CELLULAR BASIS OF COUPLING BETWEEN THE CENTRAL AND THE PERIPHERAL PROTHORACIC GLAND CLOCK IN *DROSOPHILA MELANOGASTER*

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A mi familia y a Brenda.

“Las neuronas son células de formas delicadas y elegantes, las misteriosas mariposas del alma, cuyo batir de alas quien sabe si esclarecerá algún día el secreto de la vida mental” Santiago Ramón y Cajal.

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LIST OF ABBREVIATIONS

Abbreviation	Term
20-E	20-hydroxyecdysone
ACTH	Adenocorticotropic hormone
AD	Alzheimer's disease
ALK	Anaplastic lymphoma kinase
ANF	Atrial natriuretic factor
ARC-Luc	Activity-Regulated Gene-Luciferase reporter
AstA	Allatostatin A
AstAR	AstA receptor
AVP	Arginine vasopressin
BD	Bipolar disorder
cAMP	Cyclic adenosine monophosphate
$[Ca^{+2}]_i$	Intracellular calcium
CaLexA	Ca^{+2}-dependent nuclear import of LexA
CaMKII	Ca^{+2}/calmodulin-dependent protein kinase II
CasK	Calmodulin-dependent protein kinase activity
ChAT	Choline acetyltransferase
CK1ε	Casein kinase 1 epsilon
CRE	Ca^{+2}/cAMP-responsive elements
CREB	CRE-binding protein
CRH	corticotropin-releasing hormone
CRY	Cryptochrome
Crz	Corazonin
CrzR	Crz receptor
CT	Circadian time
$cyc^{\Delta 901}$	dominant negative form of *cycle* gene

dcr2	*dicer-2*
DD	Constant darkness
DHR4	*Drosophila* Hormone Receptor 4
DLP	*Drosophila* insulin-like peptide
Dmca1a	Calcium-channel protein α1 subunit A
Dmca1d	Calcium-channel protein α1 subunit D
DN	Dorsal neurons
DREADD	Designer receptor exclusively activated by designer drug
E	Ecdysone
EcR	Ecdysone receptor
EGF	Epidermal growth factor
EH	Eclosion hormone
EP	Early pupal
ER	Endoplasmic reticulum
ETH	Ecdysis triggering hormone
Gαq	Guanine nucleotide-binding proteins
GABA	Gamma-aminobutyric acid
GC	Glucocorticoid
GCaMP	Green fluorescent protein (GFP)-Calmodulin fusion protein
GFP	Green fluorescent protein
I/R	Ischemia-reperfusion
InR	Insulin receptor
IP_3R	Inositol 1,4,5, -tris-phosphate receptor
JEB	Jelly belly
L	Larval
LD	Light/Dark
LN	Lateral neurons
lLNv	Large ventral lateral neurons
LP	Late pupal stages

LS	Lomb-Scargle
NGS	Normal goat serum
qPCR	Quantitative real-time PCR
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PDF	Pigment dispersing factor
PDFr	PDF receptor
PER	Period
PFA	Paraformaldehyde
phosphoERK	phosphorylated ERK
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
phm	*Phantom*
PG	Prothoracic Gland
PTTH	Prothoracicotropic hormone
PTTHn	PTTH-expressing neurons
PVF	PDGF- and VEGF-related factor
PVR	PDGF- and VEGF-receptor related
RACK1	Receptor for activated C kinase-1
RI	Rhythmicity index
RNAi	RNA interference
RT	Room temperature
RTK	Receptor tyrosine kinase
RyR	Ryanodine receptor
SCN	Suprachiasmatic nucleus
SERCA	Sarco/endoplasmic reticulum Ca^{+2}-ATPase
SF	Subcutaneous fat
sLNv	Small ventral lateral neurons
sNPF	Short neuropeptide F

sNPFr	sNPF receptor
SPARK	separation of phases-based activity reporter of kinase
TIM	*timeless*
TTFL	Transcriptional/translational feedback loops
TTX	Tetrodotoxin
UAS	Upstream activating sequence
VGGCs	Voltage-gated Ca^{+2} channels
VGAT	Vesicular GABA transporter
VGLUT	Vesicular glutamate transporter
VIP	Vasoactive intestinal peptide
WPP	White prepupal stage
ZT	Zeitgeber time

ABSTRACT

Circadian clocks impose daily periodicities to behavior, physiology, and metabolism. In *Drosophila melanogaster*, the emergence of the adult fly is controlled by the circadian clock, which restricts the time of emergence to a specific window of time. This gating of emergence depends on the activity of the central circadian pacemaker in the brain and of a peripheral clock located in the prothoracic gland (PG). These two clocks are coupled by the peptidergic neurons that produce the neuropeptide, PTTH, through unclear mechanisms. Here, using GCaMP, I reported a daily variation in Ca^{+2} levels in the PTTHn (PTTH neurons), which is dependent of a functional molecular clock. In addition, using an RNAi knockdown screen directed at PTTHn, I found that SERCA and RyR, two Ca^{+2} transporters of the endoplasmic reticulum, are relevant to the rhythm of the adult emergence. Additionally, I show that PTTHn express a circadian rhythm in PTTH immunoreactivity at the terminals of PTTHn and in the expression of the PTTH receptor (*torso*) in the PG. By expressing a kinase activity sensor in the PG, I showed that there are daily variations in the levels of ERK phosphorylation, a key component in the PTTH transduction pathway. In addition to PTTH, I demonstrated that ligands, Jeb/Alk, and the autocrine Pvf2/Pvr signal in the PG also contribute to the rhythmicity of emergence. My work provides information on how the time signal is transmitted from the central clock to the peripheral PG clock, and will serve as a paradigm to understand how central and peripheral clocks are coordinated.

RESUMEN

Los relojes circadianos imponen un ritmo diario al comportamiento, la fisiología y el metabolismo. En *Drosophila melanogaster*, la emergencia de la mosca adulta está controlada por el reloj circadiano, que restringe el momento de la emergencia a una ventana de tiempo específica. Esta regulación de la emergencia depende de la actividad del reloj central en el cerebro y de uno periférico ubicado en la glándula protorácica (PG). Estos dos relojes están acoplados por neuronas peptidérgicas que producen el neuropéptido PTTH, por mecanismos poco claros. Aquí, utilizando GCaMP, yo reporto una variación diaria en los niveles de Ca^{+2} en las PTTHn (neuronas PTTH) que depende de un reloj molecular funcional. Del mismo modo, una búsqueda usando RNAi dirigida a las PTTHn muestra que SERCA y RyR, dos transportadores de Ca^{+2} en el retículo endoplásmico, son relevantes para el ritmo de emergencia. Además, muestro un control circadiano en la acumulación de PTTH en las terminales de PTTHn y en la expresión del receptor PTTH (*torso*) en la PG. Al expresar un sensor de la actividad de la quinasa ERK en la PG, reporto variaciones diarias en la fosforilación de ERK, un componente clave en la vía de transducción de PTTH. Además de PTTH, demuestro que las señales Jeb/Alk y la señal autocrina Pvf2/Pvr en la PG también contribuyen a la ritmicidad de la emergencia. La descripción aquí proporcionada sobre cómo se transmite la señal de tiempo desde el reloj central al reloj periférico PG servirá como un paradigma para entender cómo se coordinan los relojes central y periférico.

INTRODUCTION

The Circadian clock, an endogenous timekeeper

The Earth's rotation period is approximately 24 hours, which causes day/night cycles and temperature fluctuations. To adapt to these changes in their environment, multicellular organism evolved an endogenous timekeeping system termed circadian clocks. These endogenous clocks impose a rhythm close to 24 hours to a wide variety of molecular, cellular, physiological, and behavioral processes. Circadian clocks sustain these endogenous rhythms in the absence of external cues (*zeitgebers*) such as light and temperature, which contribute to establishing the phase of rhythm (Pittendrigh, 1993). At the molecular level, the circadian timekeeping system is controlled by clock genes which, through a succession of expression, accumulation, and degradation of gene products, constitute an autonomous molecular oscillator (Patke et al., 2020). In order to be considered a circadian clock, a biological system needs to have four characteristics (Saunders, 2002):

A) An oscillatory event with a period close to 24 hours.
B) An oscillatory event which is entrainable by environmental cues (light, temperature).
C) An oscillatory event which is sustained in constant conditions (i.e., without environmental cues).

D) An oscillatory event which is temperature-compensated (i.e., shifts in the external temperature do not alter the periodicity of the clock).

In animals, molecular oscillators are present in multiple tissues and together constitute a highly interactive network that imposes daily rhythmicity to physiology and behavior. For instance, in mammals a master oscillator housed in the suprachiasmatic nucleus (termed central clock) synchronizes to the environmental cues and, in turn, coordinates other peripheral oscillators that reside in multiple tissues in the body (Welsh et al., 2010). Disruption in the coordination between clocks has been associated with detrimental effects on human health, such as sleep disorders, cancer, and metabolic and cardiovascular diseases (Roenneberg and Merrow, 2016). Therefore, the study of the interaction between clocks is pivotal in the field of chronobiology, and the knowledge of the mechanisms that mediate the synchronization between pacemakers may have significant implications for human health.

The circadian clock in *Drosophila melanogaster*

Drosophila melanogaster has been instrumental for the study of circadian clocks. The first circadian behavior described was eclosion, which occurs at the end of the metamorphosis when the adult fly emerges from the pupal case. In wildtype flies, this behavior occurs around dawn, but not in flies bearing null alleles of the *period* gene (*per0)*, which is a core component of the clock

(Konopka and Benzer, 1971). Subsequently, circadian behaviors have been reported in the locomotor activity (Konopka and Benzer, 1971), feeding (Xu et al., 2008), sleep (Shaw et al., 2000), courtship behavior (Hardeland, 1972), learning and memory (Lyons and Roman, 2009), oviposition (Allemand, 1976), olfactory response (Krishnan et al., 2001), cuticle deposition (Ito et al., 2008), sperm release (Giebultowicz et al., 2000). Biochemical and genetic studies have revealed that intracellular transcriptional/translational feedback loops (TTFL) are responsible for generating circadian rhythms (Hardin, 2011). At their core, TTFLs include the transcription factors, CLOCK and CYCLE, which activate the transcription of the *period* (*per*) and timeless (*tim*) genes. In the cytoplasm, PER and TIM dimerize and translocate to the nucleus where they repress the transcription of their own genes by suppressing CLOCK and CYCLYE activity. During the evening, PER and TIM start to accumulate, initially in the cytoplasm, and then around the middle of the night in the nucleus (Dubowy and Sehgal, 2017). This rhythmic accumulation is mediated, at least in part, by CRYPTOCHROME, a blue-light receptor that induces the degradation of TIM in the presence of photic inputs. Without TIM, PER is phosphorylated by the kinase Double-time (DBT), which leads to the degradation of PER. As a result, the internal biological clock is synchronized with the light-dark cycle (Figure 1).

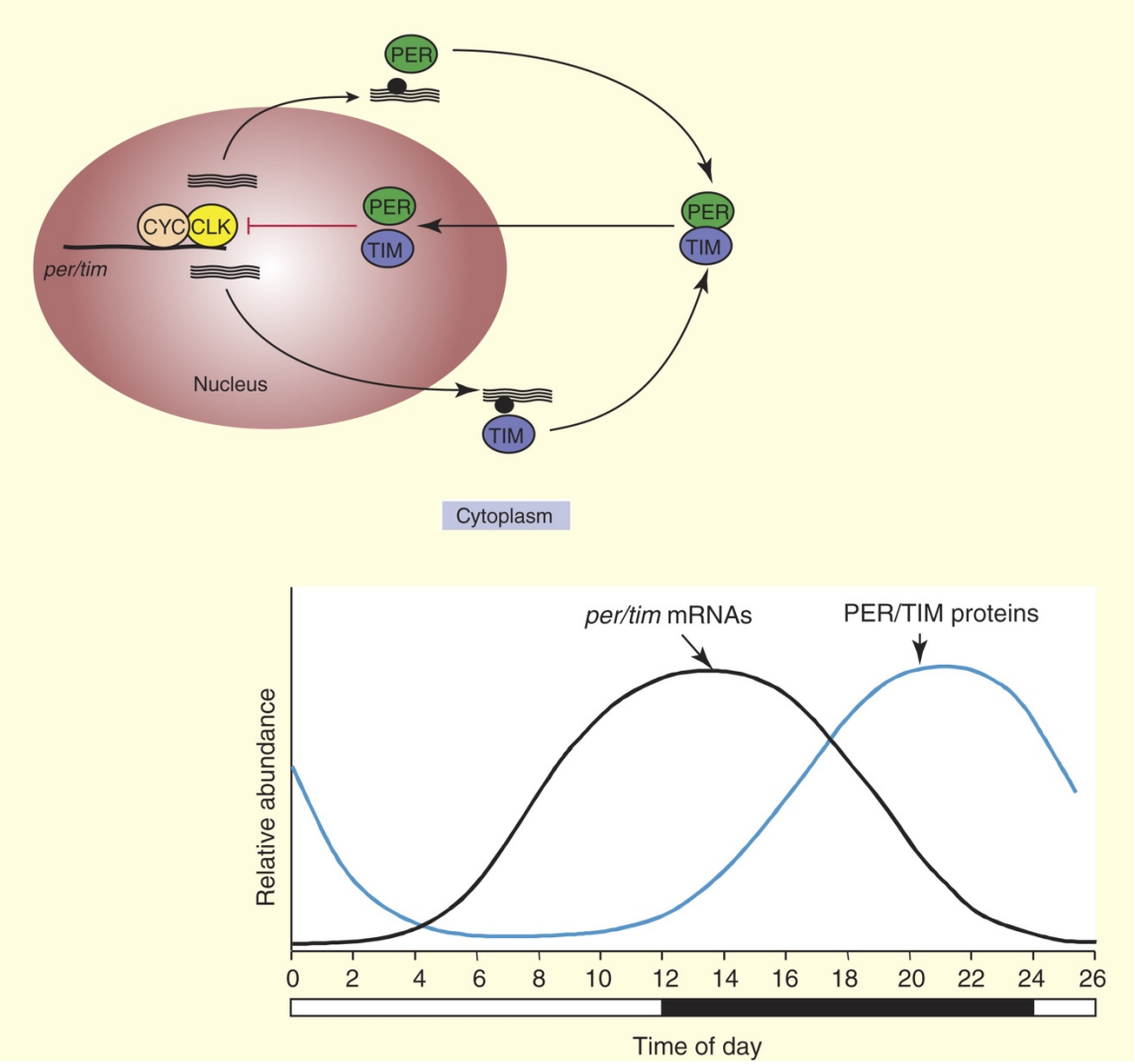

Figure 1. The Drosophila molecular clock and its synchronization with the light-dark cycle. The transcriptional activators Clock (CLK) and Cycle (CYC) induce the transcription of *per* and *tim*, which reach their highest expression levels in the early evening. The translated PER and TIM gradually accumulate and form dimers within the cytoplasm, peaking during the nighttime. The PER-TIM heterodimer then translocates into the nucleus, where it represses the transcription of *clk* and *cyc* genes, establishing a transcription-translation feedback loop. Adapted from (Nitabach and Taghert, 2008).

At the anatomical level, the central clock in flies is composed of approximately 150 circadian neurons, which are classified into two major groups: the lateral neurons (LN) and the dorsal neurons (DN) (Nitabach and Taghert, 2008)(Figure 2). In turn, LNs are divided into three subgroups: small and large ventral lateral neurons (sLNv and lLNv respectively), dorso-lateral neurons (LNds) and the 5th small ventral lateral neuron. Dorsal neurons are divided in three sub-groups: DN1, DN2, and DN3. On the other hand, peripheral clocks reside in a variety of tissues such as the prothoracic gland, the fat body, the antenna, Malpighian tubules, oenocytes, and epidermal cells (Ito and Tomioka, 2016). Similar to mammals, some processes that are under circadian control in flies are dependent on the complex relationship between central and peripheral clocks. For instance, in the fat body, the rhythmic expression of some genes is only dependent on the clock of the fat body; by contrast, other genes cycle in response to the central clock input (Xu et al., 2008; Erion et al., 2016).

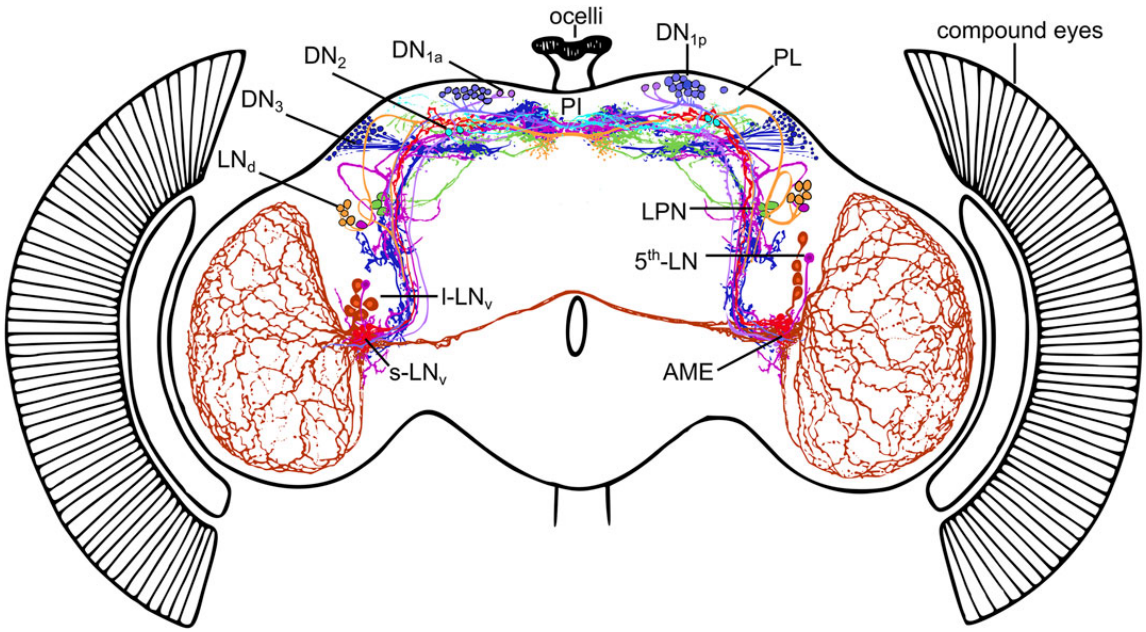

Figure 2. The clock neurons in the adult brain of *Drosophila melanogaster*. Clock-gene-expressing neurons in the adult brain are classified in lateral (sLNv, lLNv, dLN and 5th sLNv, LPN) and dorsal neurons (DN1, DN2 and DN3). Many of them send projections into the *pars intercerebralis* (PI), the *pars lateralis* (PL), and the accessory medulla (AME). Figure obtained from (Reinhard et al., 2022).

The circadian rhythm of eclosion

In *Drosophila*, as in all insects, the exoskeleton is replaced during the molt (Ewer, 2007). The final step of this process is ecdysis, during which the remains of the exoskeleton from the previous stage is shed and the new one is inflated, hardened, and pigmented. In *Drosophila*, molting is present during the different stages of development. Specifically, *Drosophila* shows three stages during the development: larval stages (L1 to L3), pupae and the adult stage. Each developmental transition is accompanied by ecdysis behavior, and the final molting occurs at the end of metamorphosis, when the adult fly emerges out from the pupal case. At the endocrine level, molts are controlled by 20-

hydroxyecdysone (20E), a steroid hormone produced by the prothoracic gland (PG), an endocrine gland located, in flies, above the brain during larval stages (Figure 2). At the beginning of the molt, 20E levels increase in the hemolymph, inducing the biosynthesis of ecdysis triggering hormone (ETH) from peripheral endocrine cells and also changing the sensitivity of the central nervous system (CNS) to ETH (Zitnan et al., 1999; Zitnanova et al., 2001). At the end of the molt, 20E levels fall below a threshold level, eventually causing the release of ETH and eclosion hormone (EH) and triggering ecdysis (Ewer et al., 1997; Kingan et al., 1997). Most ecdyses are regulated to occur at the end of the molting process. By contrast, adult ecdysis, also called eclosion, also depends on the circadian clock, which restricts ("gates") eclosion to the early part of the day. In the PG, 20E signaling is critical for the gating of eclosion (Mark et al., 2021). This circadian behavior is dependent on the central clock in the brain and the peripheral clock in the PG (Myers et al., 2003). Importantly, our laboratory showed that the central and the PG clocks are coupled by a peptidergic pathway to generate the circadian control of emergence of the adult fly (Selcho et al., 2017). Specifically, this work revealed that the sLNv neurons of the central clock transmit time information via the small neuropeptide F (sNPF) to the neurons that release prothoracicotropic hormone (PTTH). PTTH is a neuropeptide that binds to its receptor tyrosine kinase (RTK), TORSO, in the PG to induce the biosynthesis of ecdysone (E) (McBrayer et al., 2007; Rewitz et al., 2009) and somehow, transmit time information to this peripheral clock (Figure 3)(Selcho et al., 2017).

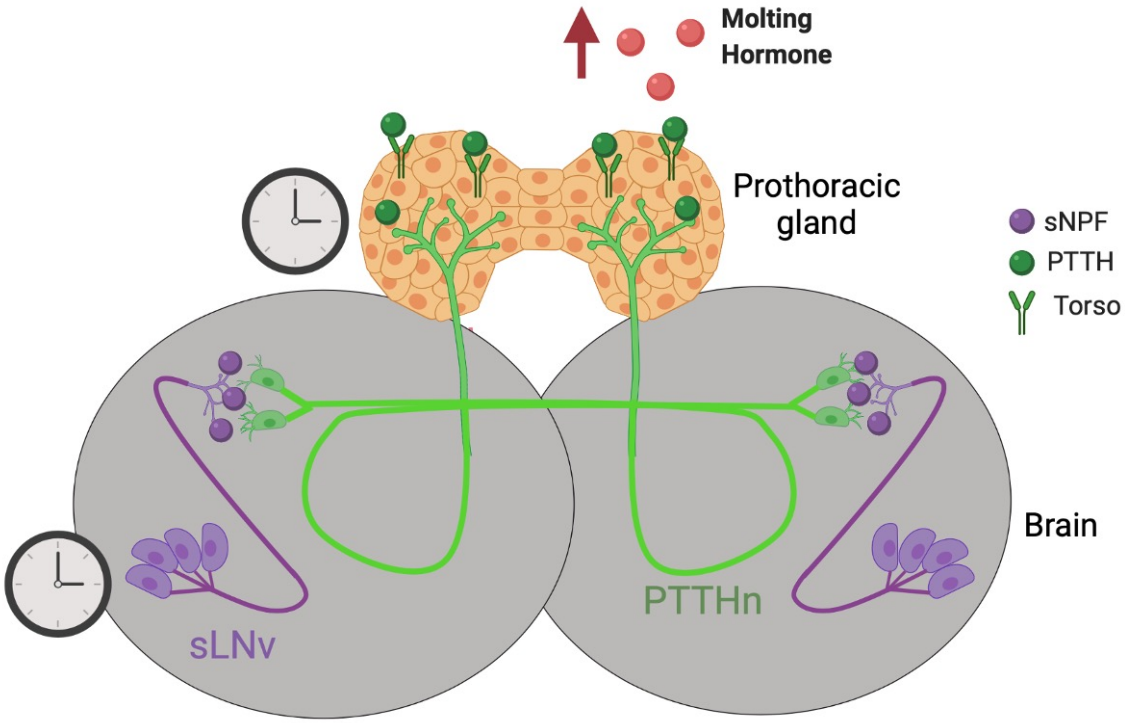

Figure 3. Neuroanatomical organization of the brain and the PG clocks at the larval stage. Representation of the brain and the prothoracic gland at the larval stage. Neuropeptides that mediate the coupling between the brain and the PG clocks are indicated. sLNv: small ventral lateral neurons. sNPF: small neuropeptide F. PTTHn: neurons that secrete prothoracicotropic hormone (PTTH).

Although neuropeptidergic signaling connecting the brain clock to the PG clock has been described, the cellular and molecular mechanisms that mediate this process are poorly understood. For instance, even though sNPF has an inhibitory effect on PTTHn that is dependent of Ca^{+2} (Selcho et al., 2017), it is not known how this translates into a mechanism that transmits time information transmission and which subcellular processes are involved. Likewise, although PTTH is required for the circadian rhythmicity of emergence, *ptth* mRNA levels

do not exhibit a circadian fluctuation (Selcho et al., 2017), indicating that the clock may operate at the translational or post-translational levels. In addition, it has been described that PTTH-independent pathways mediated by insulin, the epidermal growth factor, $G_{\alpha}q$ proteins, jelly belly, and PDGF- and VEGF-related factor regulate E production in the PG (Colombani et al., 2005; Yamanaka et al., 2015; Cruz et al., 2020; Pan and O'Connor, 2021). However, their role in the circadian control of eclosion remains to be determined.

Here, I investigated the cellular and molecular basis of the coupling between the central and the PG clocks. I report four principal findings. First, using Ca^{+2} imaging, I showed that PTTHn exhibit a daily oscillation in intracellular Ca^{+2} levels $[Ca^{+2}]_i$ and notably, two endoplasmic reticulum Ca^{+2} transporters are relevant for the daily rhythm of eclosion. Second, PTTH accumulation in the PTTHn terminals and *torso* expression under circadian control. Third, using a phase separation-based sensor of kinase activity, I showed that ERK phosphorylation levels, a key component of PTTH transduction in the PG, oscillate in a time-dependent fashion, Fourth, I showed that two additional RTKs, PDGF and VEGF receptor-related (PVR) and anaplastic lymphoma kinase (ALK), that are expressed in the PG, contribute to the rhythmicity of emergence. Together, my findings provide an extensive characterization of the cellular and molecular basis of the coupling between the brain clock and PG clock, which may provide a general mechanism behind the coordination between clocks.

This thesis is organized in two main chapters. The first is a review that we wrote during the worst of covid-19 lockdown for Frontiers in Molecular Neuroscience, which reviews the relationship between calcium signaling and clock function in mammals and insects. I wrote the first drafts of the manuscript and of the revisions made in response to reviewers' comments. The second chapter describes the experimental work I carried out investigating how time information is transmitted from the central clock to the clock located in the PG. This work is included in a manuscript that will be submitted for publication shortly. I did most of the work reported in the manuscript and wrote most of the first draft.

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Chapter I: Reciprocal Relationship Between Calcium Signaling and Circadian Clocks: Implications for Calcium Homeostasis, Clock Function, and Therapeutics.

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Reciprocal relationship between calcium signaling and circadian clocks: implications for calcium homeostasis, clock function, and therapeutics

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Abstract

In animals, circadian clocks impose a daily rhythmicity to many behaviors and physiological processes. At the molecular level, circadian rhythms are driven by intracellular transcriptional/translational feedback loops (TTFL). Interestingly, emerging evidence indicates that they can also be modulated by multiple signaling pathways. Among these, Ca^{+2} signaling plays a key role in regulating the molecular rhythms of clocks genes and of the resulting circadian behavior. In addition, the application of *in vivo* imaging approaches has revealed that Ca^{+2} is fundamental to the synchronization of the neuronal networks that make up circadian pacemakers. Conversely, the activity of circadian clocks may influence Ca^{+2} signaling. For instance, several genes that encode Ca^{+2} channels and Ca^{+2}-binding proteins display a rhythmic expression, and a disruption of this cycling affects circadian function, underscoring their reciprocal relationship.

Here, we review recent advances in our understanding of how Ca^{+2} signaling both modulates and is modulated by circadian clocks, focusing on the regulatory mechanisms described in *Drosophila* and mice. In particular, we examine findings related to the oscillations in intracellular Ca^{+2} levels in circadian pacemakers and how they are regulated by canonical clock genes, neuropeptides, and light stimuli. In addition, we discuss how Ca^{+2} rhythms and

their associated signaling pathways modulate clock gene expression at the transcriptional and post-translational levels. We also review evidence based on transcriptomic analyzes that suggests that mammalian Ca^{+2} channels and transporters (e.g., *ryanodine receptor, ip3r, serca, L-and T-type* Ca^{+2} *channels*) as well as Ca^{+2}-binding proteins (e.g., *camk, cask, calcineurin*) show rhythmic expression in the central brain clock and in peripheral tissues such as the heart and skeletal muscles. Finally, we discuss how the discovery that Ca^{+2} signaling is regulated by the circadian clock could influence the efficacy of pharmacotherapy and the outcomes of clinical interventions.

Introduction

All multicellular animals contain a biological clock that allows them to anticipate the arrival of dawn or dusk. These are endogenous mechanisms that are cell-autonomous and are invariably controlled by highly conserved intracellular transcription-translation feedback loops (Hardin, 2011; Takahashi, 2017). Circadian clocks are classified according to where they reside: central oscillators are located in the brain whereas peripheral oscillators can be housed in a wide variety of tissues, where they impose a daily rhythmicity to the physiology of each organ (Mohawk et al., 2012). Yet, the different cells that make up the central pacemaker must coordinate their activity and must also be entrainable by the daily environmental signals that synchronize the organism's pattern of activity to the appropriate time of day (usually light, but can also be temperature). In addition, peripheral clocks are coordinated with the central

clock to produce a unitary biological time for the organism. And, finally, the activity of clocks can in turn be modified by the organism's physiology and/or behavior (e.g., through feeding). At the systems level, the entrainment of central and peripheral clocks, the coordination between clocks as well as between clocks and their host, occurs via neuronal and endocrine signals (Albrecht, 2012; King and Sehgal, 2020), which, at the intracellular level, are then transduced by various signaling pathways that ultimately alter the temporal pattern of gene expression. Here, we review the role of Ca^{+2} signaling in clock function by considering the mechanisms by which (I) Ca^{+2} signaling affects clock function, and how, in turn, (II) clock function affects Ca^{+2} signaling. Finally, as a result of our growing appreciation of the importance of clocks in health and disease, we discuss (III) How our knowledge of the interlocked relationship between clocks and Ca^{+2} signaling impacts the effectiveness of drugs and is relevant to the development of improved therapeutics.

I. Ca^{+2} rhythms in biological clocks are regulated by intracellular and extracellular signals

In animals, intracellular Ca^{+2} levels ($[Ca^{+2}]_i$) are essential for the proper functioning of many cellular and physiological processes including circadian rhythms. In biological clocks, fluctuations in $[Ca^{+2}]_i$ have been associated with the circadian control of neuropeptide release, the expression of clock genes, synaptic plasticity, and the periodicity of rhythmic behaviors (Lundkvist et al., 2005; Harrisingh et al., 2007; Depetris-Chauvin et al., 2011). For this reason, an

important area of research in chronobiology is devoted to identifying changes in $[Ca^{+2}]_i$ that occur in circadian pacemaker neurons and understanding their origin. Interestingly, daily Ca^{+2} variations occur in circadian oscillators of rodents (Colwell, 2000; Ikeda et al., 2003; Enoki et al., 2012; Brancaccio et al., 2013; Enoki et al., 2017; Noguchi et al., 2017), flies *(Liang et al., 2016; Guo et al., 2017)*, and mollusks (Colwell et al., 1994).

I.1. Neuropeptide-mediated Ca^{+2} rhythms

In mice, Ca^{+2} rhythms have been described in neurons and astrocytes of the central mammalian clock, which is located in the suprachiasmatic nucleus (SCN) (Ikeda et al., 2003; Brancaccio et al., 2017). Remarkably, they are not uniform over the entire nucleus: Indeed, the phase of Ca^{+2} oscillations in dorsal regions is advanced relative to that of the ventral zone, and this spatial organization is highly dependent on neuronal network properties within the SCN (Enoki et al., 2012; Brancaccio et al., 2013). In addition, this difference in Ca^{+2} phase is abolished when the intercellular synchronization within the SCN is weak (Enoki et al., 2017), suggesting that diffusible factors such as the neuropeptides, arginine vasopressin (AVP) or vasoactive intestinal peptide (VIP), which are enriched in dorsal and ventral regions of SCN respectively, are relevant for maintaining the phase difference between Ca^{+2} oscillations in the dorsal vs. ventral SCN. Similarly, in *Drosophila*, where daily locomotor activity shows a bi-modal pattern, the central clock neurons that control the morning and the evening peaks of activity express different Ca^{+2} phases (Liang et al., 2016). In

particular, neurons that regulate the morning activity (the so-called M-cells) exhibit an advanced Ca^{+2} phase relative to circadian pacemaker neurons that control the evening activity (the so-called E-cells). Remarkably, inhibiting signaling mediated by pigment dispersing factor (PDF), a neuropeptide analogous to mammalian VIP, causes the phases of Ca^{+2} oscillations in M and E cells to be synchronous (Liang et al., 2016) and the pattern of behavior to be unimodal or arrhythmic (Hyun et al., 2005). In addition, in *Drosophila* $[Ca^{+2}]_i$ varies during the course of the day in the prothoracic gland (PG), a peripheral clock that regulates the circadian rhythm of adult eclosion (Morioka et al., 2012; Palacios-Munoz and Ewer, 2018). A study carried out in organotypic PG cultures shows that the phase of the Ca^{+2} oscillations is set by inputs from the brain (Morioka et al., 2012), which are probably mediated by prothoracicotropic hormone (PTTH), a neuropeptide that transmits time information from the central clock to the PG (Selcho et al., 2017). Of note, Ca^{+2} plays a critical role in the release of the molting hormone ecdysone (E) from the PG (Yamanaka et al., 2015), suggesting that fluctuations in $[Ca^{+2}]_i$ due to clock activity could produce a daily rhythm in hemolymph E titers and, consequently, be relevant to the circadian control of adult emergence. Nevertheless, although such oscillations occur in some insects (Ampleford and Steel, 1985), they have not been detected in *Drosophila.*

I.2. Clock genes and Ca^{+2} oscillations

Although neuropeptides can set the phase of Ca^{+2} oscillations in biological clocks, these extracellular signals are not the only relevant ones for regulating Ca^{+2} rhythms. In mice, an early study using FURA-2, a synthetic fluorescent Ca^{+2}-sensitive dye, showed that Ca^{+2} oscillations are completely abolished by tetrodotoxin (TTX) treatments, suggesting that neuronal firing of SCN neurons is essential for maintaining these rhythms (Colwell, 2000). However, FURA-2 does not allow for the long-term measurement of Ca^{+2} oscillations because it is eventually cleared from the cytoplasm. In contrast to these results, studies that have expressed genetically encoded Ca^{+2} sensors such as GCaMP or Cameleon in the SCN have shown that Ca^{+2} rhythms are only partially reduced in the absence of neuronal firing, suggesting that intracellular signaling also plays a role in regulating the oscillations of $[Ca^{+2}]_I$ (Enoki et al., 2012; Noguchi et al., 2017). Intracellular pathways could be associated with TTFLs of clock genes. Indeed, in mammals, overexpression of a dominant-negative allele of BMAL1, a transcription factor and core element of the clock, inhibits Ca^{+2} rhythms in the SCN (Ikeda and Ikeda, 2014). Similarly, in *Drosophila* a null allele of the *period* gene, which is also a core component of the clock, reduces the rhythm and coherence of Ca^{+2} oscillations in central clock neurons (Liang et al., 2016), and similar results have been described for the peripheral clock housed in the prothoracic gland (Palacios-Munoz and Ewer, 2018). Remarkably, another potential intracellular regulator of $[Ca^{+2}]_i$ in biological clocks are ryanodine-sensitive stores (Ding et al., 1998; Ikeda et al., 2003; Aguilar-Roblero et al.,

2007). Indeed, blockers of the ryanodine receptor impair Ca^{+2} oscillations in organotypic cultures of SCN, suggesting that the release of Ca^{+2} from the endoplasmic reticulum is critical for the regulation of the rhythm of $[Ca^{+2}]_i$ in the mammalian central clock.

I.3 Photic inputs set the phase of Ca^{+2} oscillations

A third regulator of intracellular Ca^{+2} rhythms is photic inputs. In circadian oscillators, light causes a time-dependent shift in the phase of the Ca^{+2} fluctuations and in the rhythm of behavior. In mammals, such light-induced phase shifts are mediated by neurons that produce VIP, which exhibit spontaneous Ca^{+2} oscillations that are critical for propagating photic inputs through the SCN as well as for resetting the daily rhythms (Jones et al., 2018). Consistent with this, mice lacking calbindin (a cytosolic Ca^{+2}-buffering protein) display increased phase delays when they receive a light stimulus in the early part of the night (Stadler et al., 2010). In *Drosophila*, photic inputs set the phase of Ca^{+2} oscillations in central clock neurons through two pathways: they either act through the visual system via PDF, or they act directly on the circadian pacemaker through the internal photoreceptor, CRYPTOCROME (CRY), to set the phase of a group of E-cell neurons (Liang et al., 2017). By contrast, in the PG, CRY-dependent photoreception inhibits Ca^{+2} activity and this effect is abolished when the brain-PG complex is treated with TTX, suggesting that in this peripheral oscillator light acts through neuronal pathways (Morioka et al., 2012). In insects light can also penetrate the translucid exoskeleton and entrain

peripheral clocks; however, whether these photic inputs can directly affect the Ca^{+2} rhythms of pacemaker neurons is currently unknown.

Overall, this evidence suggests that Ca^{+2} rhythms in circadian clocks are regulated by both intracellular and extracellular signals. In addition, extracellular signals from neuropeptides and photic inputs set the phase of the rhythm and coordinate the relative timing of the Ca^{+2} oscillations (Figure 1A), whereas TTFLs, and probably also intracellular Ca^{+2} stores, also contribute to generate Ca^{+2} oscillations in circadian clocks (Figure 2A). This model is based on the proposal that the central clock is comprised of interconnected autonomous circadian oscillators whose emerging network properties reinforce their circadian rhythmicity, synchronizing their oscillations, and adjusting them to the day-night cycles (Welsh et al., 2010).

II. Effects of Ca^{+2} signaling on the expression of clock genes and rhythmic behavior

In multicellular organisms, a variety of molecules are critical for sustaining circadian behavior. In *Drosophila* and mammals, transcription factors, microRNAs, and protein kinases, control the proper functioning of biological clocks (Patke et al., 2020). Interestingly, Ca^{+2} signaling is also involved in driving rhythmic behaviors and rhythms of gene expression. In *Drosophila,* genetic manipulations that decrease Ca^{+2} levels or reduce the expression of proteins such as Ca^{+2}/calmodulin-dependent protein kinase II (CaMKII) in circadian pacemaker neurons, lengthen the periodicity of the circadian rhythms of

locomotor activity and of adult eclosion (Harrisingh et al., 2007; Palacios-Munoz and Ewer, 2018). Similarly, a mouse bearing a mutation in the CaMKII gene that abolishes all kinase activity exhibits a longer free-running period of locomotor activity and a desynchronization in molecular rhythms between the left and right nuclei of the SCN (Kon et al., 2014). In terms of gene expression, buffering intracellular Ca^{+2} abolishes *Per1* oscillations in cultured SCN slices, and voltage-gated Ca^{+2} channel antagonists dampen the rhythm of *Per2* and *Bmal1* in an SCN cell line (Lundkvist et al., 2005; Nahm et al., 2005). Likewise, in the peripheral clock of the liver, lowering extracellular [Ca^{+2}] abolishes *Per1* rhythmicity (Lundkvist et al., 2005). Remarkably, blocking Ca^{+2} flux through inositol 1,4,5-trisphosphate receptor (IP_3R) and sarco/endoplasmic reticulum Ca^{+2} ATPase (SERCA) in liver explants lengthens the period of *Per1-luc* oscillations (Baez-Ruiz and Diaz-Munoz, 2011), which provides additional evidence that intracellular Ca^{+2} stores play a key role in the generation of circadian rhythms.

The growing evidence for the role of Ca^{+2} in circadian gene expression raises the question of how Ca^{+2} signaling would influence the TTFL. Three mechanisms have been proposed: a) regulation in via Ca^{2+}/cAMP-responsive elements (CREs), and phosphorylation of clock proteins with effects at b) the transcriptional and c) the posttranslational levels.

II.1 Ca^{+2}/cAMP-responsive elements

CRE is a sequence that binds CRE-binding protein (CREB) and is present within the promoter region of several clock genes (Zhang et al., 2005). In this context, CREB induces changes in clock gene expression in response to light and contributes to the synchronization of activity among pacemaker cells (Ginty et al., 1993; Welsh et al., 2010). Several findings suggest that CREB is critical for integrating the signaling mediated by second messengers such as Ca^{+2} into the rhythm of expression of clock genes. For instance, in the hamster SCN, the phase-shifts in *Per1* and *Per2* induced by light are mediated by CaMKII (Yokota et al., 2001), which can stimulate CRE-promotor activity through the phosphorylation of CREB (Figure 1B) (Nomura et al., 2003). In addition to actions mediated through CaMK signaling, manipulations of electrical activity in neurons reveals a close relationship between Ca^{+2}-induced changes in CREB expression and alterations in TTFL. In *Drosophila*, for example, the hyperexcitation of a cluster of clock neurons triggers a morning-like transcriptome profile whereas their hyperpolarization induces an evening-like transcription state (Mizrak et al., 2012). Genes that are differentially expressed in response to electrical activity are enriched in CRE elements, and altering pacemaker excitability changes CREB expression, supporting the idea that CREB is involved in modifying the pattern of expression of circadian genes. On other hand, assays using a CRE-luciferase reporter system have shown that CRE-mediated transcription exhibits a circadian rhythmicity in neurons and glia in multiple areas of the *Drosophila* brain (Tanenhaus et al., 2012); in the mouse

SCN, this oscillation is highly dependent on the Gq-Ca^{+2} axis (Figure 1B) (Brancaccio et al., 2013). In particular, the Ca^{+2} peak occurs earlier than the CRE-luc peak, which in turn, precedes the peaks in *Per1-luc* and *Per2*-luc activity. Remarkably, the activation of the G_q-dependent pathways using DREADDs (designer receptor exclusively activated by designer drug) reorganizes this hierarchical organization altering Ca^{+2} rhythm in clocks cells and lengthening the period of CRE-luc and *Per-luc* cycling in the SCN. These effects are not mediated through G_s or G_i, indicating that CRE-mediated transcription is exclusively activated through the Gq-Ca^{+2} axis.

II.2. Ca^{+2} signaling and transcriptional changes mediated by phosphorylation of clock proteins

The phosphorylation state of clock proteins plays a pivotal role in the functioning of circadian clocks. Indeed, mutations in a variety of protein kinase and phosphatases alter the rhythm of circadian behaviors in flies, hamsters, mice, and humans (Reischl and Kramer, 2011). At the molecular level, kinase activity can regulate the transcription levels of canonical clocks genes, their accumulation in the nucleus, their ability to bind other proteins, and their stability (Gallego and Virshup, 2007). Interestingly, Ca^{+2} signaling has been implicated in the control of the phosphorylation state of clock proteins (Figure 1C). For instance, protein kinase C (PKC) and phospholipase C are critical to light-induced clock resetting in mice (Jakubcakova et al., 2007) and *Drosophila* (Saint-Charles et al., 2016; Ogueta et al., 2018, 2020), respectively. In the SCN,

the adaptation to photic stimuli is highly dependent on the phosphorylation state of chromatin modifiers mediated by PKC. In particular, PKCα phosphorylates a lysine-specific demethylase 1 (LSD1) that controls the phase resetting of pacemaker cells and the circadian control of locomotor activity in mice (Nam et al., 2014). At the molecular level, phosphorylated LSD1 binds the BMAL1/CLOCK complex, which controls the pattern of expression of clock genes that contain an E-box sequence (a circadian transcriptional enhancer) in their promotor region. In addition, PKCα can act autonomously on core clock proteins, for instance by phosphorylating BMAL1, and this activity is enhanced by RACK1 (receptor for activated C kinase-1), a signaling protein that recruits PKC to its substrates (Robles et al., 2010). The PKCα/RACK-1 complex is recruited to BMAL1 in a circadian manner in central and peripheral mammalian pacemakers. In a fibroblast cell line, the PKCα/RACK1 complex controls the circadian period by acting as a negative regulator of BMAL1-CLOCK transcriptional activity, which supports the idea that PKC plays a key role in regulating circadian clocks. CaMKII is another protein associated with Ca^{+2} signaling that directly phosphorylates core clock proteins. Indeed, in pacemaker cells, this protein kinase phosphorylates CLOCK in a circadian manner by promoting BMAL1-CLOCK heteromerization and enhancing E-box dependent gene expression including that of *Per1-3* and *Cry1* (Kon et al., 2014). Remarkably, the pattern of expression of these genes is also altered by calmodulin inhibitors and Ca^{+2} chelators, indicating that the

Ca^{+2}/calmodulin/CaMKII-mediated phosphorylation of CLOCK is an important regulator of cell-autonomous clockwork periodicity.

II.3. Ca^{+2} signaling and posttranslational modifications mediated by the phosphorylation of clock proteins

In circadian clocks, the actions mediated through Ca^{+2} modulation of kinase/phosphatase activity are not restricted to the nucleus of pacemaker cells (Figure 1D). For instance, transfection of PKC in mammalian cell lines increases the stability of PER2 and promotes its cytoplasmic localization (Jakubcakova et al., 2007). These effects are independent of the CREB pathway and may involve CK1ε (casein kinase 1 epsilon), which is known to regulate the proteasomal degradation of PER2 and its shuttling between the cytoplasm and the nucleus (Akashi et al., 2002; Eide et al., 2005). On other hand, in *Drosophila*, calcineurin, a Ca^{+2}/calmodulin-dependent serine/ threonine phosphatase, also controls the stability of clock proteins at the posttranslational level (Kweon et al., 2018). In particular, a null mutant of *sarah*, a calcineurin regulator, reduces the levels of TIM (also a canonical clock protein in *Drosophila*) and PER proteins in head extracts but not those of their respective transcripts. Of note, the effect of calcineurin on clock proteins levels is abolished when the proteasomal machinery is inhibited, suggesting that calcineurin regulates the core clock mechanism by protecting PER and TIM from proteasomal degradation.

Collectively, these findings demonstrate that the role of Ca^{+2} signaling is not limited to transducing the actions of external inputs within circadian pacemakers.

Instead, studies in mammals and flies show that PKC, CaMK, calmodulin, and calcineurin, are integral components of clocks´ transcriptional and posttranslational feedback loops (Figure 1C and 1D). How is circadian rhythmicity decoded by these signaling protein? Here, we propose a model in which the oscillations of free Ca^{+2} in the cytoplasm impose a daily rhythm to the activity of its associated proteins (Figure 1). Thus, Ca^{+2} and its signaling may regulate the molecular machinery of pacemaker cells and, consequently, also the periodicity of the resulting circadian behavior.

III. Circadian regulation of Ca^{+2} associated proteins

III.1. Rhythmic expression of genes coding for proteins associated with Ca^{+2} pathways

In animals, the core molecular clock machinery directly or indirectly controls the expression of multiple downstream genes involved in the generation of rhythmic cellular process and circadian behaviors. Interestingly, a number of genes coding for proteins associated with Ca^{+2} pathways exhibit rhythmic transcription within biological clocks. For example, microarray and high throughput RNAseq analyses of mouse SCN reveal that genes that code for key proteins in Ca^{+2} signaling pathways such as *camkii*, *pkca*, and *calcineurin*, are under circadian control (Table 1) (Panda et al., 2002; Pembroke et al., 2015). These findings are consistent with previous studies that demonstrate that PKC activity shows a daily rhythm in SCN cells lines (Rivera-Bermudez et al., 2003) and that CaMKII is rhythmically expressed in the hamster central pacemaker (Agostino et al.,

2004). In mice, canonical endoplasmic reticulum Ca^{+2} transporters (*serca*, *ip$_3$r*, and *ryr*) also exhibit circadian regulation at the transcriptional level in the SCN (Table 1). Interestingly, the contribution of the RyR to the phase-shifting mediated by light in the central clock is restricted to the early night (Ding et al., 1998), which coincides with the time of peak protein levels in the SCN (Pfeffer et al., 2009) and, as a result, cytosolic Ca^{+2} reaches high enough levels to phase shift the core molecular clock. Importantly, the promotor regions of *camkii, pkca, serca, ip$_3$r*, and *ryr*, include E-BOX and ROREs motifs (a consensus sequence for transcriptional regulation mediated by core clock components) (Table 1), suggesting that the rhythmic oscillation in mRNA levels of these genes may result from a direct regulation by the molecular machinery of the clock. Indeed, a deletion in a promotor region of *ryr2* that includes the E-box sequence reduces the transcriptional activation induced by the BMAL1-CLOCK complex in a mouse fibroblast cell line (Pfeffer et al., 2009), supporting the idea that the molecular clockwork influences Ca^{+2} signaling.

Other genes that are rhythmically expressed are voltage-gated Ca^{+2} channels (VGCCs), which are key regulators of the excitability and functioning of neuronal clocks. In the SCN, some VGCCs subunits mediate the phase shifting induced by photic inputs as well as the daily changes in conductance (Colwell, 2011). In addition, L-type, T-type, and P/Q type VGCCs, are under circadian control at the transcriptional level in the rat SCN (Nahm et al., 2005). Interestingly, the rhythmic expression of L-type Ca^{+2} channels, the most abundant VGCCs in the SCN, is regulated by the circadian clock component, REV-ERBa (Schmutz et

al., 2014). In particular, in mouse hypothalamic tissues (including the SCN), REV-ERBa binds to the promotor region of an L-type Ca^{+2} channel (*Cacna1c*) at REOREs and direct mutagenesis of this sequence abolishes the oscillation of *Cacna1c* mRNA levels, indicating that REV-ERBa plays a critical role in this circadian regulation. Of note, this mechanism is not the only one that has been associated with the rhythmic control of expression of L-type VGGCs. Indeed, in chicken cone photoreceptors, L-type-VGCCa1Cs display a diurnal rhythm of expression, which is controlled at the posttranscriptional level by microRNAs (Shi et al., 2009). In particular, in the retina, microRNA-26a exhibits a rhythmic expression and binds to the untranslated region of an *L-type-VGGCa1C* during the subjective day, thereby imposing a rhythm to the translation of this Ca^{+2} channel. Although there is evidence that microRNAs participate in the circadian control of transcription in the mammalian central clock (Cheng et al., 2007), it is still unknown whether they act at the posttranscriptional level on genes encoding proteins associated with Ca^{+2} signaling.

Another interesting case of circadian transcriptional regulation involving Ca^{+2} pathway proteins occurs in the *Drosophila* central clock. The adult fly brain has around 150 clock neurons per hemisphere, which are classified based on their anatomical location (Helfrich-Forster et al., 2007). These include so-called dorsal (DN), ventral (VN), and lateral (LN), clusters of pacemaker neurons, and a recent study using RNA-seq demonstrated that each of these clusters exhibits a different transcriptional profile (Abruzzi et al., 2017). Interestingly, a number of genes that are differentially expressed encode proteins of the Ca^{+2} pathway. For

instance, dorsal lateral pacemaker neurons (LNd) (but not ventral lateral pacemaker neurons, LNv), display a rhythmic expression in members of the PKC pathway (*norpA, pkc53E, pkc98-E, pkca*), and in genes encoding Ca^{+2} channels (*cacophony*, *Ca^{+2}-b*) and Ca^{+2} signaling (*CaMK*, *calcineurin*) (Table 2). Many of these genes exhibit E-box or RORE sequences in their promotor region suggesting that the circadian transcriptional control is directly mediated by core clock components (Table 2). However, although LNd and DN1 dorsal neurons exhibit a similar circadian pattern of expression of genes encoding Ca^{+2}-associated proteins, the spatiotemporal patterns of Ca^{+2} activity of LNds and DN1s are quite different (Liang et al., 2016), suggesting the existence of an additional level of regulation. What could be its the underlying molecular mechanism? A recent study evaluated the alternative pre-mRNA splicing in *Drosophila* clock neurons, which is recognized as a major mechanism used to diversify the neuronal proteome (Wang et al., 2018). Combing RNAseq and computational methods, these authors quantified and categorized pre-mRNAs in pacemaker cells observing that each subgroup of pacemaker neurons possesses a unique alternative splicing profile. Of note, many transcripts of the Ca^{+2}-calmodulin-dependent family of protein kinases, PKC signaling, and Ca^{+2} channels, were differentially enriched in DN1 vs. LNd pacemaker neurons, suggesting that posttranscriptional regulation may be critical for differentiating the roles of each circadian neuronal cluster in order to produce a functional circadian clock.

III.2. Skeletal muscle and heart, two peripherals clocks displaying rhythmic changes in the transcription of elements of the Ca^{+2} signaling pathway

Ca^{+2} pathways are fundamental to the functioning of some mammalian peripheral clocks. One of them is the skeletal muscle, in which clock genes are necessary for maintaining its phenotype and metabolic homeostasis (Lefta et al., 2011). In rodents, 3-16% of the skeletal muscle transcriptome exhibits a circadian oscillation and many of these genes code for proteins involved in Ca^{+2} signaling (McCarthy et al., 2007; Dyar et al., 2014). For instance, a microarray study evaluated the transcriptomic profile of hindlimb leg muscle in adult mice and observed rhythmic expression in Ca^{+2}-calmodulin-dependent protein kinases (*camk*, *cask*), the Ca^{+2} transporter of the sarcoplasmic reticulum *(serca*), and the Ca^{+2} buffer protein, *parvalbumin*, which did not occur in the muscles of CLOCK-knockout mice (McCarthy et al., 2007). Similar results have recently been described for human primary myoblasts with reduced expression of CLOCK (Perrin et al., 2018). Interestingly, the hindlimb muscles of a muscle-specific BMAL1 knockout mouse continue to exhibit a significant proportion of cycling genes, and, when they are then denervated, 15% of the genes then lose their rhythmic expression (Dyar et al., 2015). These findings suggest that extracellular signals --probably originating from the SCN--play a major role in imposing a daily oscillation to the skeletal muscle transcriptome. Importantly, the changes in gene expression that are dependent on innervation are mediated by

Ca^{+2} signaling. Indeed, RNAseq data reveal that, in the soleus, a slow muscle fiber, one of the circadian pathways that is enriched in response to nerve activity is the Ca^{+2}-calcineurin-NFAT pathway (Dyar et al., 2015). Importantly, NFAT is a transcription factor with a key role in muscle adaptation in response to physical activity (Akimoto et al., 2005). NFAT translocates to the nucleus in a circadian manner, leading to the rhythmic expression of genes such as *rcan1*, a typical reporter of the Ca^{+2}-calcineurin-NFAT axis, thus revealing that this signaling pathway plays a critical role in integrating external stimuli with cycling gene expression.

The heart is another peripheral clock whose functioning is highly dependent on a rhythmic Ca^{+2} pathway. Indeed, Ca^{+2} homeostasis is fundamental to the functioning of the heart and alterations in Ca^{+2} signaling are associated with a variety of cardiac pathologies (Bers, 2014). In addition, the circadian clock is critical in order for myocytes to maintain their contractile and metabolic function. As a result, cardiac muscle cells exhibit rhythmic expression in many genes including those that code for proteins of the Ca^{+2} signaling pathway (Bray et al., 2008; Sachan et al., 2011). In particular, muscles of the left ventricle exhibit a daily rhythm in the translocation of NFAT into the nucleus and, as a result, also in RCAN1 expression (Sachan et al., 2011). Importantly, this effect is abolished by calcineurin inhibitors, indicating that the Ca^{+2}/calcineurin/NFAT axis has rhythmic activity. Additionally, the functioning of SERCA is also under circadian control in myocytes. In particular, phosphorylation of phospholamban (a SERCA inhibitory signaling protein) is restricted to the early part of the night, which

prevents it from binding to SERCA, thereby allowing the entry of Ca^{+2} into the endoplasmic reticulum in a circadian manner.

Collectively, current evidence reveals that Ca^{+2} modulates the expression of clock genes and is in turn modulated directly or indirectly by the molecular clock machinery (Figure 2B). This relationship results in a rhythm of cytosolic Ca^{+2} levels and of its downstream effectors. As a result, Ca^{+2} signaling and core molecular clock components make up a feedback loop that is important for the proper functioning of biological clocks as well as for that of the underlying circadian regulation of physiology (see Graphical abstract).

IV. Ca^{+2} signaling and clocks: implications for diseases and chronomedicine

IV.1. Circadian Ca^{+2} signaling and diseases

Circadian clocks impose a daily rhythm to many physiological processes and behaviors including sleep, blood hormone levels, locomotor activity, body core temperature, and metabolism (Levi and Schibler, 2007). In several neurological disorders, patients exhibit alterations in their circadian rhythms, which are associated with changes in the expression of core clock components. Such is the case for schizophrenia, depression, Parkinson's disease (PD), and Alzheimer disease (AD), where patients display altered sleep, and melatonin and body core temperature rhythms, due to alterations in clock gene expression and the loss of synchronization among pacemaker cells in the SCN (Videnovic

et al., 2014; Musiek and Holtzman, 2016). Similarly, a dysfunction in the circadian clock is linked with the pathogenesis of various types of human cancers (Sancar and Van Gelder, 2021). What molecular mechanisms could link the disruption of circadian rhythms to the development of these diseases? As we discussed above, PKC and CaMK are integral components of the molecular clock and, remarkably, abnormalities in the activity of these proteins have been described in the early stages of disorders that are associated with disturbances in clock function. For instance, higher levels or abnormal activity of PKC and CaMK isozymes leads to neuronal cell death and a disruption in neuronal transmission in PD, AD, and in neuropsychiatric disorders (Mochly-Rosen et al., 2012; Robison, 2014). Similarly, PKC overexpression promotes angiogenesis and excessive cell proliferation in stomach, colon, and breast cancers (Mochly-Rosen et al., 2012). Given that a variety of psychotropic and cancer chemopreventive agents act by modulating CaMK and PKC activity, respectively, (Celano et al., 2003; Mochly-Rosen et al., 2012), recovering the normal functioning of the circadian clocks could have important implications for the effectiveness of these treatments. Thus, future studies may focus on elucidating whether these Ca^{+2}-associated proteins mediate the link between the disruption of circadian clocks and the pathogenesis of neurological disorders and cancer.

Ca^{+2} channels also play a role in neurological disorders that cause alterations in circadian rhythms. One of them is bipolar disorder (BD), which is characterized by mood instability and abnormalities in sleep and daily activity schedules

(bedtime, waketime, mealtime)(Alloy et al., 2017). In BD, lithium is the most effective mood stabilizer and corrects daily rhythms, but not all patients are responsive to this treatment (Alda, 2015). In particular, BD patients with longer circadian periodicities do not respond to lithium and exhibit a greater polypharmacy (Sanghani et al., 2020). Additionally, mice with a dysfunctional clock do not display typical lithium-induced behavioral changes. These findings suggest that in BD patients the mood stabilizing effects of lithium could be mediated by the circadian clock. On other hand, several variants of genes coding for proteins of the Ca^{+2} pathways including *cacna1c* (L-type VGCC) are risk alleles for BD (Gershon et al., 2014; Ament et al., 2015). Interestingly, fibroblasts from healthy humans, but not from BD patients, exhibit a rhythm in *cacna1c* expression, and the inactivation of this Ca^{+2} channel prevents lithium from increasing the amplitude of circadian rhythms (McCarthy et al., 2016). But VGCCs are not the only components of Ca^{+2} pathways that are relevant to the lithium-induced circadian response in BD patients. In particular, lithium lengthens the circadian period in cultured cells, which does not occur in the presence of an IP_3R antagonist (McCarthy et al., 2019). In agreement with the potential role of IP_3R, an allelic variant of this Ca^{+2} transporter potentiates the lithium-induced period lengthening in BD patient-derived fibroblasts, suggesting that a genetic factor in Ca^{+2} signaling could influence the circadian defects seen in BD patients (McCarthy et al., 2019). Given that the effects of lithium on the clock appear to correlate with its effectiveness in mood stabilization of BD patients (Sanghani et al., 2020), considering the allelic variants of Ca^{+2} signaling

could be important for implementing personalized medicine that improves the effectiveness of BD treatments.

IV.2. Implications of Ca^{+2} signaling in chronomedicine

In recent years, a growing number of studies have documented time of day effects in the effectiveness of medical interventions. Indeed, such variations have been reported for the success of treatments such as chemotherapy (Sancar and Van Gelder, 2021) as well as in the efficacy of drugs used to treat several disease including hypertension, cancer, hypercholesterolemia, rheumatoid arthritis, allergies, sleep disturbances, and asthma (Cederroth et al., 2019). One explanation for this is that many of drugs have short half-lives and their targets exhibit circadian expression. For instance, in non-human primates, about 80% of protein-coding genes display a daily rhythm of expression and a large proportion of these genes are identified as drug targets by the U.S. Food and Drug Administration (Mure et al., 2018). Similarly, a genome-wide transcriptome study in humans identified thousands of genes with tissue-specific circadian expression in 13 different organs (Ruben et al., 2018). A notable example is the various L-type Ca^{+2} channel subunits, which display a rhythmic expression in the heart (Table 3 and Figure 4) suggesting that their sensitivity to drugs would vary during the course of the day. And indeed, nifedipine and verapamil, two Ca^{+2} channels blockers used as antihypertensive drugs, show an improved efficacy when administered before bedtime (White et al., 1995; Hermida et al., 2008). In addition, a variety of Ca^{+2} channels blockers have half-

lives <6 h (Elliott and Ram, 2011) suggesting that taking these drugs at peak times of Ca^{+2} channel expression could improve the efficacy of the pharmacological therapy (this could also reduce side effects caused by actions on other tissues where the relevant targets cycle with a different phase). Consistent with this hypothesis, harmonizing the timing of administration of a drug with the time of peak expression of its target improves the effectiveness of treatments for cardiovascular disease, hypercholesterolemia, and obesity (Awad et al., 2017). On other hand, a circadian database generated by (Ruben et al., 2018) shows that many genes encoding Ca^{+2} signaling proteins show daily rhythms of expression in a variety of peripheral clocks (Figure 3), which is consistent with previous transcriptomic analyses of different tissues and brain regions of mice (Zhang et al., 2014) and baboons (Mure et al., 2018). Taking into account the daily changes in the abundance of these drug targets may have significant implications for medicine because proteins from the Ca^{+2} pathway are therapeutic targets in multiple disorders. For instance, a SERCA inhibitor prodrug (mispsagargin) is being assessed in clinical trials for the treatment of various types of cancers including prostate and liver (Mahalingam et al., 2016; Mahalingam et al., 2019). Similarly, recent pre-clinical studies propose that the RyR antagonist, dantrolene, would have a neuroprotective role in Huntington and Alzheimer's models (Chen et al., 2011; Liang and Wei, 2015).

Considering the daily changes in Ca^{+2} signaling is relevant not only for deciding on the best time for drug administration but also for scheduling medical interventions. For example, in humans, a common outcome of cardiac surgery is

myocardial injury due to ischemia-reperfusion (I/R) (Hausenloy and Yellon, 2016). A recent study revealed that the injury by I/R following aortic valve replacement is greatest when the surgery is done in the morning compared to the evening, and in a mouse model of hypoxia/reoxygenation this difference is abolished by Rev-ERBα antagonists, suggesting a critical role of this circadian clock component in the myocardial tolerance to I/R (Montaigne et al., 2018). Interestingly, this circadian regulation would not only be controlled by Rev-ERBα but also by the calcineurin/rcan1 axis. As we discussed above, in mice the heart calcineurin/NFAT/Rcan1 pathway displays rhythmic activity (Sachan et al., 2011) and, remarkably, this process could influence the daily variations in the myocardial tolerance to I/R. Unlike that reported by (Montaigne et al., 2018), injury by I/R following coronary artery surgery in mice is greater in animals operated at the end of their active phase (PM) compared to those operated at the beginning of this phase (AM) (Rotter et al., 2014). This time of day difference in tolerance to I/R is not present in Rcan1 knockout mice. Since these mutant mice display a rhythmic expression in canonical clock genes in the heart, these results suggest that Rcan1 mediates this effect without altering the global functioning of this peripheral clock. In addition, inhibiting calcineurin activity decreases the severity of the I/R injury in animals operated at PM whereas it does not confer additional protection in AM-operated mice, supporting the idea that the calcineurin/Rcan1 pathway plays a key role in determining the time of day-dependent susceptibility of the heart to I/R. Even though additional studies

are needed to elucidate whether a similar regulation occurs in humans, this finding positions the calcineurin/rcan1 axis as a therapeutic target for cardioprotection and for blunting the effect of the time of day on the outcome of cardiac surgery.

Collectively these findings provide strong evidence that the circadian control of Ca^{+2} signaling in biological clocks may have significant implications for medicine. Taking into account the 24-h dynamics of Ca^{+2} signaling in pharmacological therapies and medical interventions could improve drug efficacy and postoperative clinical outcomes, supporting the emerging idea that circadian biology should be an integral part of translational research (Cederroth et al., 2019).

Closing remarks

We currently have a very detailed understanding of how circadian clocks function. By comparison, much less is known about how they are synchronized and coordinated; how they transmit time information to the host: and, in turn, how they can be affected by the host's physiology and behavior. All of these system-level functions ultimately converge on intracellular signals that affect gene expression, which in turn, alter the activity of clocks, and the physiology of cells, organs, and organisms. Future work aimed at increasing our knowledge on how intracellular signaling regulates, and is regulated, by clocks will be important for furthering our understanding of how this intricate network of interactions is effected and how a unified time for the organism is maintained yet

can be modulated by the host. It will also be critical for the development of more effective chronomedicine prescriptions aimed at improving the effectiveness of drugs while reducing their negative and off-target effects.

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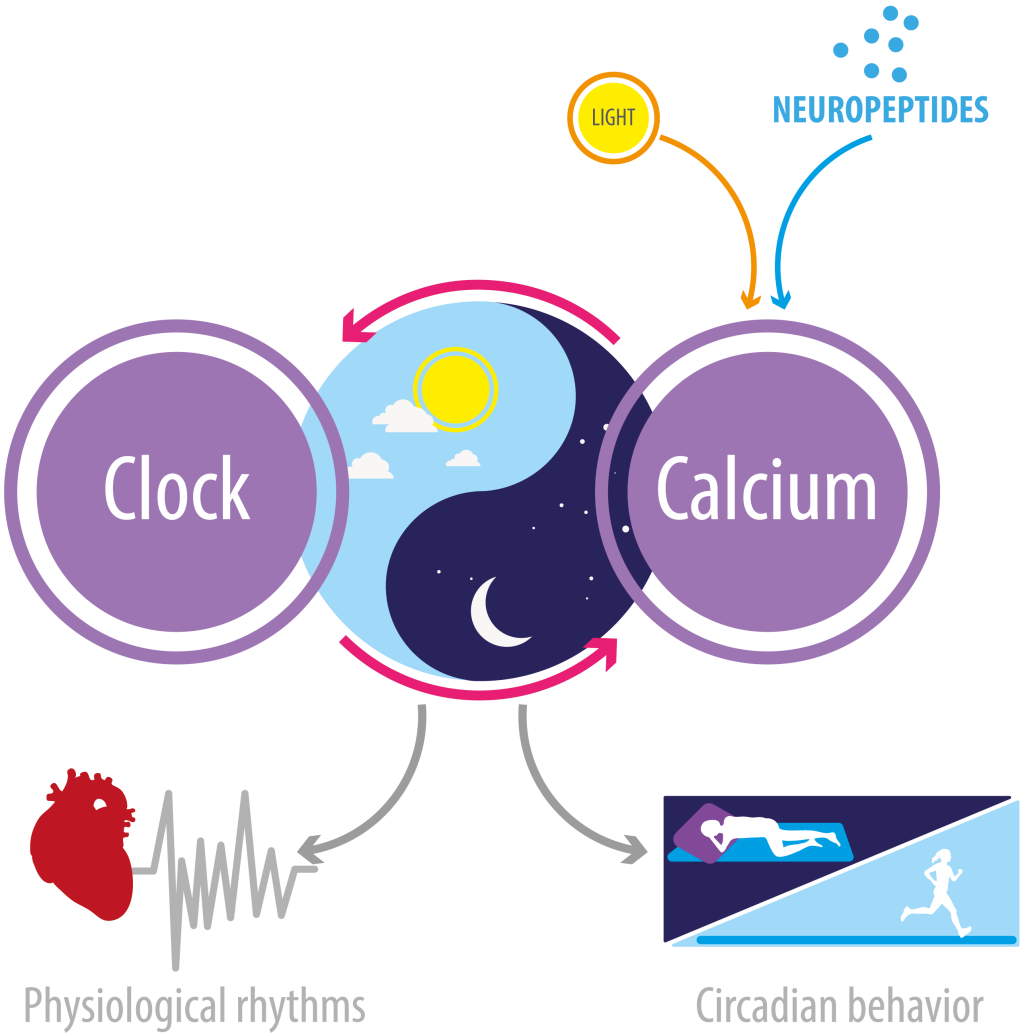
Figures



Graphical abstract. Reciprocal relationship between Ca+2 signaling and the circadian clock. Ca^{+2} rhythms contribute to transmitting daily external signals to the clock TTFL and Ca^{+2} signaling regulates the expression of core clock components. Conversely, circadian clocks control the daily expression of a wide variety of components of the Ca^{+2} signaling pathway. This bidirectional regulation is critical to the daily rhythm of many physiological and behavioral processes.

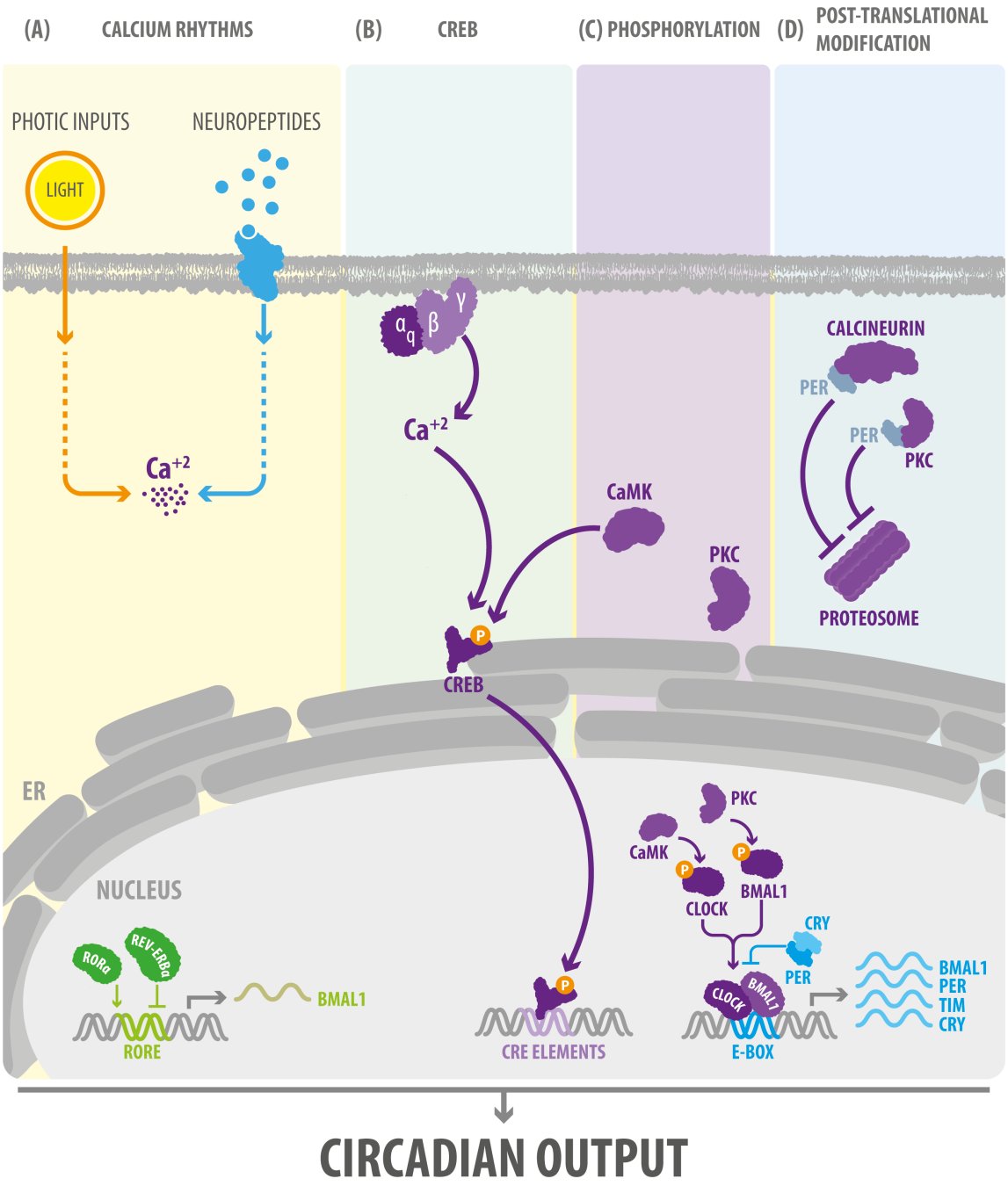


Figure 1. Ca^{+2} signaling modulates the core components of the circadian clock. (A) Intracellular Ca^{+2} rhythms are regulated by external signals such as light stimuli and neuropeptides. In turn, Ca^{+2} signaling via **(B)** CREB and the regulation of both **(C)** the phosphorylation state and **(D)** proteasomal degradation of clock proteins, orchestrate the effect of Ca^{+2} on the circadian clock.

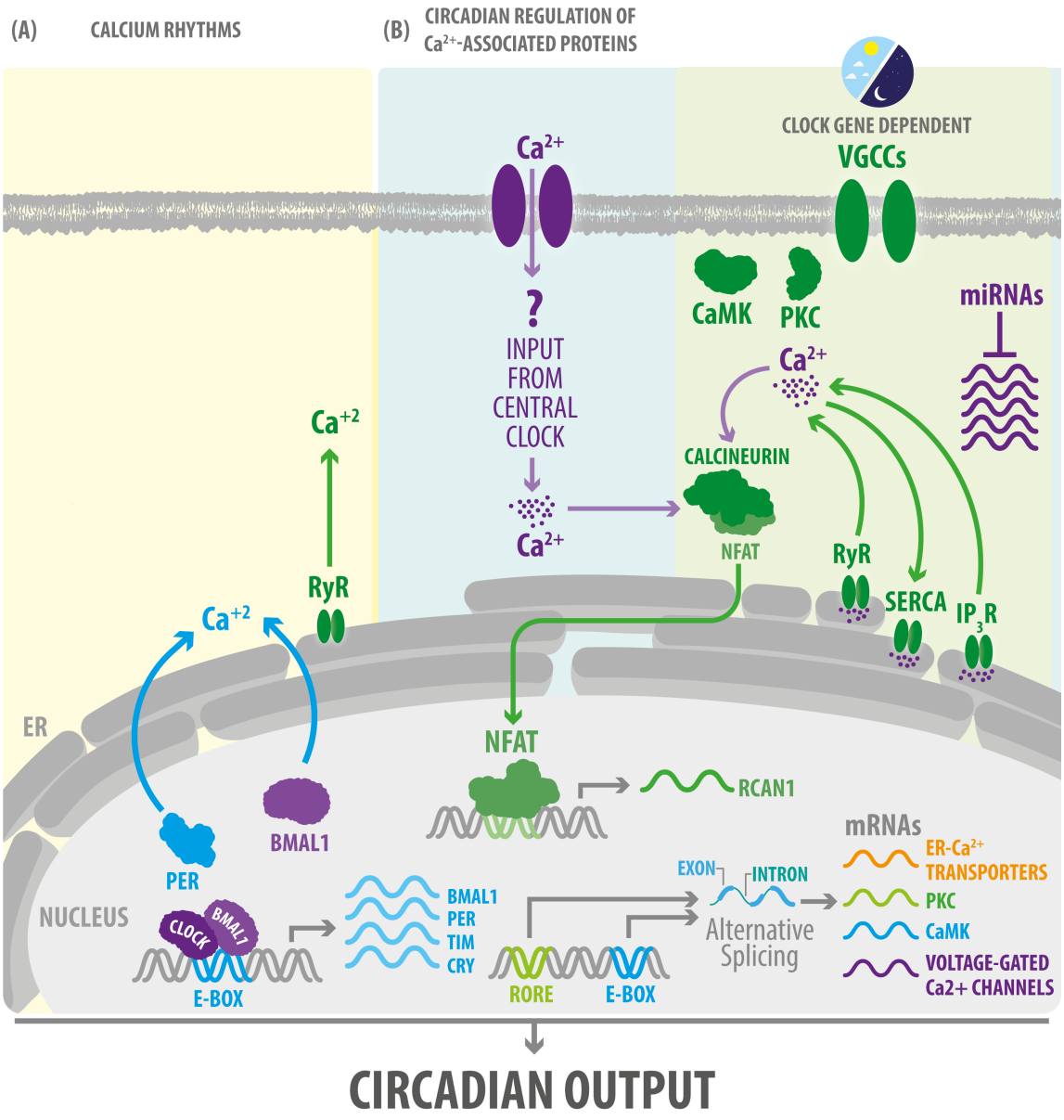


Figure 2. Circadian clocks impose a daily rhythm on intracellular Ca^{+2} signaling. (A) Ca^{+2} rhythms are regulated by molecular clock components and by the mobilization of Ca^{+2} from endoplasmic reticulum. **(B)** Circadian clocks impose a rhythm of expression to a large number of components of Ca^{+2} signaling by acting at transcriptional or post-transcriptional levels. In addition, the Ca^{+2}/calcineurin/NFAT pathway exhibits a rhythmic activity in peripheral clocks such as skeletal muscle or heart, which is probably mediated by inputs from central clock.

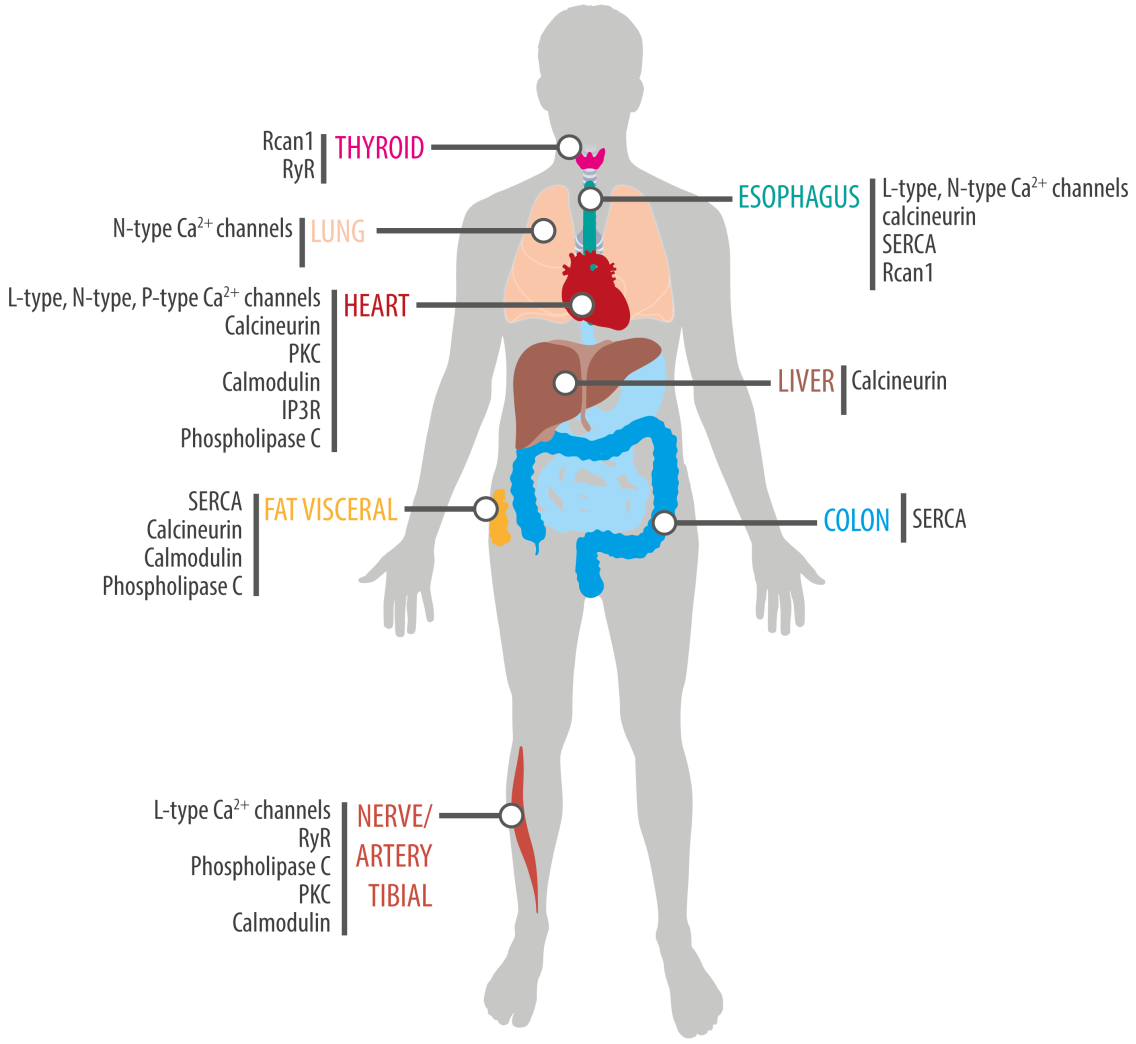


Figure 3. Rhythmic expression of components of the Ca^{+2} signaling pathway in human tissues. Schematic showing tissue-specific circadian expression of Ca^{+2}-associated proteins and of Ca^{+2} channels and transporters in peripheral human tissues Based on data from (Ruben et al., 2018)(http://circadb.hogeneschlab.org/human).

Tables

Table 1. Genes coding for proteins associated with Ca^{+2} signaling and their circadian regulation in mice.

Gene name	Description of encoded protein	E-box[a]	REREs[b]	Oscillates in SCN[c]
ip3r	IP3-sensitive $Ca2^{+}$ channel localized to the ER	√ (1169)	√(1020)	√
pkcα	Protein kinase that can be activated by Ca^{+2}	√ (15780)	√ (680)	√
camkiiα	Protein kinase that is regulated by the Ca^{2+}/calmodulin complex	√ (3420)	√(1200)	√
rack1	Intracellular protein receptor for PKC	√(720)	√(700)	√
plcβ-1	Enzyme mediator of signal transduction through Ca^{+2} pathways	X	√(1260)	X
serca	Ca^{+2} transporter ATPase (cytosol to ER)	√(10.173)	√ (1680)	X
calcineurin	Ca^{+2}-dependent protein phosphatase involved in signal transduction	√(4340)	√(1740)	√
cask	Protein kinase that regulates signal transduction in multiple pathways including Ca^{+2}	√(7340)	√(2280)	√
cacna1c	Subunit of L-type voltage-dependent Ca^{+2} channel	√(6400)	√(420)	X
cacna1g	Subunit of T-type voltage-dependent Ca^{+2} channel	√(3610)	√(3680)	√
ryr3	Ca^{+2} channel in the ER that releases Ca^{+2} in response to depolarization	√(13140)	√(2200)	√

[a] When present, number of base pairs upstream of the transcription start site where the E-BOX sequence (CACGTG)(Hao et al., 1997) is found.

[b] When present, number of base pairs upstream of the transcription start site where the ROR response sequence (AGGTCA) (Preitner et al., 2002) is found.
[c] Presence of circadian oscillation in the transcript levels of genes in the SCN, according to database generated using HTseq and DESeq2 (http://wgpembroke.com/shiny/SCNseq/) (Pembroke et al., 2015).

Table 2. Coding genes for proteins associated with Ca^{+2} signaling and its circadian regulation in *Drosophila*.

Gene name	Description of the encoded protein	E-box[a]	RevRE[b]	Oscillates in central clock[c]
ip3r	IP3-sensitive Ca^{2+} channel localized to the ER	√ (120)	√ (4080)	Not described
pkc53E	Protein kinase that can be activated by Ca^{+2}	√ (4400)	√ (10920)	√(LNd)
norpA	Protein kinase that can be activated by Ca^{+2}; associated with phototransduction	√ (7900)	√ (3840)	√(LNd)
camkii	Protein kinase that is regulated by the $Ca^{2+/}$calmodulin complex	X	X	√(LNd)
rack1	Intracellular protein receptor for PKC	X	X	Not described
plc21c	Enzyme mediator of signal transduction through Ca^{+2} pathways	√ (1020)	√ (900)	√(LNv)
serca	Ca^{+2} transporter ATPase from cytosol to ER	√ (480)	X	Not described
calcineurin B	Ca^{+2}-dependent protein phosphatase involved in signal transduction	X	X	√(LNd/DN1)
cask	Protein kinase that regulates signal transduction in multiple pathways including Ca^{+2}	√ (7800)	√ (16680)	√(LNd/DN1)
$Ca^{+2}\alpha$-1D	α subunit of an L-type voltage-gated Ca^{+2} channel expressed in neurons	X	√ (2700)	Not described
cacophony	Subunit of a voltage-gated Ca^{+2} channel located at presynaptic active zones	X	√ (1320)	√(LNd)
ryr	Ca^{+2} release channel in the ER in response to depolarization of the cell	√ (2400)	X	Not described

[a] When present, number of base pairs upstream of the transcription start site where the E-BOX sequence (CACGTG)(Hao et al., 1997).
[b] When present, number of base pairs upstream of the transcription start site where the RevRE sequence (AGGTCA) (Preitner et al., 2002).
[c] Presence of circadian oscillation in the transcript levels of genes in the central clock (specific neuronal group is indicated in parenthesis) according to database generated using RNAseq (Abruzzi et al., 2017).

Table 3. Rhythmic expression of elements of the Ca^{+2} pathway that are therapeutic targets, and its implications for medicine.

Therapeutic target	Tissue-specific circadian expression[a]	Drug	Clinical use
L-type Ca^{+2} channel subunits	Heart, artery tibial, nerve tibial, subcutaneous fat	Verapamil, nifedipine,	Antihypertensive, cardiac arrhythmia, angina (Striessnig et al., 2015)
N-type Ca^{+2} channel subunits	Heart, esophagus, lung	Ziconotide, nicardipine	Analgesic (McGivern, 2007), antihypertensive (Elliott and Ram, 2011)
P-type Ca^{+2} channel subunits	Heart, pituitary	Verapamil, flunarizine, levetiracetam	Antihypertensive (Elliott and Ram, 2011), epilepsy (Hasan et al., 2013)
calcineurin	Heart, fat visceral, esophagus, liver	Cyclosporin	Immunosuppression, rheumatoid arthritis (Tedesco and Haragsim, 2012)
Serca	Esophagus, colon, fat visceral	mipsagargin	Cancer (clinical trials) (Mahalingam et al., 2016)
Ryr	Thyroid, nerve tibial	dantrolene	Neuroleptic malignant syndrome (Krause et al., 2004)
PKC	Heart, artery tibial, skin	Tamoxifen	Cancer (Mochly-Rosen et al., 2012)
Rcan1	Thyroid, esophagus	Not described	Not described
Calmodulin	Heart, artery tibial, fat visceral	Loperamide, fluvoxamine	Schizophrenia (Celano et al., 2003), diarrhea (Hanauer, 2008)
IP3R	Heart	Not described	Not described
Phospholipase C	Heart, nerve tibial, fat visceral	Not described	Not described

[a]Circadian expression according to database generated by RNAseq and cyclic ordering by periodic structure (CYCLOPS) (Ruben et al., 2018)(http://circadb.hogeneschlab.org/human).

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Chapter II: Timed receptor tyrosine kinase signaling couples the central and a peripheral circadian clock in *Drosophila*

This chapter is a manuscript that will be submitted for publication shortly. I did most of the work reported in the manuscript and wrote most of the first draft. Emad Amini in Christian Wegener's lab did the work reported in Figs. 1, 5, and S6. Work reported in Figure 5, C-F was done by Emad in Ralf Stanewsky's lab. Dick Nässel provided an unpublished anti-PTTH antiserum.

Timed receptor tyrosine kinase signaling couples the central and a peripheral circadian clock in *Drosophila*

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Summary

Circadian clocks impose daily periodicities to behavior, physiology, and metabolism. This control is mediated by a central clock and by peripheral clocks, which are synchronized to provide the organism with a unified time through mechanisms that are not fully understood. Here, we characterized in *Drosophila* the cellular and molecular mechanisms involved in coupling the central clock and the peripheral clock located in the prothoracic gland (PG), which together control the circadian rhythm of emergence of adult flies. The time signal from central clock neurons is transmitted via small neuropeptide F (sNPF) to neurons that produce the neuropeptide, PTTH, which is then translated into circadian oscillations of Ca^{+2} concentration and daily changes in PTTH levels. Rhythmic PTTH signaling is required at the end of metamorphosis, and transmits time information to the PG by imposing a daily rhythm to the expression of the PTTH receptor tyrosine kinase (RTK), TORSO, and of ERK phosphorylation, a key component of PTTH transduction. In addition to PTTH, we demonstrate that signaling mediated by other RTKs contribute to the rhythmicity of emergence. Interestingly, the ligand to one of these receptors (Pvf2), plays an autocrine role in the PG, which may explain why both central brain and PG clocks are required for the circadian gating of emergence. Our findings show that the coupling between the central and the PG clock is unexpectedly complex and involves several RTKs that act in concert, and could serve as a paradigm to understand how circadian clocks are coordinated.

Introduction

Circadian rhythms allow multicellular organisms to anticipate daily changes in the environment such as the arrival of dawn or dusk. In animals, these behavioral and physiological rhythms are generated by multi-oscillator systems composed of a central pacemaker housed in the brain as well as of peripheral pacemakers located in a wide variety of tissues. The coordination of these clocks is critical for the organism to express a unified circadian time (Mohawk et al., 2012). In mammals, the so-called “master pacemaker” located in the suprachiasmatic nucleus (SCN) synchronizes to the environmental day-night cycles and coordinates peripheral oscillators through neural, endocrine, behavioral, and thermal signals (Welsh et al., 2010). In turn, a variety of peripheral signals feedback to adjust and stabilize SCN rhythmicity (Buijs et al., 2016; Harder and Oster, 2020). However, our understanding of the cellular and molecular mechanisms and rules that mediate this coupling is still fragmentary (Pilorz et al., 2018).

In the fruit fly, *Drosophila melanogaster,* the central clock is comprised of about 150 neurons that are critical for imposing a daily periodicity to behaviors such as locomotor activity and sleep (Nitabach and Taghert, 2008). A key component of the central clock are the small and large ventral lateral neurons (s- and lLNvs, respectively), which produce the neuropeptide pigment-dispersing factor (PDF) and are critical for circadian timekeeping (Shafer and Yao, 2014). In addition, peripheral clocks reside in a variety of tissues and are mostly autonomous and

entrained directly by external inputs (Ivanchenko et al., 2001; Glaser and Stanewsky, 2005; Ito and Tomioka, 2016). However, in other cases, and similarly to mammals, the central clock can coordinate its activity with peripheral oscillators. In particular, the rhythm of emergence of adult flies (used here interchangeably with the term, eclosion), is mediated by the coupling of the brain clock and a peripheral oscillator that resides in the prothoracic gland (PG), the endocrine gland that produces the steroid molting hormone, ecdysone (Myers et al., 2003; Morioka et al., 2012; Selcho et al., 2017). We previously reported that this coupling is mediated by a peptidergic signaling pathway in which small neuropeptide F (sNPF) from the sLNv clock neurons inhibits the neurons that express prothoracicotropic hormone (PTTH) (Selcho et al., 2017). In turn, the PTTH neurons (PTTHn) secrete PTTH, which binds to the receptor tyrosine kinase (RTK) encoded by *torso* in cells of the PG, to control the biosynthesis of the molting hormone, ecdysone (McBrayer et al., 2007; Selcho et al., 2017). Although the titers of this steroid must fall below a threshold level to trigger emergence (Truman, 1984; Porter and Collins, 2009), we recently showed that the clock does not exerts its action by regulating the levels of ecdysone but by controlling its actions, which are mediated by the ecdysone receptor in the PG (Mark et al., 2021). Indeed, although injections of 20E delay the time of eclosion they do not affect its circadian gating. By contrast, disabling 20E actions in the PG renders arrhythmic the pattern of adult emergence.

Although the neuropeptide circuit that connects the brain clock to the PG clock has been identified, how and when during development the time signal is

transmitted from the central circadian pacemaker to this peripheral clock remains unclear. For example, although sNPF from sLNvs suppresses Ca^{+2} activity in the PTTHn (Selcho et al., 2017), it is not known whether this translates into a rhythmic activity and output from PTTHn. Similarly, although PTTH is required for the circadian rhythmicity of emergence, *ptth* mRNA levels do not exhibit a circadian fluctuation (Selcho et al., 2017), indicating that timing information from the central clock may be encoded by a different mechanism. In addition, PTTHn are targets of peptidergic inputs other than sNPF at least in larvae (Deveci et al., 2019; Imura et al., 2020; Hao et al., 2021), yet it is not known whether any of these peptides also affect the circadian rhythm of emergence. Finally, signaling molecules other than PTTH, including Jelly belly, PDGF- and VEGF-related factor, Egf, and insulins, also regulate ecdysone production by the PG (Colombani et al., 2005; Cruz et al., 2020; Pan and O'Connor, 2021) but their role in the circadian control of the eclosion is currently unknown.

In order to understand how and when the time signal is transmitted from the central clock to the PG clock, we examined the anatomical, cellular, and molecular basis of the central clock-PTTHn-PG axis. We first determined the connectivity between sLNv clock neurons and the PTTHn by pre- and postsynaptic tracing, and found that this connection is unidirectional and likely to be exclusively peptidergic. We also show that PTTHn exhibit a daily oscillation in intracellular Ca^{+2} levels ($[Ca^{+2}]_i$) and of PTTH immunoreactivity at the terminals of PTTHn in the PG. Unexpectedly, we found that PTTH is required for the

circadian rhythmicity of *torso* expression and of PTTH transduction in the PG. Direct imaging of the activity of PTTHn and the PG revealed that the final peak of activity in PTTHn occurred around 16h before eclosion and was followed 6h later by a peak of activity in the PG . This timing is consistent with our recent findings on the events that control the timing of eclosion (Mark et al., 2021), and we further show here that PTTHn signaling is required at the end of metamorphosis for the expression of a circadian rhythm of adult emergence. In addition, the presence of activity peaks in the PG that occurred prior to the activation of PTTHn suggests that the PG responds to additional factors, and we show here that PDGF- and VEGF-receptor related (Pvr) and Anaplastic lymphoma kinase (Alk), two RTKs that are also expressed in the PG, contribute to the circadian control of emergence. Thus, our detailed characterization of the transduction pathway from the sLNv clock neurons to the PG clock reveals that the transmission of time information between clocks is surprisingly complex and involves multiple actors. It also defines a new role for RTK signaling in the coordination of circadian clocks. Our work may provide a general mechanism for understanding how circadian clocks are coordinated.

Results

All PDF-positive sLNv signal non-synaptically to the PTTHn

The dorsal projections of PDF-expressing LNv clock neurons terminate in close proximity to PTTHn arborizations (Fig. 1A-A''). We previously showed that sLNvs transmit time information to PTTHn via peptidergic sNPF signaling, but

not PDF (Selcho et al., 2017). To determine which LNv neurons signal to PTTHn and whether this contact includes synaptic transmission, we performed a connectomic analysis by GFP reconstitution across synapses (syb-GRASP) (Macpherson et al., 2015), BAcTrace (Cachero et al., 2020), and *trans-Tango* MkII (Sorkac et al., 2022), in adult pharate animals. This work was necessary because the PTTHn can no longer be detected starting shortly after eclosion (Liu et al., 2016) and are therefore not included in the available EM-based connectomic datasets that are based on older adult flies (Scheffer et al., 2020). Despite the close proximity of the sLNv terminals to the dendritic arborisations of PTTHn, the reconstituted GFP signal between these neurons using syb-GRASP was weak and restricted to the primary process (Figure 1B), suggesting that sLNv transmit time information to the PTTHn primarily via non-synaptic connections. We then used BAcTrace and *trans-Tango MkII*, which are retrograde and anterograde neuronal tracers, respectively, to determine the directionality of the connection, and to identify the LNvs that provide input to the PTTHn. As shown in Figures 1C and 1D, BAcTrace labeled all sLNv and none of the lLNv, whereas *trans-Tango MkII* labeled both pairs of PTTHn. Finally, we used receptor-specific intragenic driver lines to show that PTTHn express the receptor for sNPF (sNPFR) but not that for PDF (PDFR) (Fig. 1E and 1F), consistent with our previous finding that transmission is mediated via sNPF and not PDF (Selcho et al., 2017).

Ca^{+2} signaling in PTTHn is relevant to the circadian control of adult emergence

Since sNPF released from sLNv reduces [Ca^{+2}] in PTTHn, we explored whether Ca^{+2} levels [Ca^{+2}] in PTTHn neurons changed in a time-dependent manner. For this we used the genetically encoded Ca^{+2} sensor, GCaMP6M (Chen et al., 2013), to measure changes in [Ca^{+2}] in PTTHn during the course of the day (and subjective day) at the beginning of metamorphosis (white pre-pupal stage, WPP; because the circadian clock is intact and fully functional at this time (Morioka et al., 2012). By contrast, the PG of animals close to emergence is undergoing apoptosis (Dai and Gilbert, 1991), making them difficult to image). As shown in Figure 2A-C, the cell bodies of PTTHn showed a daily rhythm in [Ca^{+2}] under a 12h light: 12h dark regime (LD), with maxima at the beginning of the night (Zeitgeber time (ZT) 12) and minima 4h after lights-on (ZT4). This daily oscillation was maintained under constant darkness (DD), but the peak was delayed by about 6h (circadian time (CT) 18; where CT12 is the start of the subjective night) (Figure 2E) (this delay may occur because photic inputs can set the Ca^{+2} phase in a group of clock neurons (Liang et al., 2017)). Importantly, this daily rhythm was lost under both LD (Figure 2D) and DD conditions (Figure 2F) in animals bearing null alleles for the *period* gene (per^0), which is a core component of the clock. Expression of GCaMP in the PTTHn did not affect the rhythm or period of emergence (Table S1).

In order to determine if there is a temporal relationship between the rhythm of sNPF release from sLNv and the Ca^{+2} oscillations in PTTHn, we measured the

temporal pattern of neuropeptide abundance at the terminals of sLNv by expressing ANF-GFP, a transgenic neuropeptide reporter (Husain and Ewer, 2004), in PDF-expressing LNv. As shown in Figure S1A-S1B, and similar to previous findings for PDF in adult flies (Park et al., 2000), the ANF-GFP signal in the terminals of sLNv at the WPP stage was highest at the beginning of the day, which is coincident with the time when [Ca^{+2}] levels in PTTHn were minimal (Figure 2C). Thus, our results suggest that the phase of the Ca^{+2} rhythm in PTTH neurons is set by the inhibitory sNPF input from sLNv (although *pdf*>ANF-GFP reports the abundance of both sNPF and PDF, PTTHn do not express PDFr).

In order to evaluate the relevance of Ca^{+2} in the PTTHn to the circadian control of adult emergence, we knocked-down elements involved in Ca^{+2} signaling in PTTHn and determined the consequences on the rhythm of adult emergence (Figure 2G-2J). We found that the knockdown of voltage-gated Ca^{+2} channels (Ca^{+2}-α-1D, Ca^{+2}-α-1T, and *cacophony*) and of Ca^{+2}-binding proteins (CasK, and PKC) did not affect the rhythm or the periodicity of eclosion (Figure 2J). By contrast, knocking down the expression of RyR (ryanodine receptor), an endoplasmic reticulum Ca^{+2} channel, caused a significant weakening of the circadian pattern of emergence (Figure 2G and 2J). (In these and all RNAi-mediated knockdown experiments, at least 2 different RNAi transgenes were tested; see Materials and Methods.) A similar result was obtained following the knockdown of SERCA (sarco/endoplasmic reticulum calcium ATPase) (Figures

2H and 2J). Importantly, knockdown of RyR or SERCA did not affect the gross morphology of PTTHn (Figure S2A-S2C) suggesting that the weakening of the rhythm of the emergence may be caused by alterations in Ca^{+2} homeostasis in PTTHn. . Together, these results suggest that Ca^{+2} signaling in PTTHn through the RyR and SERCA transporters is part of the cellular mechanism that imposes a daily rhythm to the pattern of adult emergence

The PTTH/*torso* axis is under circadian control

Although PTTH transmits time information from the sLNv to the PG clock, this timing signal does not involve the circadian regulation of *ptth* transcript levels (Selcho et al., 2017). However, whether PTTH abundance cycles in the PTTHn axon terminations in the PG is unknown. To explore this possibility, brain-PG complexes from WPP were dissected at different times of day and immunostained for PTTH. As shown in Figure S3A, PTTH immunosignal showed a rhythm in the cell bodies; however we found that this rhythm was not dependent on the clock. (This cycling of PTTH staining in the cell bodies may be dependent on photic inputs, similar to the role of PTTH in light avoidance during larval stages (Yamanaka et al., 2013; Sorkac et al., 2022).) By contrast, PTTH immunoreactivity of the axonal terminals onto the PG showed clock-dependent daily rhythmicity. Indeed, PTTH immunoreactivity was maximal during the night and minimal during the day (Figure 3A-3C), and persisted under DD conditions and was abolished in per^0 null mutant animals under both LD and DD conditions (Figure 3D-3F). Together, these results suggest that the circadian clock acts at

the post-translational level to impose a daily rhythm of PTTH accumulation at the site of innervation of the PG and suggest that the PTTH neuropeptide may be rhythmically released onto this gland.

Another critical step in the transmission of time information from the brain clock to the PG clock is the PTTH receptor, TORSO (Selcho et al., 2017). To test whether *torso* expression is regulated by the clock, we first examined *torso* mRNA abundance in the PG at different times of day, in wildtype and in per^0 null mutant animals. As shown in Figure 3G-3H and S3B, *torso* transcript levels exhibited a daily rhythm under LD and DD conditions in wildtype but not in per^0 null animals. Intriguingly, this oscillation peaked during the subjective day and reached a minimum at the beginning of the subjective night, which is in antiphase relative to the oscillations observed for the PTTH-immunoreactivity of the terminals of PTTHn onto the PG. Interestingly, *torso* mRNA levels did not cycle in the PG of *ptth* null mutant animals under DD condition (Figure 3I). Taken together our findings suggest that the circadian rhythmicity of *torso* transcript abundance is dependent on the central clock input, and that this timing signal is transmitted by PTTH. As expected, given that knockdown of *torso* in the PG causes the expression of an arrhythmic eclosion pattern (Selcho et al., 2017), *ptth* null mutant animals showed an arrhythmic pattern of emergence (Figure S3C and S3D).

Circadian regulation of PTTH transduction in the PG

Our findings reveal that PTTH immunoreactivity of PTTHn terminals and *torso* transcript levels oscillate in antiphase. In order to obtain a readout of the temporal pattern of the resulting activation of the Torso pathway we asked whether the levels of phosphorylated ERK (phosphoERK), a key component of PTTH transduction (Rewitz et al., 2009), express a daily rhythmicity. For this we expressed in the PG a genetically-encoded ERK kinase reporter (ERK-SPARK), which is based on phase separation-based kinase activity (Zhang et al., 2018)(Figure S4A). As shown in Fig. 4, ERK activity was highest during the early part of the day under LD conditions (Fig. 4F); it was also highest during the early part of the subjective day (CT0-2) under DD conditions (Figure 4A, 4B and 4H). Importantly, this daily rhythm in phosphoERK levels was lost in per^{0} null mutant animals (Figure 4G) indicating that ERK activity in the PG cells is dependent on the circadian clock. This rhythm was also lost when *torso* was knocked down in the PG (Figure 4C, 4D and 4I) (Although rhythmicity itself is lost, a SPARK signal was still detected, as would be expected because this pathway can be activated by other ligands, e.g., insulins). Since early in the day, PTTH immunoreactivity in the PTTHn was lowest and *torso* transcript levels and phosphoERK levels in the PG were highest, our results suggest that PTTH release and Torso activation occur at this time of day. Expression of this reporter in the PG did not affect the circadian rhythmicity of emergence (Figure S4B-S4D).

ERK shuttling between cytoplasm and nucleus is relevant for ecdysone biosynthesis and for the control of developmental timing during the larval stages (Ou et al., 2011). As shown in Fig. S5C, nuclear SPARK signal was higher during the day than during the night. This daily oscillation persisted under DD conditions, although the maximum levels were phase-advanced (Figure S5A, S5B and S5BE), which is consistent with the rhythm of the total SPARK signal in the PG cells. In addition, *per*^{0} and PG>*torso*-RNAi animals showed an arrhythmic pattern of nuclear SPARK signal (Figure S5D and S5F) suggesting that daily change in the phosphoERK shuttling between cytoplasm and nucleus in the PG is dependent on a functional circadian clock and a rhythmic PTTH input.

Neuronal activity of PTTHn is required at the end of pupal development for the circadian gating of eclosion

To determine when during pupal development the rhythmic activity of PTTH is required for a circadian gating of emergence, we investigated the consequences on the timing of emergence of conditionally silencing the PTTHn. To do so, we expressed the inward rectifying K^+ channel, Kir2.1, at different times of development using the temperature dependent TARGET system (McGuire et al., 2004). As shown in Fig. 5A and 5B, silencing the PTTHn during the embryonic and larval stages or during early pupal development did not affect the circadian rhythmicity of emergence. By contrast, silencing these neurons during the entire or only the second half of metamorphosis caused the expression of an

arrhythmic pattern of emergence (These results indicate that the activity of PTTHn is required during the final stages of metamorphosis for the clock to impose circadian rhythmicity to the temporal pattern of adult emergence.

In order to directly assess the activity of PTTHn and of the PG during the second half of metamorphosis, and considering that we are not able to perform *in vivo* Ca^{2+} imaging of the PG at this time, we turned to an Activity-Regulated Gene-Luciferase reporter (ARG-Luc)(Chen et al., 2016) for long-term monitoring of neuronal activity in intact developing pupae (Figure 5C and 5D). Strikingly, we found that PTTHn under DD conditions expresses a monophasic circadian rhythm of activity that started 2 days prior to adult emergence, with a second peak of activity preceding eclosion by 6h (Figure 5E and 5F). This timing is consistent with the peak in intracellular Ca^{2+} signaling detected in the middle of the subjective night in DD at the WPP stage (Fig. 2E), as well as with the increased signal detected just before emergence using the calcium-dependent sensor, CaLexA (Masuyama et al., 2012) (Fig. S6). In parallel experiments, we also used ARG-Luc to monitor the activity of the PG during pupal development. As shown in Fig. 5F, the PG expressed a peak of activity at around -25h and a large peak at around the time of eclosion, each lagging PTTHn activity by around 6h. In addition, our records showed an earlier peak of activity in the PG at around -40h that was not preceded by a peak in PTTHn activity, suggesting that activity in the PG depends on other factors in addition to PTTH but that its phase close to the time of emergence is set by PTTHn activity. (In the PG, increases in ARG-LUC signal may be due to changes in Ca2+ levels (Morioka et

al., 2012) but we cannot rule out that they may be caused by a different mechanism.)

PTTH-independent RTK signaling pathways contribute to the rhythm of the adult emergence.

Although PTTH is the best known ecdysteroidogenic factor, the peak of activity detected in the PG 40h before eclosion preceded the first peak of activity in PTTHn, which may occur in response to other signals that act on the PG (Colombani et al., 2005; Cruz et al., 2020; Pan and O'Connor, 2021). In particular, a recent study reported that anaplastic lymphoma kinase (Alk) and PDGF and VEGF receptor-related (Pvr), two receptor tyrosine kinases (RTK) that are expressed in the PG, act in an additive manner to regulate the timing of metamorphosis (Pan and O'Connor, 2021). To determine whether these RTKs contribute to the rhythm of adult emergence, we determined the consequences on the timing of eclosion of knocking down or of expressing dominant-negative versions of these receptors, in the PG. As shown in Figures 6A-B and 6I, overexpression of a dominant-negative version of *Pvr* in the PG weakened the rhythm of the emergence relative to its control. A similar phenotype was produced by expressing two different *Alk* RNAi constructs (Figure 6C), as well as when both receptors were knocked down together (Figures 6D and 6I). Flies bearing only UAS-RNAi transgenes for Alk and Pvr expressed normal circadian rhythmicity of emergence (Figure S7A and S7B). It has been shown that Alk acts via both PI3K and Ras/Erk signaling pathways (Pan and O'Connor, 2021).

Although we previously showed that Ras/Erk is critical for the rhythm of emergence (Selcho et al., 2017), we found that knockdown of *pi3k* in the PG did not affect the rhythm or period of emergence (Table S1). These results suggest that Alk contributes to the circadian control of eclosion exclusively via the Ras/Erk signaling pathway. Nevertheless, the contribution to the ERK pathway from receptors other than TORSO is comparatively minor since SPARK rhythmicity was not detected in the PG of PTTH null mutant animals (Fig. 3I) nor when torso was knocked down in the PG (Fig. 4I).

Recent work has revealed that PTTHn express the Alk ligand, Jeb, and one of three known ligands for Pvr, Pvf3, (Pan and O'Connor, 2021). Consistent with the consequences of knocking down the Alk receptor in the PG, knockdown of its ligand, *Jeb*, in PTTHn using the strong *NP423-GAL4* driver (Yamanaka et al., 2013) weakened the rhythmicity of emergence (Figures 6E and 6J). By contrast, the corresponding knockdown of *Pvf3* was without effect (Figure 6F and 6J). Finally, given that Pvf2 (another ligand for Pvr) and Pvf3 are expressed in the PG (Pan and O'Connor, 2021), we explored the possibility that these ligands might regulate the circadian rhythmicity of emergence in an autocrine manner. Interestingly, knockdown of *Pvf2* (but not of *Pvf3*) in the PG weakened the rhythmicity of adult eclosion (Figures 6G, 6H, and 6K) indicating that *Pvf2* contributes to the circadian rhythm of emergence *via* an autocrine pathway. Together, these results reveal that PTTH/Torso axis is a major, but not exclusive, regulator of the rhythmicity of emergence. Indeed, our results suggest

that Jeb/Alk and autocrine signaling involving Pvf2/Pvr also contribute to the rhythmicity of eclosion.

We also explored whether other ecdysteroidogenic signals including Allatostatin A (Deveci et al., 2019), Corazonin (Imura et al., 2020), insulin signaling (Colombani et al., 2005), as well as Gq proteins encoded by CG30054 and CG17760(Yamanaka et al., 2015), might contribute to the circadian control of adult emergence. As shown Table S1, RNAi knockdown or null mutations of components of these signaling pathways in the PG did not affect the rhythmicity or the periodicity of adult eclosion.

Since peptidergic neurons often co-express small molecule neurotransmitters (Nassel, 2018), we also investigated whether any of such signaling molecules are involved in the circadian control of emergence. As shown in Suppl. Fig. S8, we found that PTTHn do not express the signature markers of signaling by glutamate (VGLUT), GABA (VGAT) or acetylcholine (ChAT), suggesting that PTTHn may be exclusively peptidergic. Finally, we found that stopping the clock located in the fat body(Xu et al., 2008) by overexpressing the dominant negative form of *cycle* gene (cyc[Δ901])(Tanoue et al., 2004) did not affect the timing of adult fly emergence (Table S1), indicating that the fat body, which also houses a circadian clock and could influence the PG via endocrine signaling, does not contribute to the circadian regulation of adult emergence.

Discussion

Circadian clocks impose daily periodicities to behavior, physiology, and metabolism (Roenneberg and Merrow, 2005; Herzog, 2007; Pilorz et al., 2018). This control is mediated by the activity of a central clock and of peripheral clocks, which are housed in a variety of relevant organs. Although these various clocks are known to be synchronized to provide the organism with a unified time, how this coordination occurs is not fully understood. Here, we characterized the cellular and molecular mechanisms involved in coupling two circadian clocks in *Drosophila*, the central clock of the brain and the peripheral clock located in the PG, which together restrict the time of emergence of the adult fly to the early part of the day. We showed that time information is propagated from the central sLNv pacemaker neurons to PTTHn through a peptidergic non-synaptic connection mediated by sNPF, and is then translated into a circadian regulation of Ca^{+2} levels and PTTH accumulation in PTTHn. Although these features were measured in animals initiating metamorphosis (white puparium stage, WPP), we found that Ca+2 levels in PTTHn also varied in the pharate adult and peaked just before adult emergence. (The fact that PTTHn showed a similar timecourse in Ca+2 levels at both developmental stages also suggests that the WPP is a relevant stage for investigating the clock control of behaviors that occur many days later, at adult emergence). Importantly, we found that neuronal activity of PTTHn at the end of pupal development is necessary for the circadian gating of eclosion. The PTTHn then transmit time information to the PG, where we show that PTTH imposes a daily rhythm to the transcript levels of *torso* and ERK

phosphorylation, a key component of PTTH transduction. Although PTTH plays a critical role in the coupling between the central and the PG clock, we demonstrate here that Jeb/Alk and Pvf2/Pvr signaling also contribute to regulate the rhythm of emergence, revealing that this circadian behavior is the result of timed signals from several RTKs that converge onto the PG.

The transduction of the time signal in the PG is unexpectedly complex

Our findings reveal a complex relationship between the timing of PTTH abundance in the terminals of PTTHn and the transduction of PTTH action in the PG (However, the timing of PTTH release remains hypothetical and will need to be directly demonstrated). Beyond that point, our findings reveal a complex relationship between the timing of PTTH abundance in the terminals of PTTHn, and the transduction of PTTH action in the PG. Indeed, PTTH immunoreactivity in PTTHn terminals and somatic Ca^{2+} levels were lowest at the start of the day, coincident with the highest *torso* expression and a transient maximum of phosphoERK in the PG. In turn, PTTH levels were highest in the terminals of PTTHn during the middle of the night, a time when *torso* expression and phosphoERK levels in the PG reached their lowest levels. Assuming that TORSO receptor expression follows torso mRNA levels (there are currently no reagents to reliably measure TORSO protein levels in situ), this temporal relationship is consistent with an anti-cooperative model proposed for the PTTH/Torso interaction in the silkworm (Jenni et al., 2015), where the actions of the ligand are dampened by low receptors levels, leading to a low sustained

output, whereas high receptor levels induce a transient output burst. A similar regulation has also been described in *Drosophila* and in mammalian cells (Puig and Tjian, 2005) for the insulin receptor, InR, another RTK family member. Indeed, under high nutrients conditions, a known inductor of insulin secretion, cells down-regulate *InR* expression via the transcription factor FOXO. A similar mechanism could also apply to the clock control of glucocorticoid (GC) production by the mammalian adrenal gland, where the clock of the adrenal gland regulates the sensitivity to adrenocorticotrophic hormone (ACTH) (Oster et al., 2006).

Convergence of RTK signaling onto the PG

Across insects including *Drosophila* (Mele and Johnson, 2019), RTK signaling plays a major role in the control of growth and the timing of the molts. In the PG, PTTH-TORSO, Jeb-ALK, and Pvf/Pvr signaling, act to regulate the timing of ecdysteroid production (Pan and O'Connor, 2021), which causes the animal's developmental transitions. Here we showed that all three RTK signaling pathways also contribute to the circadian control of emergence. Eclosion requires ecdysteroid titers to fall below a certain threshold level (Sláma, 1980; Schwartz and Truman, 1983; Zitnan and Adams, 2012). However, the timing of emergence is not determined by the time when ecdysteroid titers drop below this threshold (Handler, 1982; Lavrynenko et al., 2015). Instead, it is PTTH (this work) and ecdysteroid signaling (Mark et al., 2021) that control the circadian gating of eclosion in *Drosophila* by committing the insect to complete

metamorphosis around 14h before emergence (Mark et al., 2021). Since we observed peaks of activity in PTTHn around 31h and 6h before emergence, it may be the timing of the first rather than the second peak of activity in PTTHn that is critical for determining the timing of emergence. In this scenario, PTTH/Jeb signaling may not only promote ecdysteroid production, but also regulate the phase of the PG clock. Although our study does not provide mechanistic evidence for this scenario, it has been shown that RTK/MAPK signaling serves as both an output and an input pathway to the mammalian circadian clock (Goldsmith and Bell-Pedersen, 2013). Furthermore, a mutation in *Alk* was found to significantly alter the circadian period of locomotor activity in *Drosophila* (Kumar et al., 2021). In addition, it is known that *pvr* and *alk* expression is under circadian control in ventral and dorsal lateral clock neurons in the brain of the adult fly (Abruzzi et al., 2017), suggesting that these RTKs are downstream of the clock. It is therefore possible that the central-clock driven PTTHn-derived RTK signaling ensures proper eclosion timing by aligning the phases of the central and PG clocks.

Insulins are another class of ligands that act on the PG via an RTK (Colombani et al., 2005). An RNAseq study reported that insulin signaling modulates the expression of clock genes in the PG, which in turn regulates *torso* expression (Di Cara and King-Jones, 2016). Nevertheless, we found that insulin signaling in the PG does not affect the rhythm of emergence. Thus, not all signaling pathways mediated by RTKs are involved in the circadian control of adult emergence. Similarly, even though ALK acts through Ras/ERK and PI3K

signaling pathways in the PG (Pan and O'Connor, 2021), we showed that PI3K signaling is not involved in the circadian gating of emergence, suggesting that, similar to TORSO, ALK and PVR contribute to the rhythm of emergence via the Ras/ERK pathway. Interestingly, the involvement of multiple signals in the transmission of the time information is consistent with the general principle of coherent coupling between multiple oscillators, which helps to stabilize and strengthen period, phase distribution, and amplitude in the SCN, and between central and peripheral body clocks (Pilorz et al., 2018; Schmal et al., 2018).

Autonomous role of the PG clock in the timing of eclosion

We found that knockdown of the ligand, Pvf2, in the PG itself reduced the rhythmicity of emergence. This identifies elements of the circadian control of emergence that are autonomous to the PG, and may be responsible for the peak of ARC-LUC activity detected in the PG two days prior to eclosion, which occurred before the activation of PTTHn. These findings may explain why functional clocks in both the central brain and the PG are required for the daily gating of eclosion (Myers et al., 2003). Indeed, since *torso* levels do not cycle in the absence of PTTH, it appears that the PG clock does not control the sensitivity to PTTH, raising the question of what role the clock of the PG plays in regulating the timing of adult emergence. In addition, it is intriguing that the peak of activity in the PG occurs at eclosion because it is undergoing apoptosis at this time (Dai and Gilbert, 1991), raising the possibility that apoptosis is part of a

sequence of physiological events that are regulated by the circadian clock and are critical for the timing of eclosion.

Much is currently known about how the circadian clock functions in a variety of multicellular organisms (Bell-Pedersen et al., 2005). By contrast, much less is known about how the clocks housed in different organs are coordinated. Interestingly, although animal clocks are universally controlled by intracellular transcriptional/translational feedback loops (Hardin, 2011; Takahashi, 2017), the mechanisms that mediate the coupling of clocks appears to be diverse and fine grained, such that in some cases some genes within a peripheral clock cycle autonomously whereas the cycling of others depends on a central input (Xu et al., 2011; Erion et al., 2016; Versteven et al., 2020). In addition, the relationship between central and peripheral clocks is even more complex if the integration of external stimuli such as light, temperature or feeding is considered (Ivanchenko et al., 2001; Glaser and Stanewsky, 2005; Mohawk et al., 2012; Ito and Tomioka, 2016). In the case of the circadian gating of adult emergence, we provide here a detailed analysis of the mechanism that couples a central and a peripheral clock, which may provide principles that apply to other circadian clocks. Knowing how clocks are coordinated will be critical to understand how circadian rhythmicity is generated at the cell, systems, and organism levels.

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Author contributions

Conceptualization: J.C.-L., E.A., C.W., J.E., Experiments: J.C.-L, E.A. Antiserum production: D.R.N. Supervision: R.S., C.W., J.E. Wrote the manuscript: J.C.-L., E.A., C.W., J.E., with review from D.R.N. and R.S. Funding Acquisition, C.W., J.E.

Declaration of interests

The authors declare no competing or financial interests.

Star*Methods

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact.

Material Availability

This study generated a new polyclonal rabbit antiserum against PTTH. Requests should be directed to D.R.N.

Data and code availability

All relevant data are included in this report; the results of all statistical tests are shown in Table S3. No codes were generated in this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly stocks and husbandry

Flies were raised on standard cornmeal/yeast media and maintained at room temperature (20 to 22 °C) under a 12h:12h LD schedule. All UAS and GAL4 stocks have previously been described; unless noted, they were obtained from the Bloomington *Drosophila* stock center (Bloomington, Indiana, USA; BL) or the Vienna *Drosophila* Resource Center (Vienna, Austria; VDRC). They included: wildtype (Canton-S strain), *white*[1118], *ptth*-GAL4(45A3,117b3) (McBrayer et al., 2007), *np423*-GAL4 (Yamanaka et al., 2013), *phm*-GAL4 (obtained from Michael O'Connor), *pdf*-GAL4 (obtained from Paul Taghert), *lsp*-GAL4 (BL6357), UAS-cyc[Δ901] (Tanoue et al., 2004), 20xUAS-IVS-GCaMP6m (Chen et al.,

2013), UAS-ANF-GFP (Husain and Ewer, 2004), UAS-EGFR (BL9535), UAS-*Pvr* DN (BL58431), *ptth delta* (Shimell et al., 2018), UAS-EGFP-Kir2.1 (Baines et al., 2001) (obtained from Sebastian Hückesfeld and Michael Pankratz), tubP-Gal80^{ts} (BL7019), lexAop-nSyb-spGFP1-10, UAS-CD4-spGFP11 (Macpherson et al., 2015) (obtained from Peter Soba), LexAop2-Syb.GFP.P10 LexAop2-Syb.GFP^{N146I}.TEV^{T173V} LexAop2-QF2.V5.hSNAP25.HIVNES.Syx1A/CyO, 20XUAS-B3R.PEST UAS(B3RT.B2)BoNTA QUAS-mtdTomato-3xHA/TM6B, Tb^{1} (obtained from Sebastian Cachero) (Cachero et al., 2020), CaLexA (BL66542), 20XUAS-flp; lola-frt-stop-frt-luc (Chen et al., 2016) (obtained from Matthias Schlichting and Michael Rosbash), hsFLP UAS-mCD8::GFP.L QUAS-mtdTomato-3xHA; trans-TangoMkII/SM6b (BL95317), QUAS-nlsDsRed (obtained from Meet Zandawala) (Snell et al., 2022), *dilp2, 3, 5* and *dilp 7* mutants (Gronke et al., 2010) (BL30889 and kind gift of Peter Soba), *dilp8* and *Lgr3* mutants (Garelli et al., 2012; Garelli et al., 2015) (kind gift of Alisson Gontijo). Flies bearing UAS-SPARK (Zhang et al., 2018) and UAS-SPARK (T→A) (Zhang et al., 2018) were provided Tom Kornberg, All genotypes involving the use of UAS-RNAi transgenes included a copy of a UAS-*dcr2* transgene. When performing RNAi knockdown, several UAS-RNAi lines were tested and the one producing the most severe phenotype was used. The lines used and their source is indicated below; the line chosen for the results reported here are indicated in bold: *IP$_3$R* (Inositol 1,4,5, -tris-phosphate receptor; CG1063): **BL#25937**, VDRC#6486; *Dmca1A* (*cacophony* or Calcium-channel protein α1 subunit A; CG1522): **BL#27244**, VDRC #104168; *Dmca1D* (*Calcium-*

channel protein α1 subunit D; CG4894): BL#25830; *SERCA* (*sarcoendoplasmic reticulum calcium ATPase* or *Calcium ATPase 60A*; CG3725): **VDRC#4474**, VDRC#107446, BL#25928; *RyR* (*ryanodine receptor*): **VDRC#109631**, BL#29445, *CASK* (*calmodulin-dependent protein kinase activity*; CG6703): **BL#27556**, BL#35309; *PKC* (*protein kinase C*); **VDRC#27699**, BL27491; *AstAR1*: **VDRC#39222**, VDRC#48495; *CrzR*: **VDRC108506**, VDRC#44310; *torso*: VDRC#36280; *Alk*: **BL#27518**, VDRC#107083; *Pvf2:* **BL#61195**, VDRC#7629 *Pvf3*: VDRC#373933**;** *Jeb:* **VDRC#103047**, CG30054: **BL#51823**, VDRC#4643; CG17760: **BL#64919**, VDRC#52308; *InR*: VDRC#992; PI3K: VDRC#107390; PTEN: VDRC#35731.

METHOD DETAILS

Emergence monitoring

Flies were raised at 20ºC under 12 h light and 12 h darkness (LD 12:12). After 12–17 days, pupae were collected and fixed on eclosion plates with Elmer's glue or methyl cellulose glue (Tapetenkleister #389, Auro, Braunschweig, Germany) and mounted on Trikinetics eclosion monitors (Trikinetics, MA, USA). Emergence was then monitored for 7 days at 20ºC under DD conditions, in climate- and light-controlled chambers. Rhythmicity of eclosion profiles was analyzed using MATLAB (MathWorks, Inc., Natick, USA) and the appropriate Matlab toolbox (Levine et al., 2002). Using autocorrelation analyses, records were considered rhythmic if the rhythmicity index (RI) was $\geq$0.3, weakly rhythmic if 0.1$\leq$RI<0.3 and arrhythmic if RI<0.1 (Sundram et al., 2012). In the case of

experiments in which the temperature was increased to 29ºC during metamorphosis, the increase in temperature causes a faster development, leading to a reduced number of eclosion peaks. In such cases autocorrelation analysis produced low RI values for controls so Lomb-Scargle (LS)(Ruf, 1999) and eJTK (Hughes et al., 2010) analyses were used instead.

***Ex vivo* measurements of Ca^{+2} and phosphorylated ERK signals**

Animals were reared under 12h:12h LD conditions with lights-on at noon or midnight. Animals used were always at the start of metamorphosis (white pre-pupal stage, WPP). Thus, developmental stage was kept constant; the only variable was time of day. For measurements made under DD conditions, cultures were wrapped in aluminum foil at lights-off and maintained covered until the desired time. In order to determine Ca^{+2} levels in PTTHn, we measured the GFP fluorescence of these neurons expressing the Ca^{+2} sensor, GCaMP6m. For this, WPP stage animals were collected, and their brains dissected under ice-cold calcium-free fly saline (46mM NaCl, 5mM KCl, and 10mM Tris pH 7.2). They were then mounted on slides coated with poly-lysine in an Attofluor chamber (A-7816, Invitrogen) and imaged on an Olympus Spinning Disc microscope using an UMPlanFI 20X/0.50 water immersion objective and CellSens software. Samples were visualized using the GFP filter (excitation 485nm and emission at 515nm) and imaged using a Hamamatsu camera (model ORCA IR2). Regions of interest (ROIs) were drawn over the cell bodies of

PTTHn. Background was subtracted for every ROI and the changes in fluorescence intensity were calculated as:

$$\Delta F/F_0 = (F-F_0)/F_0$$

(1)

Where F is the fluorescence in the cell bodies of PTTHn and F_0 is the baseline fluorescence value in the brain. Mean fluorescence intensities were measured using Fiji (Schindelin et al., 2012). Data were analyzed in Microsoft Excel.

Levels of phosphorylated ERK in the PG were determined by driving expression of the SPARK sensor in the PG and measuring the GFP fluorescence emitted by individual PG cells. PGs dissections and imaging were performed as described above. For quantitative analysis of the SPARK signal, images were processed using Fiji (Schindelin et al., 2012). The droplets included in the analyses were selected using the ‘‘threshold’’ function, considering only those with a size larger than that of the residual droplets observed when the mutant (inactive) version of ERK-SPARK was expressed in the PG. Normalized SPARK was calculated as:

Normalized SPARK= (Σdroplets’ pixel intensity/ΣPG pixel intensity) (2)

The sum of droplet pixel fluorescence intensity and the PG pixel intensity were calculated using Analyze Particle function in the software. For the quantification of nuclear droplets, only droplets with a clear nuclear localization were considered; droplets in a region between nucleus/cytoplasm were excluded.

CaLexA Ca^{2+} imaging

Eggs of *ptth>CaLexA* flies were collected for five hours and kept at 12:12 LD, 25°C, and 65% humidity. Pupal brains were dissected at pupal stage P14 in 4-hour intervals at ZT0, 4, 8, 12, 16, and 20, and immediately fixed and stained for GFP- and PTTH- immunoreactivity as outlined above. After mounting, the brains were imaged under a confocal microscope using the same settings for all preparations. Staining intensities were determined using Fiji.

ARG-Luc imaging

*ptth>*20X*UAS-flp; lola-frt-stop-frt-luc* larvae were raised in the dark on normal food supplemented with 15 mM luciferin (Carbosynth Ltd., Newbury, UK) until pupariation. A 1h light pulse was given at ZT0 to synchronize the clocks of the animals used. The next day puparia were transferred to light during the subjective photophase and washed from the bottles, dried, and glued on adhesive transparent plastic sheets (TopSeal™ A Plus (PerkinElmer LAS, Rodgau, Germany) on top of a 96 well OptiPlate™ (PerkinElmer LAS, Rodgau, Germany). Three days before the estimated time of eclosion, puparia were glued on every other well to minimize light spill-over. Bioluminescence was recorded every 30 min in DD at 25°C and 65% humidity using a TopCount Multiplate Reader (Perkin Elmer). An sCMOS camera (DMK33UX178, ImagingSource, Bremen, Germany) on top of the plate recorded a picture every 5 minutes under red light to monitor the eclosion state. Rhythmicity of the ARG-

Luc signals was analyzed using MESA and JTK_Cycle implemented in BioDare2 (Zielinski et al., 2014).

PTTH antiserum production

Rabbit polyclonal PTTH antiserum to PTTH was produced against the synthetic peptide CQSDHPYSWMNKDQPWamide (residues 207-221, with an N-terminal Cys added), not amidated which was coupled to thyroglobulin via the N-terminal Cys residue using maleimide. The immunization was carried out by Pineda-Antikörper Service (Berlin, Germany) and lasted over 3 months, with a booster before the final bleed.

Immunocytochemistry

Dissected brains or PGs were fixed in 4% paraformaldehyde for 15 minutes at room temperature (RT). Tissues were washed in PBS containing 0.3% TritonX-100 (PBT), blocked with 5% normal goat serum (NGS) in PBT, and incubated overnight at 4ºC in primary antibodies. Primary antibodies used are listed in Table 1. Tissues were then washed in PBT and incubated for 2h at RT in secondary antibody, rinsed three times in PBS, and mounted on a poly-L-lysine coated coverslip using Fluoromont-G T (Electron Microscopy Science, USA). Alexa Fluor conjugated secondary antibodies (Alexa Fluor 488 conjugated goat anti-guinea pig, and goat anti-rabbit were from Invitrogen, MA, USA) were used at 1:500.

Tissues were imaged on an Olympus Spinning Disc microscope using either UPLFLN20X/0.5NA or UPLSAP040X/0.9NA objectives, and were analyzed

using ImageJ. Mean fluorescence intensity in sLNv terminals or PTTH signal in the PTTHn cell bodies was quantified and the signal from brain areas devoid of specific immunoreactivity was subtracted as background. For the quantification of the number of PTTH immunoreactive boutons on the PG, boutons were selected using the “threshold” function and quantified using ImageJ’s “analyze particles” function.

Immunocytochemistry for (chemo)connectomics, BacTrace, and syb-GRASP

Brains of P14 pupal stage adults were dissected in HL3.1 fly ringer (70 mM NaCl, 5mM KCl, 1.5mM $CaCl_2.2H_2O$, 4mM $MgCl_2.6H_2O$, 10mM $NaHCO_3$, 115mM sucrose, 5mM Trehalose.$2H_2O$, and 5mM HEPES (Feng et al., 2004)) and collected on ice. The HL3.1 solution was then replaced by 4% paraformaldehyde in phosphate-buffered saline (100 mM PBS, pH 7.4) and fixed for 30 minutes. Tissues were then washed three times with PBS with 0.3% Triton-X100 (PBT) for 10 minutes, followed by blocking in 5% normal goat serum (NGS) in PBT for 90 minutes. After blocking, the tissues were transferred into primary antibody diluted in PBT containing 3% NGS for 3 days at 4°C on a shaker, washed 6x20 min with PBT, and incubated for 24h at 4°C with secondary antibody in PBT containing 3% NGS. Finally, the tissues were washed 4x10min with PBT and 3x10min with PBS, and mounted on poly-lysine-coated slides (Thermo Scientific) with Vectashield (Vector Laboratories, California, United States), and stored at 4°C until imaged.

To visualize the syb-GRASP signal, freshly dissected brains were briefly (5-7s) depolarized three times using HL3.1 containing 70 mM KCl, then returned for 10 min in HL3.1 to allow reconstruction of GFP at active synaptic sites prior to fixation. For *trans-Tango* MkII, animals were raised at 18°C until P14 in order to maximize the accumulation of signal in post-synaptic neurons. In both cases, tissues were fixed for 30 minutes with 4% PFA in PBS and processed as described above.

Tissues were imaged on a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with 20x and 40x high aperture immersion objectives.

qRT-PCR

For each timepoint point, 40 PGs from WPP animals were dissected under ice-cold phosphate-buffered saline (PBS) and stored in RNAlater solution (Qiagen). Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions, and resuspended in 20 ml of RNAse-free water.

cDNA synthesis was carried out using SuperScript II Reverse Transcriptase (Invitrogen) following manufacturer's instructions. One μg of RNA was first treated for 15 min at room temperature with 1 μl DNAse I (2U μl^{-1}), 10X DNAse I Buffer (100 mM Tris, pH 7.5; 25 mM $MgCl_2$; 5 mM $CaCl_2$); DNAse I was then inactivated adding 1.5 μl 25 mM EDTA and the reaction incubated for 10 min at 65 ºC. Oligo dT (0.5 μg) and dNTP's (10mM each) were then added to each

sample, incubated at 65 ºC for 5min, then placed on ice. Four μl of 5X First-Strand Buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM MgCl2) and 2 μl of DTT (100 mM) were then added, and samples incubated for 2min at 42 ºC. Finally, 1 μl of Reverse Transcriptase was added, and the reaction incubated at 42 ºC for 50 min, followed by 15 min at 70 ºC. Control reactions were processed in parallel except that the Reverse Transcriptase was omitted.

Quantitative PCR with reverse transcription (qRT-PCR) was carried out using *torso*-specific primers suitable for qRT-PCR; *rp49* was used as reference (see Supplementary Table 1 for the sequence of the primers used). Reaction mix included 5 μl Maxima SYBR Green/ROX qPCR Master Mix 2X (Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.25 ml primers (10 μM), 1 μl cDNA, and 3.5 μl nuclease-free water. PCRs were carried out using an Agilent Mx3000P QPCR System thermocyler (Santa Clara, CA, USA) using the following regime: 25 ºC for 5 s, 95 ºC for 5 min, followed by 40 cycles of 15 s at 95 ºC, 15 s at 60 ºC and 15 s at 72 ºC. A melting curve was done at the end of the reaction, which consisted of 10 s at 95 ºC, 5 s at 25 ºC, 5 s at 70 ºC, and ending with 1 s at 95 ºC. All analyses were carried out using MxPro QPCR Software (Agilent, Santa Clara, CA, USA). At least three independently isolated cDNAs were used, and each cDNA was qRT-PCR amplified in triplicate.

Quantification and statistical analyses

Parametric datasets were first analyzed for normal distribution using the D'Agostino-Pearson or Shapiro-Wilk normality test using Prism 8.0 (GraphPad,

USA), then analyzed using unpaired Student's t-test or one-way ANOVA followed by Tukey's multiple comparisons tests. For non-parametric datasets, we used Kruskal-Wallis tests followed by Dunn's multiple comparisons tests. The results of all statistical tests are shown in Table S3.

Figures

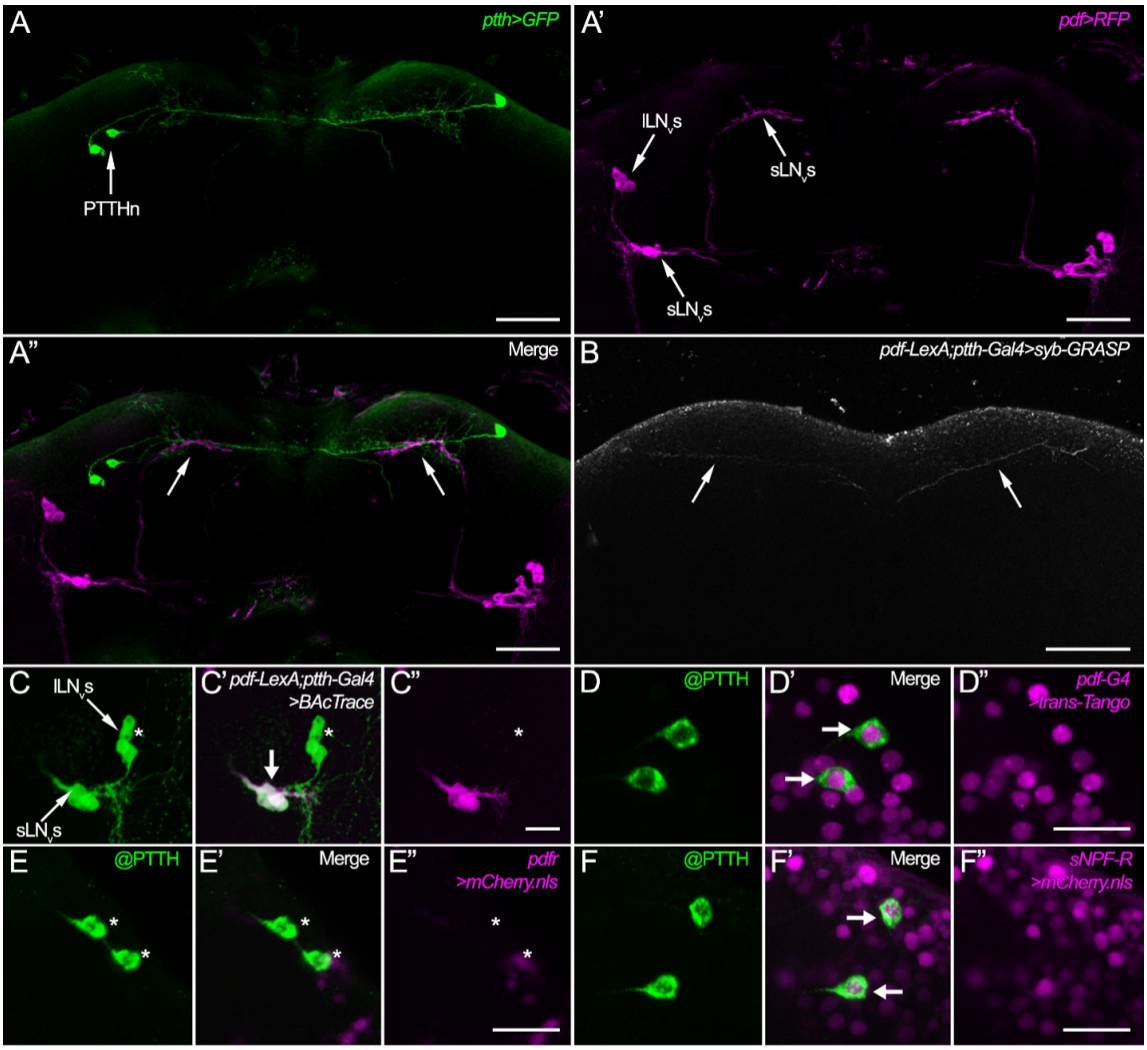

Figure 1 Connectivity between PDF-expressing sLNv clock neurons and PTTHn. (A-A") The arborizations of PTTHn in the superior protocerebrum **(A)** are in close contact (arrows in **(A")**) with the PDF-expressing sLNv clock neurons **(A')**. **(B)** *syb*-GRASP reconstruction between PTTHn and PDF-expressing sLNv clock neurons produced only a faint reconstruction of GFP (arrows), which did not include small branches in the contact area between PTTHn and sLNv located in the superior protocerebral region. **(C-C")** BAcTrace-labeling showing that all PDF-expressing sLNv but not the large lLNv synapse onto the PTTHn. Whereas *pdf*-LexA drives GFP expression in the sLNv and lLNv **(C)**, the BAcTrace signal **(C')** was only detected in the sLNv **(C")**. **(D-D")** Trans-Tango labeling showed that all PTTHn are postsynaptic to the sLNv since the positive trans-Tango signal was visible in PTTHn present in each hemisphere **(D')**. **(E-E").** A PDF receptor-specific intragenic driver line **(E")** did

not label the PTTHn **(E)** in pharate adults **(E')**. Asterisks mark the position of the PTTHn. **(F-F").** By contrast, an sNPF receptor-specific intragenic driver line **(F")**, labeled the PTTHn **(F)** in pharate adults **(F')**. Scale bars: A-B: 50 *μ*m*;* C-F: 20 *μ*m.

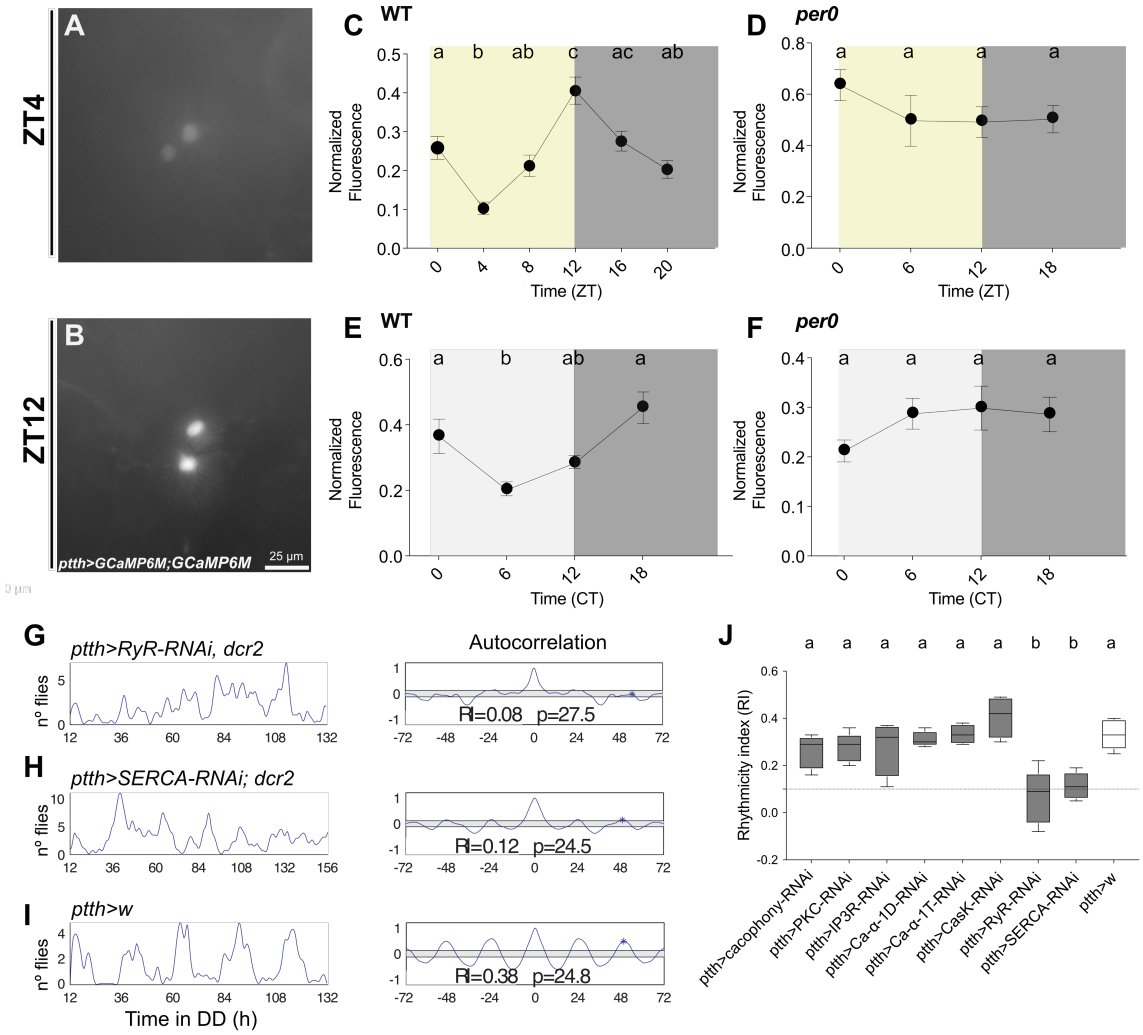

Figure 2. Ca^{+2} signaling in PTTHn is relevant to the circadian control of adult emergence. (A-B) Representative images of GCaMP fluorescence in PTTHn in the brains of WPP examined at ZT4 (A) and ZT12 (B). (C-F) Levels of GCaMP fluorescence in PTTHn of: wildtype (WT) animals at different times of day under LD (C) and DD conditions (E), and in the soma of PTTHn of *per[0]* mutants under LD (D) and DD (F) conditions. Different letters indicate statistically different groups. (ANOVA with Tukey's *post hoc* test). Between 8 and 10 animals were analyzed for each timepoint. ZT: zeitgeber time. CT: Circadian time. (G-I) Records showing the time course of emergence of a single population of flies under DD conditions (left) and corresponding autocorrelation analysis (right) of populations bearing a knockdown in PTTHn of Ryanodine Receptor (RyR)(G), SERCA (H) and in corresponding controls (I). Periodicity (p, in hours) and associated rhythmicity index (RI) are indicated. (J) Average RI values from knockdown in PTTHn of mediators of Ca^{+2} signaling. The dashed line at RI=0.1 indicates cutoff below which records are considered

arrhythmic. Different letters indicate statistically different groups. ($p<0.05$; one-way ANOVA, Tukey's *post hoc* multiple comparison analyses).

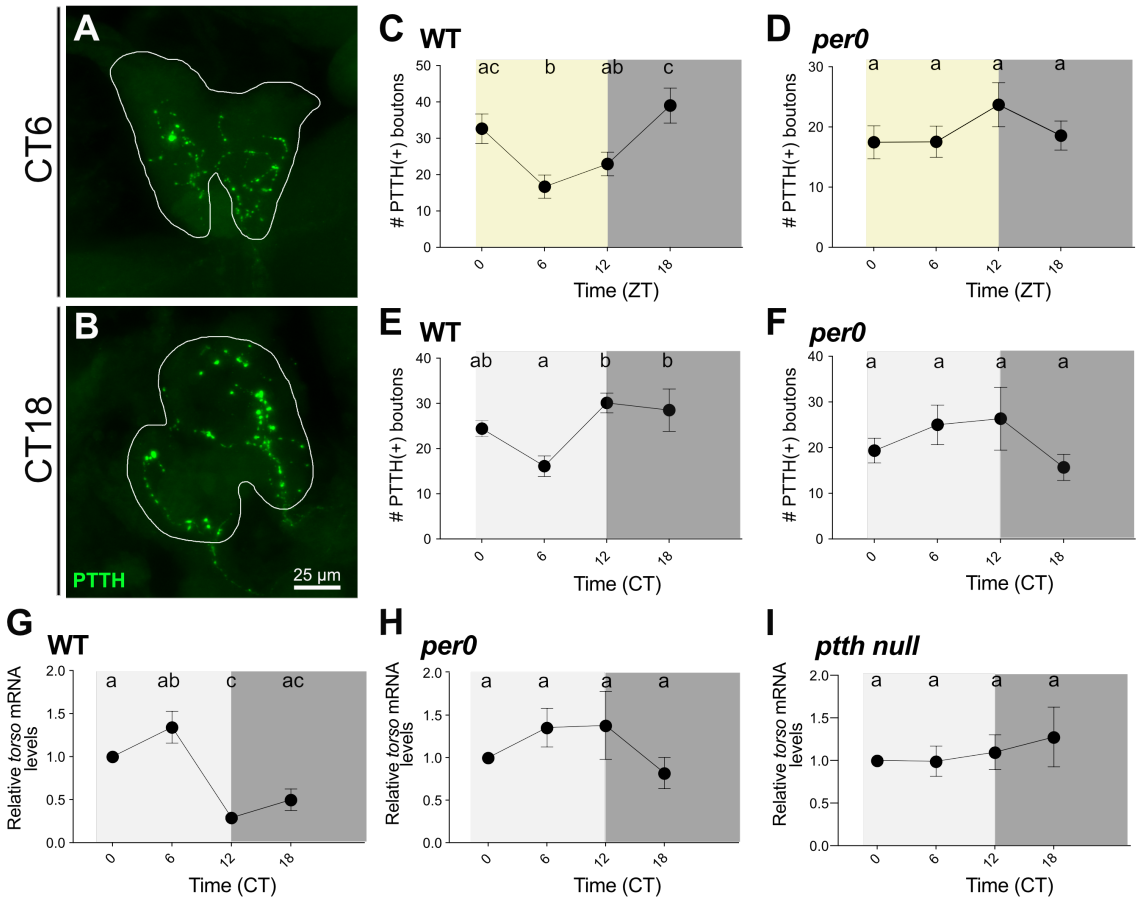

Figure 3. Circadian regulation of PTTH/TORSO axis. (A-B) Pattern of PTTH-immunoreactivity of terminals of PTTHn on the PG of WPP animals at CT6 (A) and CT18 (B). White line delimits the PG. (C-F) Average number of PTTH-immunoreactive boutons showing above-threshold intensity on the PG at different times of day in wildtype (C, E) and in *per[0]* mutants (D,F) under LD (C,D) and DD (E,F) conditions. 8–10 brains were analyzed per time-point. (G-I) Relative expression of mRNA levels of *torso* in PGs isolated from WPP stage wildtype (G), *per[0]* (H), and *ptth* null mutant (I) animals under DD conditions. Different letters indicate statistically different groups ($p<0.05$; one-way ANOVA, Tukey's *post hoc* multiple comparison analyses).

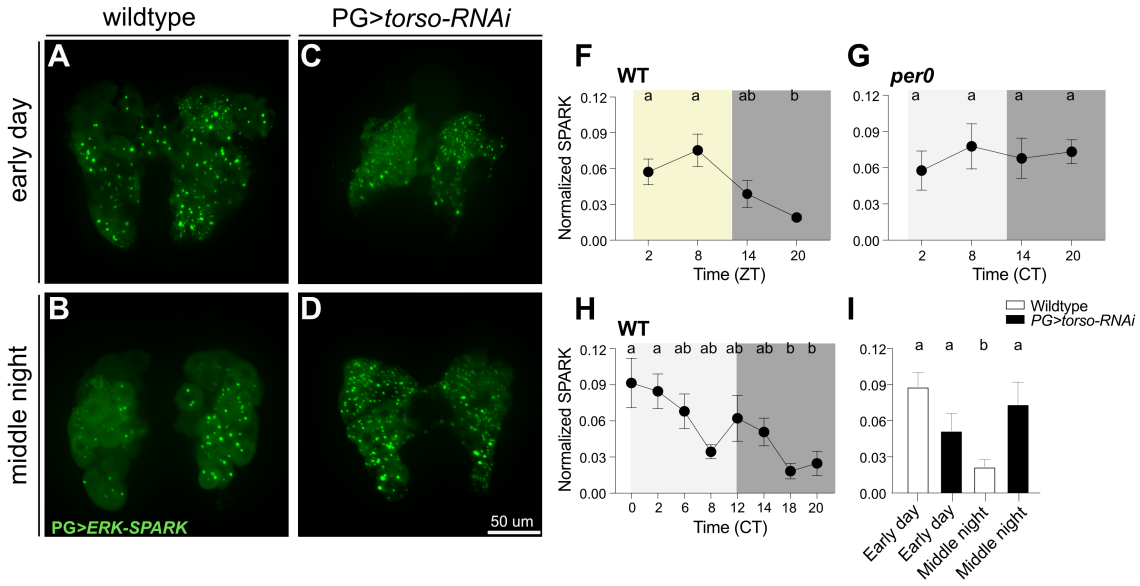

Figure 4. ERK phosphorylation in the PG shows a PTTH-dependent daily rhythm. (A-D) ERK-SPARK signal in whole mount PGs (*phm>ERK-SPARK*) of wildtype (WT) and PG>*torso-RNAi* animals at the WPP at CT0-2 (early subjective day) and CT18-20 (middle of subjective night). (F-H) Average of normalized ERK-SPARK signal in the PG at different times of day in wildtype PG under LD (F) and DD (H) conditions, and in *per[0]* mutants (under DD condition; G). (I) Average normalized ERK-SPARK signal in wildtype and PG>*torso-RNAi* animals at CT0-2 (early subjective day) and CT18-20 (middle of subjective night). Eight to 10 brains were analyzed per time-point. Different letters indicate statistically different groups ($p<0.05$; one-way ANOVA, Tukey's post hoc multiple comparison analyses).

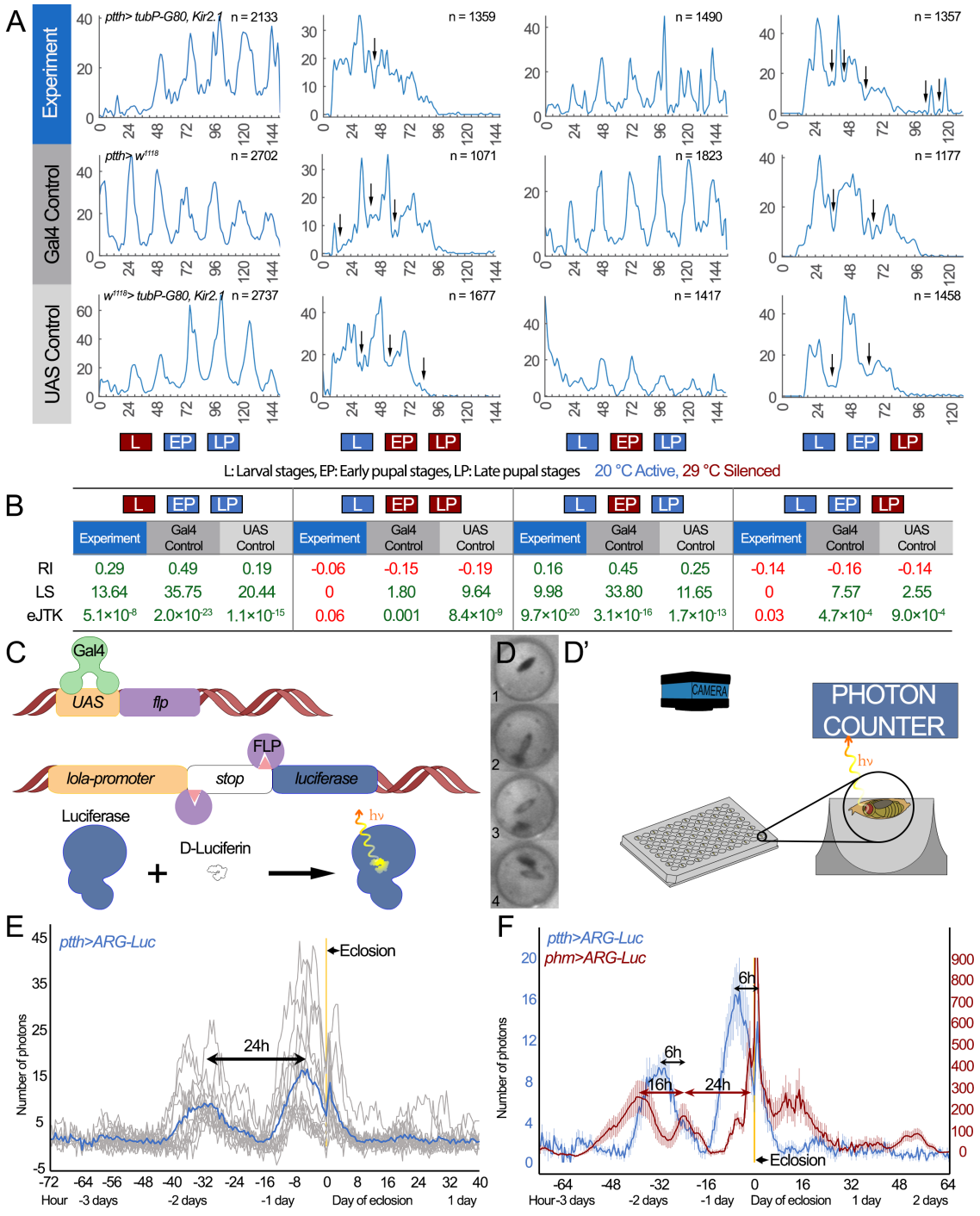

Figure 5. PTTHn activity is required during the end of pupal development. Record of the time course of emergence in ptth>tubP-Gal80ts, Kir2.1 flies with PTTHn conditionally silenced at 29°C during larval (L), entire pupal (EP+LP), early pupal (EP), and late pupal stages (LP) and in corresponding controls. Rhythmic records show peaks followed by valleys (arrows), which are separated by □24 hours. (B) Rhythmicity index (RI), Lomb-

Scargle (LS) and eJTK_Cycle values of experiments shown in (A). Rhythmic records are coded in green, arrhythmic ones in red. Raising the temperature to 29°C during metamorphosis accelerates development, and when extended to the late pupal stage (LP) causes emergence to occur in only two to three peaks eclosion peaks. This renders autocorrelation inappropriate for rhythmicity analysis. Hence values are given for LS and eJTK_Cycle only. (C-F) In vivo imaging of PTTHn and PG activity prior to emergence. (C-D´) Schematic representation of the ARG-Luc system and in vivo imaging setup. (D) Images of intact animals captured at different stages: pharate [1]; eclosing [2]; eclosed; prior to [3], and after [4], wing expansion. (E) ARG-Luc activity of the PTTHn for the 14 out of 21 individual flies that showed signal changes during monitoring. (In the seven non-responding flies, PTTHn may either have remained inactive during the pupal period, or may have not been close enough to the detector). The curves are plotted relative to the time of emergence (yellow line). (F) Average ARG-Luc activity for the PTTHn (blue) and for the PG (red, n=22). Error bars indicate SEM. The peaks directly following eclosion in (E-F) are likely artifacts due to higher photon yields once flies have left their absorbing puparium.

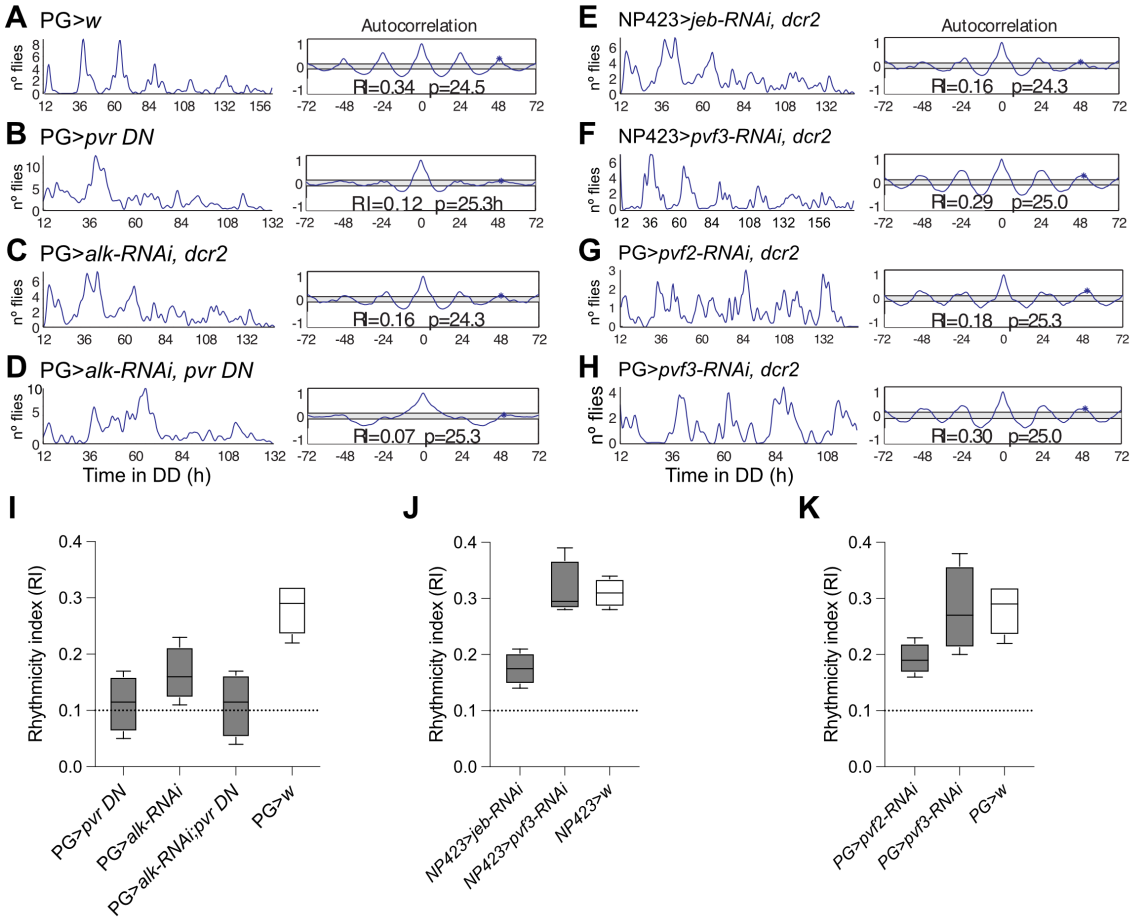

Figure 6. Alk and Pvr contribute to the circadian rhythmicity of adult emergence. (A-H) Records showing the time course of emergence under DD (left) and corresponding autocorrelation analysis (right) of a single population of flies expressing in the PG: a dominant negative form of *pvr* (B), *alk* RNAi (C), simultaneous knockdown of PVR and ALK receptors (D), *pvf2* RNAi (G) and *pvf3* RNAi (H). In addition, flies expressing in the PTTHn: *jeb* RNAi (E) and *pvf3* RNAi (F). Periodicity (p, in hours) and associated rhythmicity index (RI) are indicated. (I-K) Average RI values for results shown in (A-H) and their respective controls; dashed line indicates cutoff below which records are considered arrhythmic. Different letters indicate statistically significant differences (*p < 0.05; one-way ANOVA, Tukey *post hoc* analysis.

Supplementary Figures

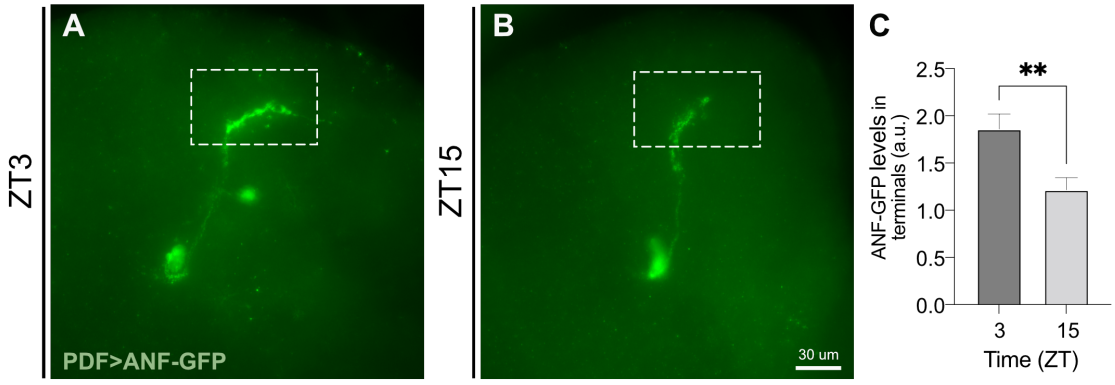

Figure S1. Rhythm in neuropeptide accumulation in sLNv terminals. (A-B) ANF-GFP signal in PDF neurons from wildtype animals at the WPP stage at ZT3 and ZT15. Dashed box indicates the sLNv terminals. (C) Average ANF-GFP fluorescence levels in the sLNv terminals. Eight to 10 brains were analyzed per time-point (**: $p<0.05$; Student t-test).

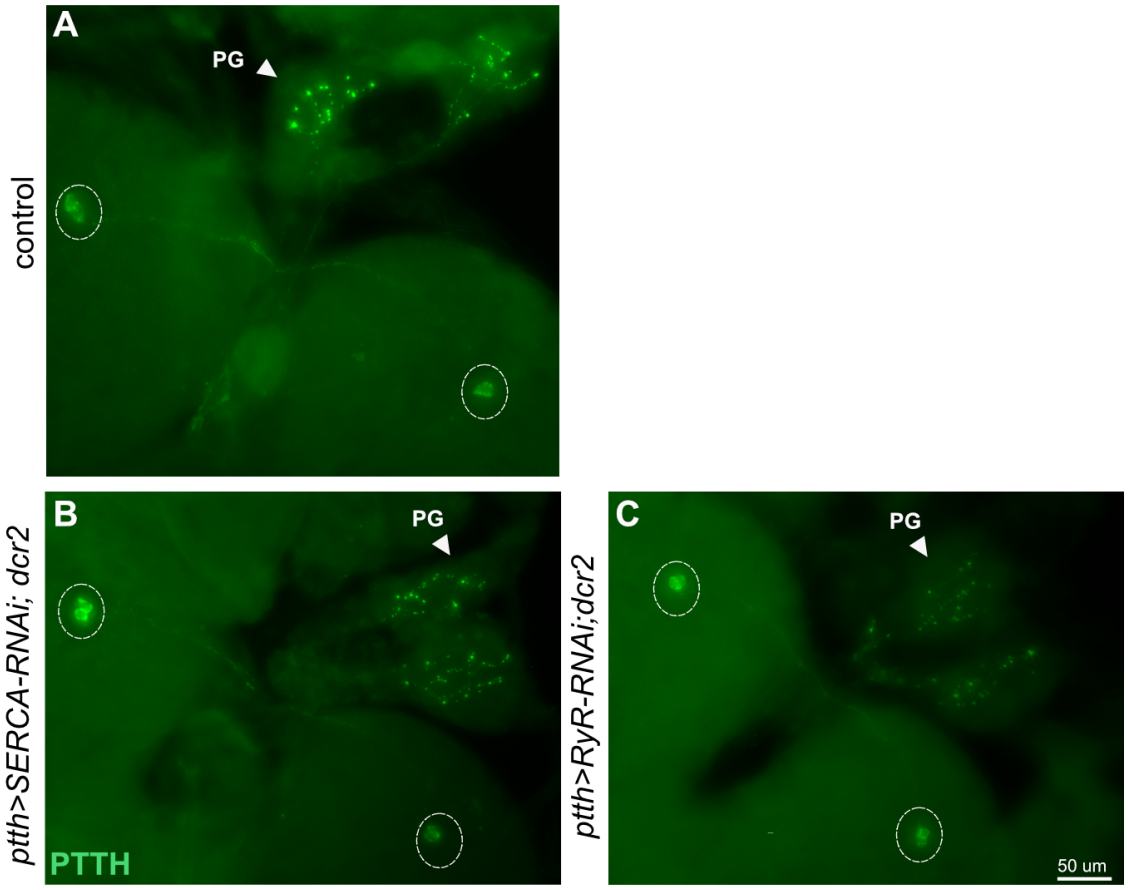

Figure S2. Morphology of PTTHn is not grossly affected by knockdown of SERCA or RyR. (A-C) Whole mount of brain-PG complex immunostained for PTTH from control (ptth>w) (A), ptth>SERCA-RNAi;dcr2 (B) and ptth>RyR-RNAi,dcr2 (B) animals at the WPP stage. Dashed circles indicate PTTHn cell bodies. Bright spots on PG (arrowhead) correspond to the terminals of PTTHn. Both images were not acquired with the same exposure time and thus, fluorescence levels are not comparable.

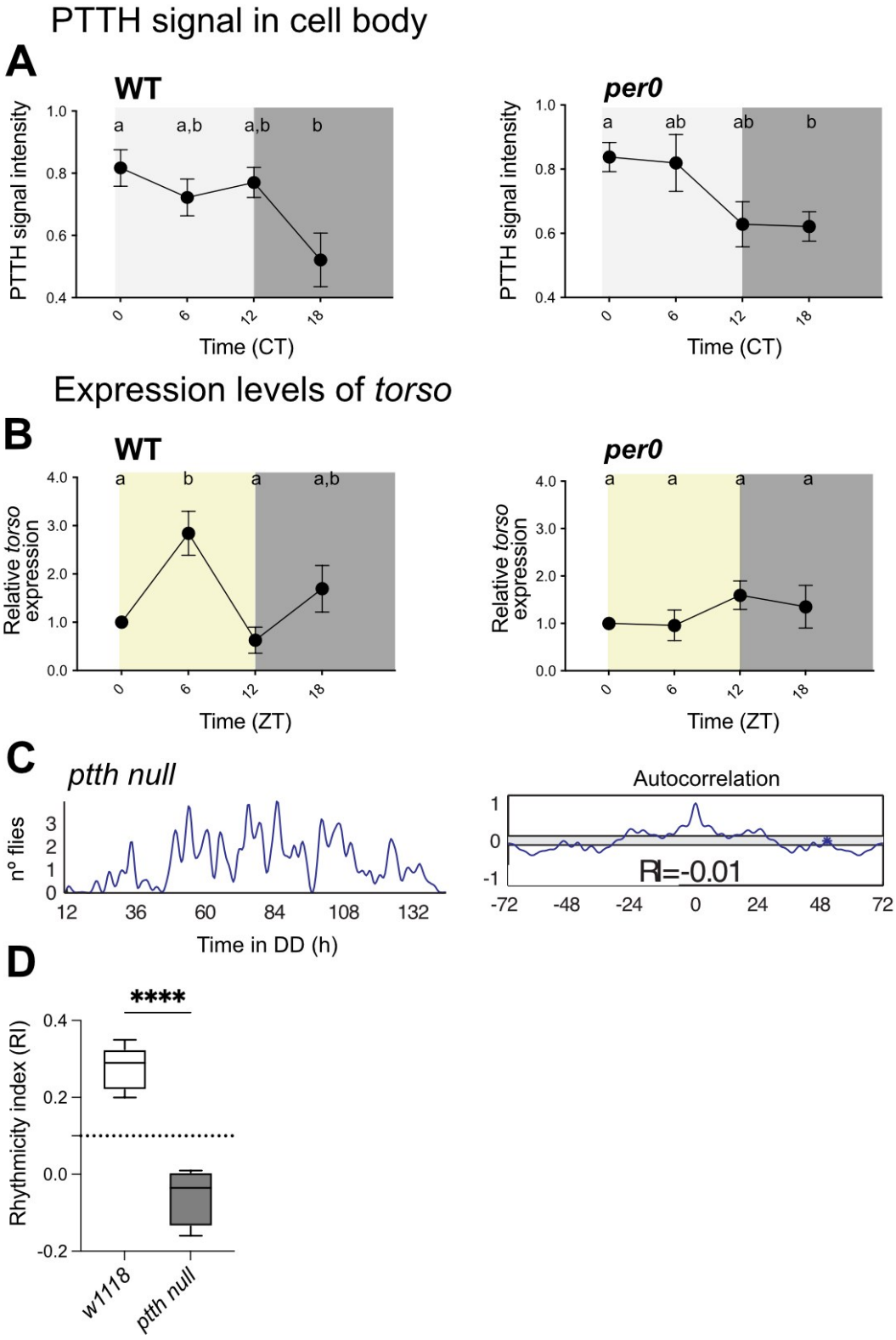

Figure S3. Daily rhythm in PTTH levels in PTTH neurons and torso

expression in the PG. (A) Quantification of PTTH immunoreactivity in the cell bodies of PTTHn in wildtype (left) and *per[0]* mutant (right) animals at the WPP stage at different times of (subjective) day under DD conditions. (B) qRT-PCR analysis of *torso* expression in PGs dissected from WPP stage wildtype (left) and *per[0]* mutant (right) animals under LD conditions. *rp49* was used as reference gene. (C) Record showing timecourse of emergence of a single population of flies under DD conditions (left) and corresponding autocorrelation analysis (right) in *ptth* null mutant animals. Rhythmicity index (RI) associated is indicated. (D) Average values for RI (left) for *ptth null* mutant animals and controls (****: $p<0.0001$; Student t-test).

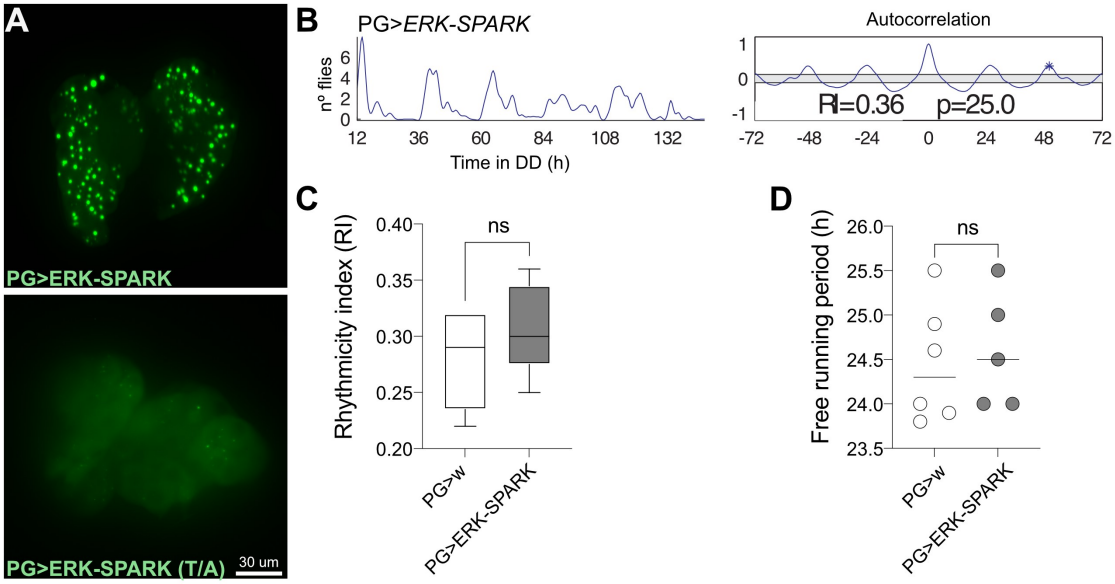

Figure S4. SPARK reports ERK activity in the PG without affecting the circadian control of adult emergence. (A) PG whole mount of animals expressing ERK-SPARK sensor (top) or ERK-unresponsive mutant sensor (bottom). (B) Record showing time course of emergence of a single population of flies under DD conditions (left) and corresponding autocorrelation analysis (right) of a population of flies expressing ERK-SPARK in PG. Periodicity (p, in hours) and associated rhythmicity index (RI) are indicated. (C-D) Average values for RI (C) and periodicity (D) for PG>*ERK-SPARK* and for control. Points represent the results from a separate experiment (ns: p>0.05; Student t-test).

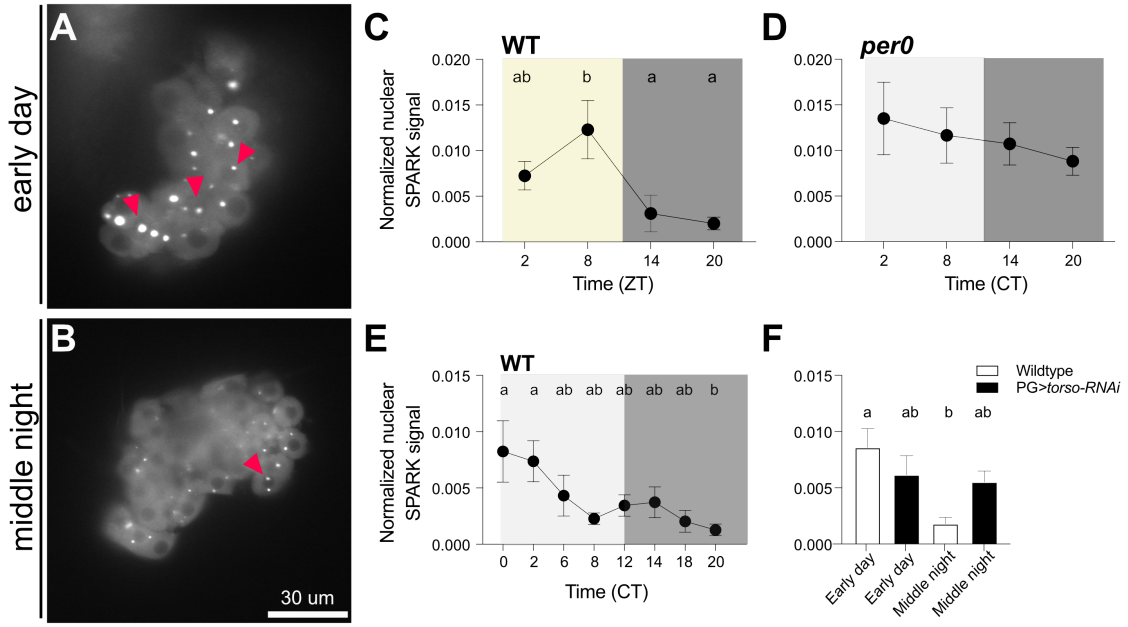

Figure S5. Nuclear localization of phosphorylated ERK in the PG exhibits a daily rhythm. (A-B) PG whole mount of animals expressing ERK-SPARK in the PG (*phm>ERK-SPARK*) at CT0-2 (early subjective day) and CT18-20 (middle of subjective night. Red arrowheads indicate droplets with nuclear localization. (C-E) Average of normalized nuclear SPARK signal at different times of day in wildtype under LD (C) and DD (E) conditions, and in *per0* mutants under DD condition (D). Different letters indicate statistically different groups ($p<0.05$; ANOVA, Dunn's multiple comparison analyses). (F) Average of normalized nuclear SPARK signal in wildtype and PG>*torso-RNAi* animals at CT0-2 (early subjective day) and CT18-20 (middle of subjective night). 8-10 brains were analyzed per time-point. Different letters indicate statistically different groups ($p<0.05$; one-way ANOVA, Tukey's post hoc multiple comparison analyses).

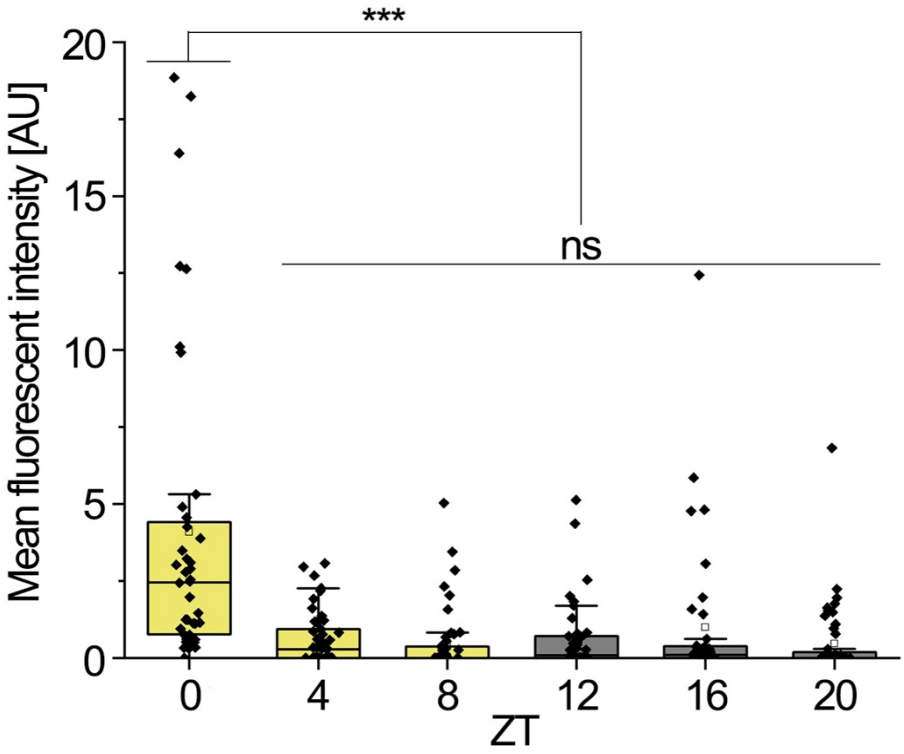

Figure S6. Ca2+-dependent CaLexA signals from the PTTHn suggests a peak of PTTHn activity preceding the emergence peak at dawn. *ptth>CaLexA* flies were raised in 12:12 LD condition and brains from pharate adults were dissected in 4-hour intervals in the light phase (yellow) or under red light in the dark phase (gray). The highest GFP signal occurred at the expected time of emergence.

Figure S7.

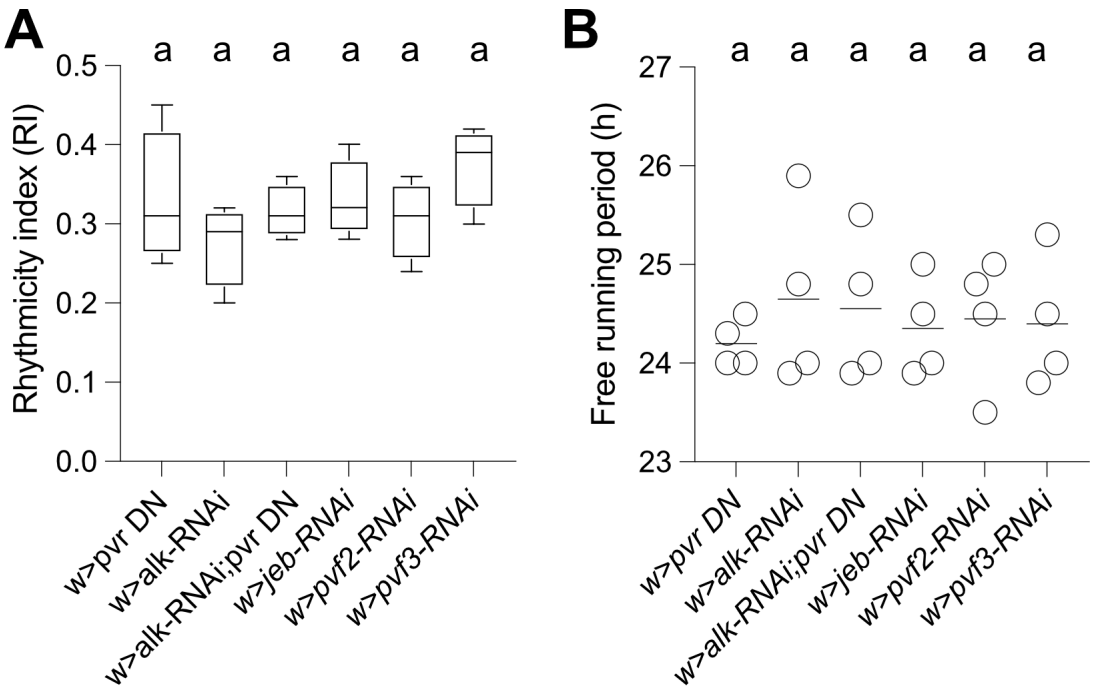

Figure S7. Flies bearing single copies of UAS-RNAi insertions for alk or pvr signaling express normal circadian rhythmicity of eclosion. (A) Average rhythmicity index (RI) values (± SEM) of flies heterozygous for UAS-RNAi transgenes for *pvr*, *alk*, *alk*;*pvr*, *jeb, pvf2*, and *pvf3*. Same letters indicate that there are no statistically significant differences between groups (one-way ANOVA, Tukey's post hoc multiple comparison analyses). (B) Free-running period (h) values for genotypes indicated in (A); each point represents the results from a separate experiment; horizontal lines indicate the average. Same letters indicate that there are no statistically significant differences between groups (one-way ANOVA, Tukey's post hoc multiple comparison analyses.

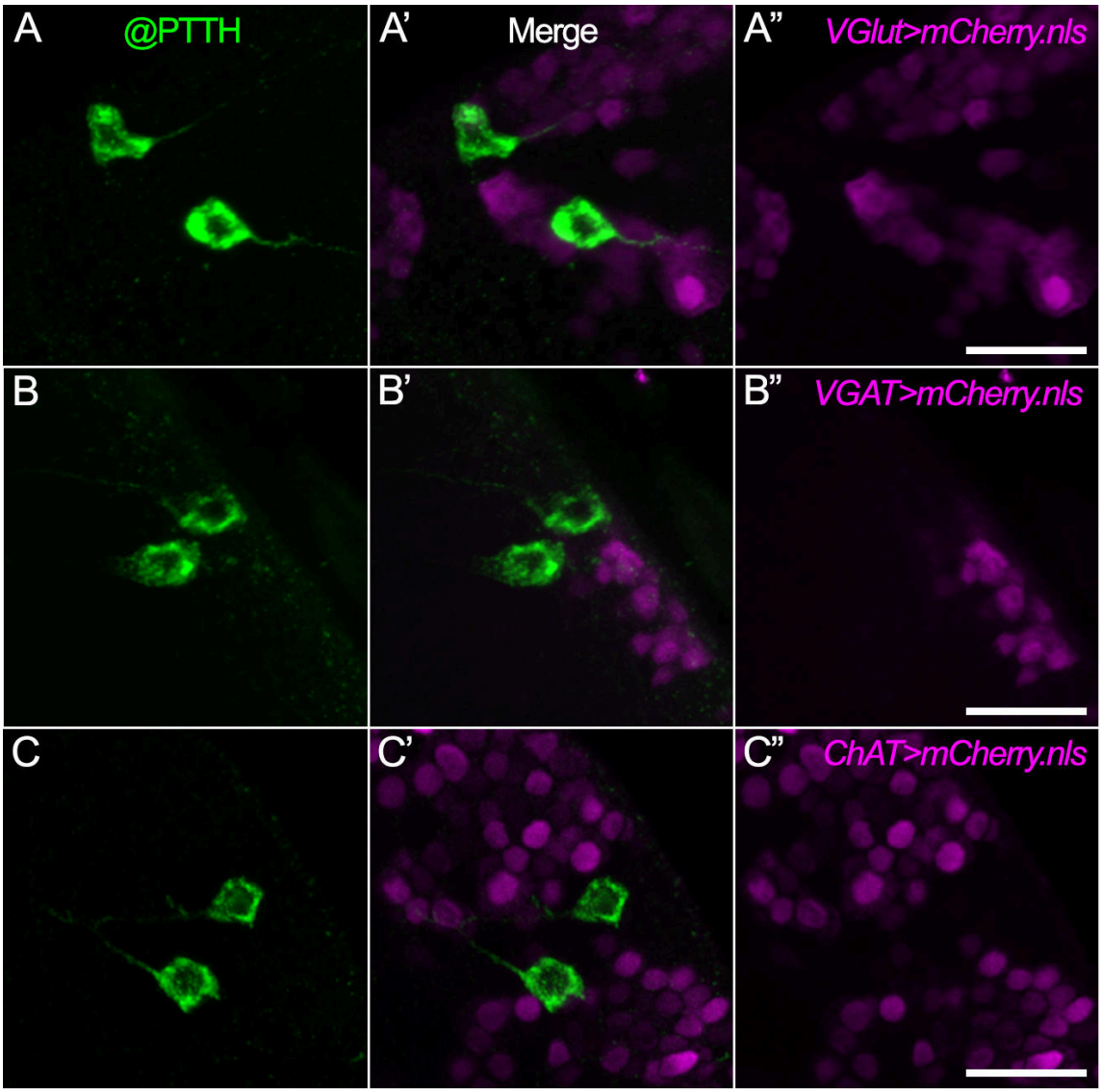

Figure S8 PTTHn do not express the neurotransmitters glutamate, GABA or acetylcholine. (A") Expression pattern of driver lines for vesicular glutamate transporter (*VGlut-Gal4*), (B") vesicular GABA Transporter (*VGAT-Gal4*) and (C") choline acetyltransferase (*ChAT-Gal4*). In all cases, there was no colocalization between the PTTHn (A, B, C,) and labeling for glutamatergic (A'), GABAergic (B'), or cholinergic neurons (C'), suggesting that the PTTHns are exclusively peptidergic and do not signal via classic neurotransmitters. Scale bars*:* 20 *μm*.

Tables

Table S1. Adult emergence rhythmicity phenotypes following genetic manipulations in the PTTH/PG axis or in the clock function.

Genotype	Associated biological process	N	RI ± SEM	Period (h) ± SEM
ptth>GCaMP6M	Ca^{+2} imaging	3	0.32 ± 0.02	24.1 ± 0.20
ptth>AstAR1-RNAi	PTTH secretion (Deveci et al., 2019)	5	0.30 ± 0.04	24.6 ± 0.22
ptth>CrzR-RNAi	PTTHn activity (Imura et al., 2020)	5	0.35 ± 0.04	24.5 ± 0.17
phm>CG30054-RNAi	developmental delay (Yamanaka et al., 2015)	7	0.25 ± 0.03	24.8 ± 0.78
phm>CG17760-RNAi	Gαq protein (Yamanaka et al., 2015)	5	0.27 ± 0.04	24.2 ± 0.88
phm>egfr	precocious pupariation (Cruz et al., 2020)	4	Eclosion failure	Eclosion failure
phm>pten-RNAi	PG growth (Mirth et al., 2005)	5	0.24 ± 0.02	24.0 ± 0.36
phm>pi3k-RNAi	20-hydroxiecdysone titers (Caldwell et al., 2005; Colombani et al., 2005)	5	0.31 ± 0.03	24.4 ± 0.20
phm>InR-RNAi	ecdysone biosynthesis (Colombani et al., 2005)	3	0.25 ± 0.05	24.1 ± 0.21
dilp2-3, 5 null	nutritional input to the PG (Kannangara et al., 2021)	1621	0.16 ± 0.04	24.0 ± 0.44
dilp 7 null	insulin signaling (Kannangara et al., 2021)	2231	0.32 ± 0.03	24.0 ± 0.16
dilp 8 null	ecdysone biosynthesis (Garelli et al., 2012)	1023	0.39 ± 0.04	24.5 ± 0.25
lgr3 null	developmental delay (Colombani et al., 2015)	1145	0.40 ± 0.04	23.5 ± 0.28
lsp2>Δ901	fat body clock (Xu et al., 2008)	5	0.36 ± 0.03	24.3 ± 0.26

Periods were calculated using Autocorrelation analysis. (N) Total number of different experiment performed except for *dilp* and *lgr3* mutants in which (N)

indicates the number of flies emerged out from the pupal case; (SEM) standard error; (RI) rhythmicity index. Records are considered arrhythmic if RI<0.1.

Table S2. Sequence of PCR Primers used.

Oligonucleotides	Sequence of primer pair
torso forward primer	CCAGTGATCTCTTGCAGCTAC
torso reverse primer	AGTCTGTGTTTAAGGGCGG
rp49 forward primer	TGTGATGGGAATTCGTGGG
rp49 reverse primer	ATCTTGGGCCTGTATGCTG

Table S3. Primary antibodies used

Primary antibody	Procedure	Source	Dilution	Catalogue #/ Reference
Rat α-mCherry monoclonal 16D7	Double staining, BAcTrace	Invitrogen	1:1000	M11217
Rabbit α-GFP polyclonal	Double staining, BAcTrace, syb-GRASP, CaLexA	Chromotek	1:1000	PABG1
Guinea pig α-RFP polyclonal	*trans-Tango*	Meet Zandawala	1:10000	
Rabbit α-PTTH-polyclonal	Double staining, *trans-Tango*, CaLexA	this paper	1:10000	this paper
Guinea pig, α-PTTH polyclonal	Immunocytochemistry	Michael O'Connor	1:500	(McBrayer et al., 2007)
Rabbit α-GFP polyclonal	Immunocytochemistry	Thermofisher	1:1000	A11122

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DISCUSSION

Circadian rhythms help multicellular organisms adapt to environmental day/night cycles. In circadian clocks, multiple cellular signals are involved in imposing a daily rhythm to behavior and physiology. In this doctoral thesis, I reviewed the reciprocal relationship between Ca^{+2} signaling and the circadian clock, which is critical for many physiological and behavioral processes and its potential implications for chronomedicine (Chapter 1). On other hand, a variety of circadian behaviors in animals are mediated by a complex relationship between central and peripheral clocks. In *Drosophila*, the timing of adult eclosion is a circadian behavior that depends on a central clock located in the brain and a peripheral clock located in the PG. The work reported in Chapter 2 provides an extensive characterization of the cellular and molecular mechanisms that mediate the coupling between the brain and the PG clocks. In addition, I demonstrated that the PG exhibits autocrine signaling, which contributes to the rhythm of adult emergence.

Ca^{+2} signaling in the PTTHn mediates the transmission of time information

In many animals, Ca^{+2} activity plays a crucial role in propagating time information downstream from the central clock (Chapter 1)(Cavieres-Lepe and Ewer, 2021). In Chapter 2, I showed that Ca^{+2} levels exhibit a daily rhythm in the PTTHn at the white prepupal stage (WPP). The lowest levels of $[Ca^{+2}]$ occurred

in the middle of day, which coincides with the peak of neuropeptide release from the sLNv (Figure S1, Chapter 2). Since sNPF is an inhibitory Ca^{+2} signal in the PTTHn (Selcho et al., 2017), my findings suggest that the central clock sets the phase of Ca^{+2} oscillation in the PTTHn. These findings are consistent with those from others neural circuits downstream from the central clock. For instance, in flies, different groups of central pacemaker oscillators drive rhythmic Ca^{+2} activity in dopaminergic neurons and neuroendocrine cells, which are critical for imposing rhythms in behaviors such as sleep, feeding, mating, and locomotor activity (Liang et al., 2019; Liang et al., 2023). Similarly, in mammals, the central circadian pacemaker modulates, via vasoactive intestinal polypeptide-expressing (VIP) neurons, the rhythm of Ca^{+2} levels in corticotropin-releasing hormone (CRH) neurons, which are crucial for sustaining daily rhythms in glucocorticoid release in the adrenal gland (Jones et al., 2021). Therefore, my findings and those of others support the idea that the rhythmic Ca^{+2} activity in neural circuits downstream from the central pacemaker neurons is critical for circadian behavior and physiology.

I found that Ca^{+2} flux from the ER via SERCA and RyR in the PTTHn is relevant to the rhythm of emergence. This situation mirrors the situation for circadian behavior of locomotor activity in *Drosophila*, where a knockdown for ITPR, another ER Ca^{+2} channel, in PDF neurons caused strong arrhythmicity by modulating Ca^{+2} rhythms in these cells (Liang et al., 2022). How could SERCA and RyR expression in the PTTHn contribute to the circadian timing

mechanism? SERCA and RyR mediate opposite Ca^{+2} flux: SERCA controls the transport of Ca^{+2} from cytosol to the ER whereas RyR mediates the release of Ca^{+2} from the ER. One possibility is that SERCA and RyR expression contribute to the Ca^{+2} rhythms in the cytosol and consequently to the rhythm of PTTH accumulation in PTTHn terminals. Although this work does not provide mechanistic evidence for this scenario, it is known that the expression of IP3R in clock neurons in the adult fly brain is necessary to generate daily rhythms in basal Ca^{+2} levels (Liang et al., 2022). Future studies will be necessary to establish whether SERCA and RyR are relevant for the daily Ca^{+2} oscillations in the PTTHn.

The circadian clock regulates PTTH accumulation and signaling at different steps

A previous study from our laboratory showed that *ptth* transcript levels do not oscillate in the brain of animals prior to the emergence (Selcho et al., 2017). Here, I extended this observation to include PTTH levels in the soma of PTTHn in constant darkness, but importantly, I showed that PTTH immunoreactivity in PTTHn terminals is under circadian control. The timing disparity between PTTH levels in the soma vs. the terminals suggest that the circadian clock imposes a daily rhythm in PTTH accumulation via a post-translational mechanism, which may involve axonal transport or Ca^{+2} influx at the terminals. Interestingly, it has recently been shown that LNv clock neurons exhibit neuropeptide secretion from the soma with a different timing compared to neuropeptide secretion from the

terminals, revealing the existence of a compartmentalized mechanism that differentially regulates the neuropeptide release from the soma vs. the terminals (Klose et al., 2021). On other hand, my results show that PTTH accumulation in terminals and somatic Ca^{+2} rhythms have similar phases, suggesting a potential role for Ca^{+2} in imposing a daily oscillation in the PTTH release onto the PG. However, it is also possible that PTTH accumulation in terminals does not imply that neuropeptide is being released. Instead, high levels of PTTH immunoreactivity in the terminals may indicate that the neuropeptide has not yet been released, whereas the low levels seen at CT6 may indicate that the release has already occurred. This latter scenario is supported by the time course of phosphoERK levels in the PG, which are maximal early in the subjective day, suggesting that PTTH release onto the PG and consequently PTTH/TORSO signaling activation occurs at this time of day (Figure 1).

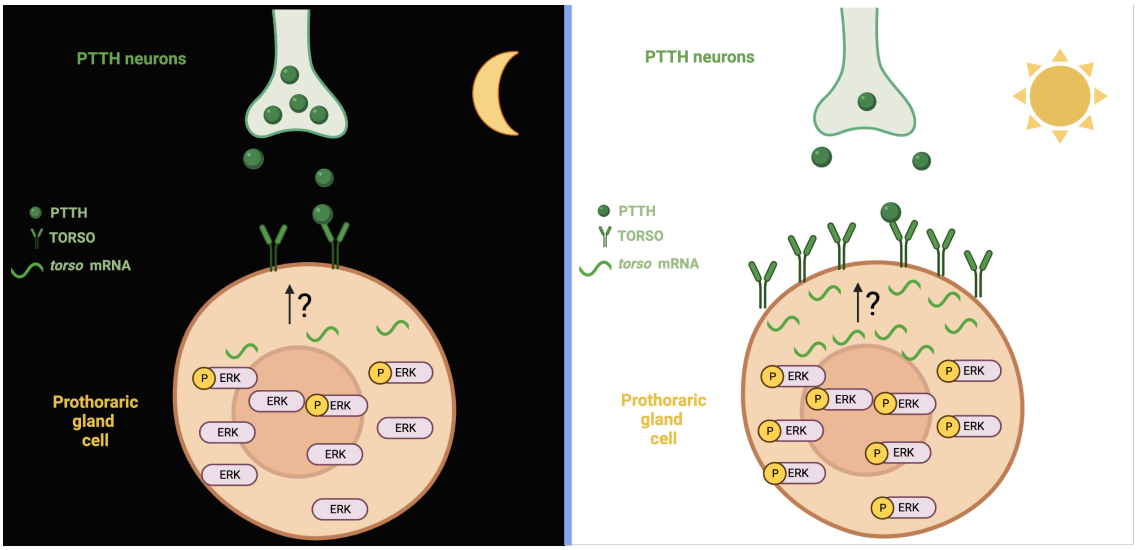

Figure 1. Temporal relationship between PTTH levels in PTTHn terminals, *torso* expression, and ERK phosphorylation levels in the PG. In the middle of the night (CT18, left panel), PTTH accumulates in the terminals and *torso* expression and phosphoERK levels in the PG reach their lowest levels. During the first hours of the day (CT6, right panel), PTTH accumulation in PTTHn terminals drops, which coincides with the high levels of *torso* expression in the PG. At the start of the day (CT2), phosphoERK levels reach maximum levels in the PG.

I showed that the transcript levels of PTTH receptor, *torso*, are under circadian control in the PG. This finding is consistent with a previous RNAseq study, which showed that insulin signaling modulates the expression of clock genes in the PG, which in turn regulate *torso* expression (Di Cara and King-Jones, 2016). Although I report here that *torso* expression is modulated by the circadian clock, my findings demonstrate that insulin signaling in the PG does not affect clock function. Rather, I show that the daily changes in *torso*

expression depend on the brain clock acting through PTTH. Indeed, high PTTH immunoreactivity levels in terminals are temporally coincident with low *torso* transcript levels in the PG and vice versa. This type of ligand-dependent transcriptional feedback control has been described for InR, another RTK family member, in which *Drosophila* and mammalian cells change their insulin sensitivity in response to nutrient availability (Puig and Tjian, 2005). Interestingly, sensitivity to PTTH in the PG changes during the course of the day in the bug, *Rhodnius prolixus* (Vafopoulou and Steel, 1999). Specifically, during the larval-adult development, the PG shows high responsiveness to PTTH around dusk, whereas it is largely unresponsive during the daytime. Future studies will be needed to elucidate whether a similar cycling mechanism is present in *Drosophila.*

Three RTK signaling converge onto the PG to impose a daily rhythm to the emergence

This thesis reveals that, in addition to TORSO, expression in the PG of two other RKTs (ALK and PVR) contribute to the rhythmicity of the emergence. Since the ALK ligand (Jeb) is expressed in the PTTHn (Pan and O'Connor, 2021), it is likely that this peptide is co-released with PTTH under the same signaling

pathway regulated by the circadian clock. Notably, I demonstrated that the rhythm of emergence is also modulated by Pvf2/Pvr signaling via autocrine activity within the PG, suggesting that the proper circadian function of the PG includes a component that is autonomous to this endocrine gland. This autonomous role is consistent with a previous study using cultured PGs that revealed that rhythmic nuclear expression of TIM, but not of PER, is independent of the central nervous system (Morioka et al., 2012). One potential relationship between these findings is that TIM regulates the expression of PVR, and consequently, the rhythmicity of the ERK signaling pathway. Although this thesis does not provide mechanistic evidence for this scenario, an RNAseq study revealed that *pvr* expression is under circadian control in LNv and LNd in the adult fly brain (Abruzzi et al., 2017), suggesting that the expression of PVR is downstream of the core clock components. Importantly, the autonomous role of the PG may explain why two functional clocks in the brain and in the PG are necessary for the circadian gating of emergence.

According to previous findings (Selcho et al., 2017) and also this doctoral thesis, the circadian control of adult emergence is modulated by three RTK in the PG: TORSO, PVR, and ALK (Figure 2). Importantly, the signal transduction of these receptors is mediated by the ERK pathway (Pan and O'Connor, 2021) raising the question of how three timed signals are integrated into a common signaling pathway. Knockdown of *torso* in the PG leads to an arrhythmic pattern of eclosion, whereas knockdown of Alk/Pvr weakens rhythmicity, suggesting that

PTTH/TORSO signaling is a major activator of ERK pathway, whereas JEB/ALK and PVF2/PVR play a secondary role. This idea is also supported by the finding that reducing TORSO expression in the PG abolishes the maximum and minimum peak in phosphoERK levels during the course of the day.

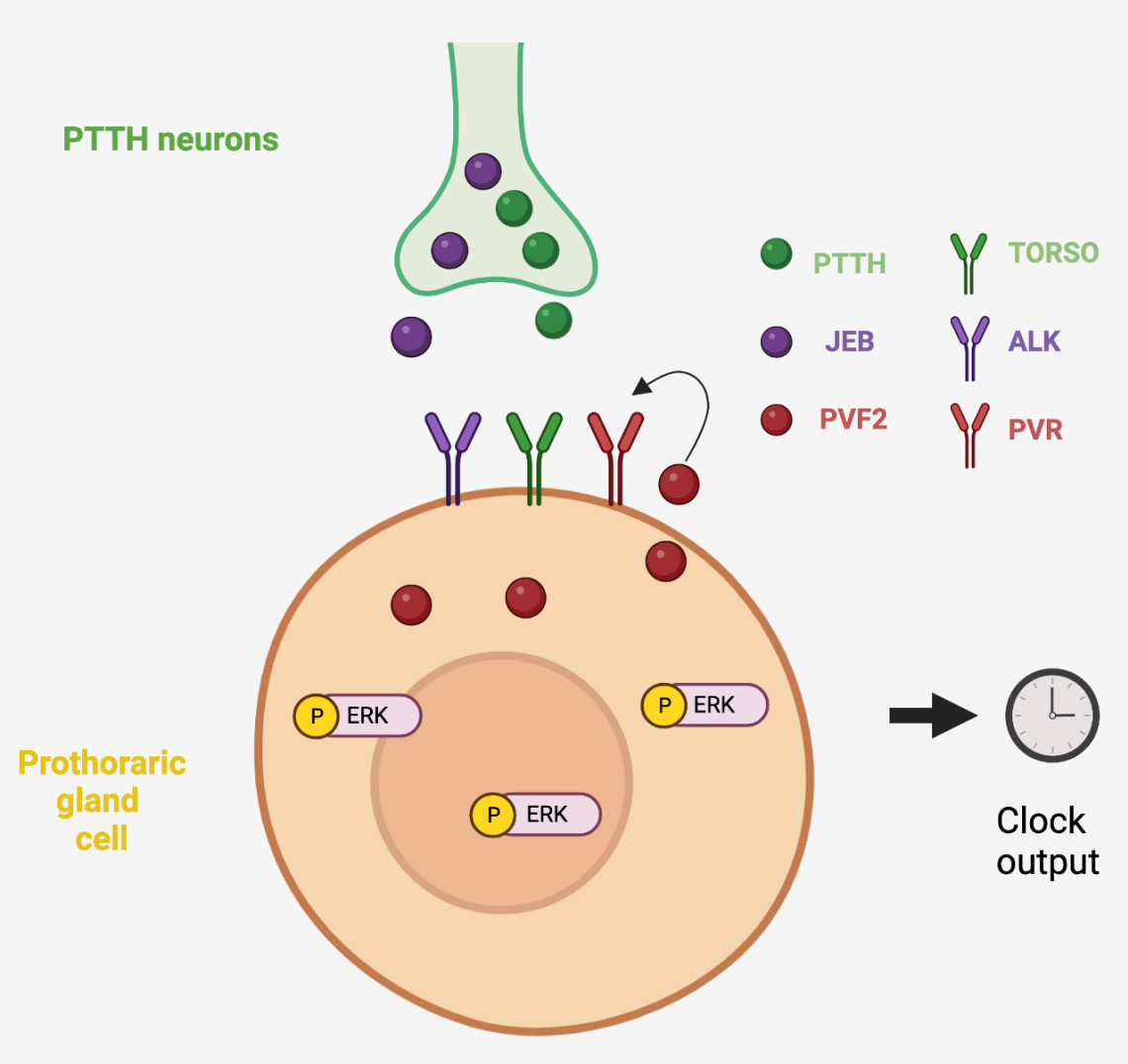

Figure 2. Three RTK signaling pathways converge onto the PG to impose a daily rhythm to the timing of emergence. PTTH and JEB are released by the PTTHn and bind to their receptors, TORSO and ALK respectively, in the PG. PVF2 is produced by the PG, and acts in an autocrine manner, binding to its receptor, PVR. These three RTKs modulate the rhythm of emergence via the ERK pathway, with the PTTH/TORSO axis serving as the major regulator of this circadian behavior.

Although our characterization of the multiple signals that converge in the PG to impose a daily rhythm on emergence is extensive, many question remain. For instance, it is currently unclear whether PG cells respond uniformly to PTTH, JEB, and PVF2 or if there is a spatial pattern of activation among them. Interestingly, it was recently reported that the nuclear size of PG cells located near PTTHn terminals is larger than that of non-innervated PG cells, indicating that PTTH acts autonomously to control PG cell size (Shimell et al., 2018). However, it is currently unknow whether the spatial distribution of PG cells relative to PTTHn terminals is relevant for circadian function. In addition, it will be important to determine whether PG cells communicate with each other. The intercellular coupling of the network is a critical component of circadian oscillators that integrate multiple timed signals. For instance, Ca^{+2} levels and *per* expression exhibit a topological organization throughout the entire central mammalian clock (Enoki et al., 2012). Notably, this topological organization is dependent on the intrinsic communication of the SCN cells, which is mediated by the vasoactive intestinal peptide (Brancaccio et al., 2013). Interestingly, intercellular bridges have been described in *Manduca sexta* PGs (Dai et al., 1994), but it is unknown if these structures are present in the *Drosophila* PG and if they allow the spread of signaling molecules between neighboring cells. Finally, it is not known how ERK phosphorylation levels modulate clock function in the PG. In terms of development, it has been described that the nuclear receptor, DHR4, is a mediator between PTTH signaling and ecdystereoid biosynthesis (Ou et al., 2011). The authors describe that DHR4 acts as a

repressor in the promoter regions of genes associated with ecdysone biosynthesis in the PG. However, in presence of PTTH, phosphoERK translocates into the nucleus, which eventually displaces DHR4 into the cytoplasm, and consequently, derepresses ecdysone biosynthesis. Recently, our lab demonstrated that the knockdown of *dhr4* in the PG eliminated the rhythmic pattern of emergence (Mark et al., 2021) suggesting that phosphoERK levels could impose a daily rhythm to the subcellular localization of DHR4 and modulate clock function. However, future studies will be needed to test this hypothesis.

Brain and PG clocks as a paradigm to study the circadian control glucocorticoid production in mammals

Our detailed characterization of the transmission of time information from the brain clock to the PG clock is reminiscent of the control of the circadian rhythm in the glucocorticoid (GC) production in mammals. In this case, the central clock in the SCN controls the activity of corticotropin-releasing hormone (CRH)-producing neurons to promote the release of adrenocorticotropin hormone (ACTH) from the pituitary. In turn, ACTH induces the production of GC in the peripheral clock of the adrenal gland (Lightman and Conway-Campbell, 2010). Under non-stress conditions, the central clock is one of the modulators of rhythmic Ca^{+2} activity in CRH neurons and of corticosterone release (Jones et al., 2021). However, is not well established how this time information is decoded

by the pituitary gland and, in turn, how this signal is propagated to the peripheral clock in the adrenal gland. On the other hand, the adrenal gland exhibits a daily change in its sensitivity to ACTH. This circadian regulation seems to have autonomous (adrenal gland)(Oster et al., 2006) and non-autonomous (central clock)(Engeland and Arnhold, 2005) components. Therefore, the mechanisms underlying the circadian release of GC in mammals are complex but surprisingly analogous to the circadian pathway that modulates the rhythm of adult emergence in *Drosophila*. Consequently, this doctoral thesis may provide valuable knowledge for understanding how circadian rhythmicity of GC is produced in mammals.

CONCLUSION

1. $[Ca^{+2}]_I$ levels in PTTHn are under circadian control.
2. Expression of SERCA and RyR in the PTTHn is relevant for the rhythm of adult emergence.
3. At the WPP stage, there is a rhythm of neuropeptide accumulation in sLNv terminals.
4. There is a circadian regulation of PTTH immunoreactivity in the PTTHn terminals onto the PG.
5. *torso* transcript levels in the PG exhibit circadian regulation that is dependent on PTTH.
6. ERK phosphorylation in the PG shows a PTTH-dependent daily rhythm.
7. The nuclear localization of phosphorylated ERK in the PG exhibits a daily rhythm
8. *Alk* and *Pvr* in the PG contribute to the circadian rhythmicity of adult emergence
9. In the PG, Pvf2 acts in an autocrine manner to modulate the rhythm of emergence.
10. Jeb expression in the PTTHn contributes to the rhythm of emergence.
11. AstA and Crz signaling in the PTTHn are not relevant for the circadian control of emergence.
12. In PG, insulin signaling and two G proteins (CG17760 and CG30054) do not contribute to the circadian control of emergence.

13. The fat body clock is not involved in the circadian control of emergence.

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