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Regulation of sleep and circadian rhythms by TARANIS

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Trabalho efectuado sobre a orientação da
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e da
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STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 04/01/2016

Assinatura: Dinis José Silva Afonso

"If every single man and woman, child and baby, acts and conducts itself in a known pattern and breaks no walls and differs with no one and experiments in no way and is not sick and does not endanger the ease and peace of mind or steady unbroken flow of the town, then that unit can disappear and never be heard of."

In "The Pearl" of John Steinbeck

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Resumo

O sono é essencial à vida, e quando perturbado tem um efeito extremamente prejudicial na saúde humana. Apesar do crescente reconhecimento da importância do sono, não há muito para oferecer aos pacientes que sofrem de distúrbios do sono. Os tratamentos atuais não produzem os mesmos resultados que o sono normal satisfaz, são altamente inespecíficos e rapidamente tolerados pelos doentes. Encontrar mecanismos reguladores do sono, certamente melhorará os padrões de qualidade de vida.

Neste trabalho em *Drosophila*, identificámos TARANIS (TARA), um gene em homólogo da família de coreguladores de transcrição Trip-Br (SERTAD), necessários para os padrões normais de sono. Através de um rastreio genético, isolámos mutantes de *tara* com uma redução marcada na quantidade de sono. *tara* codifica uma proteína do ciclo celular que contém um domínio conservado de ligação à Ciclina-A (CycA). TARA regula os níveis da proteína CycA e interage genetica e fisicamente com a CycA para promover o sono. Além disso, a diminuição dos níveis da proteína Cinase dependente da Ciclina 1 (Cdk1), um parceiro da CycA, resgata o fenótipo de sono nos mutantes de *tara* e *CycA*, enquanto o aumento da actividade de Cdk1 reproduziu os fenótipos de *tara* e *CycA*, o que sugere que a proteína Cdk1 medeia o papel de TARA e CycA na regulação do sono.

Além do papel na regulação do sono, os mutantes *tara* apresentam um comportamento locomotor arrítmico em condições de escuridão constante (DD). Apesar dos mecanismos pelos quais a TARA regula o comportamento circadiano serem desconhecidos, mostrámos que TARA regula a velocidade do

oscilador molecular circadiano e o débito neuropeptidico dos neurónios pacemaker. À semelhança dos mutantes *Clock* (*Clk*), os mutantes *tara* exibem um padrão de atividade noturna em condições dia/noite (LD). Além disso, a abolição da expressão de *tara* ou a sua sobreexpressão nos neurónios sLN_vs alteram a morfologia da sua projeção dorsal. Em conjunto, estes resultados sugerem que a TARA é importante em vários passos que comunicam as oscilações moleculares para o comportamento locomotor circadiano.

No presente trabalho, descrevemos uma nova via genética que controla o sono em *Drosophila*. Esta via genética é conservada em mamíferos e tem o potencial de regular o sono em seres humanos, por um mecanismo semelhante. Além disso, neste trabalho desvendámos outros fenótipos sob o controlo da TARA, que podem ser usados para projectar experiências futuras direccionadas à compreensão da morfologia neuronal e dos padrões diários de vigília/sono.

Abstract

Sleep is essential for life, and when disturbed has a deleterious effect in human health. Despite the increased awareness of the importance of sleep, not much can be offered to patients suffering from sleep disorders. The current treatments do not produce the same results as real sleep does, are highly unspecific, and quickly tolerated by patients. Finding novel sleep regulatory mechanisms will certainly improve the quality of life.

In this work, we identify TARANIS (TARA), a *Drosophila* homolog of the Trip-Br (SERTAD) family of transcriptional coregulators, as a molecule that is required for normal sleep patterns. Through a forward-genetic screen, we isolated *tara* as a novel sleep gene associated with a marked reduction in sleep amount. *tara* encodes a conserved cell-cycle protein that contains a Cyclin A (CycA)-binding homology domain. TARA regulates CycA protein levels and genetically and physically interacts with *CycA* to promote sleep. Furthermore, decreased levels of Cyclin dependent kinase 1 (Cdk1), a kinase partner of CycA, rescue the short-sleeping phenotype of *tara* and *CycA* mutants, while increased Cdk1 activity mimics the tara and CycA phenotypes, suggesting that Cdk1 mediates the role of TARA and CycA in sleep regulation.

In addition to the role in the regulation of sleep, *tara* mutants exhibit arrhythmic locomotor behavior in constant darkness (DD). However, the mechanisms through which TARA regulates circadian behavior are unknown. Here, we show that TARA regulates the speed of the molecular oscillator and the neuropeptide output of the pacemaker neurons. Similarly to *Clock* (*Clk*) mutants, *tara* mutants display a nocturnal pattern of activity in light:dark

conditions (LD). Furthermore, *tara* knockdown or overexpression in sLN_vs alters the morphology of their dorsal projection. Taken together, our data suggest that *tara* functions in multiple steps that link molecular cycling to overt circadian locomotor behavior.

In these work, we describe a novel genetic pathway that controls sleep in *Drosophila*. This genetic pathway may be conserved in mammals and holds the potential to regulate sleep in humans as well. Furthermore, in this work we unravel other phenotypes under the control of *tara*, which can be used to design future experiments aimed at understanding neuronal morphology and the daily patterns of wakefulness/sleep.

Contents

Abbreviations

GABA – γ-aminobutyric acid

REM – Rapid eye movement

NREM – Non-rapid eye movement

EEG – Electroencephalography

DAM – *Drosophila* Monitoring System

per – *period*

Pdf – *Pigment dispersing Factor*

Gal4 – Transcription factor derived from yeast

UAS – Upstream Activating Sequence. GAL4 binds UAS to initiate transcription of downstream sequences

LexA – Transcription factor derived from bacteria

LexAop – LexA operator. LexA binds LexAop sequence

CycA – *Cyclin-A*

cAMP – Cyclic adenosine monophosphate

CLK – CLOCK

CYC – CYCLE

timeless – *tim*

sLN_v – small ventral lateral neuron

lLN_v – large ventral lateral neuron

LNd – dorsal lateral neuron

DN – dorsal neuron clusters (DN1, DN2, and DN3)

LPN – lateral posterior neuron

DD – constant darkness conditions

LD – light and dark conditions

LL – constant Light conditions

CREB – cAMP response element binding protein

CRY – CRYPTHOCHROME

tara – taranis

PHD – Plant homeo domain

TRIP-Br – Transcriptional Regulators Interacting with PHD zinc fingers and/or Bromodomains

Cdks – Cyclin-dependent kinases

PL – *pars lateralis*

MB – Mushroom bodies

PI – *pars intercerebralis*

NaChBac – Bacterial Sodium Channel

TrpA1 – Warm activated cation channel

Cdk1-AF – Cdk1 mutant with elevated kinase activity due to mutations in inhibitory phosphorylation sites

TNT-Imp – Inactive form of tetanus neurotoxin

TNT-G – Active form of tetanus neurotoxin

ZT – Zeitgeber time

CT – Circadian time

Ub-tara – Ubiquitous promoter upstream of *tara*

DAT^{fmn} – Dopamine transporter mutant

PP2A – Protein Phosphatase 2A

Chapter 1

Introduction

I. Importance of sleep in health and disease

The brain is complex and generates a wide range of behaviors. Of all the behaviors required for survival, sleep is the most time-consuming, and its loss has many deleterious effects on human physiology [1, 2]. Sleep deprivation disrupts metabolism, increasing glucose intolerance and insulin resistance [3], and shift workers have higher rates of cancer [4]. Sleep deprivation also decreases intellectual ability and motor function, greatly increasing the risk of fatal accidents for sleep deprived drivers. It is estimated that 16.5% of all fatal motor vehicle crashes in the United States are due to drowsy drivers [5]. Finally, sleep disturbances are also highly prevalent in patients with psychiatric diseases, including anxiety, depression, obsessive compulsive disorder and intellectual disability [6, 7], and are an early sign of neurodegenerative diseases in Parkinson's [8] and Alzheimer's [2] diseases. Importantly, sleep loss is not simply a symptom of these diseases but a major risk factor in their cause and/or perpetuation [2, 6, 9]. Despite being important for almost all aspects of life, the reasons why we sleep and how sleep is regulated remain largely unknown [10, 11].

While the functions of sleep are not yet clear, several theories have been proposed [12]. First, increasing evidence suggests that sleep is important for memory consolidation and synaptic plasticity [12]. In humans, learning a task leads to an increase in synaptic strength (cortical evoked field potentials) that is followed by a higher slow-wave activity (marker of sleep need) in the subsequent sleep period [12]. Evidence from mice also shows a net increase in synaptic spines during wake and a net decline during sleep [13]. These data are consistent with a theory of sleep function called the synaptic homeostasis hypothesis, which postulates that, during

sleep, synapses are downscaled to renormalize neural connectivity [12]. Second, lower energy consumption during sleep is also viewed as a function of sleep [12]. The brain is the most energy-demanding organ in the body, requiring 25% of consumed glucose [12]; however, during slow-wave sleep the energetic demands of the brain are reduced by 25-44% [14]. Finally, a recently proposed function for sleep is brain detoxification where, during sleep, the brain removes waste products to improve cellular homeostasis. Work in rats has demonstrated that during sleep the interstitial space increases by 60%, which leads to a higher exchange of cerebrospinal fluid, improving the removal of potential neurotoxic byproducts that accumulated during wakefulness [15]. Given the evidence supporting these various hypotheses, it is possible that sleep serves multiple functions.

That sleep is essential for life and that its disorders must be treated is evident. However, the currently available treatments for sleep are highly unsatisfactory. The widely used drugs in the treatment of insomnia, benzodiazepines, are unspecific and have a high risk of dependence [16]. Benzodiazepines bind to $GABA_A$ receptors and potentiate the postsynaptic response to γ-aminobutyric acid (GABA), decreasing neuronal activity globally. However, reducing neuronal activity is not the same as inducing sleep, as the brain electrical activity during rapid eye movement (REM) sleep is as high as during wakefulness [17]. In addition, under the influence of the benzodiazepines, the overall sleep architecture is altered, with less REM sleep and less N3 non-rapid eye movement sleep (NREM) sleep (slow-wave sleep). The current limitations of the available treatments for sleep disorders highlight the need for the discovery of new sleep regulatory mechanisms that can be used as novel drug targets. In addition, determining the molecular mechanisms of sleep may

contribute to our understanding of related processes including memory and cognition.

II. Sleep research across species

Sleep is conserved from flies to humans [18] and *Drosophila* shares with humans several important features of sleep [19, 20]. During sleep, flies become immobile and adopt a specific sleep posture. Similar to humans, flies sleep mostly at night and exhibit a compensatory response to sleep deprivation. In addition, drugs that promote wakefulness in humans such as caffeine [19], modafinil [21], and amphetamine [22], also promote arousal in flies, suggesting that the underlying regulatory mechanisms may be conserved from flies to humans.

In humans, sleep is defined on the basis of brain electrical activity as detected by electrodes placed on the scalp. Polysomnographic profiles identify two states of sleep: REM and NREM sleep. NREM sleep is further subdivided into three stages, N1 to N3, characterized by increasing arousal thresholds and slowing of cortical electroencephalographic (EEG) activity. Sleep in *Drosophila* is typically monitored using the *Drosophila* Monitoring System (DAM) or video recording, which allows automated quantification of locomotor activity [23, 24]. After 5 min of immobility, flies exhibit increased arousal thresholds, which provides the rationale for the commonly accepted definition of sleep as immobility lasting 5 min or longer [19].

In contrast to human research, which is expensive with significant technical and ethical limitations, *Drosophila* enables the use of sophisticated genetic tools that, combined with a fast life cycle and easy handling, allow systematic and unbiased screens for new genes, neurons, and circuits. Mutational analysis and targeted manipulations of neuronal excitability have the potential to uncover novel signaling pathways and regulatory mechanisms that underlie a specific process or behavior. Furthermore, *Drosophila* has the advantage of less genomic redundancy and

compensation [25]. A simpler genome is useful because a single genetic alteration is more likely to produce a phenotype that can be observed and quantified; it is a much easier entry point for new signaling pathways. Importantly, *Drosophila* has functional orthologs of ~75% of the human disease-related genes [26]. Therefore it is not surprising *Drosophila* is gaining momentum in the study of various human diseases, ranging from intellectual disability [7], depressive syndromes and bipolar disorders [27] to cardiomyopathies [28], and neurodegenerative diseases, including Parkinson's, Alzheimer's, and amyotrophic lateral sclerosis [29].

The genetic tools provided by the fruit fly *Drosophila melanogaster* allow the dissection of complex and largely unknown behaviors such as sleep. Forward genetic screens allow unbiased approaches to be directed at the discovery of new regulatory mechanisms and signaling pathways underlying complex processes. In forward genetic screens, random, genome-wide mutations are systematically screened in search of a phenotypic alteration of interest. Perhaps one of the most significant contributions to behavioral neuroscience was the discovery of *period* (*per*) mutants in 1971 through a forward genetic screen [30]. This discovery was remarkable because it caused a dramatic change in our view of how genes control behaviors. Until then scientists were resistant to accept that a single gene would by itself control a behavior, claiming "genetic architecture of behavior is complex and multigenic" [31], and were highly skeptical of fly studies [32, 33]. The discovery of *per* mutants and the work that followed led to the discovery of the molecular clock, highlighting the power of the genetic tools available in *Drosophila*.

In a little over 100 years, research in *Drosophila* has contributed tremendously to the understanding of vertebrate neuroscience [34]. The fly community has developed a wide variety of tools ranging from libraries of *Drosophila* mutants with

genome-wide random transposon insertions to binary gene expression systems including Gal4/UAS [35], derived from yeast, and LexA/LexAop [36, 37], derived from bacteria. Binary systems are composed of a transcription factor and a DNA sequence to which the transcription factor binds, leading to the expression of whatever sequence is downstream. The binary systems are powerful genetic tools that allow targeted alterations of gene expression in living organisms, with spatial and temporal control. The phenotypic consequences of these specific manipulations can be monitored through high-throughput and automated behavioral paradigms.

Since the first description of a sleep state in *Drosophila* in 2000 [19, 20], the field has identified a number of genes that play important roles in sleep. The functions of the diverse array of genes include: neurotransmission (dopamine*,* octopamine, serotonin, and GABA pathway genes) [38-42], neuropeptide signaling (*diuretic hormone 31*, *Pigment-dispersing factor* (*Pdf*), *short neuropeptide F precursor, Sex peptide receptor*) [43-45], regulation of neuronal excitability (*Shaker*, *quiver*, *wide awake*, and *nicotinic Acetylcholine Receptor α4*) [46-49], protein degradation (*insomniac*) [42, 50], control of cell cycle progression (*Cyclin-A* (*CycA*) and *Regulator of Cyclin-A*) [51], intracellular signaling (*protein kinase A*, *cAMP response element binding protein* (*CREB*), *crossveinless c*) [52-55], synaptic structure (*Neurexin 1*, *Neuroligin 4*, *Fmr1*) [56-58], and chromatin remodeling (*Tat interactive protein 60kDa*) [59]. Given the variety of genes involved, it is important to understand how these genes interact with each other to regulate sleep and not simply how each by itself affects sleep.

The mechanistic insights gained in *Drosophila* may help elucidate the molecular basis of sleep regulation in vertebrates. *Shaker*, one of the first genes identified in sleep regulation, mediates a voltage-activated fast-inactivating I_A current

that has a major role in membrane repolarization in vertebrates [60]. Importantly, mice lacking the closest mammalian homolog of *Shaker*, the *Kv1.2* channel, also sleep less [61]. Another important insight gained in *Drosophila* that was successfully translated to mammals was the identification of the cyclic adenosine monophosphate (cAMP)-CREB pathway that is important for learning, memory, and intellectual ability. Initial work in *Drosophila* identified *rutabaga* (*rut*), which encodes a Ca^{2+}/calmodulin-activated adenylyl cyclase [62] and *dunce* (*dnc*), which encodes a cAMP-specific phosphodiesterase [63]. Remarkably, several genes underlying intellectual disability, which is a common neurodevelopmental disorder affecting 3% of the population [64], disrupt the cAMP-CREB pathway [7]. Furthermore, most genes affected by sleep deprivation have a cAMP-responsive element [65], and sleep deprivation impairs cAMP-CREB signaling [66], which may explain the memory deficits observed with sleep loss [67].

III. Circadian regulation of sleep-wake rhythms

Sleep is regulated by two main mechanisms [18]: a circadian mechanism that concentrates sleep to a specific period of the day and a homeostatic mechanism that controls sleep amount. Life on earth has evolved to anticipate recurring changes in light and temperature driven by earth's spin. Animals have adapted their behavior to these changes, and sleep is one of the most prominent behaviors that exhibit circadian rhythms. The circadian control of behavior is mediated through complex molecular oscillators that can maintain daily cycling even in constant environmental conditions. Interestingly, these molecular mechanisms are highly conserved across evolution, highlighting its selective advantage.

At the core of the molecular oscillators are transcriptional-translational feedback loops, where transcriptional activators drive the expression of their own repressors. In *Drosophila*, the expression of the *per* and *timeless* (*tim*) transcripts are driven by CLOCK (CLK) and CYCLE (CYC), and PER and TIM suppress the transcriptional activity of CLK and CYC. Additional feedback loops, post-transcriptional regulations, and post-translational modifications contribute to the precise time course of molecular oscillations [23]. These molecular oscillations take place in ~150 neurons in the adult *Drosophila* brain which can be divided into several symmetric clusters of distinct anatomy and physiology: the small and large ventral lateral neurons (sLN_v and lLN_v, respectively), the dorsal lateral neurons (LNd), three dorsal neuron clusters (DN1, DN2, and DN3) and the lateral posterior neurons (LPN) [23]. The sLN_vs are necessary and sufficient to drive circadian locomotor rhythms in constant darkness conditions (DD) [68, 69]. In light and dark conditions (LD), the sLN_vs drive the morning peak of activity while the LNds and the 5^{th} sLN_v drive the

evening peak [68, 69]. The DN1 neurons receive input from the sLN_vs and integrate information about light and temperature [70, 71]. The lLN_vs communicate with the sLN_vs through PDF and are waking promoting cells. The function of the DN2 and DN3 clusters is less well understood.

An important feature of the circadian mechanism is its ability to synchronize itself to the external environmental conditions. The circadian mechanism can be divided into three components: the input pathway that relays information from the external environment to the core molecular clock machinery, the molecular clock itself, and the output pathway that translates the molecular clock oscillations to overt behaviors and physiologic functions. The input pathway and the core molecular machinery are fairly well dissected [72, 73]. Briefly, the entrainment of the *Drosophila* clock to light happens through the light-induced degradation of TIM [74-76], that is transmitted to TIM through the blue-light photoreceptor CRYPTHOCHROME (CRY) [77]. TIM and CRY are degraded by the proteasome in response to light, and the E3 ubiquitin ligase, JETLAG, mediates the light-dependent degradation of TIM [78]. Much less is understood about the output pathway and it remains unclear how circadian signals are integrated with other signals to control behavior.

In mammals, the molecular timekeeper is remarkably similar to that of *Drosophila*. Mammalian CLOCK and BMAL1 positively regulate the mammalian *period* genes (*per1*, *per2*, and *per3*) and the *Cryptochrome* genes (*Cry1* and *Cry2*). PER and CRY proteins dimerize, form a complex and translocate to the nucleus, repressing the transcriptional activity of CLOCK and BMAL1 [79]. In mammals, the suprachiasmatic nucleus, composed of ~20,000 neurons, acts as a master pacemaker for circadian rhythms.

Peripheral clocks have been observed throughout the body of mammals [80], and it is estimated that ~10% of their genes have circadian expression [81, 82]. Therefore, it is not surprising that the circadian mechanism contributes to many aspects of health and disease. Unstable angina, myocardial infarction, and sudden cardiac death have been reported to occur more frequently in the morning within a few hours of awakening [83], and it has been postulated that circadian genes have a major role in synchronizing cardiomyocyte metabolic activity. Asthma symptoms are higher at night and the degree of bronchoconstriction of the airway tree has a circadian profile [84]. Moreover, patients with bipolar disease have altered circadian rhythmicity and lithium therapy appears to be beneficial through the resynchronization of circadian rhythms [85]. It is likely that *Drosophila* studies will continue to enlighten our understanding of how sleep and circadian rhythms impact health and disease.

IV. *taranis*, cell cycle genes, and sleep regulation

Through a forward genetic screen, we discovered a novel mutant with reduced sleep amount and disrupted rhythmicity in locomotor activity. Genotypic mapping of this new mutant revealed that the locus of the previously identified *taranis* (*tara*) gene was affected. Previous work described TARA as a transcriptional co-regulator [86]. *tara* encodes two isoforms, TARA-A and TARA-B, that are functionally interchangeable; both isoforms have several evolutionarily conserved domains: a CYCLIN-A-binding homology domain, a SERTA motif with unknown function, a plant homeo domain (PHD)-bromo binding domain, and a C-terminal domain implicated in transcriptional regulation [86]. More recently, *tara* was demonstrated to play a role in neuronal development through its interaction with E2f/Dp1 cell cycle regulators [87]. A recent study has proposed that *tara* has a role in tissue regeneration by preventing other signaling pathways to alter the fate of the regenerating cells [88], while another recent study showed that *tara* is upregulated in the *Drosophila* gut after pathogen-induced wounds [89]. However, no behavioral role has previously been assigned to *tara*.

Mammalian homologs of TARA, the TRIP-Br (Transcriptional Regulators Interacting with PHD zinc fingers and/or Bromodomains) family of proteins, also play important roles in cell cycle control through their interaction with E2f/Dp1 [90] and direct binding of Cyclin-D/Cdk4 [91], which shows *tara* functions are conserved from flies to humans. Importantly, additional roles have been described for Trip-Br molecules including: regulation of lipolysis by Trip-Br2 [92] and function of pancreatic β-cells byTrip-Br1 [93]. However, it is not known whether the *tara* mammalian homologs regulate sleep or circadian rhythms.

TARA and its mammalian homologs have a CycA-binding homology domain, which is relevant given that *CycA* and its regulator *Rca1* control sleep in *Drosophila* [51]. These observations suggest that *tara* and *CycA* may work in the same molecular pathway to regulate sleep. Cyclins regulate cell cycle progression through its modulation of cyclin-dependent kinases (Cdks). Previous work showed that CycA can physically interact with Cdk1 [94] which also raises the possibility that *Cdk1* may have a role in sleep as well. Importantly, it has been shown that cell cycle regulators have additional functions in adult neurons. *Cyclin E*, for instance, plays a role in memory formation and synaptic plasticity [95]. In addition, *Cdk4* and *Cyclin-B* knockdown in *bas*^{1} (*bang-sensitive*^{1}) mutants rescue duration of seizures [96] suggesting these cell-cycle regulators also modulate ion channel activity in the adult *Drosophila* brain. In humans with temporal lobe epilepsy, *Cyclin-B1* is upregulated in the hypothalamus [97], further demonstrating a role beyond cell cycle control.

V. Working hypothesis and aims

Several genes involved in sleep regulation have been found through genome-wide forward genetic screens [46, 47, 51]. *tara* was found in a forward-genetic screen for short-sleeping mutants. The overall goal of this thesis project was to investigate the role of *tara* in sleep and in circadian behaviors.

Specific aims:

1. **Characterize sleep and circadian rhythms in *tara* mutants;**
2. **Identify the anatomical *loci* in which *tara* regulates sleep and rhythm behavior;**
3. **Identify the molecular pathways in which TARA regulates sleep and circadian rhythms.**

In here, we describe how *tara* regulates sleep and circadian rhythms through distinct molecular mechanisms in separate neuronal populations. In chapter 2.1 we describe a novel role for *tara* in sleep regulation through its interaction with *CycA* and *Cdk1* in a novel arousal center. In chapter 2.2 we describe a role for *tara* in circadian regulation through its modulatory action over the intrinsic speed of the molecular clock and PDF levels. Chapter 3 presents an overall discussion of the results with future perspectives.

VI. References

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Chapter 2

Experimental work

Chapter 2.1

TARANIS functions with Cyclin A and Cdk1 in a novel arousal center to control sleep in *Drosophila*

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Article

Current Biology

TARANIS Functions with Cyclin A and Cdk1 in a Novel Arousal Center to Control Sleep in *Drosophila*

Highlights

- *tara* is a novel sleep-regulatory gene in *Drosophila*
- TARA regulates CycA levels and interacts with CycA to control sleep
- TARA promotes sleep in CycA-expressing PL neurons, a novel arousal center
- Cdk1 interacts with *tara* and CycA and acts in PL neurons to suppress sleep

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In Brief

The molecular and neural mechanisms of sleep regulation are not well understood. Afonso et al. show that TARANIS promotes sleep by regulating CycA protein levels and inhibiting Cdk1 activity in a novel arousal center.

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Article

TARANIS Functions with Cyclin A and Cdk1 in a Novel Arousal Center to Control Sleep in *Drosophila*

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SUMMARY

Sleep is an essential and conserved behavior whose regulation at the molecular and anatomical level remains to be elucidated. Here, we identify TARANIS (TARA), a *Drosophila* homolog of the Trip-Br (SERTAD) family of transcriptional coregulators, as a molecule that is required for normal sleep patterns. Through a forward-genetic screen, we isolated *tara* as a novel sleep gene associated with a marked reduction in sleep amount. Targeted knockdown of *tara* suggests that it functions in cholinergic neurons to promote sleep. *tara* encodes a conserved cell-cycle protein that contains a Cyclin A (CycA)-binding homology domain. TARA regulates CycA protein levels and genetically and physically interacts with CycA to promote sleep. Furthermore, decreased levels of Cyclin-dependent kinase 1 (Cdk1), a kinase partner of CycA, rescue the short-sleeping phenotype of *tara* and *CycA* mutants, while increased Cdk1 activity mimics the *tara* and *CycA* phenotypes, suggesting that Cdk1 mediates the role of TARA and CycA in sleep regulation. Finally, we describe a novel wake-promoting role for a cluster of ~14 CycA-expressing neurons in the *pars lateralis* (PL), previously proposed to be analogous to the mammalian hypothalamus. We propose that TARANIS controls sleep amount by regulating CycA protein levels and inhibiting Cdk1 activity in a novel arousal center.

INTRODUCTION

Most animals sleep, and evidence for the essential nature of this behavior is accumulating [1–3]. However, we are far from understanding how sleep is controlled at a molecular and neural level. The fruit fly, *Drosophila melanogaster*, has emerged as a powerful model system for understanding complex behaviors such as sleep [4, 5]. Mutations in several *Drosophila* genes have been identified that cause significant alterations in sleep [5–13]. Some of these genes were selected as candidates because they were implicated in mammalian sleep [10, 11]. However, others (such as *Shaker* and *CREB*) whose role in sleep was first discovered in *Drosophila* [6, 12] have later been shown to be involved in mammalian sleep [14, 15], validating the use of *Drosophila* as a model system for sleep research. Since the strength of the *Drosophila* model system is the relative efficiency of large-scale screens, we and other investigators have conducted unbiased forward-genetic screens to identify novel genes involved in sleep regulation [6–9, 16]. Previous genetic screens for short-sleeping fly mutants have identified genes that affect neuronal excitability [6, 7], protein degradation [9, 16], and cell-cycle progression [8]. However, major gaps remain in our understanding of the molecular and anatomical basis of sleep regulation by these and other genes.

Identifying the underlying neural circuits would facilitate the investigation of sleep regulation. The relative simplicity of the *Drosophila* brain provides an opportunity to dissect these sleep circuits at a level of resolution that would be difficult to achieve in the more complex mammalian brain. Several brain regions, including the mushroom bodies, *pars intercerebralis*, dorsal fan-shaped body, clock neurons, and subsets of octopaminergic and dopaminergic neurons, have been shown to regulate sleep [17–23]. However, the recent discovery that Cyclin A (CycA) has a sleep-promoting role and is expressed in a small number of neurons distinct from brain regions detailed above [8] suggests the existence of additional neural clusters involved in sleep regulation.

From an unbiased forward-genetic screen, we discovered *taranis* (*tara*), a mutant that exhibits markedly reduced sleep amount. *tara* encodes a *Drosophila* homolog of the Trip-Br (SERTAD) family of mammalian transcriptional coregulators that are known primarily for their role in cell-cycle progression [24–27]. TARA and Trip-Br proteins contain a conserved domain found in several CycA-binding proteins [26]. Our research shows that *tara* regulates CycA levels and genetically interacts with *CycA* and its kinase partner *Cyclin-dependent kinase 1* (*Cdk1*) [28] to regulate sleep. Furthermore, we show that a cluster of CycA-expressing neurons in the dorsal brain lies in the *pars lateralis* (PL), a neurosecretory cluster previously proposed to

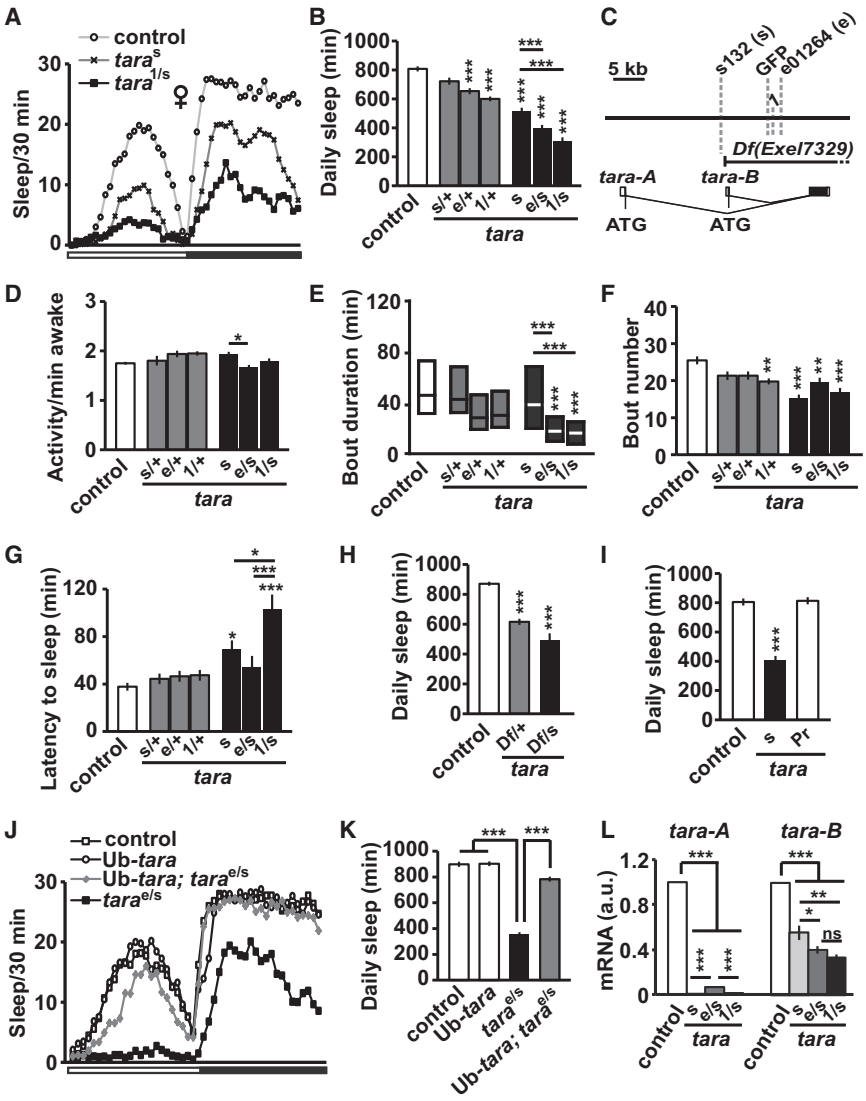

Figure 1. Sleep Phenotypes of *tara* Mutants

(A) Sleep profile of background control (white circles), $tara^{s132}$ ($tara^{s}$, black X's), and $tara^{1/s132}$ ($tara^{1/s}$, black squares) female flies (n = 50–64) in 30-min bins. The white and black bars below the x axis represent 12-hr-light and 12-hr-dark periods, respectively.

(B) Total daily sleep amount for control and *tara* female flies of the indicated genotypes (n = 44–72). In this and subsequent figures, s132 and e01264 alleles are referred to as s and e, respectively.

(C) Schematic of the genomic region of the *tara* locus. Gray dashed lines indicate transposon insertion sites. The Exel7329 deficiency removes most of *tara*-A and all of *tara*-B coding regions as indicated.

(D–G) Waking activity (activity counts per waking minute) (D), sleep-bout duration (E), sleep-bout number (F), and sleep latency (time from lights off to the first sleep bout) (G) for the same female flies shown in (B). Sleep-bout duration is not normally distributed and is shown in simplified box plots, where the median and interquartile range are represented.

(H) Total daily sleep amount of control and Df(3R)Exel7329 female heterozygotes in *trans* to either a wild-type (Df/+) or $tara^{s132}$ (Df/s) allele (n = 35–102).

(I) Total daily sleep of control, $tara^{s132}$, and precise excision ($tara^{Pr}$) female flies (n = 16–36).

(J) Sleep profile of female flies of the indicated genotypes (n = 53–58). The white and black bars below the x axis represent 12-hr-light and 12-hr-dark periods, respectively.

(K) Total daily sleep amount for the same flies showed in (J).

(L) *tara*-A and *tara*-B mRNA levels relative to *actin* mRNA levels in head extracts of indicated genotypes (n = 3–6). For each experiment, relative *tara* mRNA levels of the control flies were set to 1.

Mean ± SEM is shown. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant, one-way ANOVA followed by Tukey post hoc test (B, D, F, G, K, and L) or Dunnett post hoc test relative to control flies (H and I); Kruskal-Wallis test (E). For simplicity, only significant differences between the control and each mutant genotype (above the bar for the mutant) and those between $tara^{s132}$, $tara^{e01264/s132}$, and $tara^{1/s132}$mutants (above the line for the mutant pair) are indicated. See also Figure S1.

be analogous to the mammalian hypothalamus, a major sleep center [29, 30]. Knockdown of *tara* and increased Cdk1 activity in CycA-expressing PL neurons, as well as activation of these cells, reduces sleep. Collectively, our data suggest that TARA promotes sleep through its interaction with CycA and Cdk1 in a novel arousal center.

RESULTS

Identification of *tara* as a Sleep-Regulatory Gene in *Drosophila*

In an ongoing forward-genetic screen for sleep and circadian mutants in *Drosophila* [31], we identified a novel transposon insertion line (s132) that resulted in a substantial reduction in daily sleep (Figures 1A, 1B, S1A, and S1B). Sleep was reduced in both female and male mutants relative to background controls. Using inverse-PCR, we mapped the s132 P-element insertion to the *tara* locus (Figure 1C), which suggests that TARA has a previously unappreciated role in sleep regulation. The *tara* transcription unit generates two transcripts (A and B) with alternative transcriptional and translational start sites [26] (http://flybase.org; Figure 1C). The two protein isoforms are identical except for a small number of N-terminal amino acids and appear to be functionally interchangeable [26].

For detailed characterization of the sleep phenotypes of *tara* mutants, we obtained two additional transposon insertions in the *tara* locus ($tara^{1}$ and $tara^{e01264}$) from *Drosophila* stock centers (Figure 1C). s132 homozygotes are viable, but $tara^{1}$ and $tara^{e01264}$ homozygotes are lethal, suggesting that 1 and e01264 are stronger alleles than s132. Consistent with this view, when combined in *trans* with s132, the lethal alleles exhibited a greater reduction in sleep than s132 (Figures 1A, 1B, S1A, and S1B). The strong *tara* alleles resulted in a significant reduction in sleep even as heterozygotes (Figures 1B and

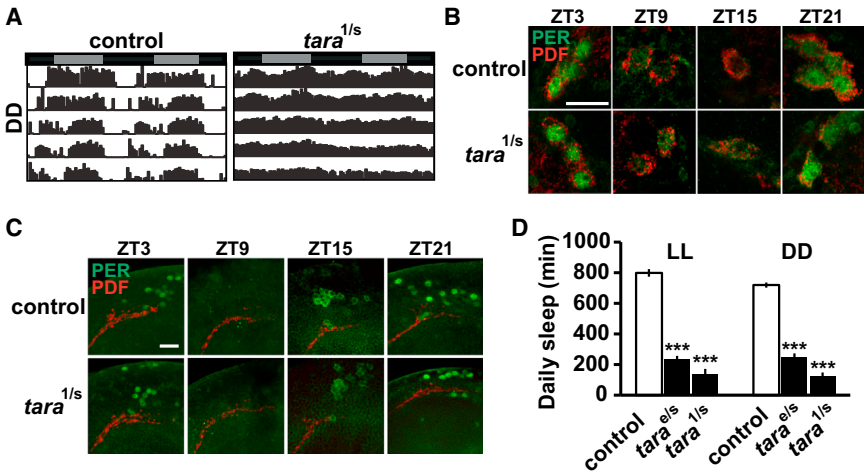

Figure 2. Circadian Phenotypes and Clock-Independent Sleep Loss of *tara* Mutants

(A) Representative circadian actogram of individual control and *tara*$^{1/s132}$ male flies in DD. Gray and black bars above the actogram indicate subjective day and night, respectively.

(B) Cycling of PER protein (green) in small ventral lateral neurons (s-LNvs) is normal in *tara*$^{1/s132}$ brains. Samples were dissected at indicated Zeitgeber times (ZT) and stained for PER and PDF (red), which was used to identify s-LNvs. Scale bar, 10 μm.

(C) Cycling of PER is also normal in a cluster of dorsal neurons (DN1s). Scale bar, 20 μm.

(D) Total daily sleep amount in LL and DD for females of indicated genotypes (n = 32–79 for LL; n = 39–96 for DD). Sleep levels on the third day in constant conditions are shown. Mean ± SEM is shown. ***p < 0.001, Dunnett post hoc tests relative to control flies (D).

See also Figure S2.

S1B). Whereas waking activity (activity counts per minute awake) was slightly increased in some *tara* mutants, it was not increased in strong allelic combinations (Figures 1D and S1C). Sleep-bout duration in both females and males was reduced in strong allelic combinations (Figures 1E and S1D), which suggests that TARA plays a role in sleep maintenance. The number of sleep bouts was markedly reduced in females with strong *tara* mutations (Figure 1F) and was unchanged in *tara* males (Figure S1E). In addition, sleep latency (time from lights off to the first sleep bout) was significantly increased in strong *tara* mutants (Figures 1G and S1F), revealing a role for TARA in sleep initiation. Taken together, our data demonstrate that *tara* is a novel sleep gene essential for sleep initiation and maintenance.

We undertook additional experiments to rule out the possibility that secondary, background mutations are responsible for the sleep phenotype in *tara* mutants. First, a deficiency line deleting the *tara* locus did not complement the s132 allele (Figures 1H and S1G). Second, precise excision of the s132 insertion by transposase-mediated mobilization restored normal sleep (Figures 1I and S1H). Third, ubiquitous expression of *tara*-B [26] restored sleep to nearly normal levels (Figures 1J, 1K, and S1I). These data confirm that disruption of *tara* is indeed the underlying cause of the severe sleep reduction in *tara* mutants.

As shown in Figures 1B and S1B, three *tara* allelic combinations (s132, e01264/s132, and 1/s132) yielded varying degrees of sleep reduction, suggesting that *tara*^{1} is the strongest allele and *tara*^{s132} is the weakest. To determine whether differences in *tara* mRNA levels mediate varying phenotypic strengths, we performed qRT-PCR using primers designed to distinguish between the two *tara* isoforms. *tara*^{s132} homozygous mutants had almost no detectable *tara*-A mRNA and an ~50% reduction in *tara*-B mRNA levels relative to control flies (Figure 1L). Like *tara*^{s132} mutants, *tara*$^{1/s132}$ flies had almost no detectable *tara*-A mRNA, but *tara*-B transcripts were further reduced, indicating that *tara*^{1} is a null or strongly hypomorphic allele. In *tara*$^{e01264/s132}$ flies, *tara*-A mRNA levels were slightly higher than in *tara*$^{1/s132}$ flies while *tara*-B mRNA levels were lower than in *tara*^{s132} homozygous flies. These results demonstrate that the amount of daily sleep correlates with *tara* levels. Collectively, the above data establish *tara* as a novel sleep regulatory gene.

Sleep Loss in *tara* Mutants Is Independent of the Circadian Clock and Light

To examine whether *tara* mutants exhibit circadian phenotypes, we monitored their locomotor activity in constant darkness (DD). Most *tara*$^{1/s132}$ mutants were arrhythmic or weakly rhythmic and the amplitude of their circadian rhythmicity was reduced, but the period length of all *tara* mutants was indistinguishable from that of control flies (Figures 2A and S2A). Moreover, daily cycling of the core clock protein PERIOD (PER) in *tara*$^{1/s132}$ mutants was similar to that in wild-type controls in two sets of clock neurons (Figures 2B and 2C), which suggests that dampened rhythmicity in these mutants is not due to a defect in the core molecular clock. Since arrhythmicity does not necessarily lead to short sleep (e.g., *per* and *timeless* mutants do not have reduced sleep [32]), the rhythm phenotype of *tara* mutants may not be the cause of the sleep phenotype. Our data showing that *tara*$^{e01264/s132}$ mutants displayed almost as severe a sleep reduction as *tara*$^{1/s132}$ but were largely rhythmic (Figures 1B, S1B, and S2A) support the view that the sleep and circadian phenotypes in *tara* mutants may not be linked. To test whether the sleep phenotype in *tara* mutants was due to arrhythmicity, we assayed sleep in constant light (LL), in which both control and mutant flies are arrhythmic. Indeed, *tara* mutants had greatly reduced sleep compared with controls in LL, demonstrating that the short-sleeping phenotype is not caused by arrhythmicity (Figures 2D and S2B). The short-sleeping phenotype was also observed in DD (Figures 2D and S2B), suggesting that TARA's role in sleep is independent of light. Of note, in both LL and DD, *tara*$^{1/s132}$ mutants lost over 80% of sleep relative to control flies, which is one of the most severe phenotypes documented among sleep mutants. These data show that *tara* mutants exhibit a striking reduction in sleep amount, independent of the circadian clock and light conditions.

TARA Is Required in Neurons to Control Sleep Levels

To examine the spatial requirements for TARA in regulating sleep, we generated a polyclonal antibody against the TARA protein (see Experimental Procedures). In western blots, the antibody recognized a band that is upregulated when TARA is overexpressed in *Drosophila* S2 cells. As expected, this band was

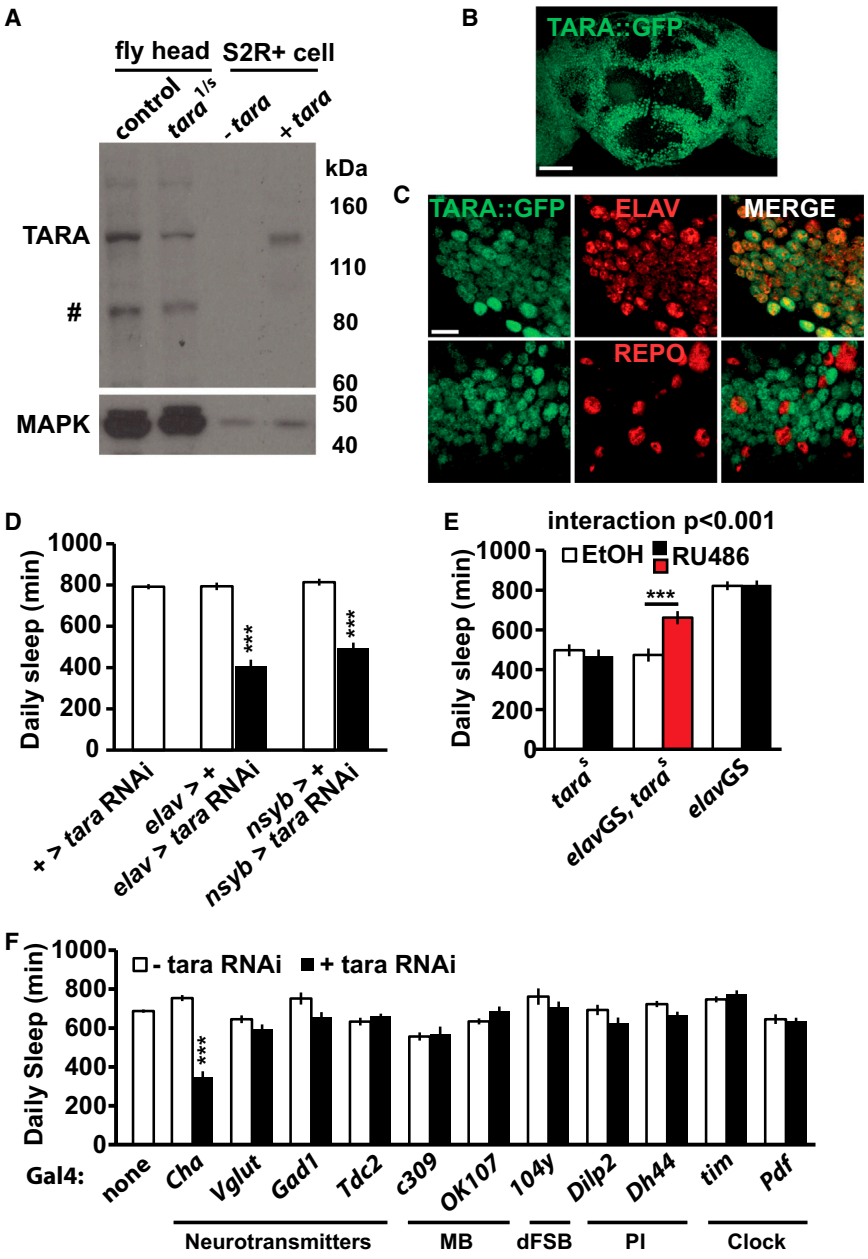

Figure 3. TARA Regulates Sleep in Neurons

(A) Western blot showing a marked reduction of TARA in *tara*$^{1/s132}$ mutants compared with control flies. Head extracts of control flies and *tara* mutants (lanes 1 and 2) as well as S2 cell extracts transfected with an empty UAS vector or a UAS-*tara* construct under the control of *actin*-Gal4 (lanes 3 and 4) were probed with a polyclonal antibody to TARA. The band that corresponds to TARA can be readily recognized by the upregulation in S2 cells transfected with *tara* cDNA. # denotes non-specific labeling or a degradation product of TARA. MAPK was used to control for loading.

(B) Maximal-intensity projection of confocal slices of the adult brain showing widespread expression of TARA::GFP. Scale bar, 50 μm.

(C) Representative confocal sections of *tara::GFP* adult brains costained with antibodies to GFP and ELAV or REPO (neuronal or glial marker, respectively). Each panel shows a single confocal slice of a region ventral to the mushroom bodies. Scale bar, 10 μm.

(D) Pan-neuronal knockdown of *tara* markedly reduces sleep. Pan-neuronal *elav*-Gal4 or *nsyb*-Gal4 was used to drive a combination of two UAS-*tara* RNAi constructs and UAS-*dcr2* (*elav*>*tara* RNAi and *nsyb*>*tara* RNAi, respectively). Flies harboring the two UAS-*tara* RNAi constructs and UAS-*dcr2* without a driver (+ > *tara* RNAi) and those harboring a driver and UAS-*dcr2* (*elav* > + or *nsyb* > +) served as controls (n = 31–58).

(E) Adult-stage expression of *tara* partially rescues the *tara* short-sleeping phenotype. Daily sleep is presented for females of the indicated genotypes in the absence (white bar) or presence (black or red bar) of RU486 (n = 24–32). Data from parental control flies show that RU486 by itself did not affect sleep.

(F) Knockdown of *tara* in cholinergic neurons reduces sleep. For each Gal4, total daily sleep of females expressing *tara* RNAi under the control of the driver (black bar) was compared to parental controls (white bar) (n = 30–173). The sleep phenotype of flies in which *tara* was knocked down in dopaminergic neurons was not determined due to lethality. Mean ± SEM is shown.

***p < 0.001, Dunnett post hoc test relative to both parental controls (D), t test with Bonferroni correction (E), Tukey post hoc test relative to both parental controls (F). See also Figure S3.

markedly downregulated in head extracts of *tara*$^{1/s132}$ mutants compared with those of control flies (Figure 3A). The identity of the band was further examined by western analysis of a previously generated GFP fusion trap in the *tara* locus (YB0035) [33], which we termed *tara::GFP*. The *GFP* exon is located upstream of the common second coding exon of both *tara*-A and *tara*-B isoforms (Figure 1C) and is expected to be incorporated into both isoforms close to the N terminus. The presumed TARA band in western blots was shifted by the addition of GFP in head extracts of *tara::GFP* flies (Figure S3A), which confirms that the band indeed represents the TARA protein. Because the polyclonal antibody did not yield a specific signal when used for immunohistochemistry, we employed the TARA::GFP fusion protein to determine the expression pattern of TARA. Homozygotes for the *tara::GFP* allele did not exhibit altered sleep levels or circadian phenotypes (Figures S2A, S3B, and S3C), indicating that the TARA::GFP fusion protein is functional. Since the *GFP* coding region is inserted into the *tara* locus in the genome, the TARA::GFP expression pattern is likely to reflect endogenous TARA expression accurately. We thus examined the localization of TARA::GFP in the adult nervous system using an anti-GFP antibody. TARA::GFP was widely expressed throughout the adult brain (Figure 3B). Costaining with neuronal and glial markers (ELAV and REPO, respectively) demonstrated that TARA is expressed in most, perhaps all, neurons but excluded from glial cells (Figure 3C).

Given the expression pattern, we sought to demonstrate a role for neuronal TARA in regulating sleep. We used RNAi to

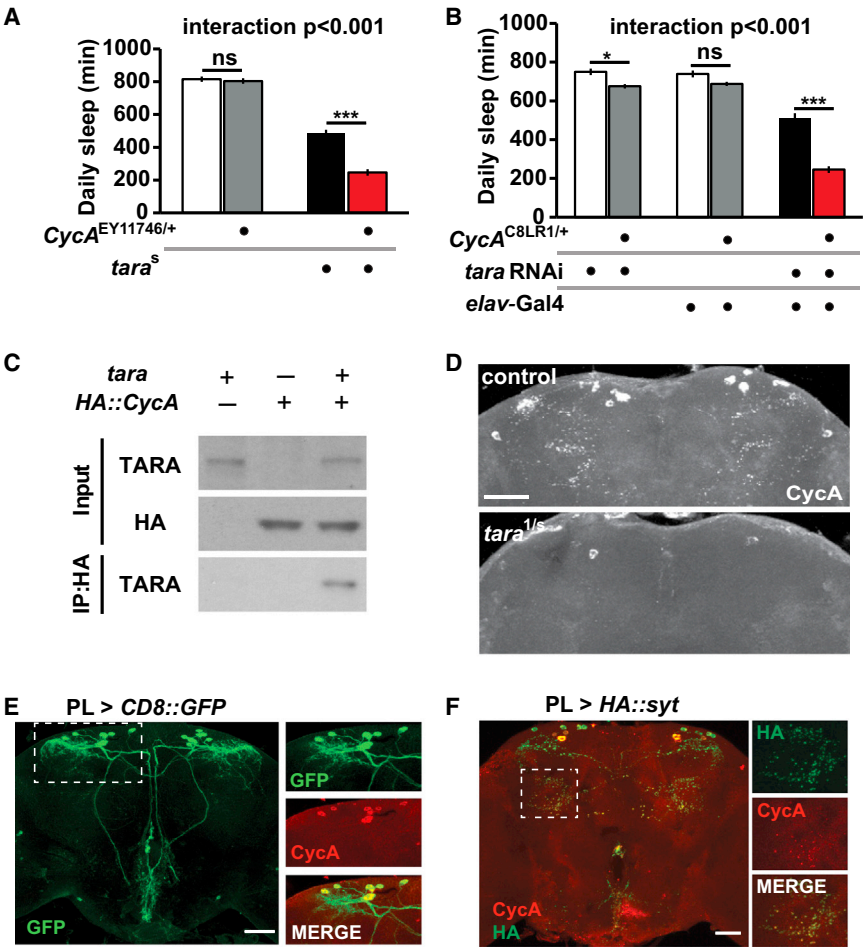

Figure 4. *tara* Interacts with *CycA* and Regulates CycA Levels

(A) Daily sleep for female flies of indicate genotypes demonstrating a synergistic interaction between $cycA^{EY11746/+}$ and $tara^{s132}$ (n = 43–60).

(B) Daily sleep for female flies of the indicated genotypes. Pan-neuronal knockdown of *tara* by RNAi (*elav*>*tara* RNAi) was more effective at suppressing sleep in a $CycA^{C8LR1/+}$ background than in a control background (n = 46–52).

(C) TARA and CycA form a complex in S2 cells. HA-tagged CycA was immunoprecipitated with an anti-HA antibody, and anti-TARA and anti-HA antibodies were used for western blotting. The experiment was repeated three times with similar results.

(D) Maximal-intensity confocal projections of the dorsal half of the central brain of representative control and $tara^{1/s132}$ adult females immunostained with an antibody to CycA. Scale bar, 50 μm.

(E) The central brain of an adult fly in which PL-Gal4 was used to express membrane-targeted CD8::GFP. Scale bar, 50 μm. Images on the right show colocalization of CycA and GFP driven by PL-Gal4 in the brain region indicated by the rectangle.

(F) The central brain of a fly in which the synaptic marker HA::SYT was expressed using PL-Gal4. The brain was costained with anti-HA and anti-CycA. The rectangle indicates the region magnified in the images on the right. Scale bar, 50 μm.

Mean ± SEM is shown. *p < 0.05; ***p < 0.001; ns, not significant, Tukey post hoc test. See also Figure S4.

reduce TARA expression specifically in neurons. As expected, driving *tara* RNAi with pan-neuronal drivers *elav*-Gal4 and *nsyb*-Gal4 resulted in a substantial reduction in daily sleep levels (Figures 3D and S3D). Reduced TARA expression by RNAi-mediated knockdown was confirmed by western analysis (Figure S3E). We next examined whether *tara* functions in the adult fly to regulate sleep by utilizing a UAS site in the s132 insertion to drive *tara* expression. We used GeneSwitch (GS), an RU486-dependent GAL4 protein that allows temporal control of transgenic expression [34]. Adult specific pan-neuronal expression of *tara* using the *elav*-GS driver partially rescued the short-sleeping phenotype of *tara* mutants (Figure 3E), demonstrating an adult function of *tara,* although the incomplete adult-stage rescue suggests a potential developmental role as well.

To identify neuronal groups where TARA acts to control sleep, we utilized the Gal4/UAS system to target *tara* RNAi expression to subsets of neurons. We examined several neurotransmitter systems as well as brain regions involved in sleep regulation such as the mushroom bodies (MB), dorsal fan-shaped body (dFSB), *pars intercerebralis* (PI), and clock cells. Only *tara* knockdown by *Cha*-Gal4 produced a significant reduction in sleep (Figure 3F). These data suggest that cholinergic neurons likely mediate the effects of TARA on sleep.

tara Interacts with *CycA* to Control Sleep and Regulates CycA Levels

Since CycA has been shown to promote sleep in *Drosophila* [8], and since TARA contains a conserved CycA binding homology motif, we tested whether *tara* and *CycA* act in a common genetic pathway to regulate sleep. To do so, we generated double mutants and compared their sleep behavior with those of wild-type control and single mutant flies. The $CycA^{EY11746/+}$ heterozygous mutation did not cause reduced sleep on its own, but it led to a significant reduction in sleep when combined with the $tara^{s132}$ hypomorphic mutation that has a moderate sleep phenotype (Figures 4A and S4A). This interaction was confirmed using a second allele of *CycA* ($CycA^{C8LR1/+}$) and *tara* RNAi (Figure 4B). Further, *CycA* did not exhibit a genetic interaction with the DAT^{fmn} short-sleeping mutant [13] (Figure S4B), demonstrating the specificity of the interaction between *tara* and *CycA*. These data reveal a synergistic interaction between *tara* and *CycA* and suggest they act in the same pathway to influence sleep.

Given the genetic interaction between *tara* and *CycA,* the presence of a CycA-binding homology domain in TARA, and the fact that Trip-Br1/2, mammalian homologs of TARA, have been shown to bind CycA [24], we tested whether TARA physically binds CycA in a co-immunoprecipitation assay. Indeed, we found that TARA co-immunoprecipitated with CycA in

Drosophila S2 cells (Figure 4C), suggesting that they can form a complex.

We next asked whether CycA levels are altered in *tara* mutants. We performed whole-mount immunostaining of adult brains using a CycA antibody previously shown to detect a dorsal set of CycA-positive neurons [8] (another CycA antibody previously used to detect a few additional clusters of CycA-expressing neurons is no longer available). We found that CycA protein levels were greatly reduced in the adult brain of *tara* mutants (Figure 4D). In contrast, CycA protein levels were not reduced in DAT^{fmn} mutants (Figure S4C), which demonstrates the specificity of the regulation of CycA levels by TARA. *CycA* mRNA levels were not affected in *tara* mutants (Figure S4D), indicating that TARA regulates CycA levels post-transcriptionally. Our data suggest that TARA promotes sleep in part through regulation of CycA protein levels.

We noticed that the dorsal CycA cluster might correspond to the *pars lateralis* (PL) [35], so we drove expression of CD8::GFP using PL-Gal4, a driver expressed in the PL neurons [36], while simultaneously labeling brains for CycA. Both GFP and CycA were expressed in ~14 neurons with large cell bodies in the dorsal brain (Figures 4E and S4E). The striking overlap seen between the GFP and CycA signals demonstrates that the dorsal CycA neurons indeed lie in the PL. This is significant because the PL, along with the *pars intercerebralis*, shares several features with the mammalian hypothalamus, a major sleep center [29, 30]. However, a possible contribution of the PL to sleep regulation has not been previously explored.

We employed the PL driver to determine whether the CycA-expressing cells were present in *tara* mutants. By examining flies expressing CD8::GFP under the control of PL-Gal4, we confirmed that the PL neurons were indeed present (Figure S4F). Interestingly, CycA protein was observed not only in cell bodies, but also in discrete puncta that appeared to be synapses (Figure 4D). This is noteworthy because according to the synaptic homeostasis hypothesis, waking activity leads to a net increase in synaptic strength, whereas sleep leads to overall downscaling of synapses [37]. To determine whether these puncta represent synapses, we used PL-Gal4 to express a synaptic marker (HA::SYT) [38] and demonstrated that CycA indeed localized to synaptic regions (Figure 4F). We note that CycA protein levels were downregulated in both cell bodies and synaptic regions in *tara* mutants (Figure 4D). CycA levels and function at synapses, under the control of TARA, may be important for normal sleep.

TARA Regulates Sleep in CycA-Expressing PL Neurons, which Define a New Arousal Center

To address whether TARA is required in CycA-expressing cells for sleep regulation, we examined the sleep phenotype of flies in which *tara* was knocked down using the PL driver. We found that PL-specific *tara* knockdown significantly reduced sleep (Figure 5A). We note that this manipulation produced a weaker sleep reduction than pan-neuronal knockdown of *tara* (Figure 3D), which suggests that TARA likely functions in additional groups of neurons to regulate sleep.

Our results pointed to a possible role of the PL neurons in sleep regulation. Indeed, we found that activation of the PL neurons via expression of the bacterial sodium channel NaChBac [39] led to decreased sleep (Figures 5B, 5C, S5A, and S5B). In contrast, activity levels during waking periods were not affected by PL activation (Figures 5D and S5C). Sleep-bout duration was markedly decreased while sleep-bout number showed little change, and sleep latency was significantly increased in flies with activated PL neurons (Figures 5E–5G, and S5D–S5F). These data suggest that activation of PL neurons promote wakefulness by delaying sleep onset and impairing sleep maintenance. Adult-stage specific activation of these neurons using the warmth-activated cation channel TrpA1 [40] also reduced sleep, demonstrating that this cell cluster functions in adult animals to promote wakefulness (Figures 5H, 5I, and S5G). Further, blocking the activity of PL neurons with tetanus toxin [41] significantly increased sleep (Figure 5J), which confirms the wake-promoting role of these neurons. The above data identify the PL neurons as a novel arousal center and demonstrate that TARA acts, at least in part, in CycA-positive PL neurons to promote sleep.

tara and *Cdk1* Interact Antagonistically to Regulate Sleep

CycA has been shown to bind Cdk1 and can either increase or decrease Cdk1 activity depending on the cellular context [28, 42]. We therefore asked whether *Cdk1* also interacts with *tara* for sleep regulation. We introduced a heterozygous $Cdk1^{GT\text{-}000294/+}$ mutation (the GT-000294 insertion is in the coding region and is likely to be a null allele) into a *tara* mutant background and compared their sleep with *tara* and *Cdk1* single mutants as well as with wild-type control flies. We found that the $Cdk1^{GT\text{-}000294/+}$ heterozygous mutation did not cause a sleep phenotype in a wild-type background, but it resulted in a substantial rescue of the *tara* sleep phenotype (Figures 6A and 6B). We confirmed the antagonistic interaction between *tara* and *Cdk1* using a second allele of *Cdk1* ($Cdk1^{c03495/+}$) (Figure S6A). The $Cdk1^{GT\text{-}000294/+}$ heterozygous mutation did not rescue the short-sleeping phenotype of *insomniac* (*inc*) mutants (Figure S6B) [9, 16], which demonstrates that the interaction between *tara* and *Cdk1* is not due to additive effects. In contrast, the *Cdk1* mutation did rescue the sleep phenotype of heterozygous *CycA* null mutants (Figure 6C), consistent with a model in which *tara* and *CycA* act together to antagonize *Cdk1*. Transcript levels of *Cdk1* were not significantly affected in *tara* mutants (Figure S6C), suggesting that the interaction between *tara* and *Cdk1* is not likely to be due to transcriptional regulation of *Cdk1* by TARA. The antagonistic interaction between *tara* and *Cdk1* suggests that *Cdk1* has a previously unrecognized wake-promoting role.

To investigate the potential wake-promoting role of Cdk1, we assayed sleep in flies overexpressing wild-type Cdk1 (Cdk1-WT). Since activity of Cyclin-dependent kinases is tightly controlled by a number of regulatory molecules [42–44], we also examined flies overexpressing Cdk1-AF, a mutant Cdk1 protein that has elevated kinase activity due to mutations in inhibitory phosphorylation sites [42]. Because overexpression of Cdk1-AF under the control of *elav*-Gal4 resulted in lethality, we used the RU486 inducible *elav*-GS to express Cdk1 specifically in the adult stage. Whereas RU486 had little effect on control flies, flies in which Cdk1-AF was expressed under the control of *elav*-GS exhibited significantly reduced sleep when fed RU486 (Figure 6D), which indicates that increased Cdk1 activity indeed promotes wakefulness. In contrast, overexpression of Cdk1-WT had little effect on sleep (Figure S6C), presumably because

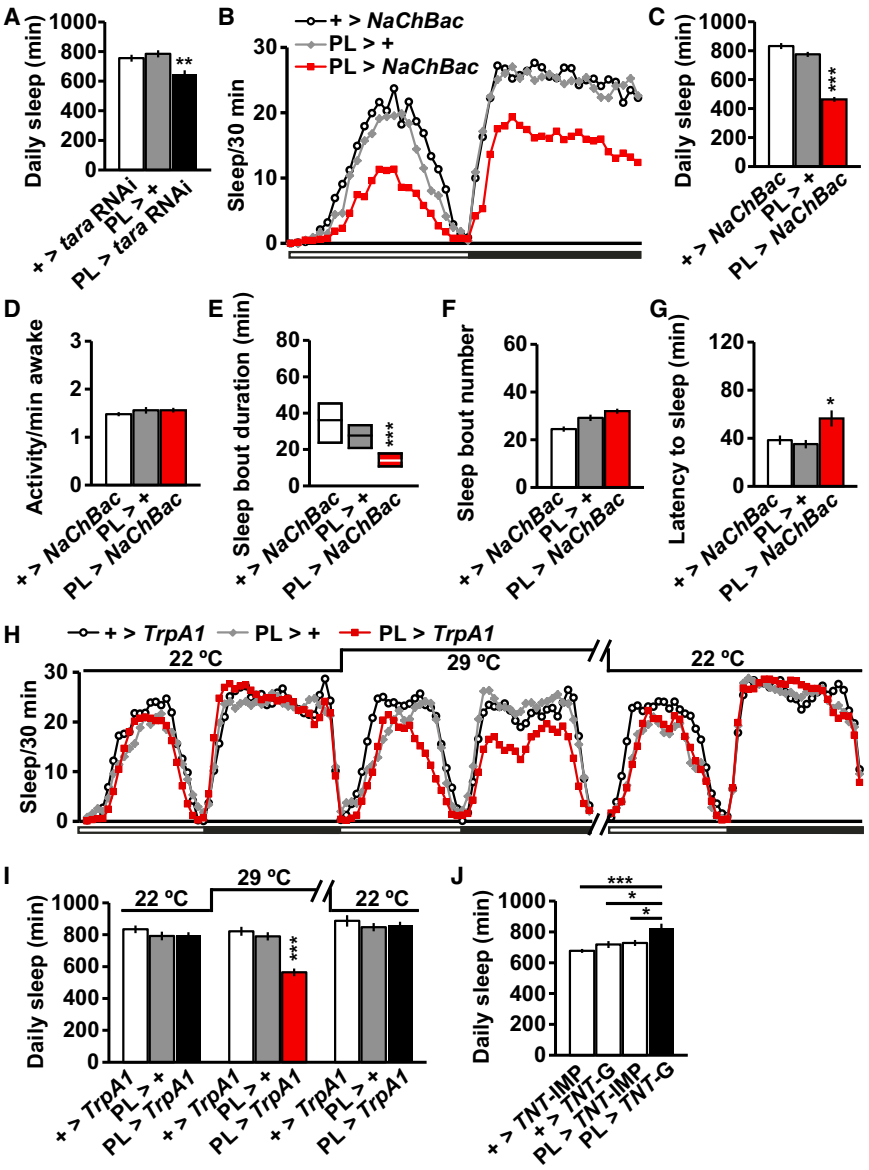

Figure 5. TARA Regulates Sleep in CycA-Expressing PL Neurons that Define a New Arousal Center

(A) Total daily sleep of female flies of the indicated genotypes (n = 32).

(B) Sleep profile for female flies expressing the NaChBac sodium channel under the control of PL-Gal4 (PL > *NaChBac*) and parental controls (n = 48–63).

(C–G) Total daily sleep (C), waking activity (D), sleep-bout duration (E), sleep-bout number (F), and latency to sleep after lights off (G) for the flies shown in (B).

(H) Sleep profile for female flies expressing *TrpA1* under the control of PL-Gal4 (PL > *TrpA1*) and parental controls (n = 16–32). Flies were monitored at 29°C, which activates the TrpA1 channel, and at 22°C, which inactivates the TrpA1 channel. Sleep profile for the second day at 29°C is omitted for simplicity.

(I) Total daily sleep for flies shown in (H).

(J) Female flies expressing functional tetanus toxin under the control of PL-Gal4 (PL > *TNT-G*) exhibited a significant increase in sleep relative to flies expressing inactive tetanus toxin (PL > *TNT-IMP*) or those carrying either form of tetanus toxin transgene without the PL driver (+ > *TNT-G* and + > *TNT-IMP*) (n = 30–32).

Mean ± SEM is shown. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant; Dunnett post hoc test relative to parental controls (A, C, D, F, G, and I) or PL > *TNT-G* flies (J); Kruskal-Wallis test (E). See also Figure S5.

overexpression of wild-type Cdk1 alone was not sufficient to increase its kinase activity. To examine whether Cdk1 acts in CycA-expressing cells to regulate sleep, we assayed sleep in flies expressing the *Cdk1*-AF transgene under the control of PL-Gal4. These flies had significantly reduced sleep compared with parental control flies (Figure 6E). These data provide strong evidence for a novel role of Cdk1 in suppressing sleep.

Since CycA is expressed in synaptic regions (Figure 4F), we next asked whether Cdk1 colocalizes with CycA at synaptic regions. To address this question, we expressed MYC-tagged wild-type Cdk1 [45] in PL neurons and found that Cdk1::MYC exhibited marked overlap with CycA puncta at synaptic regions (Figure 6F). Although the synaptic localization of Cdk1::MYC could be an artifact of overexpression, the potential colocalization of Cdk1 and CycA at synaptic regions raises the interesting possibility that synaptic Cdk1 activity may be important for maintaining normal sleep amount. Together, our data demonstrate that Cdk1 interacts antagonistically with TARA and CycA and acts in PL neurons to promote wakefulness.

DISCUSSION

From an unbiased forward genetic screen, we have identified a novel sleep regulatory gene, *tara*. Our data demonstrate that TARA interacts with CycA to regulate its levels and promote sleep. We have also identified Cdk1 as a wake-promoting molecule that interacts antagonistically with TARA. Given the fact that TARA regulates CycA levels, the interaction between TARA and Cdk1 may be mediated by CycA. Our finding that *Cdk1* and *CycA* also exhibit an antagonistic interaction supports this view. The previous discovery that CycE sequesters its binding partner Cdk5 to repress its kinase activity in the adult mouse brain [46] points to a potential mechanism, namely that TARA regulates CycA levels, which in turn sequesters and inhibits Cdk1 activity. TARA and its mammalian homologs (the Trip-Br family of proteins) are known for their role in cell-cycle progression [24–27]. However, recent data have shown that Trip-Br2 is involved in lipid and oxidative metabolism in adult mice [47], demonstrating a role beyond cell-cycle control. Other cell-cycle proteins have also been implicated in processes unrelated to the cell cycle. For example, CycE functions in the adult mouse brain to regulate learning

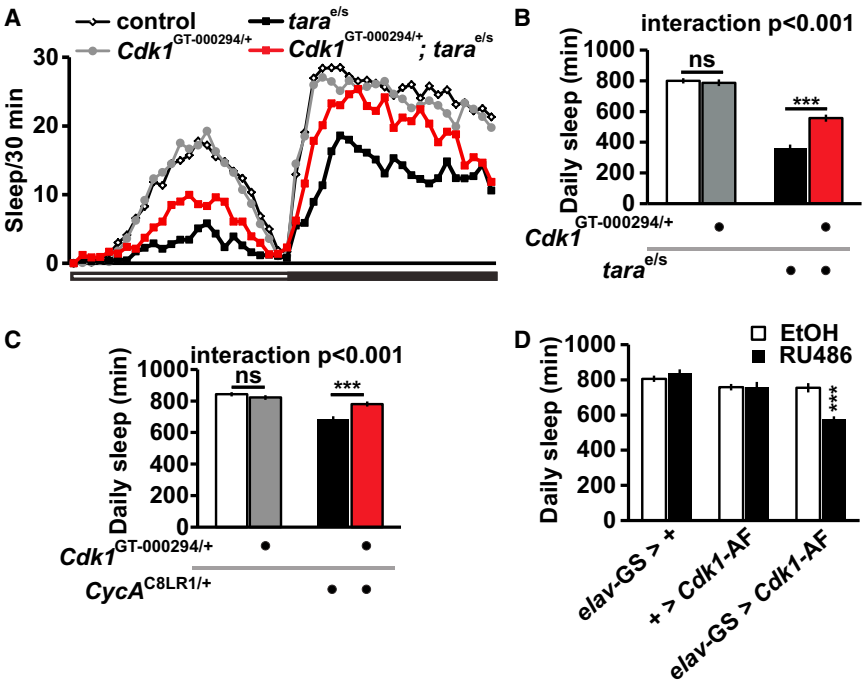

Figure 6. *Cdk1* Interacts Antagonistically with *tara* and *CycA*, and Increased Cdk1 Activity Suppresses Sleep

(A) Sleep profile for control (white diamonds), *Cdk1*$^{GT\text{-}000294/+}$ (gray circles), *tara*$^{e01264/s132}$ (black squares), and *Cdk1*$^{GT\text{-}000294/+}$; *tara*$^{e01264/s132}$ (red squares) female flies (n = 50–64).

(B) Total daily sleep for the flies shown in (A).

(C) Total daily sleep of female flies of the indicated genotypes (n = 81–88).

(D) Adult-stage specific expression of Cdk1-AF induced by feeding flies food that contain RU486 diluted in ethanol (EtOH) reduced sleep (n = 31–32).

(E) Cdk1-AF expression specifically in CycA-expressing PL neurons resulted in reduced sleep (n = 70–75).

(F) The central brain of a fly in which UAS-*Cdk1-myc* was expressed using PL-Gal4. The brain was costained with anti-MYC and anti-CycA. The rectangle indicates the region magnified in the images on the right. Scale bar, 50 μm.

Mean ± SEM is shown. ***p < 0.001; ns, not significant; Tukey post hoc test (B and C), Dunnett post hoc test relative to all other controls (D); relative to both parental controls (E). See also Figure S6.

and memory [46]. Based on the finding that CycA and its regulator Rca1 control sleep, it was hypothesized that a network of cell-cycle genes was appropriated for sleep regulation [8]. Our data showing that two additional cell-cycle proteins, TARA and Cdk1, control sleep and wakefulness provide support for that hypothesis. Moreover, the fact that TARA and CycA, factors identified in two independent unbiased genetic screens, interact with each other highlights the importance of a network of cell-cycle genes in sleep regulation.

There are two main regulatory mechanisms for sleep: the circadian mechanism that controls the timing of sleep and the homeostatic mechanism that controls the sleep amount [48]. We have shown that TARA has a profound effect on total sleep time. TARA also affects rhythmic locomotor behavior. Since TARA is expressed in clock cells (our unpublished data), whereas CycA is not [8], it is possible that TARA plays a non-CycA dependent role in clock cells to control rhythm strength. Our finding that *tara* mutants exhibit severely reduced sleep in constant light suggests that the effect of TARA on sleep amount is not linked to its effect on rhythmicity. Instead, TARA may have a role in the sleep homeostatic machinery, which will be examined in our ongoing investigation.

To fully elucidate how sleep is regulated, it is important to identify the underlying neural circuits. Here, we have shown that activation of the CycA-expressing neurons in the PL suppresses sleep while blocking their activity increases sleep, which establishes them as a novel wake-promoting center. Importantly, knockdown of *tara* and increased Cdk1 activity specifically in the PL neurons leads to decreased sleep. A simple hypothesis, consistent with our finding that both activation of PL neurons and increased Cdk1 activity in these neurons suppress sleep is that Cdk1 affects neuronal excitability and synaptic transmission. Interestingly, large-scale screens for short-sleeping mutants in fruit flies and zebrafish have identified several channel proteins such as SHAKER, REDEYE, and ETHER-A-GO-GO [6, 49, 50] and channel modulators such as SLEEPLESS and WIDE AWAKE [51, 52]. Thus, it is plausible that Cdk1 regulates sleep by phosphorylating substrates that modulate the function of synaptic ion channels or proteins involved in synaptic vesicle fusion, as has previously been demonstrated for Cdk5 at mammalian synapses [53].

Whereas our data mapped some of TARA's role in sleep regulation to a small neuronal cluster, the fact that pan-neuronal *tara* knockdown results in a stronger effect on sleep than specific knockdown in PL neurons suggests that TARA may act in multiple neuronal clusters. PL-specific restoration of TARA expression did not rescue the *tara* sleep phenotype (data not shown), further implying that the PL cluster may not be the sole anatomical locus for TARA function. Given that CycA is expressed in a few additional clusters [8], TARA may act in all

CycA-expressing neurons including those not covered by PL-Gal4. TARA may also act in non-CycA-expressing neurons. Our data demonstrate that *tara* knockdown using *Cha*-Gal4 produces as strong an effect on sleep as pan-neuronal knockdown (Figures 3D and 3F). This finding suggests that TARA acts in cholinergic neurons, although we cannot rule out the possibility that the *Cha*-Gal4 expression pattern includes some non-cholinergic cells. Taken together, our data suggest that TARA acts in PL neurons as well as unidentified clusters of cholinergic neurons to regulate sleep.

Based on genetic interaction studies, *tara* has been classified as a member of the *trithorax* group genes, which typically act as transcriptional coactivators [26, 54]. However, TARA and Trip-Br1 have been shown to up- or downregulate the activity of E2F1 transcription factor depending on the cellular context, raising the possibility that they also function as transcriptional corepressors [24, 27]. Interestingly, TARA physically interacts with CycA and affects CycA protein levels but not its mRNA expression. These findings suggest a novel non-transcriptional role for TARA, although we cannot rule out an indirect transcriptional mechanism. The hypothesis that TARA plays a non-transcriptional role in regulating CycA levels and Cdk1 activity at the synapse may provide an exciting new avenue for future research.

EXPERIMENTAL PROCEDURES

Details of experimental procedures are available in the online Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.05.037.

AUTHOR CONTRIBUTIONS

K.K. conceived the study, and D.J.S.A. and K.K. designed the experiments and analyzed the data. D.J.S.A. performed the experiments with the help of D.L., D.R.M., J.E.C.J., and H.P., and D.R. identified the dorsal CycA-expressing cells as the *pars lateralis* cluster. The manuscript was written by K.K. and D.J.S.A. with editorial input from D.R. and J.E.C.J.

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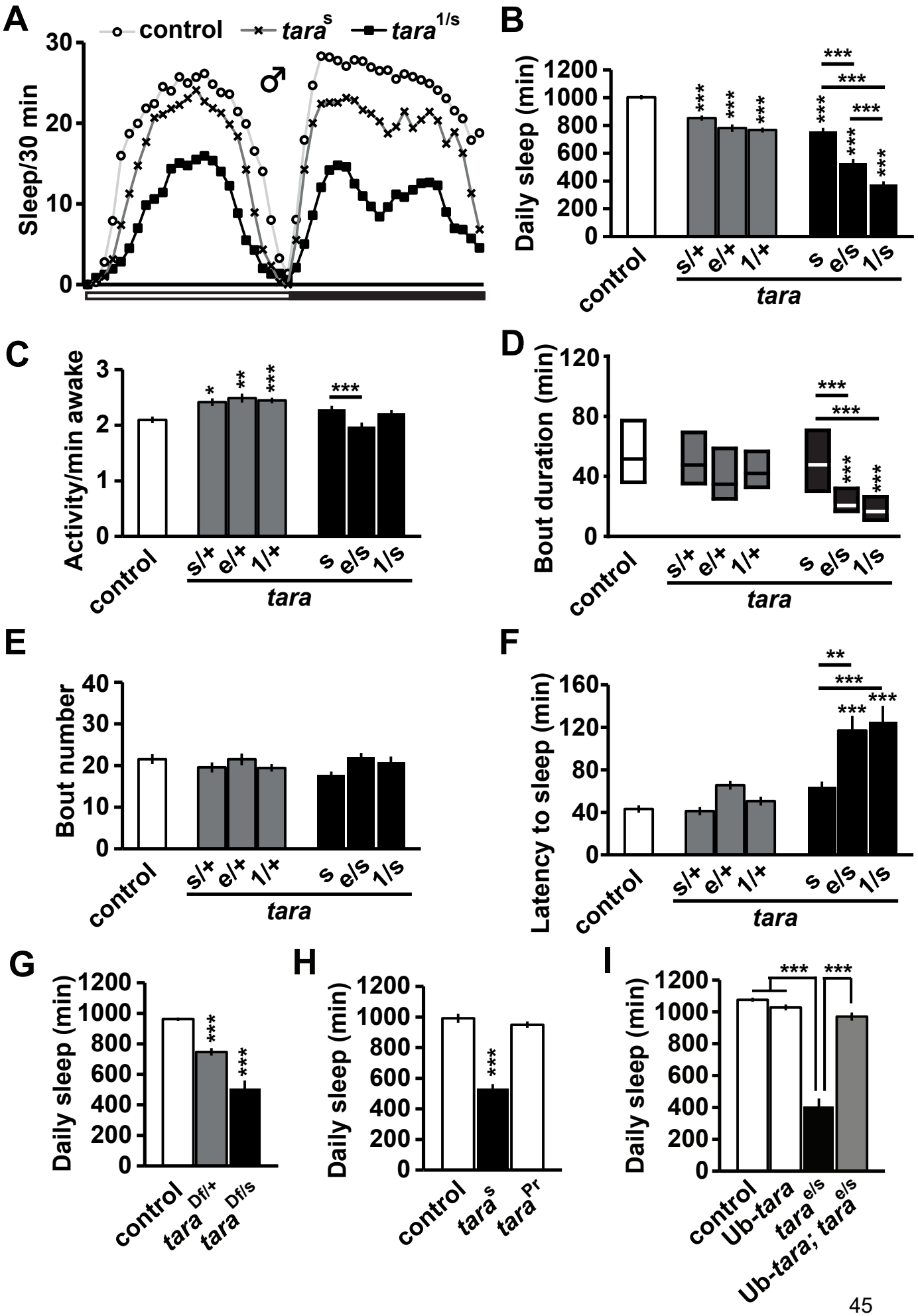
Current Biology

Supplemental Information

TARANIS Functions with Cyclin A and Cdk1 in a Novel Arousal Center to Control Sleep in *Drosophila*

Dinis J.S. Afonso, Die Liu, Daniel R. Machado, Huihui Pan, James E.C. Jepson, Dragana Rogulja, and Kyunghee Koh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tara^s
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B
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Daily sleep (min)
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tara Df/+
tara Df/s
H
Daily sleep (min)
control
tara^s
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Figure S1. Sleep Phenotypes and Genetic Analysis of *tara*: Male Data. (A) Sleep profile of homozygous *tara*^{s132} (black X's), transheterozygous *tara*$^{1/s132}$ (black squares), and their background control males (white circles) (n=60-73). The white and black bars below the X-axis represent light and dark periods, respectively. **(B)** Control and *tara* mutant males of the indicated genotypes (n=41-73). **(C)** Waking activity, **(D)** sleep bout duration, **(E)** sleep bout number, and **(F)** sleep latency at lights off for the same male flies shown in (B). Sleep bout duration is shown in simplified box plots, where the median and interquartile range are represented. **(G)** Total daily sleep of control and Df(3R)Exel7329 male heterozygotes in trans to either a wild type (Df/+) or *tara*^{s132} (Df/s) allele (n=35-70). **(H)** Total daily sleep of control, *tara*^{s132}, and precise excision male flies (n=15-34). **(I)** Total daily sleep of male flies of the indicated genotypes (n=35-42). Ubiquitous expression of *tara*-B (Ub-*tara*) rescued the *tara* sleep phenotypes. Mean ± SEM is shown. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA followed by Dunnett post hoc test (G, H); Tukey post hoc test (B, C, E, F, I); Kruskal-Wallis test (D). For simplicity, only significant differences between each mutant and the control and those between *tara*^{s132}, *tara*$^{e01264/s132}$, and *tara*$^{1/s132}$ mutants are indicated. Related to Figure 1.

A

Genotype	N	% R	% WR	% AR	tau (h) ± SEM	Power ± SEM
control	155	100	0	0	23.59 ± 0.02	133.9 ± 2.76
tara$^{s/+}$	48	89.6	6.3	4.2	23.24 ± 0.26	129.4 ± 6.60
tara$^{e/+}$	59	94.9	1.7	3.4	23.59 ± 0.03	142.2 ± 5.11
tara$^{1/+}$	62	90.3	8.1	1.6	23.51 ± 0.03	129.9 ± 5.98
tara^{s}	76	77.6	9.2	13.2	23.51 ± 0.03	95.1 ± 6.45
tara$^{e/s}$	86	64.0	9.3	26.7	23.44 ± 0.26	65.0 ± 5.99
tara$^{1/s}$	55	18.2	16.4	65.5	23.57 ± 0.06	18.4 ± 7.04
control *tara*^{GFP}	56	92.9	3.5	3.6	23.62 ± 0.05	126.7 ± 4.82
tara^{GFP}	88	92	3.4	4.5	23.45 ± 0.16	144.8 ± 5.19

N: number of flies; **R:** rhythmic; **WR:** weakly rhythmic; **AR:** arrhythmic
tau: free-running period; **Power:** measure rhythm strength;

B

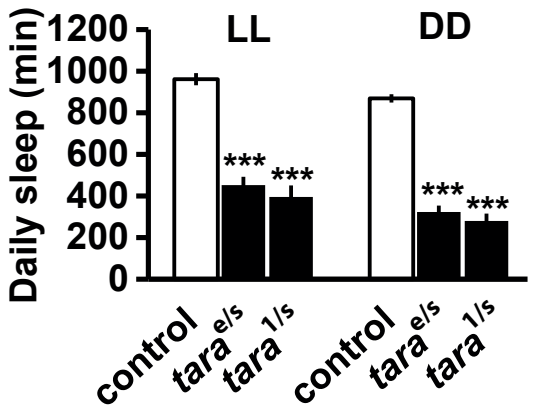

Figure S2. Circadian Phenotypes and Clock-Independent Sleep Loss of *tara* Mutant Males. (A) Free-running circadian phenotypes of male flies of the indicated genotypes in DD. χ^2 periodogram analysis was performed for each fly using the FaasX software to determine the free-running period, tau. Power, a measure of rhythmicity, corresponds to peak – significance value at p = 0.05. **(B)** Total daily sleep amount in LL and DD for control, *tara*$^{e01264/s132}$, and *tara*$^{1/s132}$ males (n=29-77 for LL; n=55-94 for DD). Sleep levels on the 3rd day in constant conditions are shown. Mean ± SEM is shown. ***p < 0.001, one-way ANOVA followed by Dunnett post hoc test relative to control flies (B). Related to Figure 2.

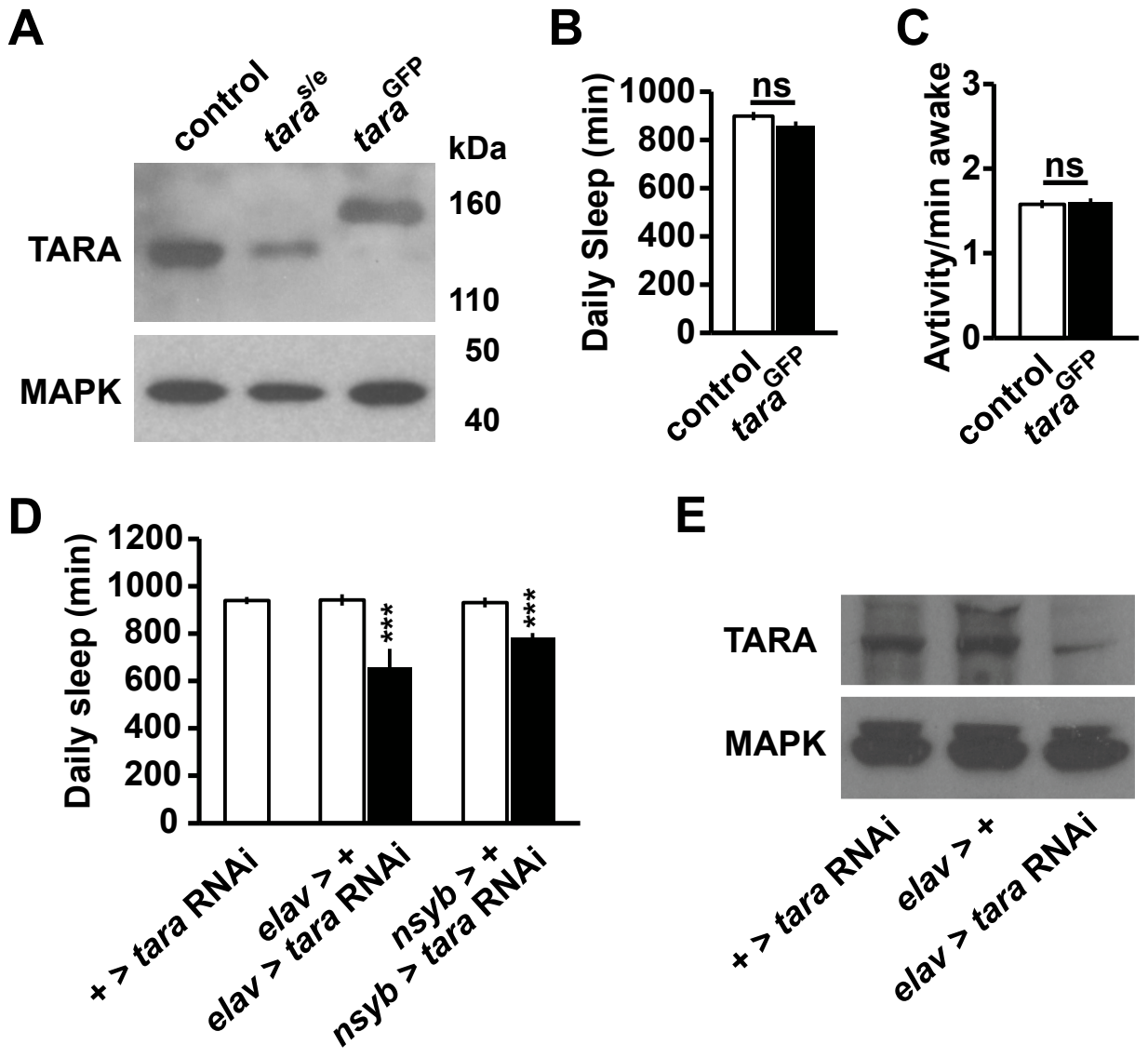

Figure S3. Characterization of *tara*^{GFP} Flies. (A) Western blot showing a band shift in homozygous *tara*^{GFP} lysates. **(B)** Total daily sleep and **(C)** waking activity of *tara*^{GFP} and control females (n=47-64). *tara*^{GFP} flies did not exhibit sleep abnormalities, suggesting TARA::GFP is functional. **(D)** Pan-neuronal *tara* knockdown using two independent Gal4 lines (*elav* and *nsyb*) resulted in a significant reduction of sleep in males (n=33-63 except for *elav* > *tara* RNAi, for which n=11). **(E)** Western blot shows a marked reduction of TARA levels in females in which *tara* was knocked down pan-neuronally (*elav* > *tara* RNAi). The experiment was performed three times with similar results. MAPK was used as loading control (A,E). Mean ± SEM is shown. ***p < 0.001, ns: not significant, Student's t-test (B,C); one-way ANOVA followed by Dunnett post hoc test relative to controls (D). Related to Figure 3.

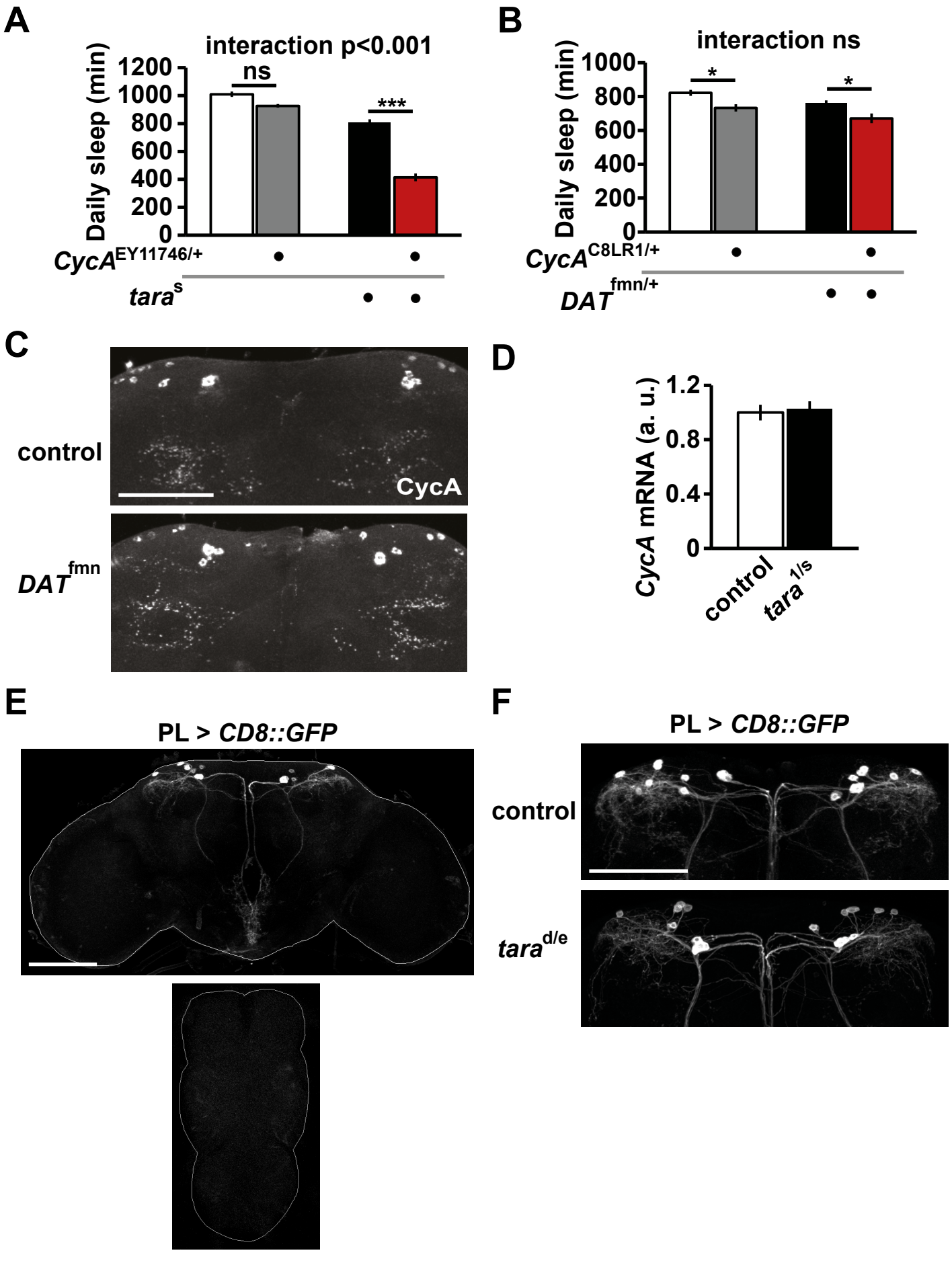
A
interaction p<0.001
ns
***
Daily sleep (min)
1200
1000
800
600
400
200
0
CycA EY11746/+
tara s
B
interaction ns
*
*
Daily sleep (min)
1000
800
600
400
200
0
CycA C8LR1/+
DAT fmn/+
C
control
CycA
DAT fmn
D
CycA mRNA (a. u.)
1.2
0.8
0.4
0
control
tara 1/s
E
PL > CD8::GFP
F
PL > CD8::GFP
control
tara d/e

Figure S4. Genetic Interaction between *tara* and *CycA*. (A) Daily sleep for male flies of the indicated genotypes (n=54-64). **(B)** Daily sleep for female flies of the indicated genotypes (n=40-56). **(C)** Maximal-intensity confocal projections of the dorsal half of the central brain of representative control and *DAT*^{fmn} adult flies immunostained with an antibody to CycA. In contrast to *tara* mutants, CycA levels were not altered in *DAT*^{fmn} mutants. Scale bar: 100 μm. **(D)** *CycA* mRNA levels relative to *actin* mRNA levels were not significantly different between *tara*$^{1/s132}$ and control flies (n=3). **(E)** Confocal projection of a female brain (top) and ventral nerve cord (bottom) expressing CD8::GFP under the control of PL-Gal4. GFP expression was observed in a small number of neurons in the dorsal brain. Scale bar: 100 μm. **(F)** Confocal projection of a representative control or *tara*$^{d40/e01264}$ central brain expressing CD8::GFP under the control of PL-Gal4. The CycA-expressing PL neurons were present and grossly normal in morphology in *tara* mutants. d40 (d, for short) is an imprecise excision allele with a sleep phenotype similar to s132 (see Experimental Procedures). Scale bar: 100 μm. Mean ± SEM is shown. *p < 0.05, ***p < 0.001, ns: not significant, two-way ANOVA followed by Tukey post hoc test (A,B); Student's t-test (D). Related to Figure 4.

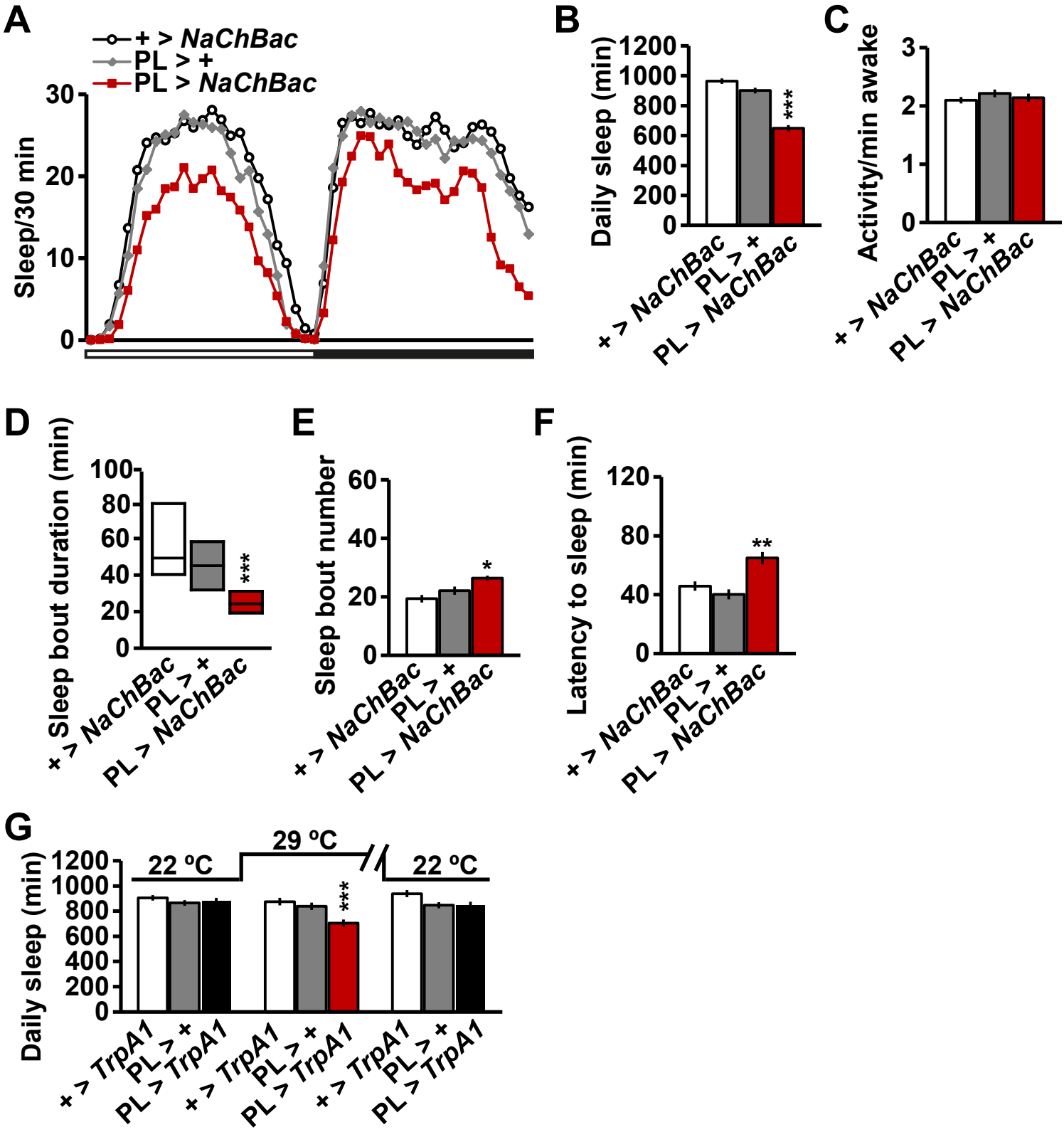

Figure S5. Activation of CycA-Expressing PL Neurons Suppresses Sleep. (A) Sleep profile for male flies expressing the NaChBac sodium channel under the control of PL-Gal4 (PL > *NaChBac*) and parental controls (n=48-64). **(B)** Daily sleep, **(C)** waking activity, **(D)** sleep bout duration, **(E)** sleep bout number, and **(F)** sleep latency at ZT12 for the flies shown in (A). **(G)** Total daily sleep for male flies carrying both UAS-*TrpA1* and PL-Gal4 (PL > *TrpA1*, n=32) relative to parental controls (n=16-32). Flies were monitored at 29°C, which activates the TrpA1 channel, and at 22°C, which inactivates the TrpA1 channel. Mean ± SEM is shown. *p < 0.05, **p < 0.01, and ***p < 0.001, one-way ANOVA followed by Dunnett post hoc test relative to controls (B-C, E-G); Kruskal-Wallis test (D). Related to Figure 5.

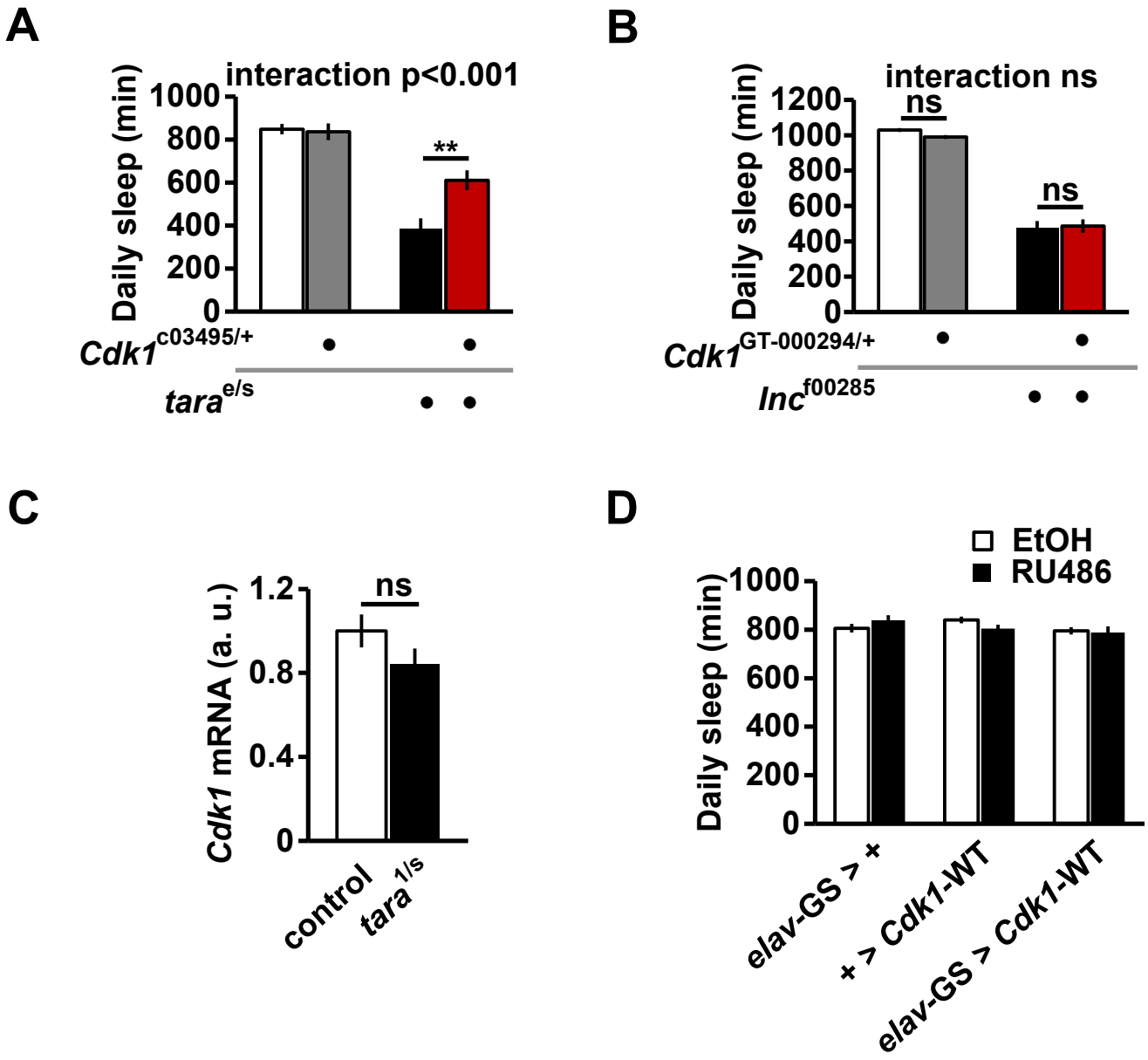

Figure S6. *tara* and *Cdk1* interact antagonistically to control sleep. (A) Daily sleep for control, *Cdk1*$^{c03495/+}$, *tara*$^{e01264/s132}$, and *Cdk1*$^{c03495/+}$; *tara*$^{e02164/s132}$ females (n=23-32). **(B)** Daily sleep for male flies of the indicated genotypes (n=26-43). **(C)** *Cdk1* mRNA levels of *tara*$^{1/s132}$ mutants were comparable to those of control flies. **(D)** Adult-stage pan-neuronal overexpression of wild-type *Cdk1* has little effect on sleep. Flies were fed either RU486 or vehicle (EtOH) (n=31-32). Mean ± SEM is shown. **p < 0.01, ns: not significant, one-way ANOVA followed by Tukey post hoc test (A, B, D); Student's t-test (C). Related to Figure 6.

Supplemental Experimental Procedures

Fly stocks

Flies were maintained at room temperature (RT) on standard food containing molasses, cornmeal, and yeast. UAS-*tara*-RNAi (JF01421), *tara*^{1}, *tara*$^{Df(3R)Exel7329}$, *CycA*^{EY11746}, *CycA*^{C8LR1}, *inc*^{f00285}, *Cdk1*$^{GT-000294}$, *Cdk1*^{c03495}, UAS-*Cdk1-myc*, UAS–*mCD8-GFP*, UAS-*NaChBac*, UAS-*TNT-G*, UAS-*TNT-IMP*, UAS-*dicer2* (*dcr2*), *elav*-Gal4, *nsyb*-Gal4, *Cha*-Gal4, *VGlut*-Gal4, *Tdc2*-Gal4, c309-Gal4, OK107-Gal4, and UAS-*TrpA1* were obtained from the Bloomington Stock Center. 104y-Gal4, *Dilp2*-Gal4, *tim*-Gal4, *Pdf*-Gal4, and *elav*-GS lines were obtained from Dr. Amita Sehgal's lab; *Dh44*-Gal4 from the Vienna *Drosophila* Resource Center; UAS-*tara*-RNAi (6889R-1) from the National Institute of Genetics, Japan; *tara*^{e01264} from the Harvard Exelixis collection; *tara::GFP* (*tara*^{yb0035}) from Dr. Lynn Cooley; Ub-*tara* and *tara*^{EP3463} from Dr. Henri-Marc Bourbon; PL-Gal4 from Dr. Jae Park; UAS-*HA::syt* from Dr. Thomas Schwarz; *DAT*^{fmn} from Dr. Kazuhiko Kume; and UAS-*Cdk1*-WT-VFP and UAS-*Cdk1*-AF-VFP from Dr. Shelagh Campbell. PL-Gal4 contains a *Corazonin* promoter fragment (504$^{\Delta 311-249}$) [36]. For improved efficiency of *tara* knockdown, two RNAi lines (JF01421 and 6889R-1) were combined with UAS-*dcr2*. All fly lines were outcrossed to an isogenic background control line (iso31) for at least four generations, except for the UAS-*tara*-RNAi lines.

Generation of excision lines

The s132 insertion maps to 573 bp upstream of the *tara-B* transcription start site. Mobilization of the P-element in the s132 line using Δ2-3 recombinase produced precise excision lines with normal sleep patterns but failed to produce imprecise excision lines.

To generate an imprecise excision line, we employed a neighboring P-element EP3463, which maps to ~50bp upstream of the *tara*-B transcriptional start site (www.flybase.org). By mobilizing the EP3463 insertion using Δ2-3 recombinase, we obtained an imprecise excision line d40, in which a portion of the P element was removed. The d40 line was outcrossed 6 times into the control iso31 background. Homozygous $tara^{d40}$ as well as transheterozygous $tara^{d40/e01264}$ flies exhibit reduced sleep.

Sleep and circadian assays

Four to seven day old flies entrained to a 12h:12h LD cycle for at least 3 days were individually placed in small glass tubes containing 5% sucrose and 2% agar at 25°C except where noted. For the GS experiment, 500 μM RU486 or vehicle (ethanol) was added to the sucrose-agar food. For experiments involving the warmth-induced TrpA1 channel, flies were raised at RT (~21°C) and entrained in LD at 22°C for at least 3 days before being monitored for 1 day at 22°C to establish a baseline, 2 days at 29°C to activate the TrpA1 channel, and 1 day at 22°C to examine recovery. Data from the first day at 29°C as well as the baseline and recovery day are presented. Activity counts were collected in 1-min bins using *Drosophila* Activity Monitoring (DAM) System (Trikinetics), and sleep was defined as a period of inactivity lasting at least 5 min [S1]. Sleep parameters were analyzed using the PySolo software [S2], except for sleep latency which was analyzed using Sleeplab (William Joiner).

Circadian assays were performed essentially as described [31]. Briefly, male flies were monitored in DD using the DAM system (Trikinetics) for six days after being entrained to an LD cycle for at least 3 days. Activity counts collected in 30-min bins were

analyzed using the FaasX software (M. Boudinot and F. Rouyer). The software uses χ^2 analysis to calculate period length and rhythm power. Rhythm power was determined for all flies including arrhythmic ones, whereas circadian period was determined only for rhythmic flies. Actograms were generated using ClockLab (Actimetrics).

Quantitative real-time reverse-transcriptase PCR (qPCR)

Total RNA was extracted from 20-30 female fly heads using TRIzol (Life Technologies), and cDNA was generated using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). qPCR was performed using SYBR green (Applied Biosystems). The following primers were used: 5'-GAA AAA GGC GCC AAA CTT AAA TTA-3' and 5'-TCG CGG AAT TCA CAT TGG AT-3' for *tara-A*; 5'-GAA AAT GTG CAC TGA GGT GAA T-3' and 5'-GTG TTG GCA TCC TTG CTG T-3' for *tara-B*; 5'-GCG GAT GAC ATA AGT GAT GG-3' and 5'-CAT GAC GCT GTA TAT TTC CGA-3' for *CycA*; and 5'-ATG GCG TGG TGT ATA AGG GT-3' and 5'-AAA TTT CTC TGA TCG CGG TT-3' for *Cdk1*.

Antibody production and Western analysis

For antigen production, a PCR product coding for 167 amino acids of TARA common to both isoforms was subcloned into the pET-28a protein expression vector using the following primers: 5'-ATG AAT TCT CGC CAT CGG AGC C-3' and 5'-ATC TCG AGA TGC GGT ACA AAG GGA TG. The His-tagged protein was purified at the Wistar Institute Protein Expression Facility, and injected into rats to generate polyclonal antibody TJR51 (Cocalico Biologicals). TJR51 recognized a ~130kD that was markedly

reduced in *tara* mutants. Since the TARA protein is predicted to be smaller at ~100kD, we examined the possibility that the annotated *tara* transcripts are incomplete. To this end, we generated a full-length UAS-*tara*-B construct, which contains the entire coding region of the *tara*-B isoform as annotated in FlyBase (www.flybase.org) but does not contain any intronic or untranslated regions. We transfected this UAS-*tara*-B construct along with an *actin*-Gal4 construct in *Drosophila* S2 cells. Overexpression of the *tara*-B transcripts in S2 cells resulted in upregulation of the ~130kD band, demonstrating that the band corresponds to the predicted TARA-B protein. As the two TARA isoforms (A and B) differ in size by only 4 amino acids, the single band likely represents both isoforms.

Western blot analysis of head extracts and quantification of immunoreactive bands were performed essentially as described [51]. Anti-TARA antibody (TJR51) was used at 1:750, anti-HA (Covance) at 1:1000, and anti-MAPK (Sigma) at 1:10000.

Transient transfection and co-immunoprecipitation (co-IP)

For Western analysis, *Drosophila* S2 cells were transfected with indicated DNA constructs in 24-well plates (150 ng of total DNA) using Effectene (Qiagen). For co-IP experiments, S2 cells were transfected with various combinations of UAS-*tara* (200 ng) and UAS-*HA-CycA* constructs [S3] (150 ng) along with *actin*-Gal4 (100 ng) in 6-well plates using Effectene (Qiagen). UAS vector DNA was included in some conditions to make the total amount of DNA equal in all conditions. Transfected cells were kept at 25°C for 2 days before being harvested. Co-IP was performed essentially as described [51] except that an antibody to HA (Covance) was used for IP and cells were lysed in

extraction buffer containing 150 mM KCl, 50 mM Tris-Cl at pH 7.0, 10 mM EDTA, 0.2% Triton X-100, 10 mM DTT, and protease inhibitor cocktail (Roche).

Immunohistochemistry

Dissected brains were fixed in 4% paraformaldehyde for 30 min at RT. Samples were blocked in 5% normal chicken serum for experiments involving CycA staining, 1% BSA for PER staining, and 5% normal goat serum for other antibody staining. The following primary antibodies were used: rabbit anti-PER [S4] at 1:8000, mouse anti-PDF (DSHB) at 1:2000, rabbit anti-GFP (Invitrogen) at 1:500, goat anti-CycA (Santa Cruz Biotechnology, #15869) at 1:50, anti-ELAV (DSHB) at 1:200, anti-REPO (DSHB) at 1:100, anti-HA (Covance) at 1:1000, and anti-MYC (Invitrogen) at 1:1000. The secondary antibodies, Alexa Fluor 647 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse, and Alexa Fluor 647 chicken anti-goat (Invitrogen) were used at 1:400. Primary and secondary antibodies were incubated at 4°C overnight. Images were obtained on an Olympus Fluoview confocal microscope.

Statistical Analysis

Data sets with two groups were compared using *t* tests. For multiple pairwise comparisons in a data set, *t* tests with Bonferroni correction were used. For comparisons of multiple groups, one-way ANOVA tests were performed, followed by Dunnett or Tukey post-hoc tests. For genetic interaction experiments, two-way ANOVA tests were performed to test for the interaction. For comparisons of non-normally distributed data,

Kruskal-Wallis tests were performed, followed by Dunn's post hoc tests with Bonferroni correction.

Supplemental References

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Chapter 2.2

TARANIS regulates circadian rhythms, nocturnal activity, and neuronal morphology

Dinis J.S. Afonso and Kyunghee Koh

Title: TARANIS regulates circadian rhythms, nocturnal activity, and neuronal morphology

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Summary

Daily cycling of light and temperature modulates life on Earth. Most organisms have evolved internal molecular clocks to timely anticipate these recurring changes. The core molecular oscillator is composed of interconnected feedback loops that cycle daily in accord with Earth's rotation. Previously we found that *tara* interacts with *CycA* and *Cdk1* to regulate sleep and that *tara* mutants exhibit arrhythmic locomotor behavior in constant darkness (DD). However, the mechanisms through which TARA regulate circadian behavior is unknown. Here, we show that TARA regulates the speed of the molecular oscillator and the neuropeptide output of the pacemaker neurons. We observe that the pace of PERIOD (PER) oscillations is faster in *tara* mutants and slower in flies overexpressing TARA in the small ventral lateral neurons (sLN_vs). Additionally, levels of the neuropeptide PIGMENT DISPERSING FACTOR (PDF), which synchronizes the clock cell network, are markedly decreased in *tara* mutants. Similarly to *Clock* (*Clk*) mutants, *tara* mutants display a nocturnal pattern of activity in light:dark conditions (LD). Furthermore, *tara* knockdown or overexpression in sLN_vs alters the morphology of their dorsal projection. Taken together, our data suggest that *tara* functions in multiple steps that link molecular cycling to overt circadian locomotor behavior. This is an ongoing project and future research will clarify how *tara* regulates locomotor rhythms, nocturnal activity, and neuronal morphology.

Introduction

The repertoire of behaviors of an animal should be timed and optimized for recurring changes in environmental conditions to promote its survival. Molecular clocks have evolved and are present in different organisms ranging from bacteria to humans [1]. The discovery of the *per* gene in *Drosophila* became the foundation to the current knowledge of the molecular mechanisms underpinning circadian rhythms [2]. The core molecular oscillator is composed of transcriptional negative feedback loops where the transcriptional activators CLK and CYCLE (CYC) are negatively regulated by their transcriptional targets *per* and *timeless* (*tim*) [1].

The neural substrates of molecular oscillations are well described in the fly brain. The PER protein is expressed in ~150 clock neurons classified in different clusters based on their anatomy, location and physiology [1]. These 150 neurons can be divided into bilateral and symmetric clusters including the dorsal lateral neurons (LNds), the dorsal neurons (DN1s, DN2s, and DN3s), the lateral posterior neurons (LPNs), the sLN_vs, and the large ventral lateral neurons (lLN_vs). The sLN_vs are believed to be necessary and sufficient to drive circadian locomotor rhythms in non-recurring environmental conditions (constant darkness and temperature) [3, 4]. In LD cycles, the sLN_vs (“morning cells”) drive the peak of activity at dawn, while the 5^{th} sLN_v and the LNds (“evening cells”) drive the peak of activity at dusk [3, 4].

The circadian mechanism includes three components that ensure proper interactions between the living organism and the environment: the input component that communicates external light and temperature conditions to the molecular clock [5]; the core molecular clock itself, which is composed of oscillatory changes in the mRNAs and proteins of core clock genes; and the output components that transduce

molecular oscillations to overt behaviors or physiologic processes. Circadian proteins such as PER and TIM can be used to address where, in the circadian mechanism, a new mutant is acting. If a new mutant is arrhythmic, but exhibits normal PER cycling, we can conclude that the output arm of the circadian mechanism is affected. But, if the PER protein does not cycle or has a different oscillatory pace, the core molecular clock machinery is impacted. Furthermore, a mutant with defects in the input pathway will show anomalous phase response curves and altered TIM degradation in response to light [6], or asynchronous cycling of the PER protein within the clock cells.

Here, we describe *tara* as a novel circadian regulatory molecule. *tara* was previously described as a regulator of transcription and is the *Drosophila* homolog of the TRIP-Br (Transcriptional Regulator Interacting with the PHD-Bromodomain) family of proteins [7, 8]. *tara* mutants have reduced robustness in rest:activity locomotor patterns [9] and *tara* knockdown restricted to the clock cell network reduces rhythm strength. In addition, our data demonstrate that *tara* negatively regulates the intrinsic speed of the molecular clock machinery and transcriptionally and post-transcriptionally regulates *Pdf*, the main synchronizing molecule of the clock cell network. *tara* not only regulates diurnal behavior and acute responses to light, but is also necessary within the PDF+ cells to modulate the structural morphology of sLN_v dorsal projection. In summary, our data suggest that TARA plays important roles in circadian locomotor behavior, nocturnal activity and neuronal morphology.

Results

tara mutants display arrhythmic locomotor activity

Sleep characterization in *tara* mutants revealed that the sleep defects were independent of the circadian mechanism [9]. However, *tara* mutants displayed a dramatic decrease in the robustness of daily oscillations of locomotor circadian activity [9]. These results suggest that *tara* has a role in the regulation of circadian rhythms in addition to its role in sleep. Pan-neuronal knockdown of *tara* by RNAi decreased rhythm strength (Figure 1A, B, and E), recapitulating the *tara* mutant phenotype [9]. To further confirm that *tara* regulates circadian rhythms, we examined *tara* mutant flies expressing a *tara* transgene ubiquitously [7, 9]. Ubiquitous expression of a *tara* transgene led to a complete rescue of the arrhythmic locomotor phenotype of *tara* mutants (Figure 1C, D, and E). Interestingly, in contrast to *tara* mutants or *tara* knockdown flies whose circadian period length did not significantly differ from that of control flies, the rescued flies exhibited an increased period length (Figure 1E), suggesting that *tara* may play a role in setting the speed of the core molecular oscillator.

Role of *tara* in the circadian rhythm of locomotor activity maps to the clock cell network

To assess whether *tara* functions in clock cells to control rhythmic behavior, we turned to *tim*-Gal4, which is expressed in all clock cells [10]. *tara* knockdown in neurons labeled by *tim*-Gal4 reduced rhythm strength by ~50% compared to sibling

controls (Figure 2A, B, and E). Rhythm strength was reduced to a lesser extent using *tim*-Gal4 than using *elav*-Gal4, suggesting that *tara* may also play additional roles in other neurons outside of the clock cell network. Next we reduced *tara* expression specifically in the LN_vs using *Pdf*-Gal4. *tara* knockdown with *Pdf*-Gal4 did not reduce rhythm strength in comparison to sibling controls (Figure 2C, D, and E), which suggests that the role of *tara* in circadian rhythms is not restricted to the LN_vs. Alternatively, *Pdf*-Gal4 may be a weaker driver than *tim*-Gal4. Neither *tim*-Gal4 nor *Pdf*-Gal4 produced an observable period length phenotype when used to drive *tara* RNAi (Figure 2E). Although our data show that *tara* is necessary in clock cells to regulate circadian locomotor activity, *tara* may also play a role downstream of the clock cells in the output component of the circadian mechanism.

tara overexpression in PDF+ neurons increases the period length of circadian locomotor activity

To further characterize *tara* function in the molecular clock we next performed overexpression experiments. To this end, we generated a new transgenic fly line containing the previously described UAS-*tara* FL construct encoding the entire sequence of the B-isoform [9]. The two *tara* isoforms, A and B, are described to be functionally interchangeable [7]. To overexpress *tara,* we made use of clock cell specific drivers. *tara* overexpression with *tim*-Gal4 lead to lethality precluding behavioral analysis of adult flies. As described above, ubiquitous rescue restored rhythm strength and also increased period length, suggesting that *tara* may impact the intrinsic speed of the molecular machinery. To address this possibility we turned to *Pdf*-Gal4 to drive the expression of UAS-*tara* FL just in PDF+ neurons. Consistent

with the ubiquitous rescue, cell-specific *tara* overexpression increased the period length by ~1.5 hours compared to parental controls without significantly affecting rhythm strength (Figure 3A, B, C, and D). Because the pace of overt locomotor behavior depends on the integrated function of the entire clock cell network [11], driving the expression of *tara* FL or *tara* RNAi transgenes with *Pdf*-Gal4 in flies with a functional clock restricted to the LN_vs (e.g., by expressing a *per* transgene only in LN_vs in a *per* null mutant background) may clarify the effect of TARA levels on period length.

tara modulates the pace of cycling of the core clock component PER

Previously, we showed that the core clock protein PER cycles normally in *tara* mutants in LD conditions [9]. To further examine the integrity and speed of the molecular clock in *tara* mutants we looked at PER expression on the 3rd day in DD. We observed robust PER protein cycling even in the strongest *tara* mutants in both the sLN_vs and DN1s, which suggests that the arrhythmic phenotype of *tara* mutants maps to signaling pathways downstream of the molecular clock (Figure 4A and C). To detect subtle changes in the pace of the molecular clock, we quantified PER cycling in sLN_vs. Interestingly, our quantification analysis of PER cycling in sLN_vs revealed that the pace of molecular clock is sped up in *tara* mutants, which is consistent with the lengthened period in locomotor activity of flies overexpressing *tara* in LN_vs. In our previous work, the PER cycling in *tara* mutants was similar to that of control flies in LD conditions [9]. The modest increase in the speed of the pace of the molecular clock detected on the 3rd day in DD may be due to an accumulation of small effects over three days.

The increase in the pace of PER cycling in *tara* mutants and the lengthened locomotor activity rhythm in flies overexpressing *tara* FL in LN_vs suggest that *tara* is a negative regulator of the intrinsic speed of the core molecular clock machinery. To further test this hypothesis, we quantified PER cycling in flies overexpressing *tara* in LN_vs after three days in constant darkness conditions. Consistently with earlier results, our data demonstrate PER cycling was delayed in TARA overexpressing flies (Figure 5A and B). To address whether the function of TARA in the control of the intrinsic speed of the molecular clock could be due to changes in its levels we quantified TARA::GFP in sLN_vs. Our results show that TARA::GFP does not cycle in sLN_vs (Figure S1A and B), suggesting that its circadian function likely derives from other aspects rather than circadian changes in its levels. Taken together, these results suggest that *tara* is a negative regulator of the intrinsic speed of the molecular clock.

Daily oscillations and levels of PDF are reduced in *tara* mutants

The clock neurons form an interconnected network that gives robustness to behavioral locomotor rhythms [11], and PDF functions as a synchronizing factor within the clock cell network [12, 13]. PDF is expressed in just 16-18 out of the ~150 clock cells per *Drosophila* brain, but its receptor is expressed in ~60% of all clock cells [14]. Intriguingly, PDF levels in the sLN_v dorsal projection have a circadian profile, although rhythmic PDF expression may not be required for circadian locomotor activity [15].

In *tara* mutants, PDF levels in the sLN_v dorsal projection were reduced by ~70% (Figure 4C and Figure 6A and B), suggesting that TARA plays a role in

regulating PDF levels. To address how *tara* regulates PDF, we quantified *Pdf* mRNA from whole heads extracts of *tara* mutants. In addition to the observed reduction in the PDF protein levels, we found that *Pdf* mRNA from whole heads extracts was also reduced by ~25%, suggesting that *tara* regulates *Pdf* transcription (Figure 6C). However, given the greater reduction in the PDF protein levels compared with the *Pdf* transcript levels, *tara* likely regulates PDF at the post-transcriptional level as well.

Pdf null mutants are arrhythmic after a few days in DD, and in LD conditions their morning anticipation is absent while their evening peak of activity is advanced [16]. In LD conditions, *tara* mutants show a morning anticipation and their evening peak of activity is not advanced (Figure 6D). However, it is important to note that PDF is not completely abolished in *tara* mutants (Figure 6A and B), which suggests that the low levels of PDF are sufficient to drive morning and evening peaks of activity. Nevertheless, the arrhythmic locomotor behavior in *tara* mutants is likely in part due to low levels of PDF and desynchronization of the clock cell network.

tara mutants display nocturnal behavior in LD conditions

Previous chromatin immunoprecipitation experiments revealed that the core clock transcription factor CLK rhythmically binds to the *tara* genomic locus [17], suggesting that *tara* is a direct target of CLK and perhaps mediates some of the *Clk* mutant phenotypes. Clk^{jrk} mutants have increased activity at night [18, 19], and *Clk* has a non-circadian role in determining the *Drosophila* diurnal pattern of activity [18]. Interestingly, *tara* mutants appear to have a switch in their daily activity profile similar to that of Clk^{jrk} mutants, with increased locomotor activity at night (Figure 6D). In

Clk^{jrk} mutants, CRYPTOCHROME (CRY) and dopamine levels are elevated [20], suggesting that *tara* mutants may also have high levels of CRY and enhanced dopamine signaling, which will be interesting to study in the future.

In the standard LD conditions, the morning peak of activity has two components: an anticipatory component driven by the circadian clock and a startle response to light onset characterized by a rapid and transient increase in locomotor activity [21]. In several arrhythmic mutants, such as *per* and *tim*, startle responses are still observed, but in *Clk*^{jrk} mutants the startle responses to light onset is absent. Interestingly, *tara* mutants phenocopy *Clk*^{jrk} mutants and lack the startle response (Figure 6D). This observation suggests that *tara* may work with *Clk* not only to regulate diurnal activity but also to regulate acute responses to light.

tara modulates the structural morphology of the sLN$_v$ dorsal projection

The sLN$_v$ dorsal projection undergoes daily morphological changes [22-24]. Early in the morning in LD cycles, the sLN$_v$ dorsal projection has an “open” conformation while at the end of the day it has a “closed” conformation. Our PDF immunostaining data suggest that the sLN$_v$ dorsal projection may be structurally altered, but due to the severe reduction in PDF levels, it was difficult to determine the morphology of the sLN$_v$ projection accurately. To test whether *tara* has a role in the circadian remodeling of sLN$_v$ dorsal projection, we used a membrane targeted version of GFP (mCD8::GFP) to label the entire structure of the sLN$_v$ dorsal projection, while using *Pdf*-Gal4 to drive *tara* RNAi or *tara* FL specifically in PDF positive cells. In the early morning (ZT3), overexpression of *tara* FL increased the length and branching of the sLN$_v$ dorsal projection, suggesting that *tara* may promote

the expansion of the sLN_v dorsal projection (Figure 7A, B, and C). Consistent with this interpretation, *tara* knockdown in PDF+ neurons blunts the length and the branching of the sLN_v dorsal projection (Figure 7A, B, and C). Additional work is required to determine whether these are developmental defects or whether TARA has an adult role in the sLN_v dorsal projection remodeling.

tara knockdown, specifically in PDF+ neurons did not alter rhythm strength (Figure 2C, D, and E), which suggests that the role of *tara* in the morphology of the sLN_v dorsal projection by itself does not cause dampened locomotor rhythms. However, it is possible that altering the structure of the entire network of clock neurons has a cumulative effect on the rhythm strength, as observed with *tara* knockdown with *tim*-Gal4.

Discussion

We have previously shown that most severe *tara* mutants are arrhythmic in DD, suggesting that *tara* regulates circadian locomotor activity in addition to sleep [9]. Here, we provide insights into the molecular and neural mechanisms underlying the regulation of locomotor rhythms by TARA. Knockdown and rescue experiments confirmed that TARA is responsible for the arrhythmic phenotype observed in *tara* mutants. Importantly, *tara* knockdown in all clock cells using *tim*-Gal4 reduced rhythm strength, which suggests that TARA acts in clock neurons to regulate locomotor rhythms. Interestingly, our data also demonstrate that TARA acts as negative regulator of the intrinsic speed of the molecular clock and controls the levels of PDF. Lack of *tara* switches daily patterns of activity so that flies start to behave as nocturnal organisms. Furthermore, *tara* has a dramatic role in the morphology of the sLN_v dorsal projection. Altogether, the evidence points for multiple roles of TARA in controlling circadian rhythms, diurnal patterns of activity, and neuronal morphology.

As described above, *tara* is a novel negative regulator of the intrinsic speed of the molecular clock. Intriguingly, TARA in itself does not appear to cycle in sLN_vs. How is TARA regulating the circadian oscillatory pace of PER without cycling itself? Recent work has shown that TRIP-Br1, one of the *tara* mammalian homologs, forms a protein complex with Protein Phosphatase 2A (PP2A) [25]. In flies PP2A regulates the circadian oscillation of PER [26], and two of the PP2A regulatory subunits: *twins* and *widerborst* are expressed in a circadian fashion. It is possible that PP2A regulates TARA phosphorylation in flies as well, and may be the regulatory factor that adds a circadian component to the function of TARA.

In flies, PP2A antagonizes GLYCOGEN SYNTHASE KINASE 3β (GSK-3β)/SHAGGY (SGG) to regulate active zone development at the NMJ [27]. This suggests that *tara* may interact with PP2A and SGG to regulate synaptic morphology in the adult fly brain. It will be interesting to address whether TARA interacts with PP2A and SGG to modulate the morphology of the sLN_v dorsal projection.

In longer photoperiodic conditions (summer days), PDF is responsible for separating the peaks of crepuscular activity [28], contributing to the adaptation of organismal physiology to seasonal changes in the duration of light and temperature cycles. Previous work has also demonstrated that, in the absence of CRY, PDF signaling is required for the maintenance of the evening peak of activity [29]. Despite the low levels of PDF observed in *tara* mutants, their morning and evening peaks of activity do not differ from those observed in control flies. In future work we will search for other behavioral consequences of low PDF levels in *tara* mutants, including whether *tara* mutants can adapt to longer photoperiodic conditions and maintain the evening peak of activity in a *cry* null background.

Previous work has found that *tara* is a direct target of the core molecular clock component CLK [17]. Interestingly, *tara* phenocopies the increased nighttime activity of Clk^{jrk} flies [30], suggesting that *Clk* and *tara* act in the same genetic pathway to regulate light-induced arousal. Previous research has demonstrated that increased CRY activity and dopamine signaling mediate the nocturnal activity of Clk^{jrk} mutants [31]. Thus, we will test whether blocking dopamine signaling and CRY activity restores diurnal activity of *tara* mutants. Whereas *Clk* and *tara* mutants exhibit similar nighttime activity phenotypes, they appear to have opposite circadian period phenotypes; Clk^{jrk} heterozygotes exhibit a long period phenotype in DD conditions [32], while *tara* mutants have a shorter PER oscillatory pace. This suggests that CLK

and TARA interact in complex ways for the control of multiple aspects of sleep:wake cycles.

From an unbiased forward-genetic screen, we recently discovered *tara* as a new sleep regulatory molecule [9]. The role of *tara* in sleep maps in part to a novel arousal center of CycA expressing neurons. Whereas knockdown of *tara* in PL neurons results in reduced sleep without arrhythmicity, knockdown of *tara* in clock neurons reduces locomotor activity robustness without reducing sleep amount, suggesting that TARA acts in distinct neural clusters to control sleep amount and circadian locomotor rhythms. Further investigation of *tara* function will likely yield important insights into the molecular and neural mechanisms of sleep and circadian rhythms.

Materials and methods

Fly stocks

Flies were maintained at room temperature (RT) on standard food containing molasses, cornmeal, and yeast. *tara*[1], UAS-*tara*-RNAi (JF01421), UAS-*dicer2* (*dcr2*), UAS–*mCD8-GFP*, and *elav*-Gal4 were obtained from the Bloomington Stock Center. UAS-*tara*-RNAi (6889R-1) was obtained from the National Institute of Genetics, Japan; *tara*^{e01264} from the Harvard Exelixis collection; *Ub-tara* from Dr. Henri-Marc Bourbon. For improved efficiency of *tara* knockdown, two RNAi lines (JF01421 and 6889R-1) were combined with UAS-*dcr2*. All fly lines were outcrossed to an isogenic background control line (iso31) for at least five generations with exception for the UAS-*tara*-RNAi lines that were not backcrossed.

Circadian assays

Four to seven day old flies entrained to a 12h:12h LD cycle for at least 3 days were individually placed in small glass tubes containing 5% sucrose and 2% agar at 25°C except where noted. Activity counts were collected in 1-min bins using *Drosophila* Activity Monitoring (DAM) System (Trikinetics).

Circadian assays were performed essentially as described [33]. Briefly, male and female flies were monitored in DD using the DAM system (Trikinetics) for six days after being entrained to an LD cycle for at least 3 days. Activity counts collected in 30-min bins were analyzed using the FaasX software (M. Boudinot and F. Rouyer, Institut de Neurobiologie Alfred Fessard, CNRS, France). The software uses χ^2

analysis to calculate period length and rhythm power. Rhythm power was determined for all flies including arrhythmic ones, whereas circadian period was determined only for rhythmic flies. Morning and evening anticipation analysis was also performed with FaasX software. Actograms were generated using ClockLab (Actimetrics).

Immunohistochemistry

Dissected brains were fixed in 4% paraformaldehyde for 30 min at RT. Samples were blocked in 1% bovine serum albumin (BSA); primary and secondary antibodies were diluted in 0.1% BSA. The following primary antibodies were used: anti-PER at 1:8000 (rabbit 13.1), mousse anti-PDF at 1:2000 (mouse C7s) from Developmental Studies Hybridoma Bank (Iowa City, USA), and rabbit anti-GFP (Invitrogen) at 1:500. The secondary antibodies, Alexa Fluor 647 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse (Invitrogen) were used at 1:500. Primary and secondary antibodies were incubated at 4°C overnight. Images were obtained on an Olympus Fluoview confocal microscope.

Statistical Analysis

Data sets with two groups were compared using *t* tests. For comparisons of multiple groups, one-way ANOVA tests were performed, followed by Dunnett or Tukey post-hoc tests. For genetic interaction experiments, two-way ANOVA tests were performed to test for the interaction.

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Author contributions

D.J.S.A. and K.K. conceived the study, designed the experiments and analyzed the data. D.J.S.A. performed the experiments. The manuscript was written by D.J.S.A. and K.K.

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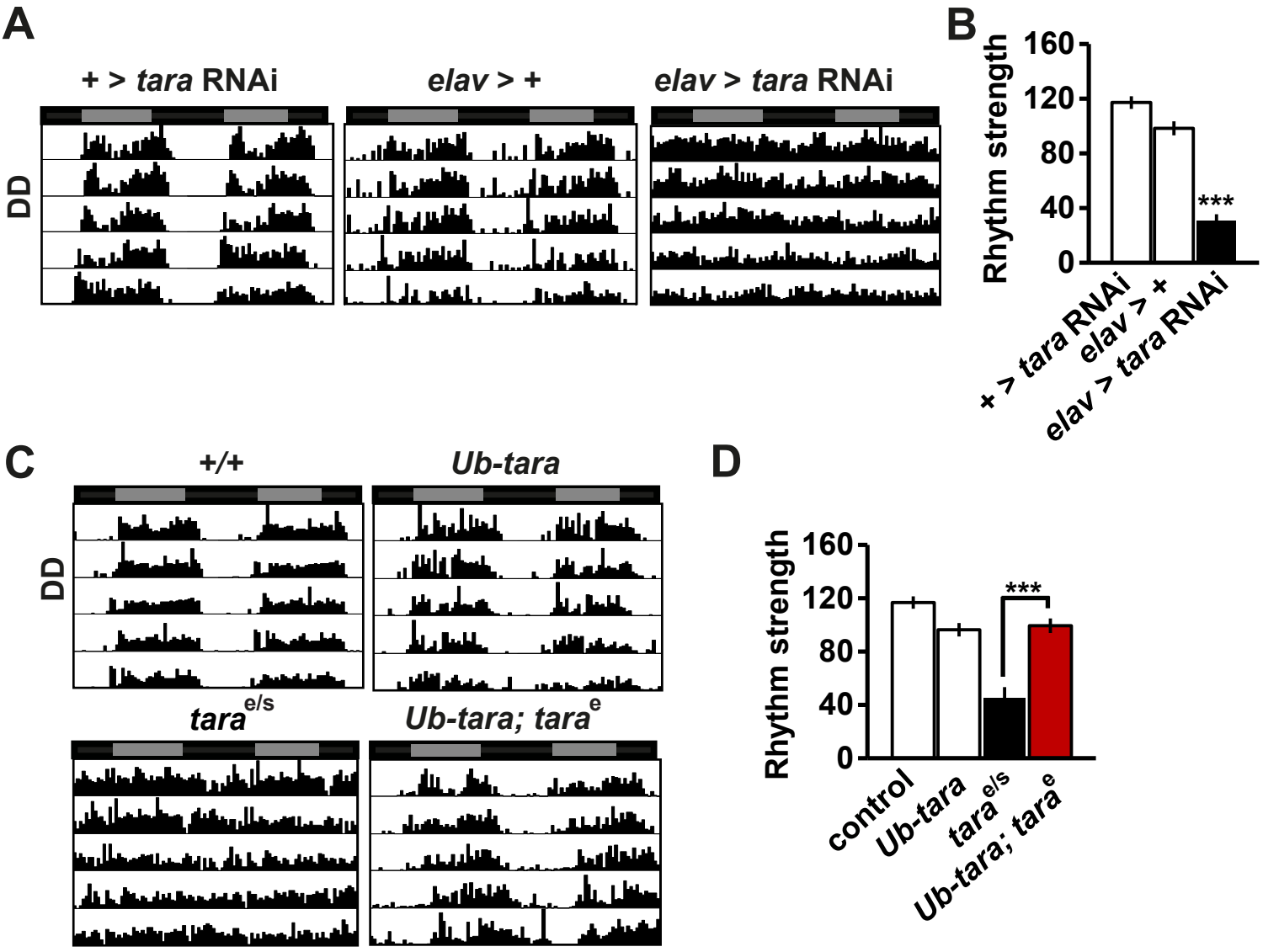
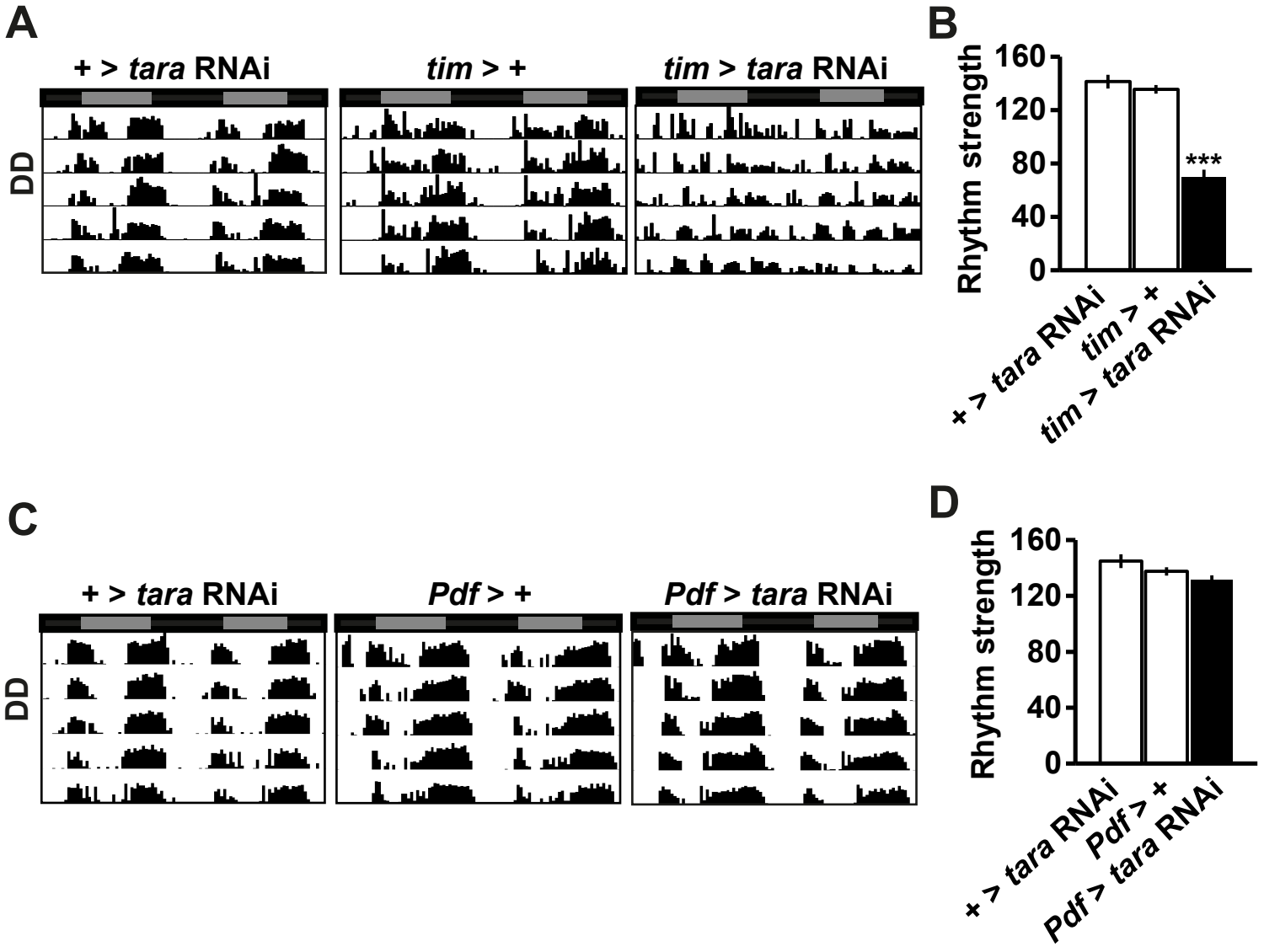


E

Genotype	N	% R	% WR	% AR	tau (h) ± SEM	Power ± SEM
Knockdown						
+ > *tara* RNAi	45	100	0	0	24.4 ± 0.07	117.1 ± 4.65
elav > +	53	88.7	3.8	7.5	24.3 ± 0.17	98.3 ± 5.14
elav > *tara* RNAi	73	26	21.9	52.1	23.9 ± 0.18	30.7 ± 4.73
Rescue						
+/+	48	95.8	4,2	0	24.1 ± 0.08	116.8 ± 4.67
Ub-tara	48	89.6	8.3	2.1	24.2 ± 0.12	96.4 ± 5.10
tara$^{e/s}$	26	50.0	23.1	26.9	24.1 ± 0.15	44.8 ± 8.50
Ub-tara; *tara*^{e}	49	85.7	10.2	4.1	25.1 ± 0.08	99.4 ± 5.45

N: number of flies; **R:** rhythmic; **WR:** weakly rhythmic; **AR:** arrhythmic
tau: free-running period; **Power:** measure rhythm strength;

Figure 1



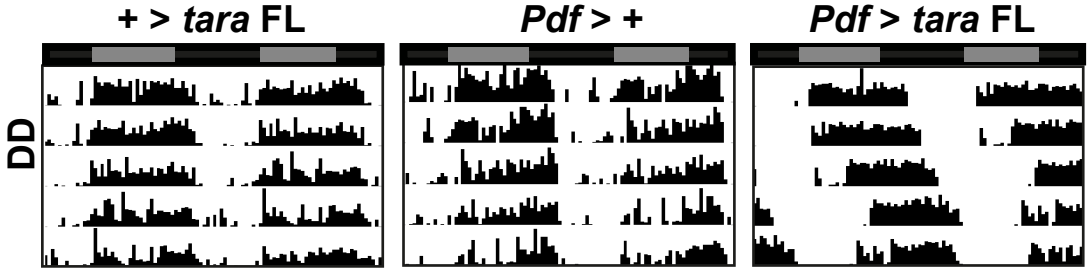
E

Genotype	N	% R	% WR	% AR	tau(h) ± SEM	Power ± SEM
+ > *tara* RNAi	46	97.8	2.2	0	23.8 ± 0.06	141.3 ± 5.12
tim > +	47	100	0	0	24.3 ± 0.05	135.6 ± 3.23
tim > *tara* RNAi	43	72.1	14	14	24.2 ± 0.08	69.3 ± 5.99
+ > *tara* RNAi	31	100	0	0	23.6 ± 0.05	144.9 ± 4.90
Pdf > +	31	100	0	0	24.3 ± 0.06	137.5 ± 2.98
Pdf > *tara* RNAi	47	100	0	0	24.0 ± 0.05	131.6 ± 3.04

N: number of flies; **R:** rhythmic; **WR:** weakly rhythmic; **AR:** arrhythmic
tau: free-running period; **Power:** measure rhythm strength;

Figure 2

A



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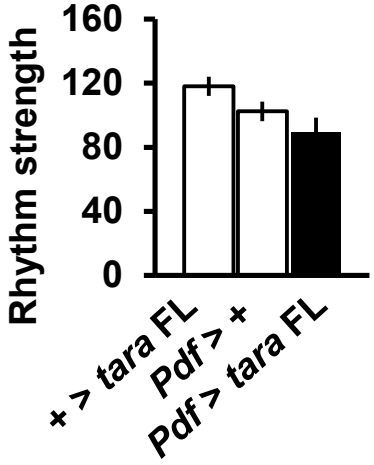

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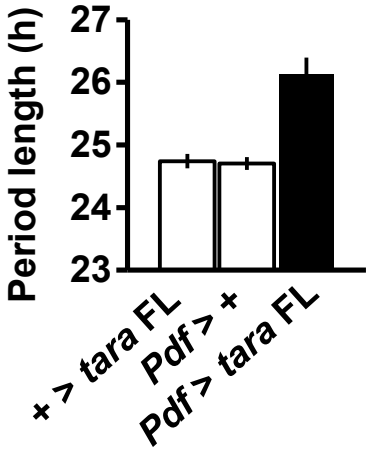

D

Genotype	N	% R	% WR	% AR	tau(h) ± SEM	Power ± SEM
+ > *tara* FL	32	96.9	3.1	0	24.7 ± 0.12	118.0 ± 6.03
Pdf > +	32	96.9	3.1	0	24.7 ± 0.10	102.4 ± 6.15
Pdf > *tara* FL	18	77.8	16.6	5.5	26.1 ± 0.28	89.7 ± 8.83

N: number of flies; **R:** rhythmic; **WR:** weakly rhythmic; **AR:** arrhythmic
tau: free-running period; **Power:** measure rhythm strength;

Figure 3

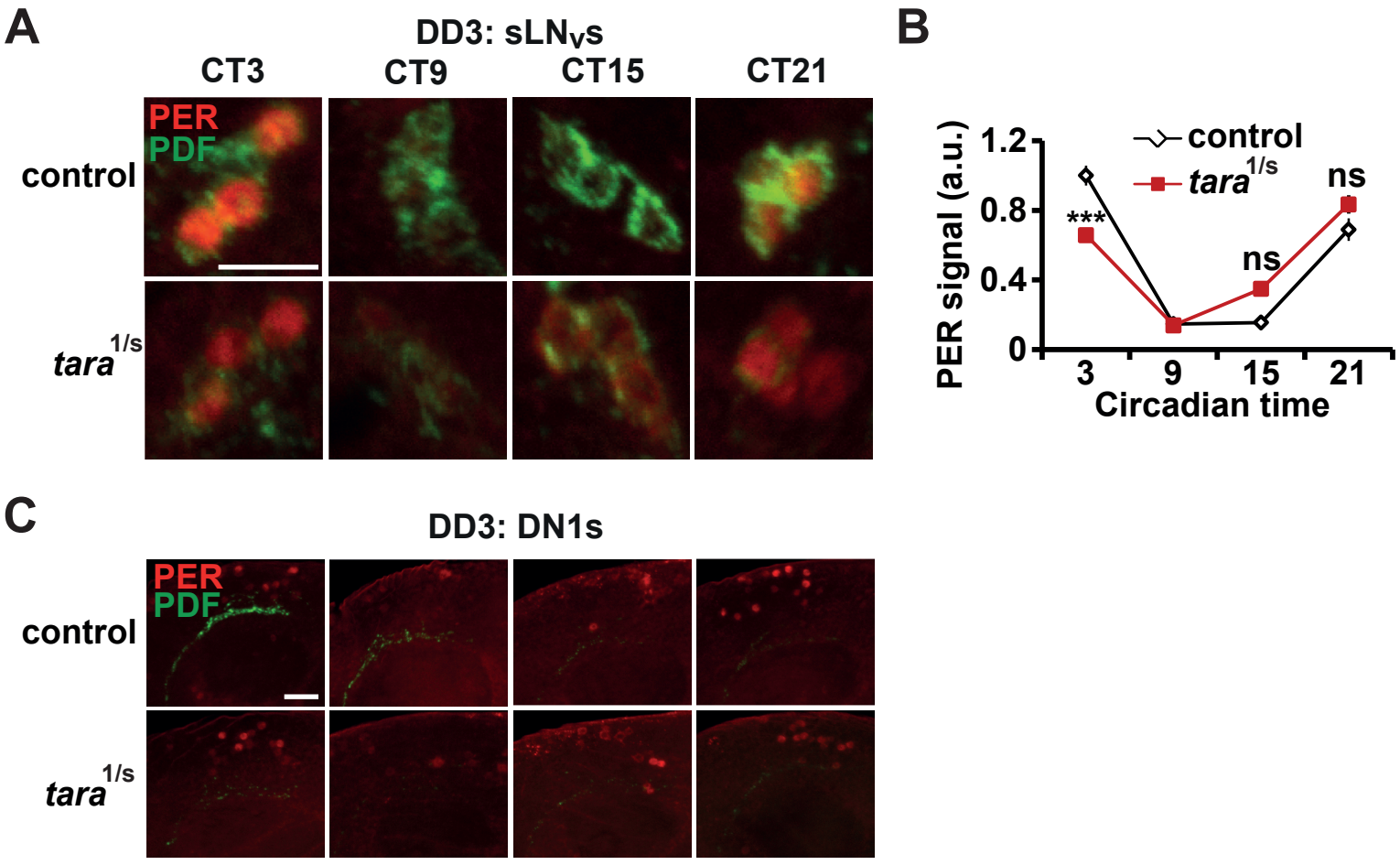
A
DD3: sLNvs
CT3
CT9
CT15
CT21
PER
PDF
control
tara1/s
B
PER signal (a.u.)
1.2
0.8
0.4
0
control
tara1/s
***
ns
ns
3
9
15
21
Circadian time
C
DD3: DN1s
PER
PDF
control
tara1/s

Figure 4

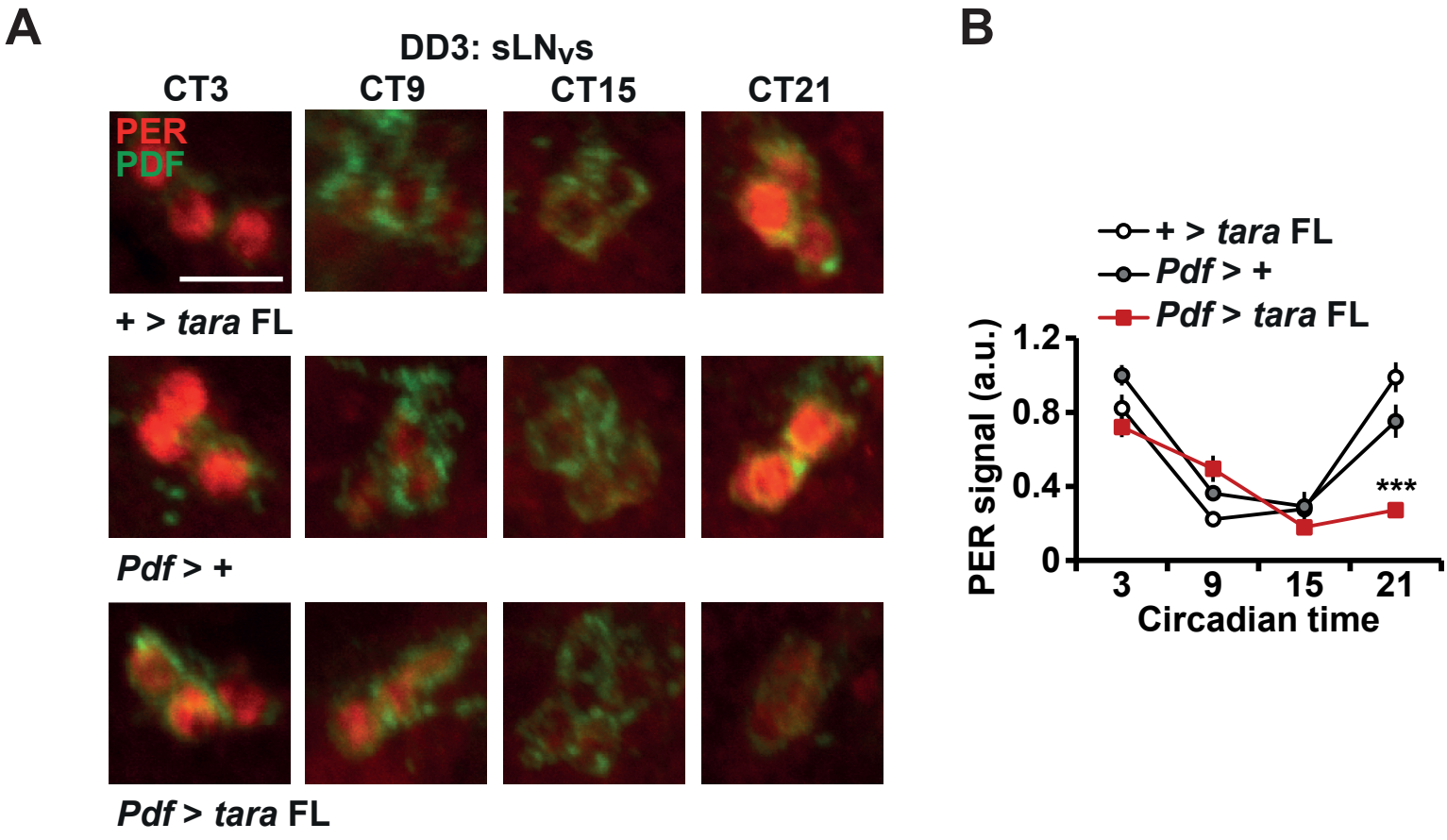
A
DD3: sLNvs
CT3
CT9
CT15
CT21
PER
PDF
+ > tara FL
Pdf > +
Pdf > tara FL
B
+ > tara FL
Pdf > +
Pdf > tara FL
PER signal (a.u.)
1.2
0.8
0.4
0
***
3
9
15
21
Circadian time

Figure 5

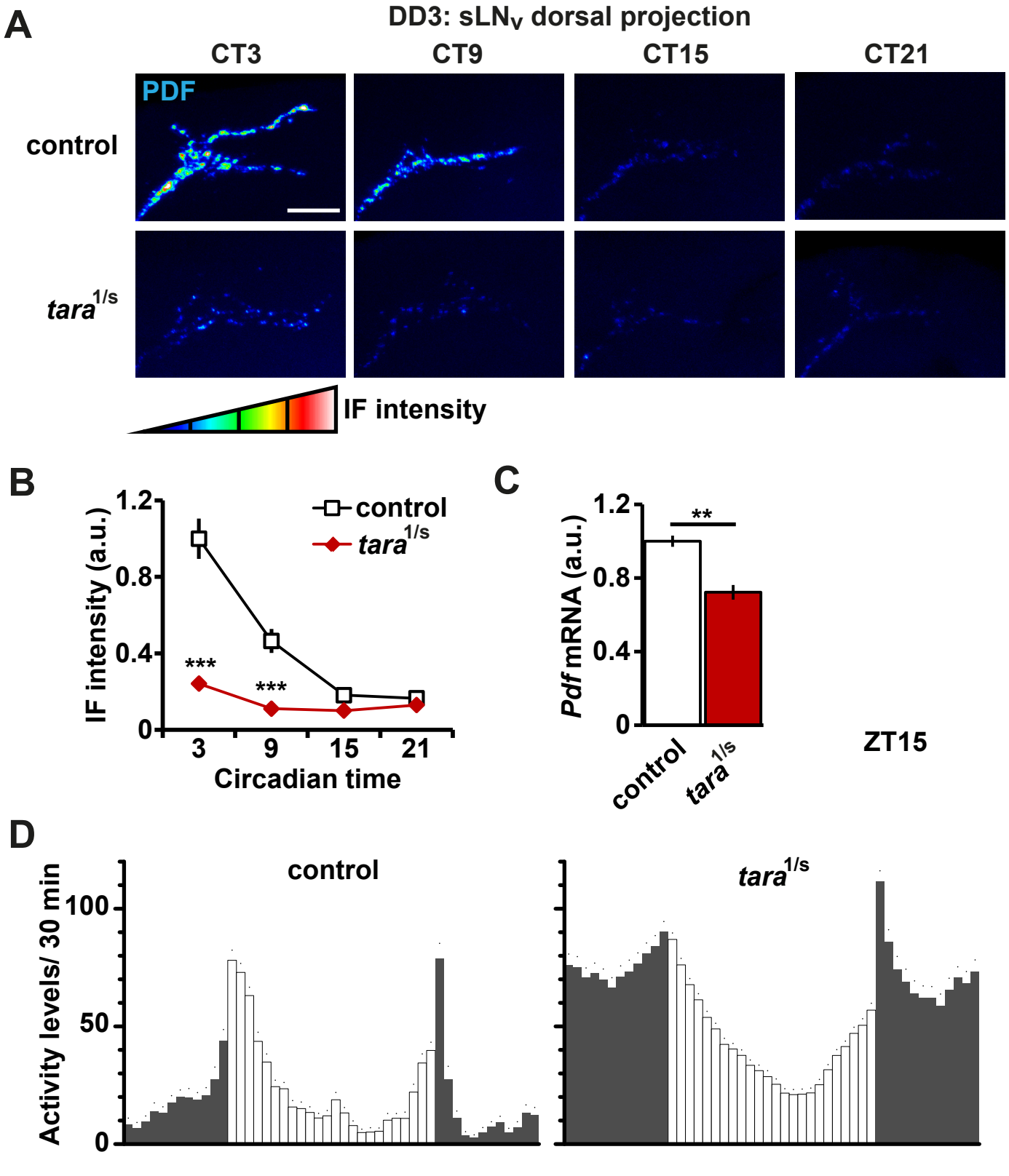
A
DD3: sLNv dorsal projection
CT3
CT9
CT15
CT21
PDF
control
tara1/s
IF intensity
B
IF intensity (a.u.)
1.2
0.8
0.4
0
control
tara1/s
***
***
3
9
15
21
Circadian time
C
Pdf mRNA (a.u.)
1.2
0.8
0.4
0
**
control
tara1/s
ZT15
D
Activity levels/ 30 min
100
50
0
control
tara1/s

Figure 6

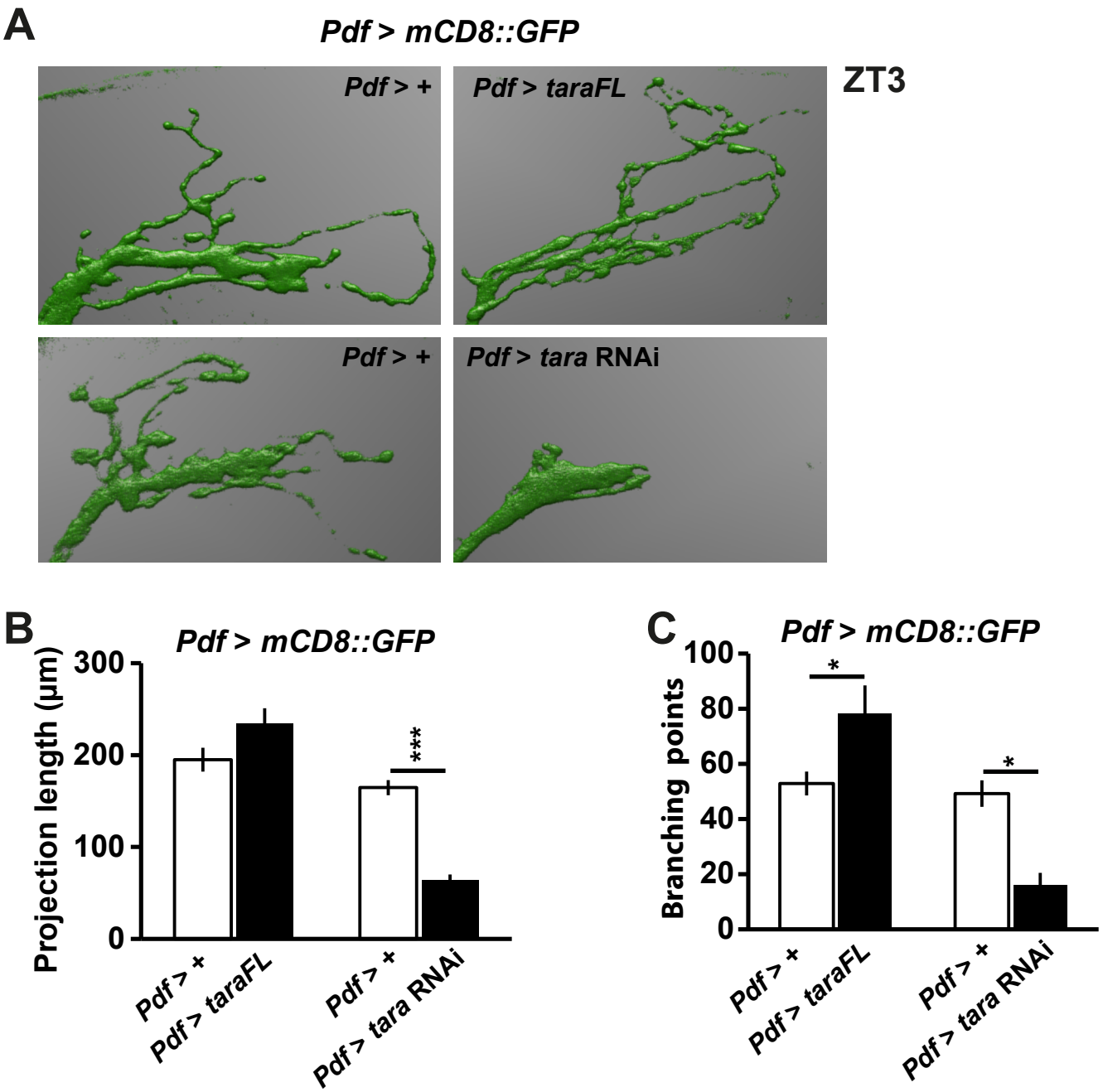
A
Pdf > mCD8::GFP
Pdf > +
Pdf > taraFL
ZT3
Pdf > +
Pdf > tara RNAi
B
Pdf > mCD8::GFP
Projection length (µm)
300
200
100
0
***
Pdf > +
Pdf > taraFL
Pdf > +
Pdf > tara RNAi
C
Pdf > mCD8::GFP
Branching points
100
80
60
40
20
0
*
*
Pdf > +
Pdf > taraFL
Pdf > +
Pdf > tara RNAi

Figure 7

FIGURE LEGENDS

Figure 1. Pan-neuronal *tara* knockdown degrades while ubiquitous rescue restores rhythm strength. (A) *tara* pan-neuronal knockdown markedly reduces rhythm strength. Representative double-plotted actograms of single background control (+ > *tara* RNAi and *elav* > +) and knockdown (*elav* > *tara* RNAi) female flies are shown. Only 2 male flies *elav* > *tara* RNAi survived for six days in free-running conditions precluding the analysis of their rhythmic behavior. Gray and black bars above the actogram indicate subjective day and night, respectively. Six 6 days in free-running conditions (constant darkness (DD) and temperature condition) are shown. **(B)** Rhythm strength of control flies (+ > *tara* RNAi and *elav* > +) and knockdown flies (*elav* > *tara* RNAi). Mean ± SEM is shown. **(C)** Representative double-plotted actograms for female flies of the indicated genotypes. **(D)** Rhythm strength for the same genotypes shown in (C). Mean ± SEM is shown. **(E)** Rest:activity locomotor rhythms for same flies shown in (B) and (D). . ***p < 0.001, one-way ANOVA followed by post hoc tests: Dunnett (B) and Tukey (D). tau indicates period length for rhythmic flies in each genotype.

Figure 2. *tara* knockdown specifically in the clock cell network degrades locomotor rhythms. (A) *tara* knockdown in clock cells markedly reduces rhythm strength. Representative double-plotted actograms of background control (+ > *tara* RNAi and *tim* > +) and knockdown (*tim* > *tara* RNAi) male flies are shown. Gray and black bars above the actogram indicate subjective day and night, respectively. Six 6 days in free-running conditions (constant darkness (DD) and temperature condition) are

shown. **(B)** Rhythm strength of control flies (+ > *tara* RNAi and *tim* > +) and knockdown flies *tim* > *tara* RNAi. Mean ± SEM is shown. **(C)** *tara* knockdown in PDF+ cells does not impact rhythm strength. Representative double-plotted actograms of background control (+ > *tara* RNAi and *Pdf* > +) and knockdown (*Pdf* > *tara* RNAi) male flies are shown. **(D)** Rhythm strength of control flies (+ > *tara* RNAi and *Pdf* > +) and knockdown flies *Pdf* > *tara* RNAi. Mean ± SEM is shown. **(E)** Rest:activity locomotor rhythms for the same flies shown in (B) and (D). ***p < 0.001 one-way ANOVA followed by Dunnett post hoc test (B).

Figure 3. *tara* overexpression in PDF+ cells increases period length of locomotor activity without significantly degrading rhythm strength. (A) Representative double-plotted actograms of background control (+ > *tara* FL and *Pdf* > +) and overexpression (*Pdf* > *tara* FL) female flies are shown. Gray and black bars above the actogram indicate subjective day and night, respectively. Six 6 days in free-running conditions (constant darkness (DD) and temperature condition) are shown. **(B)** Rhythm strength for the indicated genotypes. **(C)** Period length for the indicated genotypes. Mean ± SEM is shown. **(D)** Rest:activity locomotor rhythms for same flies shown in (B).

Figure 4. In *tara* mutants PER cycling in sLN_vs is faster. (A) PERIOD and PDF co-staining in sLN_vs for control and $tara^{1/s}$ at the circadian times shown after three days in free running conditions. Scale bar is 10µm. **(B)** PERIOD signal quantification in sLN_vs for control (n=16 to18) and $tara^{1/s}$ (n=11 to 18) male brains. Mean ± SEM is shown. **(C)** PERIOD and PDF co-staining in DN1 neurons for control and $tara^{1/s}$ at the indicated

circadian times shown, after three days in free running conditions. Scale bar is 20 µm. ***p < 0.001, ns: not significant, one-way ANOVA followed by Tukey post hoc test (B).

Figure 5. TARA overexpression in PDF+ neurons negatively regulates the pace of molecular clock. (A) PERIOD and PDF co-staining in sLN_vs for controls (+ > *tara* FL and *Pdf* > +) and overexpression (*Pdf* > *tara* FL) male flies at the indicated circadian times after three days in free running conditions. Scale bar is 10µm. **(B)** PERIOD signal quantification in sLN_vs for + > *tara* FL (n=8 to 11), *Pdf* > + (n=10 to 16), and *Pdf* > *tara* FL (n=10-12). Mean ± SEM is shown. ***p < 0.001, one-way ANOVA followed by Tukey post hoc test (B).

Figure 6. *tara* regulates *Pdf* both transcriptional and post-transcriptionally. (A) Representative sLN_v dorsal projection at the indicated circadian times are shown after three days in free running conditions for control and $tara^{1/s}$ male flies. Immunofluorescence (IF) intensity is artificially represented by a color spectrum where black represents the lowest intensity and white represents the maximum intensity. Scale bar is 20µm. **(B)** PDF signal quantification in the sLN_v dorsal projection for control (n=16 to 18) and for $tara^{1/s}$ (n=15 to 17). Mean ± SEM is shown. **(C)** *Pdf* mRNA levels in control and $tara^{1/s}$ flies. Average of three independent sets is shown. Mean ± SEM is shown. **(D)** Morning and evening anticipation activities for control and $tara^{1/s}$ flies in LD conditions. ***p < 0.001, one-way ANOVA followed by Tukey post hoc test (B). **p < 0.01, *t* test analysis (C).

Figure 7. *tara* modulates sLN_v dorsal projection structural morphology. (A) Representative sLN_v dorsal projection for the indicated genotypes are shown. sLN_v dorsal projection were reconstructed from confocal microscope files using the neurostudio software. To label the entire sLN_v dorsal projection we made use of membrane targeted GFP construct (UAS-*mCD8::GFP*). **(B)** Quantification of sLN_v dorsal projection length quantification for the same genotypes shown in (A). Quantification was automatic starting on the first branching point to the tip of the projection. *Pdf* > + (n=10), *Pdf* > *tara* FL (n=10), *Pdf* > *dicer2* (n=10), and *Pdf* > *dicer2*; *tara* RNAi (n=6). **(C)** Number of branching points for the same genotypes shown in (A). Mean ± SEM is shown. *p < 0.05, and ***p < 0.001, *t* test analysis.

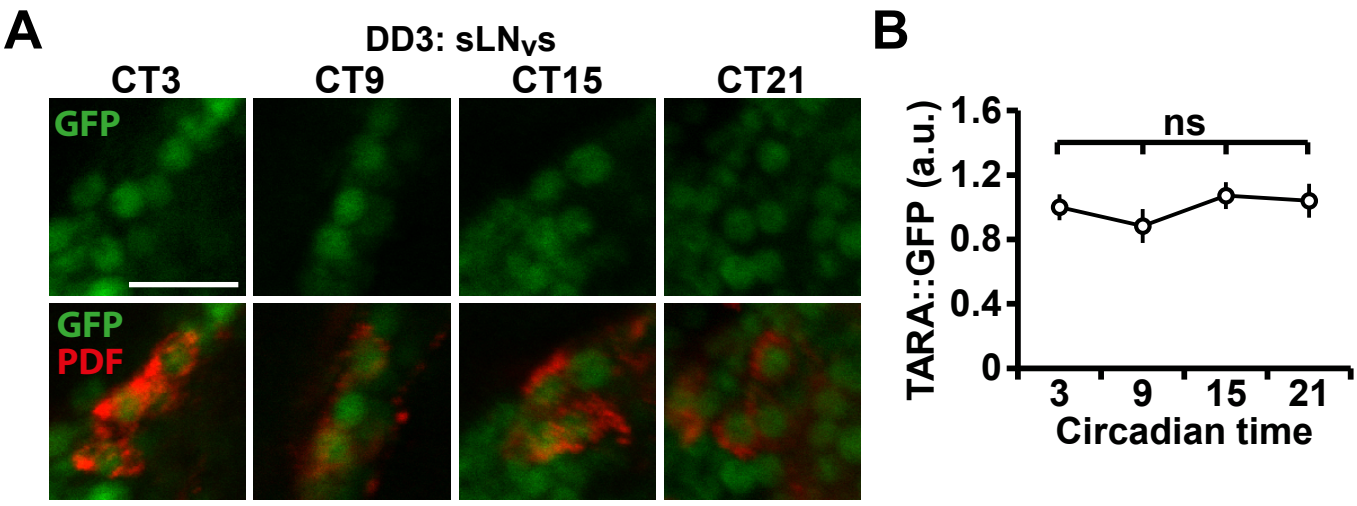

Figure S1. TARA protein levels do not cycle in circadian pacemaker neurons. (A) Immunostaining of TARA::GFP in male fly brains on the 3rd day in DD. We used transgenic flies that carry an artificial exon encoding GFP inserted into an intron of tara in the genome and therefore are expected to produce endogenous levels of TARA protein fused to GFP. Brains were dissected at indicated circadian times (CT) and stained for GFP (green) and PDF (red), which was used to identify small ventral lateral neurons (sLN_vs), the pacemaker neurons in DD. Scale bar 10μm. **(B)** Quantification of TARA::GFP signal in sLN_vs. Data from 11-16 brain hemispheres are presented. Mean ± SEM is shown. ns: not significant, two-way ANOVA followed by Tukey post hoc test (B).

Chapter 3

Discussion, Conclusions and Future Perspectives

I. *tara* is a novel regulator of sleep

Our results demonstrate that *tara* has an important role in sleep regulation. *tara* mutants display a dramatic decrease in daily sleep during the day and the night. They also have an insomnia-like phenotype with increased latency in sleep initiation. These defects can be rescued with the introduction of *tara* cDNA in the *tara* mutant background. We also found that TARA is expressed in neurons but excluded from glial cells. Importantly, the role of *tara* in sleep regulation is, in part, adult specific.

Sleep is controlled mainly by two mechanisms: a circadian mechanism that times sleep to an ecologically relevant part of the day, and a homeostatic mechanism that ensures that an adequate amount of sleep is maintained [1]. Most of the stronger *tara* mutants are arrhythmic, yet sleep levels are decreased in constant light and constant dark conditions which demonstrates that *tara* regulates sleep independently of the circadian mechanism and of the light input pathways. These observations leave the homeostatic mechanism as the probable place where *tara* function is required to control sleep amount.

Indeed our preliminary data suggest that TARA impacts the homeostatic response to sleep deprivation. When deprived of sleep for 4 hours toward the end of the night, *tara* mutants exhibited significantly reduced rebound sleep compared to control flies. However, after 12 hours of sleep deprivation, the rebound sleep of *tara* mutants was similar to that of the controls (data not shown). These data demonstrate that *tara* mutants exhibit a dose-dependent homeostatic response to sleep deprivation. Additional studies are required to determine the mechanism by which TARA controls sleep homeostasis.

II. *tara*, *CycA*, and *Cdk1* form a new sleep regulatory pathway

TARA has a CycA binding homology domain and *CycA* was shown to regulate sleep [2]. These led us to hypothesize *tara* and *CycA* regulate sleep through the same genetic pathway. Our genetic and physical interaction studies demonstrate that *tara* and *CycA* indeed interact with each other to regulate sleep. This is important because we did not simply find a new sleep regulatory molecule, but also demonstrated how this new molecule is integrated into a larger gene network consisting of cell cycle genes [2].

TARA was previously described as a transcriptional co-regulator [3]. This previous work and our finding that TARA::GFP fusion protein is expressed in neuronal nuclei are consistent with a role for *tara* in transcriptional control. However, TARA physically binds CycA and regulates its levels at the post-transcriptional level. As discussed in more detail below, these observations suggest that TARA regulates sleep through a novel non-transcriptional mechanism independent from those controlling cell cycle progression. Future experiments aimed at separating the transcriptional from the non-transcriptional roles may help determine their relative importance for sleep regulation.

Through bioinformatics analysis, Calgaro *et. al* showed that TARA has four conserved domains [3], one of which being the C-terminal domain. In the mammalian homologs of TARA, the C-terminal domain appears to be required for their transcriptional role [4]. We hypothesize that TARA controls sleep through a non-transcriptional mechanism which will be clarified by studying whether the C-terminal domain of TARA is required for transcriptional regulation but dispensable for sleep regulation.

Our data suggest that CycA regulates sleep through its modulatory action over Cdk1 activity. Cdk1 is regulated through inhibitory phosphorylation of its T14 and Y15 residues, which is dependent on the cellular context [5]. Because overexpression of wild-type Cdk1 did not affect sleep, presumably due to tight regulatory mechanisms, we made use of the Cdk1 (T14A,Y15F) mutant, termed Cdk1-AF, that cannot be inhibited [5] to show that increased activity of Cdk1 suppresses sleep. Our model is that CycA, and possibly other cyclins, suppresses Cdk1 activity within the CycA+ cells, reducing its activity and therefore increasing sleep. Other scenarios are possible, but a direct relationship between CycA and Cdk1 is likely, given that CycA and Cdk1 are known to physically interact [6]. Interestingly, both CycA and Cdk1 localize to synaptic regions, which suggest a direct modulatory role for CycA and Cdk1 over synaptic proteins. Identification of the substrates of the Cdk1 kinase activity relevant for sleep regulation is an important next step we intend to pursue in future experiments.

Recent work in *Drosophila* has demonstrated that knockdown of *Cdk1* and other Cyclin/Cdk family genes significantly rescues seizure duration in both *bas*^{1} (*bang sensitive*^{1}) and *bss*^{1} (*bang sensless*^{1}) mutants [7]. This suggests that Cdk1 may modulate ion channel activity and membrane excitability. Several lines of evidence show that ion channels have a dramatic influence over sleep. *Shaker*, a fast delayed rectifier potassium channel [8], *hyperkinetic*, the *Shaker* cytoplasmic beta subunit [9], *ether-à-go-go*, slow delayed rectifier potassium channel [10], *redeye*, nicotinic Acetylcholine Receptor α4 [11], and *Rdl*, a $GABA_A$ receptor gene [12] are all implicated in sleep and may be potential targets for *Cdk1*. Given that increased excitability of PL neurons suppresses

sleep, the net effect of Cdk1 activity over these channels may be an overall increase in neuronal excitability. Thus, these channels may serve as plausible phosphorylation targets of Cdk1.

The relationship between sleep and epilepsy is well established [13]. However, how sleep and epilepsy are interconnected at the molecular level is less clear. Interestingly, cell cycle genes are implicated in both sleep and epilepsy. Therefore, addressing how the cell cycle regulators *tara* and *Cdk1* regulate sleep may contribute to our understanding of how epileptogenic processes develop, highlighting how the finding of a basic molecular mechanism regulating one behavior contributes to the understanding of other processes.

III. A novel arousal circuit modulated by *tara*, *CycA*, and *Cdk1*

Previous work showed that CycA protein is expressed in a small number of neurons [2]. In order to manipulate the CycA expressing cells we made use of the PL (*pars lateralis*)-Gal4, which labels just the dorsal cluster of CycA expressing cells. Increasing the activity of these neurons led to a strong sleep suppression phenotype, suggesting that they serve as an arousal center. Importantly, we demonstrated their adult function by showing that adult-specific and reversible activation of these neurons suppresses sleep. Given that constitutive expression of Cdk1-AF phenocopies PL neurons activation, we envision a model where CycA regulates the activity of PL neurons through Cdk1. In *CycA* mutants, lower levels of CycA lead to higher Cdk1 activity, which in itself increases the excitability of PL neurons and, therefore, promotes wakefulness. Whether or not Cdk1 leads to an overall increase in the excitability of PL neurons is of interest to address in the future.

Different groups of neuronal populations are implicated in the regulation of sleep. These include the mushroom body [14-16], the fan shaped body [17], the pars intercerebralis [18], the dorsal paired medial (DPM) neurons [19], the PPL1 cluster of TYROSINE HYDROXYLASE positive neurons [20], and the large ventral lateral clock neurons (lLN_vs) [21, 22]. For future directions, it will be interesting to determine whether or not PL neurons form synapses with previously identified sleep/arousal centers. In order to address that, we can make use of GFP reconstitution across synaptic partners (GRASP). If PL neurons form synapses with other known sleep relevant neuronal population, it will be of further interest to examine how changes in the neuronal activity of one

group of neurons modify the activity of the other group using genetically encoded Ca^{2+} indicators [23].

IV. *tara* may control sleep by regulating synaptic structure

During sleep animals cannot engage in productive behaviors as foraging or mating and are at a higher vulnerability to predation. This apparent weakness of sleep contrasts with its near universality across different organisms, which suggests that the benefits of sleep outweigh the hazards [24]. Sleep may have more than one function; it may restore energy and supplies in the brain, rescale synapses, and consolidate memories [25].

Increasing evidence suggests that sleep is important for neural plasticity [26, 27]. In rodents, markers of synaptic strength are higher after wake and lower after sleep [28, 29]. Evidence from *Drosophila* has also shown that the volume of synapses in the sLN_v dorsal projection is increased after wakefulness and sleep deprivation [26], establishing the sLN_v dorsal projection as a useful neuronal target for the study of sleep dependent plastic changes.

Our results showing that both CycA and Cdk1 localize to the synaptic terminals suggest that they play a role in synaptic function. TARA may play a role in synaptic morphology through its interaction with CycA and Cdk1. Our data demonstrating that *tara* knockdown in PDF+ neurons dramatically affects dorsal projection morphology is proof of principle that TARA affects synaptic structure. The molecular mechanisms underlying the role of TARA in synaptic structure and function may be a fruitful area of future research.

V. *tara* negatively regulates the intrinsic speed of the molecular clock

The molecular clock synchronizes organismal physiology to external environmental conditions at a pace of ~24 hours. Different genes and circuits are responsible for maintaining the speed of the molecular clock even in the absence of external cues. Our initial analysis of circadian locomotor activity demonstrated that most of the stronger *tara* mutant flies were arrhythmic, which led us to ask whether the molecular clock was still cycling.

Previously we reported that PER still cycles robustly in the stronger *tara* mutants in LD conditions [30]. Our new data revealed that PER protein cycles at a faster pace in *tara* mutants than in control flies in DD conditions, suggesting that TARA normally decreases the speed of the molecular clock. We did not detect a short-period locomotor rhythm in *tara* mutants, but this could be because more severely affected mutants become arrhythmic, making it difficult to determine their period length. Our finding that TARA overexpression in PDF+ neurons slows down PER cycling and increases the period length of locomotor rhythm further points to a negative effect of TARA in the intrinsic speed of the molecular clock.

Multiple interlocking transcriptional feedback loops that regulate the pace of the molecular clock were identified in *Drosophila*. In addition to the main loop involving PER/TIM, CLK/CYC directly activates the transcription of their activator *PAR domain protein 1* (*Pdp1*) as well as the repressors *vrille* (*vri*) and *clockwork orange* (*cwo*) [31]. Previous chromatin immunoprecipitation experiments suggest that *tara* is a direct transcriptional target of CLK [32]. While we did not observe cycling of *tara* mRNA levels in whole head extracts, *tara*

function may cycle in clock neurons. Given the known role of TARA in transcriptional regulation, it will be interesting to investigate whether TARA interacts with previously described feedback loops or functions in a novel transcriptional feedback loop.

Most strong *tara* mutants are arrhythmic but exhibit robust molecular cycling of PER, which suggests that *tara* may play other roles downstream of the molecular clock. In contrast to the core clock mechanism, only a limited number of molecules were shown to act downstream of the core molecular clock, and those are: *Pigment dispersing factor* (*Pdf*) [33, 34], *Pdf receptor* [35-37], *neurofibromatosis1* [38], *slowpoke* [39], *narrow abdomen* [40], *ebony* [41], *miR-279* [42], *dyschronic* [43], and *diuretic hormone 44* [44]. Starting from such a selected group of genes will simplify future studies investigating genetic interactions between *tara* and genes that are pivotal to the output arm of the circadian mechanism.

VI. *tara* regulates PDF levels

The circadian locomotor rhythm of *Pdf* null mutants dampens in free-running conditions and their morning anticipation is absent while the evening anticipation is advanced [34]. *tara* mutants exhibit low levels of PDF in the sLN_v dorsal projection, yet *tara* mutants have close to normal morning and evening anticipations in LD conditions. This suggests that low levels of PDF are sufficient to drive morning and evening anticipations in these mutants. Examination of *tara* mutants in long photoperiod conditions, where *Pdf* null mutants are unable to delay the phase of the evening peak [45], may provide a more sensitive assay for the functional consequences of low PDF levels.

Robust circadian locomotor activity rhythms rely on the entire clock cell network. However, the sLN_vs set the pace of the other clock neurons in constant darkness as long as their pace is not more than 2.5 hours apart from the pace of the sLN_vs [46]. This synchronizing effect is largely mediated by PDF. PDF is expressed in the sLN_vs and lLN_vs, which represent only 16-18 cells out of ~150 clock neurons. The sLN_v dorsal projection is in close proximity to the dorsal groups of clock cells and it was shown that more than 60% of the clock cells respond to PDF [47].

Daily rhythms in the morphology of the sLN_v dorsal projection were described [48], which include not only daily cycles in fasciculation and defasciculation [49], but also gains and losses in axonal material [50]. However, the relevance of these daily structural changes remains unclear [51]. Our results showing that flies with knockdown of TARA in sLN_vs are rhythmic despite exhibiting altered morphology of the sLN_v dorsal projection suggest that

the morphological changes are not the primary cause of the circadian rhythm phenotypes in *tara* mutants. Still, low levels of PDF in these neurons likely contribute to the desynchronization of the clock cell network and to the reduced rhythm strength seen in *tara* mutants.

VII. *tara* regulates nocturnal activity and neuronal morphology

As humans, flies are diurnal organisms. Interestingly, *tara* mutants display increased nighttime activity suggesting that *tara* is important for the regulation of the diurnal pattern of activity. *Clk* mutants also have an increased nocturnal activity phenotype [52, 53], and physically bind *tara* genomic locus [32], raising the possibility that *Clk* and *tara* work in the same genetic pathway to regulate the diurnal pattern of behavior of flies. In Clk^{jrk} mutants, CRYPTOCHROME (CRY) and dopamine levels are elevated [54], Thus, we will next test whether blocking dopamine signaling and CRY activity in *tara* mutants restore their diurnal activity, and whether *tara* and *Clk* genetically interact to regulate nighttime activity.

Another interesting avenue for future research derives from a previous finding that TRIP-Br1, one of the *tara* mammalian homologs, physically interacts with and is regulated by PROTEIN PHOSPHATASE 2A (PP2A) [55]. In mammalian cells, overexpression of PP2A decreased the phosphorylated form of TRIP-Br1 and increased total TRIP-Br1 protein levels, suggesting that PP2A is a positive regulator of TRIP-Br1 [55]. Interestingly, PP2A plays a role in the molecular clock by directly dephosphorylating PER and controlling its stability and cycling [56]. PP2A is an abundant heterotrimeric enzyme composed of a highly conserved catalytic subunit, a variable regulatory subunit, and a structural subunit. In *Drosophila*, there are four genes encoding PP2A regulatory subunits, two of which, *twins* and *widerborst*, are under circadian control [56].

In addition to its role in the molecular clock, PP2A regulates active zone development and synaptic terminal morphology at the *Drosophila* NMJ [57]. This role of PP2A in synaptic morphology is particularly interesting in light of our results suggesting that *tara* regulates the morphology of sLN_v dorsal projection. It will be interesting to examine whether PP2A and TARA interact to regulate synaptic morphology and locomotor rhythms.

In conclusion, we have identified two new sleep regulatory molecules that, together with other cell cycle genes, may become important targets for the treatment of sleep disorders. Importantly, *tara*, *CycA* and *Cdk1* have mammalian homologs that may regulate sleep as well. Beyond that, this work constitutes a strong foundation for future lines of research, including the understanding of diurnal versus nocturnal behaviors and aspects of the structure and function of synapses.

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