnius* contains a discreet homogeneous population of ~200 clock cells in the PGs, which are connected and synchronized by gap junctions. It has been demonstrated here that an entire circadian axis of regulation can be isolated under *in vitro* conditions and maintained over a long

period of time. The conditions of this system can then be easily manipulated to investigate a variety of interactions for physiological analysis. The advantage of using *Rhodnius* for circadian studies cannot be ignored.

3.5. Analogies with Mammalian System

In general, mammalian oscillators are not directly photosensitive, with the clock cells in the SCN being the only mammalian clock cells which receive light/dark information from the eyes. Mammalian peripheral clocks are believed to be entrained by internal Zeitgebers released as outputs of mammalian clock cells. In contrast to this, many of the insect clock cells identified have been shown to be directly photosensitive (Plautz et al., 1997); therefore, this difference can complicate comparative investigations of circadian regulation of physiology between insects and mammals. Therefore, the PG clock in *Rhodnius* becomes ideally suited for comparative investigations between mammals and insects. While the PG clock in *Rhodnius* responds directly to light-dark cues, hormonal influences have been demonstrated previously (Pelc and Steel, 1997) and further supported here as important regulators of this system. The ability of hormonal cues from the brain to re-start the PG clock and re-initiate rhythmic synthesis of ecdysteroids is the first demonstration of its kind in insects. Additionally, neuropeptide signals appear to override direct photic cues *in vivo* and therefore the situation *in vivo* appears highly similar to that in mammals. This method of control of peripheral tissue clocks by neuropeptide influences from the brain closely resembles the entrainment of peripheral clocks in the mammalian system by the SCN. Therefore, the brain-PG clock

axis investigated here provides a simplified system which can be used to better understand hormonal and nervous entrainment of peripheral clocks in mammals.

The PTTH-ecdysteroid axis closely resembles the HPA axis found in mammals, allowing for useful comparative analogies to be drawn. Much like in the HPA axis, *Rhodnius* neuropeptides are rhythmically released from the brain to stimulate the synthesis and release of steroid hormones by a tissue known to contain a peripheral clock. Stimulation of ecdysteroid synthesis in the PGs by PTTH is directly parallel to the stimulation of corticosteroid production in the adrenal cortex by ACTH (Ottenweller et al., 1978; Henley et al., 2009). Furthermore, the PGs in *Rhodnius* are known to possess rhythmic sensitivity to stimulation of ecdysteroid synthesis by PTTH, with peak sensitivity occurring during the scotophase to coincide with PTTH release from the brain (Vafopoulou and Steel, 1999). This rhythmic sensitivity was inferred to derive from a circadian rhythm in abundance of PTTH receptor in PG cells. It has been suggested that the adrenals in mammals demonstrate a daily rhythm of ACTH sensitivity through rhythmic expression of the ACTH receptor (Oster et al., 2006); thereby further strengthening the analogy with the *Rhodnius* system. Ecdysteroids have a wide range of target tissues in order to regulate the timing of the moult (Vafopoulou et al., 2005; Vafopoulou and Steel, 2006); thus, the rhythmic release of ecdysteroids by the PG clock implicates these hormones as messengers of timing information to their target tissues. Glucocorticoid release by the adrenals also occurs rhythmically (Andrews, 1971; Lehoux and Lefebvre, 1980) and glucocorticoids are known to have a wide variety of target tissues in mammals; therefore, they appear to be acting in a similar manner to

ecdysteroids as messengers of timing information from the adrenal clock. Furthermore, the glucocorticoid analog dexamethasone has been shown to stimulate clock gene expression in target tissues such as the liver and kidneys (Balsalobre et al., 2000), indicating these rhythmic clock cell outputs to be acting as hormonal Zeitgebers to their target tissues. Additional peripheral tissue clocks have not yet been identified in *Rhodnius*; however, their wide range of target tissues and known role as rhythmic outputs of the PG clock system indicates they play a similar role as described above for gluccocorticoids in mammals.

Due to the large number of peripheral tissue clocks and oscillators found in both mammals and insects, the steroid hormone outputs of these two clock systems appear to be ideally suited to transmit timing information originating from the brain to distant and diverse target tissues, thus synchronizing the numerous clocks found throughout these organisms. The striking similarities between these two clock systems cannot be ignored; the simple framework of the *Rhodnius* brain-PG axis provides a tractable system for investigation of the underlying principles governing circadian regulation in insects and of the highly analogous mammalian HPA axis.

3.6. Conclusions

The present study provides new and compelling evidence regarding the use of internal hormonal Zeitgebers in the regulation of peripheral tissue clocks. While this idea has been largely ignored in insects due to their direct photosensitivity, it appears clear that the association of brain clocks and peripheral clocks in insects is more complicated

than previously assumed. Using immunohistochemistry I demonstrated for the first time that both photic and hormonal inputs to an arrhythmic PG clock will re-start rhythmic expression of the canonical clock protein PER, thus indicating the PG clock has been re-started. These hormonal inputs to the PG clock appear to function strictly as signals of darkness to the PGs; therefore, their role is specific as "lights-off" signals to the PG clock. Furthermore, PTTH and ILPs have been implicated as the hormonal influences acting on the PG clock and work together to stimulate the rhythmic production of ecdysteroids by the PGs. The work presented here demonstrates neuropeptide factors provide an additional level of control of peripheral clock systems in *Rhodnius* and provide valuable insights into regulation of peripheral clocks in both insects and mammals.

GENERAL DISCUSSION REFERENCES

Andrews RV (1971) Circadian rhythms in adrenal organ cultures. *Gegenbaurs Morphol Jahrb* **117:** 89-98.

Bollenbacher, W.E., Granger, A.A., 1985. Endocrinology of the prothoracicotropic hormone. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon Press, Oxford, pp. 109–151.

Brogiolo W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E. 2001. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulinlike peptides in growth control. *Curr. Biol.* **11:**213–221.

Cao, C., Brown, M.R. 2001. Localization of an insulin-like peptide in brains of two flies. *Cell Tissue Res.* **304:**317–321.

Ceddia RB, Bikopoulos GJ, Hilliker AJ, Sweeney, G. 2003. Insulin stimulates glucose metabolism via the pentose phosphate pathway in *Drosophila* Kc cells. *FEBS Lett.* **555:**307–310.

Dodd, A.N., Gardner, M.J., Hotta, C.T., Hubbard, K.E., Dalchau, N., Love, J., Assie, J.M., Robertson, F.C., Jakobsen, M.K., Goncalves, J., Sanders, D., Webb, A.A.R. 2007. The *Arabidopsis* circadian clock incorporates a cADPR-based feedback loop. *Science* **318(5857):**1789-1792.

Emery, I.F., Noveral, J.M., Jamison, C.F., Siwicki, K.K. 1997. Rhythms of *Drosophila period* gene expression in culture. *Proc Natl Acad Sci USA* **94**: 4092-4096.

Gilbert, L.I., Rybczynski, R., Warren, J.T. 2002. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu Rev Entomol* **47:**883-916.

Goltzene F, Holder F, Charlet M, Meister M, Oka T. 1992. Immunocytochemical localization of *Bombyx*-PTTH-like molecules in neurosecretory cells of the brain of the migratory locust, *Locusta migratoria*. A comparison with neuroparsin and insulin-related peptide. *Cell Tissue Res.* **269:**133–140.

Harrisingh, M.C., Wu, Y., Lnenicka, G.A., Nitabach, M.N. 2007. Intracellular Ca2+ regulates circadian clock oscillation *in vivo*. *J Neuroscience* **27(46):** 12489-12499.

Henley, D.E., Leendertz, J.A., Russell, G.M., Wood, S.A., Taheri, S., Woltersdorf, W.W., Lightman, S.L. 2009. Development of an automated blood sampling system for use in humans, *J Med Eng Tech* **33:**199-208.

Hetru C, Li KW, Bulet P, Lagueux M, Hoffmann JA. 1991. Isolation and structural characterization of an insulin-related molecule, a predominant neuropeptide from *Locusta migratoria*. *Eur. J. Biochem.* **201:**495–499.

Huang, H., Potter, C.J., Tao, W., Li, D.M., Brogiolo, W., Hafen, E., Sun, H., Xu, T.A. 1999. PTEN affects cell size, cell proliferation and apoptosis during *Drosophila* eye development. *Development* **126:**5365–5372.

Iwami M, Furuya I, Kataoka H. 1996a. Bombyxin-related peptides: cDNA structure and expression in the brain of the hornworm *Agrius convolvuli*. *Insect Biochem. Mol. Biol.* **26:**25–32.

Iwami M, Tanaka A, Hano N, Sakurai S. 1996b. Bombyxin gene expression in tissues other than brain detected by reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization. *Experientia* **52:**882–887.

Kataoka H, Nagasawa H, Isogai A, Ishizaki H, Suzuki A. 1991. Prothoracicotropic hormone of the silkworm, *Bombyx mori*: amino acid sequence and dimeric structure. *Agric. Biol. Chem.* **55:**73–86.

Kawakami A, Kataoka H, Oka T, Mizoguchi A, Kimura-Kawakami M, Adachi, T., Iwami, M., Nagasawa, H., Suzuki, A., Ishizaki, H. 1990. Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. *Science* **247:**1333-1335.

Kopec, S. 1922. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull.* **42:** 322-342.

Lagueux M, Lwoff L, Meister M, Goltzene F, Hoffmann JA. 1990. cDNAs from neurosecretory cells of brains of *Locusta migratoria* (Insecta, Orthoptera) encoding a novel member of the superfamily of insulins. *Eur. J. Biochem.* **187:**249–254.

Layalle, S., Arquier, N., Leopold, P. 2008. The TOR pathway couples nutrition and developmental timing in *Drosophila*. *Dev. Cell* **15:**568–577.

Lehoux, J.G., Lefebvre, A. 1980. *De novo* synthesis of corticosteroids in hamster adrenal glands. *J Steroid Biochem* **12:**479-485.

McBrayer, Z., Ono, H., Shimell, M.J., Parvy, JP., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-Villemant, C., Gilbert, L.I. 2007. Prothoracicotropic hormone regulates development timing and body size in *Drosophila*. *Develop Cell* **13:**857-871.

Mirth, C.K., Riddiford, L.M. 2007. Size assessment and growth control: how adult size is determined in insects. *Bioessays* **29:**344–355.

Mirth, C., Truman, J.W., Riddiford, L.M. 2005. The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster. Curr. Biol.* **15:**1796–1807

Mizoguchi A, Ishizaki H, Nagasawa H, Kataoka H, Isogai A, Tamura, S., Suzuki, A., Fujino, M., Kitada, C. 1987. A monoclonal-antibody against a synthetic fragment of bombyxin (4K prothoracicotropic hormone) from the silkmoth, *Bombyx mori*: characterization and immunohistochemistry. *Mol. Cell. Endocrinol.* **51:**227–235.

Mizoguchi, A., Ohashi, Y., Hosoda, K., Ishibashi, J., Kataoka, H., 2001. Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. *Insect Biochem. Mol. Biol.* **31:** 349–358.

Mizoguchi, A., Dedos, S.G., Fugo, H., Kataoka, H., 2002. Basic pattern of fluctuation in hemolymph PTTH titers during larval–pupal and pupal–adult development of the silkworm, *Bombyx mori. Gen. Comp. Endocrinol.* **127:** 181–189.

Morioka, E., Matsumoto, A., Ikeda, M. 2012. Neuronal influence on peripheral circadian oscillators in pupal *Drosophila* prothoracic glands. *Nature Comm.* **3 (909):** 1-11.

Nagata K, Maruyama K, Kojima K, Yamamoto M, Tanaka M, Kataoka, H., Nagasawa, H., Isogai, A., Ishizaki, H., Akinori, S. 1999. Prothoracicotropic activity of SBRPs, the insulin-like peptides of the saturniid silkworm *Samia cynthia ricini. Biochem. Biophys. Res. Commun.* **266:**575–578.

Oldham S., Montagne J., Radimerski T., Thomas, G., Hafen E. 2000. Genetic and biochemical characterization of dTOR, the *Drosophila* homolog of the target of rapamycin. *Genes Dev.* **14:**2689–2694.

O'Neill, J.S., Maywood, E.S., Chesham, J.E., Takahashi, J.S., Hastings, M.H. 2009. cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. *Science* **320:**949-953.

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffman, M.W., Eichele, G. 2006. The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metab* **4:** 163-173.

Ottenweller, J.E., Meier, A.H., Ferrell, B.R., Horseman, N.D., Proctor, A. 1978. Extrapituitary regulation of the circadian rhythm of plasma corticosteroid concentration in rats. *Enodcrinology* **103:**1875-1979.

Pelc, D., Steel, C.G.H., 1997. Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotropic hormone. *Gen. Comp. Endocrinol.* **108:** 358–365.

Plautz, J.D., Kaneko, M., Hall, J.C., Kay, S.A. 1997. Independent photoreceptive circadian clocks throughout *Drosophila*. *Science* **278:**1632-1635.

Raabe M. 1986. Comparative immunocytochemical study of release sites of insulin, glucagon, and AKH-like products in *Locusta migratoria*, *Periplaneta americana*, and *Carausius morosus*. *Cell Tissue Res.* **245:** 267–271.

Rewitz, K.F., O'Connor, M.B., Gilbert, L.I. 2007. Molecular evolution of the insect Halloween family of cytochrome P450s: phylogeny, gene organization and functional conservation. *Insect Biochem Mol Biol* **37:**741-753.

Rewitz KF, Yamanaka N, Gilbert LI, O'Connor MB. 2009. The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis. *Science* 326:1403–1405.

Richter, K., 2001. Daily changes in neuroendocrine control of molting hormone secretion in the prothoracic gland of the cockroach, *Periplaneta americana*. *J. Insect Physiol.* **47:** 333–338.

Rulifson, E.J., Kim, S.K., Nusse, R. 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science* **296:**1118–1120.

Satake S, Masumura M, Ishizaki H, Nagata K, Kataoka H, Suzuki, A., Mizoguchi, A. 1997. Bombyxin,an insulin-related peptide of insects, reduces the major storage carbohydrates in the silkworm *Bombyx mori*. *Comp. Biochem. Physiol B* **118:**349–357.

Scanga, S.E., Ruel, L., Binari, R.C., Snow, B., Stambolic, V., Bouchard, D., Peters, M., Calvieri, B., Mak, T.W., Woodgett, J.R., Manoukian, A.S. 2000. The conserved PI3'K/PTEN/Akt signaling pathway regulates both cell size and survival in *Drosophila*. *Oncogene* **19:**3971–3977.

Son, G.H., Chung, S., Choe, H.K., Kim, HD., Baik, SM., Lee, H., Lee, HW., Choi, S., Sun, W., Kim, H., Cho, S., Lee, K.H., Kim, K. 2008. Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proc Natl Acad Sci USA* **105(52):**20970-20975.

Stocco, D.M. 1999. Steriodogenic acute regulatory protein. *Vitam Horm* **55:**399-441.

Thorpe A, Duve H. 1984. Insulin-like and glucagon-like peptides in insects and mollusks. *Mol. Phys.* **5:**235–260.

Vafopoulou, X., Steel, C.G.H., 1992. In vitro photosensitivity of ecdysteroid synthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera). *Gen.Comp. Endocrinol.* **86:** 1–9.

Vafopoulou, X., Steel, C.G.H. 1998. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis in vitro. *J Comp Physiol A* **182 (3):** 343-349.

Vafopoulou, X. and Steel, C.G.H. 1996a. The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. *Pro. Natl Acad Sci* **93 (8):** 3368-3372.

Vafopoulou, X. and Steel, C.G.H. 1996b. Prothoracicotropic hormone from the brain-retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval-adult development. *Gen Comp Endocrinol.* **102 (1):** 123-129.

Vafopoulou, X. and Steel, C.G.H. 1997. Ecdysteroidogenic action of *Bombyx* prothoracicotropic hormone and bombyxin on the prothoracic glands of *Rhodnius prolixus in vitro. J Insect Physiol* **43 (7):** 651-656.

Vafopoulou, X., Steel, C.G.H., 1999. Daily rhythm of responsiveness to prothoracicotropic hormone in prothoracic glands of Rhodnius prolixus. *Arch. Insect Biochem. Physiol.* **41:** 117–123.

Vafopolou, X., Steel, C.G.H., 2006. Ecdysteroid hormone nuclear receptor (EcR) exhibits circadian cycling in certain tissues, but not others, during development in Rhodnius prolixus (Hemiptera). *Cell Tissue Res.* **323:** 443–455.

Vafopoulou, X. and Steel, C.G.H. 2012. Insulin-like and testis ecdysiotropin neuropeptide are regulated by the cicadian timing-system in the brain during larval-adult development in the insect *Rhodnius prolixus* (Hemiptera). *Gen Comp Endocrinol* **179:** 277-288.

Vafopoulou, X., Steel, C.G.H., Terry, K., 2005. Ecdysteroid receptor (EcR) shows marked differences in temporal patterns between tissues during larval–adult development in Rhodnius prolixus: correlations with haemolymph ecdysteroid titres. *J. Insect Physiol.* **51:** 27–38.

Vafopoulou, X., Steel, C.G.H., and Terry, K.L. 2007. Neuroanatomical relations of prothoracicotropic hormone neurons with the circadian timekeeping system in the brain of larval and adult Rhodnius prolixus (Hemiptera). *J Comp Neurol* **503 (4):** 511-524.

Vafopoulou, X., Cardinal-Aucoin, M., Steel, C.G.H. 2012. Rhythmic release of prothoracicotropic hormone from the brain of an adult insect during egg development. *Comp Bio Physiol A* **161 (2)**: 193-200.

Vanden Broeck J. 2001. Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster. Peptides* **22:**241–254.

Verhaert PD, Downer RG, Huybrechts R, De Loof A. 1989. A substance resembling somatomedin C in the American cockroach. *Regul. Pept.* **25:**99–110.

Warren, J.T., Sakurai, S., Rountree, D.G., Gilbert, L.I., Lee, S.-S., Nakanishi, K., 1988. Regulation of the ecdysteroid titer of Manduca sexta: reappraisal of the role of the prothoracic glands. *Proc Natl Acad Sci U S A.* **85:** 958–962.

Weinkove, D., Neufeld, T.P., Twardzik, T., Waterfield, M.D., Leevers, S.J. 1999. Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I(A) phosphoinositide 3-kinase and its adaptor. *Curr. Biol.* **9:**1019–1029.

Wigglesworth, V.B. 1934. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera) 2 factors controlling molting and "metamorphosis". *Q J Microsc Sci* **77:**191-222.

Zhang, H., Stallock, J.P., Ng, J.C., Reinhard, C., Neufeld TP. 2000. Regulation of cellular growth by the *Drosophila* target of rapamycin dTOR. *Genes Dev.* **14:**2712–2724.

Zheng, X., Sehgal, A. 2010. AKT and TOT signaling set the pace of the circadian pacemaker. *Curr Biol* **20:**1203-1208.

APPENDIX

Table 1: Summary of mean pixel intensity measurements; nuclear and cytoplasmic measurements of Figures 2, 4, 5, 6, 8, and 9.

	Nuclear Mean Pixel Intensity ± SEM							
	1AZT	**7AZT**	**13AZT**	**19AZT**	**25AZT**	**31AZT**	**37AZT**	**43AZT**
Figure 2	247.00±1.06	245.85±3.80	78.10±4.36	57.26±4.52	238.17±3.82	159.96±6.21	125.73±4.87	92.60±7.99
Figure 4	239.80±2.99	201.99±5.60	97.47±3.72	83.81±4.25	241.34±5.76	211.03±5.50	111.74±14.85	60.98±3.38
Figure 5	201.06±5.908	234.35±8.07	47.52±9.61	49.77±2.63	239.52±1.04	186.77±8.58	76.86±5.67	-
Figure 6	240.58±5.595	221.37±5.23	50.23±5.70	36.99±2.22	221.04±4.70	171.09±3.38	39.08±4.81	-
Figure 8	228.42±4.92	228.34±6.52	40.01±3.14	27.23±5.27	175.58±13.26	172.89±1.63	48.57±10.68	-
Figure 9	220.19±6.59	216.63±4.04	54.09±10.53	22.29±1.92	175.69±5.16	173.16±8.55	61.60±6.58	-
	Cytoplasmic Mean Pixel Intensity ± SEM							
Figure 2	197.71±7.98	184.07±5.80	64.69±4.54	50.02±5.35	154.35±9.18	121.38±11.28	103.56±5.32	65.74±7.29
Figure 4	187.44±7.81	144.57±7.73	90.70±5.29	62.44±2.14	172.44±5.84	130.78±15.75	70.78±6.14	49.53±1.23
Figure 5	119.58±6.05	181.38±7.75	36.37±4.93	47.48±2.23	150.41±11.00	135.48±4.44	69.75±3.75	-
Figure 6	155.59±13.71	152.89±5.42	36.98±3.04	31.01±1.73	152.08±4.87	79.05±2.90	32.70±2.15	-
Figure 8	162.54±5.77	145.46±5.41	20.59±2.53	22.19±1.63	122.96±5.56	97.91±2.72	31.98±1.76	-
Figure 9	148.06±5.01	156.08±5.38	36.26±2.75	20.89±3.13	112.84±4.21	115.69±3.92	46.26±1.64	-