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# The "Ins and Outs" of Sleep: How Internal Metabolic and External Environmental Signals Affect the Sleep Process

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# The "Ins and Outs" of Sleep: How Internal Metabolic and External Environmental Signals Affect the Sleep Process

## **Abstract**

The behavioral pattern of activity followed by rest (activity:rest) is an evolutionarily conserved trait. A prominent manifestation of rest in vertebrates consists of sleep, which is adaptive because a disruption of sleep by internal or external influences results in severe health outcomes such as obesity, mood disorders, cognitive impairment, and so forth. Despite the clinical imperative to understand how these cues influence sleep, the mechanisms by which they do so are still unclear. Internally, metabolic cues interact with biochemical pathways that regulate sleep amount and quality. Externally, numerous environmental cues such as light, temperature, and chemicals affect the timing of sleep behavior. The goal of this thesis is to understand the mechanisms by which one internal (the 'ins') and one external (the 'outs') cue regulates sleep behavior using *Drosophila melanogaster*. This work consists of two independent projects that evaluate the 'ins and outs' of sleep. We begin with the 'ins' by addressing how one metabolic enzyme GABA Transaminase (GABAT) controls energy and sleep homeostasis. GABAT is a mitochondrial enzyme found in GABAergic neurons and glial cells where it breaks down the sleep-promoting neurotransmitter, GABA, into glutamate and succinic semialdehyde, both of which can also be used for energy via the TCA cycle. In this study, we determine the mechanism by which GABAT regulates metabolic and sleep homeostasis. In our second project, we evaluate the 'outs' of sleep by examining an uncharacterized phenomenon whereby organisms lose their ability to maintain the circadian timing of sleep cycles in the presence of cold-temperature stress. In this project, we use wild-derived flies to evaluate the evolutionary significance of maintaining circadian cycles in some strains regardless of temperature variance, and then determine the cellular and molecular mechanism by which other strains lose their ability to drive circadian rhythms of behavior at low temperature. Overall, the work accomplished in this thesis puts us one step closer towards understanding the 'ins and outs' of sleep.

## **Degree Type**

Dissertation

## **Degree Name**

Doctor of Philosophy (PhD)

## **Graduate Group**

Neuroscience

## **First Advisor**

Amita Sehgal

## **Subject Categories**

Neuroscience and Neurobiology

THE “INS AND OUTS” OF SLEEP: HOW INTERNAL METABOLIC AND EXTERNAL  
ENVIRONMENTAL SIGNALS AFFECT THE SLEEP PROCESS

Sarah E. Maguire

A DISSERTATION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2014

Supervisor of Dissertation

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THE "INS AND OUTS" OF SLEEP: HOW INTERNAL METABOLIC AND EXTERNAL ENVIRONMENTAL SIGNALS AFFECT THE SLEEP PROCESS

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## DEDICATION

In 1985 my parents, Drs. Jean Bennett and Albert Maguire, were just beginning their career in science when they found out they were pregnant with me. My Dad joked that I was going to be their final thesis project at Harvard Medical School, labeling me as, “The effect of gene transfer on a human neonate.” Little did they know that unlike a science experiment (which typically doesn’t take more than a couple months) they’ve had to be responsible for this one for more than 28 years! With the advent of personal diagnostics in 2010, I’ve finally been able to see what they’ve done: thanks to them I am 3.0% Neanderthal and was the tallest person in my elementary school. But in looking over this genetic data, I did come up with one major criticism of their thesis project: I’m not just a product of the genes they’ve transferred, but also of the wonderful environment they provided for me: thank you mom and dad for encouraging my interests in science throughout the ages, for providing me with the best education, best siblings, best family dinners, wonderful house, dogs, rabbits, guinea pigs, lovebirds, lizards, mice, and even a tarantula. Although you two have the busiest careers of anyone I know, you always made time to encourage all my interests, however wacky they were.

Mom and Dad, I dedicate this thesis to you. I cannot thank you enough for everything you’ve done for me.

## ACKNOWLEDGMENTS

I would like to thank members of the Sehgal lab for all of their help and support since I first rotated in 2010. Their insights and discussions helped me not only in understanding my thesis work, but also the field of sleep and chronobiology. I would especially like to thank Richard Stone and Sue Kelchner for their administrative support; David Garbe for teaching me confocal microscopy; Mi Shi for helping me understand gene mapping; Kiet Luu for making thousands of locomotor tubes and fly bottles; Wen-Feng Chen for teaching me about GABAT; and Mallory for teaching me brain dissections. I'd also like to thank Dan, Christine, Anna, Iryna, Kat, Lei, Annika, Leksa, Dechun, Shirley, Matt, Ben, Greg, A Reum, Hiro, Christie, Abby, Jill, Chris, Sam, Zhefeng, Dechun, Shailesh, Renkse: your discussion have made me a better scientist. I would also like to thank my mentor, Dr. Amita Sehgal. When I was choosing a lab to join, I was told that Amita was a great choice because does excellent science. During my time with Amita, I have realized that she is not only a great scientist, but also a great manager and teacher. She has provided an excellent environment in which to foster my own ideas and explore questions of interest while providing guidance along the way. Thank you, Amita.

I would also like to thank Jane Hoshi, Drs. Rita Balice-Gordon, Josh Gold, and Chris Pierce for giving me the opportunity to work as an NGG student. When I transferred over to the program, you were very welcoming and made my transition very easy.

I would like to thank my co-authors: Seth Rhoades and Drs. Paul Schmidt and Aalim Weljie. You provided excellent experiments and data, without which, my projects would be not be complete. I would also like to thank my committee members, Dr. Tom Jongens and Dr. David Raizen. Your comments and insights have greatly improved my work and papers, and for that I am grateful.

Lastly, I would like to acknowledge my family: Katie and Max, you always keep me laughing. Darling: you are an amazing and brilliant grandmother. My friends: NGG and beyond.

And last but not least, I want to thank my wonderful boyfriend, Chris Reece. Chris, you have been incredible from the very beginning: you are so brilliant and kind.

## ABSTRACT

### THE “INS AND OUTS” OF SLEEP: HOW INTERNAL METABOLIC AND EXTERNAL ENVIRONMENTAL SIGNALS AFFECT THE SLEEP PROCESS

Sarah E. Maguire

Amita Sehgal, Ph.D.

The behavioral pattern of activity followed by rest (activity:rest) is an evolutionarily conserved trait. A prominent manifestation of rest in vertebrates consists of sleep, which is adaptive because a disruption of sleep by internal or external influences results in severe health outcomes such as obesity, mood disorders, cognitive impairment, and so forth. Despite the clinical imperative to understand how these cues influence sleep, the mechanisms by which they do so are still unclear. Internally, metabolic cues interact with biochemical pathways that regulate sleep amount and quality. Externally, numerous environmental cues such as light, temperature, and chemicals affect the timing of sleep behavior. The goal of this thesis is to understand the mechanisms by which one internal (the ‘ins’) and one external (the ‘outs’) cue regulates sleep behavior using *Drosophila melanogaster*. This work consists of two independent projects that evaluate the ‘ins and outs’ of sleep. We begin with the ‘ins’ by addressing how one metabolic enzyme GABA Transaminase (GABAT) controls energy and sleep homeostasis. GABAT is a mitochondrial enzyme found in GABAergic neurons and glial cells where it breaks down the sleep-promoting neurotransmitter, GABA, into glutamate and succinic semialdehyde, both of which can also be used for energy via the TCA cycle. In this study, we determine the mechanism by which GABAT regulates metabolic and sleep homeostasis. In our second project, we evaluate the ‘outs’ of sleep by examining an uncharacterized phenomenon whereby organisms lose their ability to maintain the circadian timing of sleep cycles in the presence of cold-temperature stress. In this project, we use wild-derived flies to evaluate the evolutionary significance of maintaining circadian cycles in some strains regardless of temperature variance, and then determine the cellular and molecular mechanism by which other strains lose their ability to drive circadian rhythms of behavior at low



temperature. Overall, the work accomplished in this thesis puts us one step closer towards understanding the 'ins and outs' of sleep.

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## CHAPTER 1: INTRODUCTION AND OVERVIEW

This introduction consists of three parts: the first is an historical overview of how *Drosophila melanogaster* emerged as one important model system of sleep. At its most basic level, sleep is a function of two independent processes: one that controls the quality and amount of sleep ('Process S') and one that controls the circadian timing, or more specifically the ~24h rhythm, of sleep ('Process C') (Borbély 1982). Much more is known about the circadian process (C), but the emergence of *Drosophila* as a model system has led to the identification of several molecules affecting Process S, acting on either sleep-promoting or wake-promoting pathways. Interestingly, recent studies in *Drosophila* have revealed an additional function of certain Process S molecules: not only do they act on sleep circuits but they are also intimately tied to cellular function, particularly with metabolism. In the second part of this introduction, we review how certain Process S molecules regulate both metabolic and sleep homeostasis. We then change our focus in the third part of this introduction to discuss how external, environmental cues influence the circadian timing of sleep. In addition to light, temperature is one important cue that affects the timing of sleep by modulating the oscillations of the internal circadian clock. Here we discuss how changes in ambient temperature affect the functioning of the molecular circadian clock and thus the timing of sleep.

### **Part 1: Can we use *Drosophila* to study sleep?**

The behavioral pattern of activity:rest is evolutionarily conserved in distinct animal phyla ranging from nematodes to mammals. A prominent manifestation of the resting phase in mammals consists of sleep, which we characterize according to physiological markers such as the electroencephalogram (EEG), which displays the brain's electrical activity. Traditionally, sleep researchers have used EEGs to identify sleep, which has limited the study of sleep to species within the mammalian and avian orders because EEGs are a function of the vertebrate brain (Campbell and Tobler 1984; Siegel 1995; Tobler 1995). However, early observations that human sleep characteristics are heritable (Ambrosius, et al. 2008; Dauvilliers, et al. 2005) contributed to

an intense interest in identifying genetically-traceable model organisms to understand the genetics of sleep. Mice have ~85% genetic similarity to humans (Church, et al. 2009) and display the characteristic EEG markers of sleep, so they are an excellent model to study the genetic basis of this process. However, mouse models also have a number of drawbacks: they have a long generation time, they show compensation or redundancy in several fundamental genes, and until very recently, genetic tools were not available to manipulate the spatiotemporal expression of genes in the mouse brain (Rossant and McMahon 1999). On the other hand, the fruit fly *Drosophila melanogaster* has had a rich history for the genetic dissection of complex behavior dating back to 1911<sup>1</sup>. *Drosophila* also overcome several challenges of the mouse model: they have fast generation times (an average of 10 days as compared to 63 days in *Mus musculus*!) and a relatively simple genome that can readily be manipulated using the tools of modern day molecular genetics. But, do *Drosophila* sleep?

While patterns of activity:rest are conserved in genetically distinct animal lineages (Drucker-Colin 1995), it is not always obvious whether all species experience sleep during the resting phase (Siegel 2008). Because EEGs are a function of the mammalian brain, they cannot be used to assess whether *Drosophila* sleep. However, mammals do exhibit specialized behaviors during sleep, such as: 1) circadian periods of immobility 2) a species-specific posture and/or resting place 3) an increased arousal threshold during rest 4) a homeostatic regulatory mechanism and 5) changes in central neuronal function during the rest period. Therefore, researchers can use these behavioral criteria to investigate whether invertebrates sleep during their resting phase. In 2000, studies done independently by Hendricks et al. 2000 and Shaw et al. 2000 (Hendricks, et al. 2000a; Shaw, et al. 2000) showed that *Drosophila* do sleep during their resting phase. Since then, *Drosophila* has become a widely used model organism to investigate the genetics of sleep.

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<sup>1</sup> Thomas Hunt Morgan (1866-1945) is the father of *Drosophila* genetics. Using *Drosophila*, he discovered the role that chromosomes play in heredity (Morgan 1911).

In the following review, we discuss how specific cues modulate sleep pathways in *Drosophila*. At the heart of all models describing the regulation of sleep behavior is the 'two-process model' of sleep (Borbély 1982). In this model, the sleep process is attributable to two independent phenomena: a homeostatic drive that controls sleep quality and amount (Process S) and a circadian control system that regulates the timing of sleep (Process C) (fig. 1). Although these two systems have an intimate relationship (Antle and Mistlberger 2000; Naylor, et al. 2000), one assumption of the model is that they operate independently of each other (Mistlberger, et al. 1983; Tobler, et al. 2000), which is supported by evidence showing that the homeostatic response to sleep persists even after circadian rhythmicity is disrupted (Dijk, et al. 1989), or that measures of fatigue still oscillate on a circadian basis after multiple days of sleep deprivation (Åkerstedt and Fröberg 1977).

## **Part 2: How do Process S molecules regulate metabolic and sleep homeostasis?**

According to the two process model of sleep (Borbély 1982), the drive to sleep is partially controlled by Process S, a homeostatic mechanism that predicts sleep quality and sleep amount (fig. 1). Practically nothing is known about the homeostatic mechanism that underlies sleep drive, although molecules affecting sleep amount and quality have been identified. More recently, studies have revealed that there is an intimate link between Process S molecules and cellular function, particularly with metabolism. For instance, the administration of  $\gamma$ -Aminobutyric acid (GABA) – a sleep promoting neurotransmitter in humans and flies (Agosto, et al. 2008; Chen, et al. 2014; Chung, et al. 2009; Parisky, et al. 2008; Saper, et al. 2010) – to human islet cells increases circulating human insulin and lowers blood glucose levels (Purwana, et al. 2014). However, delineating the mechanism by which Process S molecules affect metabolic homeostasis is complicated in mammals because of the complexity of their brain structure (Hendricks, et al. 2000b). On the other hand, the fruit fly has a relatively simple brain and can easily be used to understand the association between sleep and metabolic homeostasis. We

discuss below how certain molecules implicated in Process S regulate not only sleep, but also carbohydrate, lipid, or amino acid metabolism in *Drosophila*.

Process S molecules and carbohydrate/lipid metabolism in Insulin Producing Cells (IPCs) The insulin pathway is highly conserved from mammals to *Drosophila* and serves the same physiological functions (Taguchi and White 2008). During conditions when nutrients are plentiful, insulin is secreted and absorbs nutrients such as glucose and lipids and stores them for later use in the form of glycogen and triacylglycerol (TAG). Fly insulin-producing cells (IPCs), which are homologous to pancreatic beta cells, are found in the brain and produce three of eight *Drosophila* insulin-like peptides (DILPs): DILP2, 3, 5. Once released into circulation, these peptides bind to insulin receptor tyrosine kinase receptors (InR), which activate the PI3-kinase (PI3K) signaling pathway. Once activated, PI3K increases glucose uptake and storage in targeted tissue (Leto and Saltiel 2012). To date, numerous studies have analyzed how IPC activity and thus DILP secretion is regulated in the fly. Interestingly, a number of sleep molecules involved in Process S (GABA (Enell, et al. 2010; Rajan and Perrimon 2012); octopamine (Erion, et al. 2012; Luo, et al. 2014); serotonin (Luo, et al. 2014); and the neuropeptide short neuropeptide 'F' (Lee, et al. 2008)) influence or have physiological effects through DILP expression in the IPCs. The fact that sleep-relevant molecules regulate the activity of neurosecretory cells is interesting because it suggests that there is a functional link between sleep and metabolic homeostasis. In this section we will review what is known about the regulation of sleep and metabolic homeostasis in the IPCs by Process S molecules (fig. 2).

**GABA.** The inhibitory neurotransmitter GABA inhibits IPC activity through metabotropic GABA<sub>B</sub> receptors (GBRs). Flies with targeted knockdown of GBRs in the IPCs showed a faster metabolism of lipids and higher rates of lethality, indicating that GABA inhibits the production and/or release of DILPs (Enell, et al. 2010). At present, the mechanism by which GABA inhibits DILP secretion is unknown: binding of GABA<sub>B</sub> receptor commonly couples to G-protein-coupled inwardly rectifying potassium channels (GIRKS), but also inhibits Adenylate Cyclase via G<sub>ai</sub>/G<sub>α0</sub>:

both of which lead to hyperpolarization. While the signaling cascade through which GBRs control insulin signaling in the IPCs has not been identified, we have some understanding of the regulatory pathways that control transmission from GABAergic neurons to IPCs. Rajan and Perrimon 2012 (Rajan and Perrimon 2012) found that leptin-like Unpaired 2 (Upd2) is released from the fat body after feeding and acts on the receptor, Dome, which is expressed on GABAergic neurons adjacent to the IPCs. Upd2 activates JAK/STAT signaling in the Dome expressing GABAergic neurons, an effect that inhibits GABA release and thus reduces tonic inhibition on IPCs. Once GABAergic activity is inhibited, IPCs release DILPs into circulation. To date, it is unknown whether GABA regulates sleep via the IPCs.

*Octopamine.* Not only does octopamine – the *Drosophila* equivalent of norepinephrine – function as a wake-promoting signal via the IPCs, but it also regulates lipid homeostasis (Erion, et al. 2012). In *Drosophila*, octopaminergic neurons project all over the brain and can signal through several different receptors (Evans and Robb 1993). One neuronal projection is directed to the IPCs (Luo, et al. 2014) and activation of the octopamine receptor (OAMB) on the IPCs stimulates cAMP-dependent Protein Kinase A (PKA) via Adenylate cyclase, an effect that suppresses sleep (Crocker and Sehgal 2008; Crocker, et al. 2010). Recently, Erion et al. 2012 (Erion, et al. 2012) showed that octopamine acts through the insulin signaling pathway to promote fatty acid synthesis and inhibit lipid breakdown. While activation of OAMB receptors on IPC cells also inhibits *dilp3* transcription (Luo, et al. 2014), it is unknown whether octopamine regulates lipid metabolism through DILP3<sup>2</sup>. Interestingly, Erion et al. 2012 (Erion, et al. 2012) found that increased triglycerides levels associated with octopamine activation does not promote wake (or visa-versa), indicating that octopamine regulates sleep and metabolic homeostasis in the IPCs through independent mechanisms.

*Serotonin.* Serotonergic axon terminals are directly adjacent to IPCs. Binding of one of the serotonin receptors (5-HT<sub>1A</sub>) in the IPCs inhibits the expression of *Dilp2* and *Dilp5* transcripts

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<sup>2</sup> Octopamine does not regulate carbohydrate metabolism (Luo et al. 2014, *PLoS One*).



(Luo, et al. 2014)<sup>3</sup>, an effect that increases levels of hemolymph glucose, body trehalose and glycogen. At present, it is unknown which IPC signaling pathway mediates the effect of 5-HT<sub>1A</sub> binding on carbohydrate metabolism. Binding of 5-HT<sub>1A</sub> can affect the behavior of inwardly rectifying potassium channels (GIRKs) and adenylyl cyclases (Johnson, et al. 2009; Nichols and Nichols 2008) and so these are candidates for future study. Specific roles of 5-HT<sub>1A</sub> binding have also been investigated in the context of sleep in *Drosophila*. Knocking down 5-HT<sub>1A</sub> receptor significantly causes short and fragmented sleep (Yuan, et al. 2006), but it is unknown whether this affect involves the IPCs.

*short neuropeptide F (sNPF)*. Through a series of elegant experiments Lee et al. 2008 (Lee, et al. 2008) showed that sNPF promotes insulin signaling. The authors showed that sNPF receptors are expressed on 7 IPC cells, which are directly adjacent to sNPFergic neurons. Overexpressing the IPC-sNPF receptor (sNPF<sub>R1</sub>) increases transcription of *Dilp1* and *Dilp2* mRNA through MAP kinase signaling (which includes the action of ERK). To verify that sNPF induction of DILP affects insulin signaling, the authors measured carbohydrate levels in sNPF overexpression mutants and found that glucose and trehalose were reduced. Mutants of sNPF show a sleep phenotype, though it is unclear how the neuropeptide affects sleep: Chen et al. 2013 and Shang et al. 2013 show conflicting data where Chen et al. 2013 (Chen, et al. 2013) report that sNPF is wake-promoting whereas Shang et al. 2013 (Shang, et al. 2013) report that sNPF is sleep-promoting. The conflicting reports regarding sNPF action on sleep likely result from differences in baseline levels of sleep in the wild-type controls used in the two studies. Chen et al. 2013 (Chen, et al. 2013) demonstrated longer sleep times upon reduction of sNPF, which led them to propose that sNPF normally promotes wake, but their wild type males slept on average ~400min (40). On the other hand, wild-type males in Shang et al. 2013 (Shang, et al. 2013) slept on average of ~850 minutes, and loss of sNPF reduced sleep time, suggesting that sNPF promotes sleep. In our

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<sup>3</sup> Consistent with these results mutants of the serotonergic synthesis pathway show increased levels of DILP2 protein (Kaplan et al. 2008, *Genes Dev.*)

experience measuring sleep in flies, a total sleep time for male flies of ~400 minutes is comparable to that of severe short sleeping sleep mutants we have identified in our lab: e.g. *sleepless (sss)* (Koh, et al. 2008) or *redeye (rye)* (Shi, et al. 2014), so we are inclined to believe that sNPF is a sleep-promoting neuropeptide (Shang, et al. 2013). Shang et al. 2013 show that sNPF does not promote sleep through IPCs because downregulating sNPF receptors in these cells does not affect sleeptime (Shang, et al. 2013). Thus, effects of sNPF on sleep may be mediated in cells other than the IPCs, although it affects metabolism and growth via these cells (Lee, et al. 2008).

#### Process S molecules and amino acid metabolism in GABA Transaminase (GABAT)-positive cells.

In comparison to what is known about the effect of Process S molecules on sleep and lipid/carbohydrate homeostasis, it was unknown whether there is a link between Process S molecules and amino acid metabolism in *Drosophila*. We addressed this question using a mutant of the enzyme GABA Transaminase (GABAT) strain as an experimental probe. GABAT is a catabolic enzyme of  $\gamma$ -Aminobutyric (GABA), a sleep-promoting amino acid in humans and flies (Agosto, et al. 2008; Chen, et al. 2014; Chung, et al. 2009; Parisky, et al. 2008; Saper, et al. 2010). Within mitochondria, GABAT breaks down GABA into glutamate and succinic semialdehyde using alpha-ketoglutarate ( $\alpha$ -KG) as an amine acceptor. *In vitro* work has shown that GABA-derived glutamate and succinic semialdehyde can independently regulate energy homeostasis via entry into the tricarboxylic acid (TCA) cycle, but it was unknown whether any of these connections were functionally important for maintaining energy homeostasis *in vivo* (fig. 3). In an earlier paper, we demonstrated that GABAT regulates sleep homeostasis because mutant strains of GABAT sleep an average of ~200 minutes more than wild-type flies (Chen, et al. 2014). While the excess sleep observed in these mutant stress is likely due to high GABA levels, it may also be attributable to insufficient glutamate levels, one wake-promoting neurotransmitter in eukaryotes. In Chapter 2, we determine the 'ins' of sleep by addressing whether GABAT controls energy and sleep homeostasis. In this chapter, we show that the catabolism of GABA via GABAT

is functionally important for regulating both energy and sleep homeostasis *in vivo* albeit through independent mechanisms.

## **Part 2: How do external, environmental cues influence Process C?**

According to the two process model (Borbély 1982), the timing of sleep is controlled by Process C, a circadian network in the brain (fig. 1). In *Drosophila*, this network consists of ~150 neurons (fig. 4) that exhibit molecular oscillations of a specialized genetic circuit, known as the “molecular circadian clock,” the core mechanism of which consists of a negative feedback loop in which ‘clock’ proteins inhibit the transcription of their own mRNAs (fig. 5). In the principle feedback loop, two transcription factors (CLK and CYC) bind to E-boxes upstream of the *period* (*per*) and *timeless* (*tim*) genes and transcribe *per* and *tim* mRNA. These mRNAs are translated into proteins which accumulate in the cytoplasm (CT16-20), form a heterodimer complex, and translocate into the nucleus (CT0-4). Phosphorylation plays a central role in the accumulation of the proteins as well as their nuclear transfer. Once the complex translocates into the nucleus, PER-TIM bind to CLK-CYC (late night), inhibiting their binding affinity for the *per* and *tim* E-boxes, thereby negatively regulating the transcription of their own mRNAs. The negative inhibition is removed once other proteins degrade the PER-TIM complex, allowing the cycle to begin again. Transcriptional feedback is an important determinant of the circadian period and similar feedback loops operate in mammalian clocks (Takahashi, et al. 2008). In addition, several additional genes contribute to the robustness and fine-tuning of the molecular clock. For instance, a second feedback loop involving *vriille* and *Pdp1* transcription-factor genes generates rhythmic *Clk* gene expression and so contributes to high-amplitude clock-gene expression. For a review of the molecular circadian clock in *Drosophila*, refer to Zheng and Sehgal 2012 (Zheng and Sehgal 2012).

While the molecular circadian clock is an endogenous oscillator, meaning that the internal timing mechanisms persist in constant conditions, the timing mechanism of the clock can be modulated by external environmental cues, such as such as light, temperature, and chemicals. Of

these, temperature is especially interesting because it influences the oscillations and expression of the molecular circadian clock in several ways. In the following article, we will review the three ways in which the molecular clock responds to temperature under free-running conditions i.e. in the absence of other stimuli (including light) from the external environment: 1) the molecular clock entrains to heat shocks and temperature cycles 2) the molecular clock compensates for changes in temperature to maintain the same circadian period 3) the molecular clock dampens under low or high temperature stress. In discussing how the molecular clock responds to these temperature cues, we will also review what is known about the *input pathways* that sense and relay temperature information to clock neurons. To date, we do not fully understand how the clock neurons transmit signals to sleep-circuits, so we do not cover this information here.

The molecular clock is entrained by heat shock or temperature cycles. In nature, the molecular circadian clock synchronizes to natural cycles of daily light versus darkness and associated temperature fluctuations. When holding light conditions constant (e.g. constant darkness (DD) or constant light (LL)), the phase of the endogenous molecular clock and thus the output locomotor rhythm entrains to heat shocks (Kaushik, et al. 2007; Sidote, et al. 1998) or temperature cycles (Glaser and Stanewsky 2007) through independent mechanisms. In terms of heat shock, phase change of the molecular clock occurs through the blue light photoreceptor Cryptochrome (CRY) (Kaushik, et al. 2007), which degrades the PER and TIM proteins. At the biochemical level, heat shocks induce a conformational change in the PER-TIM complex, which facilitates an interaction with active CRY, which then degrades the PER-TIM complex. This degradation process via CRY resets or delays the phase of the clock.

While it is unknown molecularly how the clock synchronizes to temperature cycles, Glaser and Stanewsky 2005 (Glaser and Stanewsky 2005) found that the gene *no receptor potential A (norpA)* – which encodes phospholipase C (PLC) – is necessary for the response. In an earlier study, Majercak et al. 1999 (Majercak, et al. 1999) showed that *norpA* mediates the thermosensitive splicing of the 8<sup>th</sup> intron of the *per*, which is functionally important for the behavioral advance of activity under cold temperature: under this condition, *norpA* mediated

splicing of *per* advances *per* mRNA accumulation, protein, and thus behavioral activity (Majercak, et al. 1999). This was the motivation underlying Glaser and Stanewsky 2005 (Glaser and Stanewsky 2005) interest in testing whether *norpA*-mediated *per* splicing was important for the clock's synchronization to temperature cycles. Interestingly, the authors found that *norpA* mediated splicing of *per* is not functionally relevant for the molecular clock's synchronization to temperature cycles because wild-type animals contain equal amount of spliced and unspliced forms of the *per* transcript during temperature cycling conditions. This suggests that PLC plays an unknown role in molecular synchronization to temperature cycles.

How is heat shock or temperature cycle information transmitted to clock neurons? It is currently unknown which peripheral organs or receptors transmit heat shock cues to clock neurons, however there is evidence to suggest that *Drosophila* sense temperature cycles via the chordontal organs (Sehadova, et al. 2009), which are mechanosensory structures that operate as stretch receptors in insects. This is because knocking-down structural proteins in these organs [such as *nocte* (*no circadian temperature entrainment*)] prevents behavioral entrainment (Glaser and Stanewsky 2005). Work by Wolfgang et al. 2013 (Wolfgang, et al. 2013) suggests that chordontal organs contain different types of thermoreceptors specialized to sense different ranges of temperature cycling, such as the TRP channel encoded by the *pyrexia* (*pyr*) gene, which senses and transmits low (16-20°C) but not high (21-29°C) temperature cycles. At present, it is unknown how the peripheral structures transmit temperature information to clock neurons.

Clock neurons are divided into 8 groups based on their location, size, projection pattern, and neuropeptide content (Nitabach and Taghert 2008): s-LNVs, I-LNVs, 5<sup>th</sup> s-LNVs, LNDs, DN1-3s, and the LPNs (fig. 4). Some of these groups, such as the LPNs and some DN groups, are highly sensitive to temperature cycles because they preferentially entrain to temperature cycles over light cycles when the two zeitgebers are out-of-phase (Miyasako, et al. 2007). This indicates that these cells receive input regarding temperature cycles, but it is unknown whether this is transmitted via peripheral networks or via temperature sensitive channels on these neurons. Studies have shown that these cells are not necessary to entrain *Drosophila* to temperature

cycles because when Busza et al. 2007 (Busza, et al. 2007) restored *per* to CRY-positive or PDF-positive cells (which do not include the DNs and LNDs) in a *per*<sup>01</sup> background, these flies could be entrained to temperature cycles.

The clock can compensate for changes in temperature to maintain circadian period. One generic property of the molecular clock (and the circadian rhythm it underlies) is its ability to maintain a ~24h periodicity over a permissive temperature range, a property known as ‘temperature compensation.’ This was first demonstrated in 1954 by Colin Pittendrigh (Pittendrigh 1954), who observed that the eclosion rhythm (which is controlled by the molecular clock) has a constant period when assayed over a wide range of temperatures. This is an interesting kinetic property because biochemical reactions are predicted to speed up or slow down according to warm or cool temperatures respectively, and so the constancy of circadian period implies a unique underlying biochemistry. To date, theoretical models have been most successful in explaining the mechanism of temperature compensation: thus far, studies have found the most support for the ‘antagonistic principle’ (Hastings and Sweeney 1957; Ruoff 1992), which states that circadian period will remain stable over a range of temperatures if the reaction rates that decrease period length are balanced by those that increase it. Later, Bodenstein et al. 2011 (Bodenstein, et al. 2011) showed through theoretical modeling that this balance can occur by choosing appropriate activation energies for each reaction. From a molecular genetic perspective, it is unlikely that there is a *bona fide* temperature compensation gene, although some circadian molecules are clearly more important than others<sup>4</sup>. Interestingly, several *Drosophila* circadian mutants can temperature compensate (albeit with an abnormal period length), while some are completely defective in temperature compensation – for a complete list of these mutants, see Hong et al. 2007 (Hong, et al. 2007).

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<sup>4</sup> In 1997, Sawyer et al. 1997 found that there is natural variation in the number of threonine-glycine (Thr-Gly) encoding repeats of *per*, where flies with a longer variant (Thr-Gly<sub>20</sub>) can compensate temperature between 18-29°C, whereas flies with the shorter variant (Thr-Gly<sub>17</sub>) could not. To date, no one has followed up on how Thr-Gly encoding repeats of *per* affect temperature compensation.

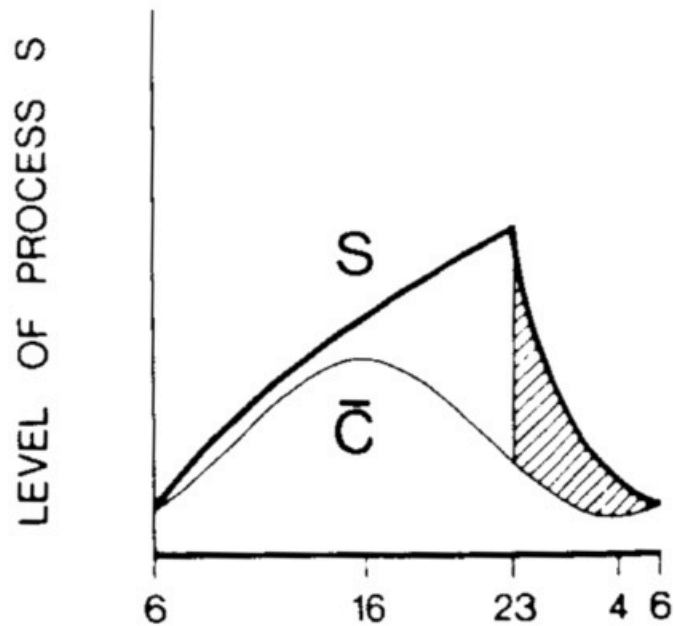
Molecular oscillations of the clock dampen under low and high temperature conditions. Circadian rhythms, such as sleep cycles, typically persist only over a permissive temperature range. As noted above, circadian period of these rhythms remains constant over this range. However, under more extreme high or low temperature conditions, rhythmic cycles are abolished in circadian organisms such as *Drosophila*. Until recently, little was understood about the functional significance of this clock property nor the mechanism underlying the loss of overt circadian behavior under extreme temperatures. The challenge inherent in studying this circadian property is the difficulty in identifying genetic lines that are capable of maintaining rhythm under low temperature stress that can serve as a basis of comparison. In Chapter 3, we overcame this challenge by sampling from natural populations of *Drosophila* that are evolving under divergent selection pressures of the environment. Using lines derived from the wild, we identified ecologically relevant natural variation in the expression of rhythmic behavior under low temperature conditions. This suggests that maintenance of circadian rhythm is directly relevant to populations in the wild that are affected by temperate selection pressures. In Chapter 3, we also highlight the advantage of using lines derived from natural populations to identify novel loci relevant to temperature-influenced circadian traits as well as to understand the molecular basis underlying the overt loss of rhythm under low temperature stress.

In conclusion, the molecular circadian clock responds to external temperature cues several different ways: some of these cues modulate the phase of the clock but do so through distinct mechanisms (e.g. temperature cycles and heat shock). In addition, there is variability in how the clock responds to a temperature ranges: within a permissive temperature range, the clock can resist the affects of temperature to maintain a consistent periodicity, whereas the clock breaks down under conditions of low temperature stress. In terms of how temperature signals are transmitted to the circadian clock, there are likely several types of signaling pathways that sense distinct temperature cues. For instance, the circuits necessary for temperature entrainment and temperature preference in *Drosophila* are distinct: antennal thermosensors are important for a

behavioral circuit dictating preference along a temperature gradient (Collins, et al. 2004; Zars 2001) whereas chordontal organs are necessary for a behavioral circuit dictating entrainment to temperature cycles. It is therefore important to recognize that *Drosophila* likely rely on distinct receptors, signaling pathways, and circuits to modulate behavior. The extreme sensitivity that *Drosophila melanogaster* exhibit to temperature might be one reason why they have been so successful in colonizing temperately diverse environmental regimes as opposed to other *Drosophila* species (e.g. Low et al. 2008 (Low, et al. 2008)).



Figure 1



**The two-process model of sleep.** Figure from Borbély 1982 (Borbély 1982). At the heart of all models describing the regulation of sleep behavior is the 'two process model' of sleep. This model proposes that the drive to sleep (Level of Process S) is attributable to two independent phenomena: Process S (S), a homeostatic mechanism that predicts sleep quality and amount, and Process C (C), which is a circadian control system that regulates the timing of sleep. Under the assumptions of this model, the drive to sleep increases with increasing wake time (time 6). Practically nothing is known about the mechanisms that underlie sleep drive although molecules affecting sleep amount and quality have been identified. The timing of sleep is controlled by C, which oscillates on a daily basis and initiates sleep at time 23<sup>5</sup>.

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<sup>5</sup> Although S and C do interact, a more detailed overview of their relationship is beyond the scope of this introduction.

Figure 2

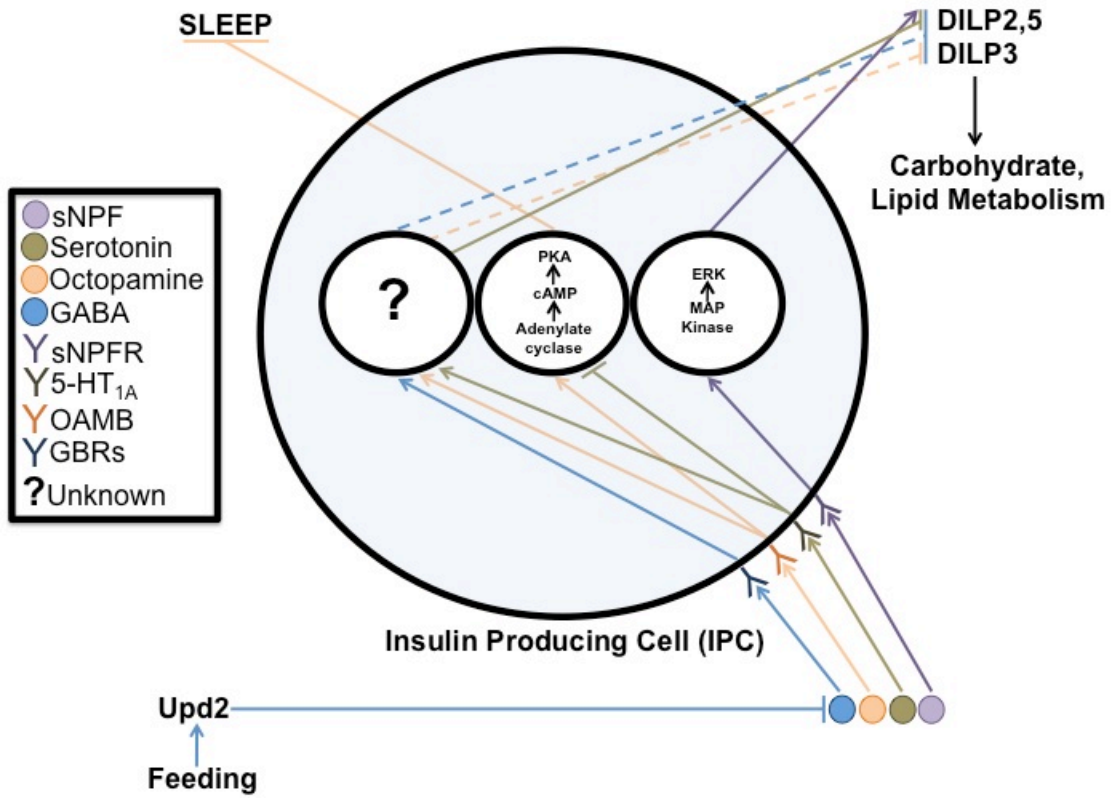
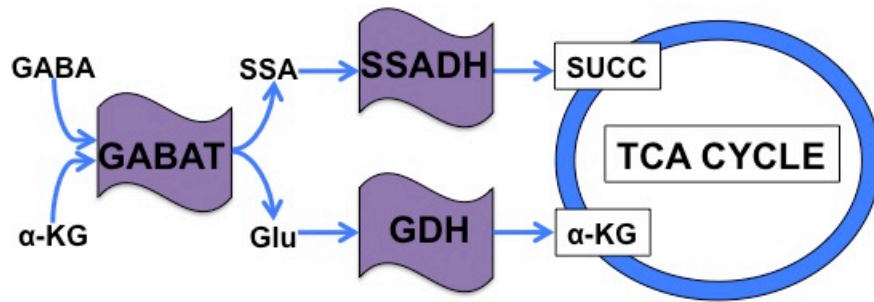


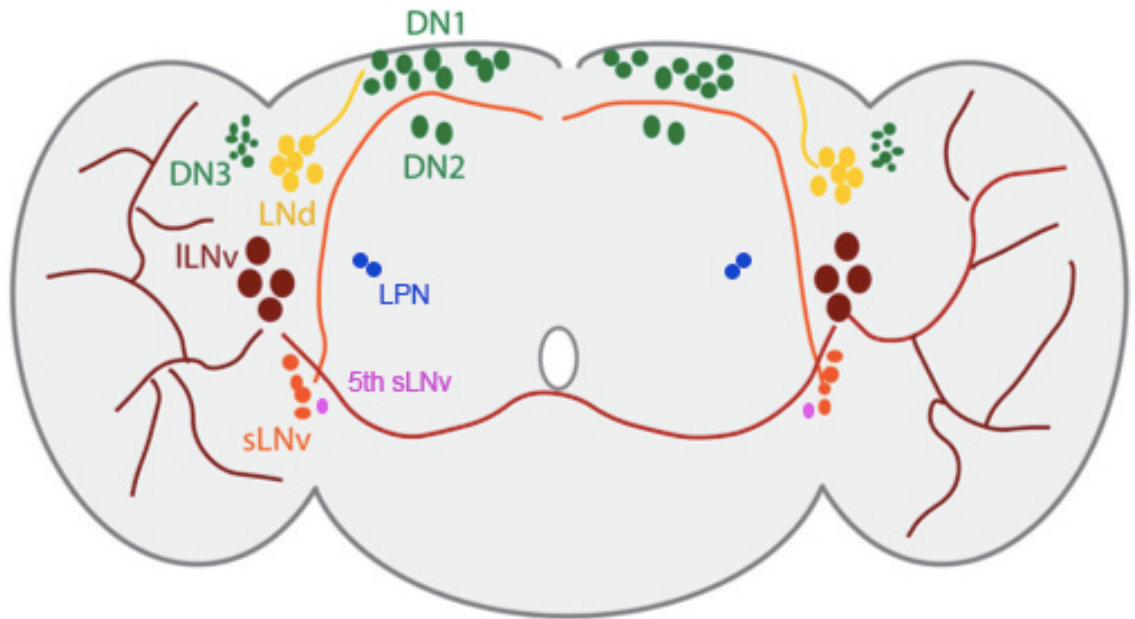
Diagram of what is currently understood about how certain Process S molecules regulate sleep and carbohydrate/lipid metabolism in the Insulin Producing Cells (IPCs). Pathways are drawn according to details in the text.

Figure 3



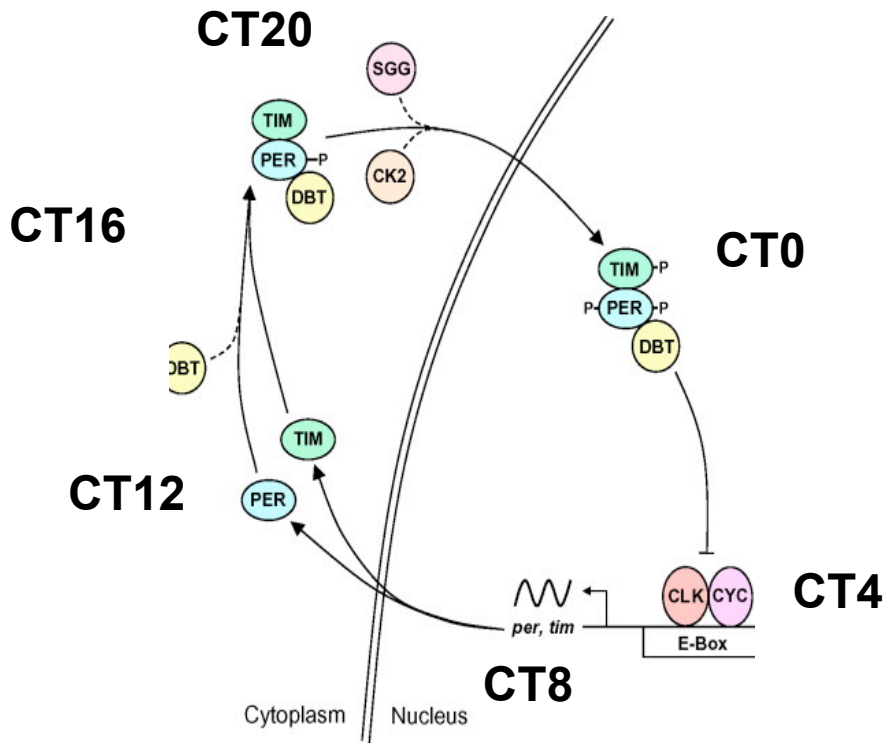
**Metabolic connections between GABA Transaminase (GABAT) and the Tricarboxylic Acid (TCA) cycle.** Once transported into mitochondria, GABA is converted by GABAT into succinic semialdehyde (SSA) and glutamate (Glu) using alpha-ketoglutarate ( $\alpha$ -KG) as an amino acceptor. SSA is then reduced by succinic semialdehyde dehydrogenase (SSADH) to form succinate (SUCC), which then enters the TCA cycle. If retained in the mitochondria, GABAT-derived GLU is catabolized by glutamate dehydrogenase (GDH) into  $\alpha$ -KG, which also enters the TCA cycle.

Figure 4



**The *Drosophila* circadian clock circuit.** Adapted from Allada and Chung 2010 (Allada and Chung 2010). *Drosophila* contain ~150 circadian clock neurons, which are identified based on their expression of a specialized genetic circuit. These clock neurons can be further subdivided into 8 groups according to their location, size, and expression neuropeptide content: the large and small ventral lateral neurons (l-LNVs, s-LNVs, respectively); the 5<sup>th</sup> small s-LNV; the dorsal lateral neurons (LNDs); three groups of dorsal neurons (DN1-3s); and the lateral posterior neurons (LPNs).

Figure 5



**The *Drosophila* molecular circadian clock.** Figure adapted from Hardin 2011 (Hardin 2011). The expression of PER and TIM proteins oscillates between the nucleus and cytoplasm on a ~24hr cycle. Chronobiologists analyze the expression of circadian genes using Circadian Time (CT), which is a time variable taken in constant conditions (light, temperature, and so forth) and repeats every 24 hrs. The expression of PER-TIM is nuclear at the beginning of the subjective day (CT0-4) and cytoplasmic towards the end of the subjective day (CT16-20).

**CHAPTER 2: Independent Effects of GABA Transaminase (GABAT) on  
Metabolic and Sleep Homeostasis in *Drosophila Melanogaster***

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**Submitted to *The Journal of Biological Chemistry*, August 1<sup>st</sup>, 2014**

**In revision at the *The Journal of Biological Chemistry*, August 31<sup>st</sup>, 2014**

## **Abstract**

The brain is the most energy-demanding tissue in mammals and so relies heavily on central metabolic pathways – such as the tricarboxylic acid (TCA) cycle – to generate fuel. The regulation of the TCA cycle (and thus energy production) is largely determined by the substrate availability of TCA cycle intermediates derived from the catabolism of fatty acids, carbohydrates, and amino acids. For instance in the brain, the breakdown of the neurotransmitter  $\gamma$ -Aminobutyric acid (GABA) in the GABA shunt generates catabolites that can enter the TCA cycle, although it is unknown which (if any) catabolic by-products support an individual's energy demands. The specific enzyme that degrades GABA – GABA Transaminase (GABAT) – has a role in sleep homeostasis, and we show here that it also affects energy balance such that flies lacking GABAT fail to survive on carbohydrate media. As GABA degradation produces two catabolic products – glutamate and succinic semialdehyde (SSA) – we sought to determine which was responsible for the metabolic phenotype. Through genetic and pharmacological experiments, we determined that glutamate rather than SSA mediates the link between GABAT and the TCA cycle. This is supported by biochemical measurements of catabolites in wild-type and mutant animals. Interestingly, effects of GABAT on sleep do not depend upon glutamate, indicating that GABAT regulates energy and sleep homeostasis through independent mechanisms. These data indicate a role of glutamate metabolism via the GABA shunt in the development of metabolic risk and suggest that neurological disorders caused by altered glutamate or GABA may be associated with metabolic disruption.

## **Introduction**

The brain is the most energy-demanding tissue in mammals. In humans, the brain is only 2% of the body's mass, yet it uses 10X more energy than what would be predicted by its weight alone (Attwell and Laughlin 2001). The majority of this energy consumption is incorporated in processes supporting synaptic signaling, especially in the reversal of ion fluxes following a post-synaptic response (Harris, et al. 2012). To support its energy demands, the brain relies on highly

active metabolic pathways, such as the tricarboxylic acid (TCA) cycle, which consists of a series of chemical reactions that generate energy in the form of adenosine triphosphate (ATP). The regulation of the TCA cycle and thus ATP production is largely determined by the substrate availability of TCA cycle intermediates derived from the catabolism of fatty acids, carbohydrates, and amino acids. The fact that several catabolic pathways converge on the TCA cycle ensures that cellular bioenergetics can be maintained, which is essential because as we have observed from human genetic disorders, a disruption in the pathway linking fatty acid, carbohydrate, or amino acid metabolism to the TCA cycle is highly neuropathic (Ciardo, et al. 2001). On the other hand, neurological disorders can have secondary effects on metabolism although the mechanistic links are not well understood (Jové, et al. 2014; van De Sande, et al. 2014).

Products of the major inhibitory neurotransmitter in the brain, GABA, can feed into the TCA cycle through a biochemical pathway known as the “GABA shunt” (Balazs, et al. 1970). This pathway generates metabolites through the activity of the catabolic enzyme GABA Transaminase (GABAT), which breaks down GABA into the products succinic semialdehyde and glutamate using a keto acid as an amino acceptor (fig. 1). *In vitro* studies have confirmed that both succinate semialdehyde and glutamate can enter the TCA cycle through independent pathways: succinic semialdehyde may do so if broken down into succinate by the enzyme Succinic Semialdehyde Dehydrogenase (SSADH) (Feehily and Karatzas 2012); glutamate can be incorporated into the TCA cycle if broken down into alpha-ketoglutarate ( $\alpha$ -KG) by Glutamate Dehydrogenase (GDH) (fig. 1) (Berg, et al. 2007). However, despite over forty years of study, the role of the GABA shunt pathway in regulating cellular energy metabolism *in vivo* has not been verified. While *in vitro* studies have confirmed that both products of GABAT can enter the TCA cycle, it is unknown which (if any) of these by-products is sufficient to support an individual's energy demands.

We recently demonstrated that GABAT plays a role in sleep homeostasis in *Drosophila* (Chen, et al. 2014). Specifically, fly strains with a null (*gabat*<sup>PL00338</sup>) or hypomorphic mutation



(*gabat*<sup>f01692</sup>) in *gabat* sleep a total of 100-200 minutes more than wild-type controls. While the excess sleep is likely due to the high GABA levels in these mutants, it may also be attributable to insufficient glutamate levels. Glutamate is one wake-promoting neurotransmitter in eukaryotes and is directly metabolized by the enzyme glutamate dehydrogenase (GAD) into GABA, the main sleep-promoting molecule in mammals (Agosto, et al. 2008; Chung, et al. 2009; Parisky, et al. 2008; Saper, et al. 2010).

In this study, we report that in addition to a sleep phenotype, GABAT mutants exhibit severe metabolic stress caused by a disruption in brain energy homeostasis. We investigated how GABAT regulates energy homeostasis by determining whether the following byproducts of GABAT – glutamate or succinic semialdehyde – regulate energy. Finally, we determined whether GABAT regulates energy and sleep homeostasis through the same mechanism.

## Methods

### *Drosophila* strains

All fruit fly strains (*Drosophila melanogaster*) were maintained at room temperature on standard cornmeal/molasses medium. An isogenic line (*w*<sup>118</sup>) was used as the wild-type strain in this study (Koh, et al. 2008). This strain, as well as *cg1640*<sup>EPgy2</sup>, *got1*<sup>wHy</sup>, ELAV-GAL4, *gabat*<sup>PL00338</sup>, *ssadh*<sup>HP37186</sup> (Bloomington stock center); *gabat*<sup>f01692</sup> (Exelixis collection at Harvard Medical School); Repo-GAL4 (gift from Gero Miesenböck, Julie Simpson, and Vanessa Auld); and the UAS-GABAT<sup>vh</sup> transgene (Chen, et al. 2014) were used in this study. All strains were outcrossed to our isogenized wild-type strain for 5-7 generations prior to behavioral analysis.

### Food preparation and behavioral analysis

For experiments analyzing the metabolic and sleep phenotype of fly strains, we placed adult male fruit flies (age: 1-5 days) into glass tubes containing either sucrose media (5% sucrose and 1.5% agar), regular food (standard cornmeal/molasses medium with 2.5% yeast), or sucrose + amino acid supplement (0.05M amino acid in 5% sucrose with 1.5% agar). The amino acids used in this study were purchased from Sigma-Aldrich: L-Alanine (A7627), L-Cysteine

hydrochloride (C1276), L-Glutamic acid (G12510), L-Glutamine (G3126), L-Isoleucine (I2752), L-Leucine (L8912), L-Phenylalanine (P2126), L-Tryptophan (T0254), L-Tyrosine (T3754), and L-Valine (V0500). For the amino acids that are soluble in water, we neutralized the solution to 7.2-0.4pH before dissolving in 5% sucrose with 1.5% agar. Flies were then placed into TriKinetics Activity Monitors (TriKinetics, Waltham, MA) housed in a temperature-regulated Precision incubator model 818 (Thermo Electron Corporation; Marietta, Ohio) under 12 hours of light followed by 12 hours of dark.

To analyze the metabolic phenotype of a strain, we assessed whether flies could survive in locomotor tubes containing sucrose food, regular food, or sucrose food + amino acid supplement for 8 days. For the sleep experiment, we monitored the locomotor activity patterns of individual flies on a limited yeast diet supplemented with an amino acid in 1-minute bins using the DAM File Scan (v.1.1.06, TriKinetics). To analyze both phenotypes, we used pySolo (Gilestro and Cirelli 2009). To compare sleeptime between treatments, we took the average total sleeptime (mins) of each fly from day 2 – day 4. Group means of a treatment were then calculated from the average sleeptime of the individual flies within the treatment.

## **Methods for High Performance Liquid Chromatography (HPLC)**

*Brain extraction.* *gabat<sup>f</sup>* and wild-type strains were entrained to 12 hours of light followed by 12 hours of dark at 25°C for 5 days. On the fifth day, 4 replicates of 25 male *gabat<sup>f</sup>* and 4 replicates of wild-type males were placed in locomotor tubes containing sucrose media. At ZT0 of the following day, we removed males from the locomotor tubes and placed them on ice until their brains were extracted. We then anaesthetized 4-5 males on CO<sub>2</sub>, placed the anaesthetized flies in 75% ethanol, and then transferred them to a dissecting dish with chilled 1X Phosphate Buffer Saline (PBS) solution. Dissected brains were transferred to an eppendorf tube with chilled 1X PBS. This process was repeated for 20 brains per genotype. We then spun the samples at 10,000g for 10 seconds, pipetted out the 1X PBS, and stored samples at -80°C until they were

shipped to the Neurochemistry Core at Vanderbilt University for tissue extraction and HPLC analysis.

*Tissue extraction.* At the Neurochemistry Core at Vanderbilt, brain samples were homogenized using a tissue dismembrator in 100-750 $\mu$ l of 0.1M TCA, which contained  $10^{-2}$  M sodium acetate,  $10^{-4}$  M EDTA, 5ng/ml isoproterenol (as internal standard) and 10.5 % methanol (pH 3.8). Samples were spun in a microcentrifuge at 10,000 g for 20 minutes. The supernatant was removed and stored at  $-80^{\circ}\text{C}$ . The pellet was saved for protein analysis using BCA Protein Assay Kit purchase from Pierce Chemical Company (Rockford, IL). Supernatant was then thawed and spun for 20 minutes. Samples of the supernatant were then analyzed for glutamate and GABA.

*HPLC methods.* Glutamate and GABA levels were determined by the Waters AccQ-Tag system utilizing a Waters 2475 Fluorescence Detector (Cohen and Michaud 1993). HPLC control and data acquisition were managed by Empower 2 software.

## **Methods for Liquid Chromatography-Mass Spectrometry (LC-MS)**

*Metabolite extraction of whole flies.* The quantity of tissue required for the LC-MS analysis prevented us from evaluating metabolite concentrations in fly brains, therefore we performed metabolite extraction on fly bodies. Three replicate samples of 30 male *ssadh*<sup>HP</sup>, *gabat*<sup>f</sup>, and wild-type flies were placed on sucrose food for four days. On ZT0 of the 4<sup>th</sup> day, males from each replicate were anaesthetized on CO<sub>2</sub> and each sample frozen in dry ice for 10 minutes. 40 $\mu$ l of 1X PBS was directly added to the eppendorf tube and then the sample was homogenized. After homogenization, 200 $\mu$ l of methanol and 100 $\mu$ l of chloroform were added and samples were sonicated for 15 minutes. 100 $\mu$ l of chloroform and distilled H<sub>2</sub>O were then added and samples subsequently centrifuged for 7 minutes at 13,000 rpm at  $4^{\circ}\text{C}$ . Once the centrifugation was completed, we pipetted 200 $\mu$ l from the upper aqueous layer of each sample into a new eppendorf tube. Samples were evaporated for 2-24hrs in a speedvac and stored at  $-80^{\circ}\text{C}$  until LC-MS analysis.

*LC-MS protocol.* LC-MS on succinate and alpha-ketoglutarate was performed on a Waters Acquity UPLC coupled to a Waters TQD mass spectrometer (Waters Corporation, Milford, MA, USA). Liquid chromatography conditions and mass spectrometer parameters were based on methods described by Yuan *et al.* 2012 (Yuan, et al. 2012), with chromatographic separation of metabolites performed using a Waters BEH Amide (100mm x 2.1mm x 2.5µm) column. Data processing was accomplished using Waters TargetLynx software (version 4.1).

### **Statistical analysis.**

All statistical analyses were performed using JMP PRO v.10 (SAS Institute, Cary, NC). To compare the differences among the proportion of individuals that were alive at the end of an experiment (proportion alive) we used a Fisher's exact test. To compare differences in the proportion of individuals alive at the end of an experiment among groups that exceeded two, we used a one-way between subjects ANOVA followed by a Tukey post-hoc test. To compare the differences in means between two genotypes, we used an independent sample *t* test. To compare differences in means among groups that exceeded 2, we used a one-way between-subjects ANOVA followed by a Tukey post-hoc test.

### **Results**

**Fly mutants of *gabat* exhibit overt signals of metabolic stress.** It is well established that the products of GABA Transaminase (GABAT) – glutamate and succinic semialdehyde (SSA) – can be incorporated in the TCA cycle (fig. 1) (Berg, et al. 2007; Feehily and Karatzas 2012). However, it is unknown whether the enzyme is functionally important for maintaining energy requirements *in vivo*. To address this, we evaluated whether *gabat* mutants could survival in the absence of nutrients (cornmeal, dry yeast, agar, molasses) whose breakdown by GABAT might generate metabolites for the TCA cycle. Therefore, we placed males of wild-type, null (*gabat*<sup>P<sup>L</sup></sup>), and hypomorphic (*gabat*<sup>f</sup>) *gabat* strains on a sucrose diet for eight days and recorded the proportion of individuals that survived the treatment. We found that both *gabat* mutant strains show reduced survival (3-4%) on sucrose food (fig. 2a). Furthermore, it appears that the increased mortality is

specifically due to a dysfunction in metabolism because transfer of *gabat*<sup>f</sup> males to regular food results in survival levels equivalent to those of wild-type flies (fig. 2b). For the remainder of the study, we show data for the hypomorphic *gabat*<sup>f</sup> strain.

Microarray expression data (St. Pierre, et al. 2014) indicate that *gabat* is expressed in neurons and non-GABAergic glial cells (Chen, et al. 2014). To determine whether neuronal or glial GABAT regulates the energy requirements of the brain, we drove the expression of a genomic *gabat* construct in either neurons or glia of the *gabat*<sup>f</sup> mutant background using the responder UAS-GABAT<sup>vh</sup> and a cell specific driver (neurons – ELAV-GAL4, glia – Repo-GAL4). We found that we could rescue the metabolic phenotype of *gabat*<sup>f</sup> in either neurons or glia (fig. 2c), indicating that it is caused by a general disruption in brain energy metabolism.

**The biochemical pathway linking GABAT-derived succinic semialdehyde (SSA) to the TCA cycle is normal in *gabat*<sup>f</sup> flies.** Within mitochondria, GABAT deaminates GABA to form SSA and glutamate (fig. 1). The aldehyde of SSA is then oxidized by succinate semialdehyde dehydrogenase (SSADH) to succinate, which may enter the TCA cycle (Feehily and Karatzas 2012; Studart-Guimaraes, et al. 2007). To test whether the metabolic phenotype of *gabat*<sup>f</sup> is caused by disrupted routing of GABAT-derived SSA to the TCA cycle, we assessed whether mutants of *ssadh* also exhibit the *gabat*<sup>f</sup> lethality phenotype on sucrose food. Interestingly, we found that *ssadh*<sup>HP</sup> mutants can survive on a sucrose food diet (fig. 3a), indicating that GABAT regulates metabolic stress independently of succinic semialdehyde. In fact, when we analyzed succinate levels of the wild-type and GABA shunt mutants using liquid-chromatography mass spectrometry (LC-MS), we did not detect a difference among the groups (fig. 3b), suggesting that alternative enzymes (such as succinyl CoA ligase, EC 6.2.1.5) compensate for a GABAT-derived reduction in succinate. Overall, these data suggest that the metabolic phenotype of *gabat*<sup>f</sup> is not caused by a disruption in the pathway linking GABAT-derived SSA to the TCA cycle.

**The biochemical pathway linking GABAT-derived glutamate to the TCA cycle is impaired in *gabat*<sup>f</sup> flies.** As our data suggest that metabolic stress of *gabat*<sup>f</sup> is not caused by a disruption of

GABAT-derived succinic semialdehyde into the TCA cycle (fig. 3), we next assessed whether glutamate metabolism is disrupted. To address this, we evaluated whether *gabat*<sup>f</sup> mutants exhibit abnormal concentrations of brain GABA and glutamate after one day of sucrose-food treatment. Using HPLC, we found that brain glutamate levels are twice as low (fig. 4a) and GABA levels are 6 times as high (fig. 4b) in *gabat*<sup>f</sup> flies maintained on sucrose. We reported previously that brain GABA levels are twice as high as wild-type on regular food (Chen, et al. 2014) and so it appears that the GABA levels of *gabat*<sup>f</sup> are exacerbated on sucrose medium.

Once formed by GABAT, glutamate could have several metabolic fates, including its direct use for glutamine formation, participation in the purine nucleotide cycle, incorporation into proteins or the tripeptide glutathione, and metabolism via the TCA cycle for energy (McKenna 2007). For use within the TCA cycle, glutamate dehydrogenase breaks down glutamate into alpha-ketoglutarate ( $\alpha$ -KG), which can then enter the TCA cycle (fig. 1). We predicted that if GABAT-derived glutamate was used for energy homeostasis,  $\alpha$ -KG levels would be low in *gabat*<sup>f</sup> mutants. Using LC-MS, we found support for this prediction because  $\alpha$ -KG levels are significantly lower in *gabat*<sup>f</sup> than in wild-type (fig. 4c). These data suggest that the biochemical pathway linking GABAT-derived glutamate to the TCA cycle is impaired in *gabat*<sup>f</sup> mutant flies.

**Deficits in glutamate cause the overt metabolic phenotype of *gabat*<sup>f</sup>.** As our data suggested that *gabat*<sup>f</sup> mutants have impaired glutamate metabolism (fig. 4), we next sought to experimentally verify that this disruption causes the *gabat*<sup>f</sup> metabolic phenotype on sucrose food. *gabat*<sup>f</sup> mutants have low brain glutamate levels (fig. 4a), so we first asked if adding glutamate to the sucrose food would rescue the metabolic phenotype. We found that this was the case (fig. 5a).

To establish specificity for disrupted glutamate metabolism in the lethality phenotype of *gabat*<sup>f</sup> mutants on sucrose, we next evaluated whether the amino acids that can be directly metabolized to glutamate through specific aminotransferases can also rescue the *gabat*<sup>f</sup> metabolic phenotype. Previous work has shown that aminotransferases play a prominent role in

regulating the synthesis and degradation of glutamate by transferring the amino group of a specific amino acid(s) to a keto-acid to produce glutamate (Brosnan and Brosnan 2009). Of the 9 amino acids that can be directly metabolized into glutamate, we found that 6 can rescue lethality (fig. 5b, table 1).

Next, we sought to determine experimentally whether the amino acids that rescue the *gabat<sup>f</sup>* metabolic phenotype (fig. 5b, table 1) do so through the action of specific aminotransferases. We were particularly interested in whether alanine degradation into glutamate by alanine aminotransferase (ALT) or indirectly by Glutamic-Oxaloacetic Transaminase (GOT) accounts for the alanine rescue of the *gabat<sup>f</sup>* mutants. Numerous epidemiological studies in humans have reported that abnormal ALT or GOT regulation is associated with metabolic disease (Sookoian and Pirola 2012), and while the mechanism by which it does so is unknown, one hypothesis proposed by Sookoian & Pirola 2012 (Sookoian and Pirola 2012) is that metabolic pathology is caused by a disruption in glutamate metabolism as ALT and GOT are major contributors of steady-state glutamate levels. Therefore, to test whether alanine degradation into glutamate by ALT or indirectly by GOT (RN: R00355, (Kanehisa, et al. 2014)) accounts for the alanine rescue of the *gabat<sup>f</sup>* metabolic phenotype, we created the double mutant strains of ALT, GABAT (cg1640<sup>EPgy2</sup>; *gabat<sup>f</sup>*) as well as GOT, GABAT (;*got1<sup>why</sup>*; *gabat<sup>f</sup>*).

In creating the double mutant lines, we found that the cg1640<sup>EPgy2</sup>; *gabat<sup>f</sup>* combination was lethal, so we could not determine whether ALT can rescue the *gabat<sup>f</sup>* metabolic phenotype on alanine food. However, we found that GOT1 is required for the rescue of *gabat<sup>f</sup>* by alanine treatment because the double mutant strain (*got1<sup>why</sup>*; *gabat<sup>f</sup>*) exhibited the *gabat<sup>f</sup>* metabolic phenotype on alanine food (fig. 5c). Overall, these data suggest that impaired glutamate metabolism causes the metabolic phenotype of *gabat<sup>f</sup>*.

**GABAT regulates sleep independently of metabolic homeostasis.** Our data suggest that a limit in glutamate causes the *gabat<sup>f</sup>* metabolic phenotype (fig. 5). Previously, we observed that *gabat<sup>f</sup>* mutants have a sleep phenotype, sleeping on average a total of 100-200 minutes more

than wild-type controls (Chen, et al. 2014). Because glutamate is one of the main wake-promoting neurotransmitters, we tested whether a limit in glutamate also accounts for the sleep phenotype of *gabat<sup>f</sup>*. Thus, we placed males (age: 1-5 days) on a sucrose diet supplemented with each of the amino acids including glutamate, which rescues the metabolic phenotype on sucrose (fig. 5a). We predicted that if a limit in brain glutamate accounts for the *gabat<sup>f</sup>* sleep phenotype, then the amino acids that rescue metabolism should also rescue sleep. We did not find support for this hypothesis, because none of the amino acids reduce the total sleeptime of *gabat<sup>f</sup>* relative to that of the negative control (fig. 6). This suggests that GABAT regulates sleep independently of metabolic homeostasis.

## Discussion

Our study is the first to report that the biochemical link between the GABA shunt and the TCA cycle is important for energy homeostasis *in vivo*. These data provide new perspectives on the pathogenesis of brain-associated metabolic disorders by introducing a role of glutamate metabolism via the GABA shunt in the development of metabolic risk (Sookoian and Pirola 2012). GABAT is located within the catabolic leg of the GABA shunt and converts GABA – the main inhibitory neurotransmitter in mammals and flies – into succinic semialdehyde and glutamate (fig. 1). *In vitro* studies have shown that products downstream of succinic semialdehyde and glutamate metabolism (succinate and  $\alpha$ -KG respectively) can be incorporated into the TCA cycle (Bouché and Fromm 2004; Feehily and Karatzas 2012), suggesting a functional link between the GABA shunt and the TCA cycle (fig. 1). However, it has not been determined *in vivo* whether this link is fundamentally important for how the brain regulates energy. We show that GABAT regulates energy homeostasis demonstrated by the finding that mutants of this enzyme cannot survive on a protein and fatty-acid restricted diet (fig. 2a). Furthermore, we show that GABAT regulates energy metabolism generally in the brain because we can rescue the metabolic phenotype of *gabat<sup>f</sup>* in either neurons or glia (fig. 2c). When we analyzed the mechanism by which GABAT regulates energy homeostasis, we found that the metabolic stress of *gabat<sup>f</sup>* on sucrose food is not caused by a disruption in SSA metabolism (fig. 3), but rather a deficiency in



glutamate. This is based upon the following: a) correlative data suggesting that the biochemical pathway linking GABAT-derived glutamate to the TCA cycle is disrupted (fig. 4) and b) experimental evidence showing that the correlation is functionally significant (fig. 5).

Given that a deficiency in glutamate causes the metabolic stress of *gabat<sup>f</sup>* on sucrose food (fig. 5), it is surprising that cysteine, isoleucine, and tryptophan – amino acids that can be metabolized into glutamate (table 1) – could not rescue the metabolic phenotype of *gabat<sup>f</sup>* (fig. 5b, table 1). One explanation for this effect is that our mutant strain could not properly ingest cysteine, isoleucine, and tryptophan. Cysteine is a particularly odorous amino acid due to its thiol side chain and so we suspect that the *gabat<sup>f</sup>* mutants were repelled by the obnoxious smell of the food supplement. We found support for this explanation because most wild-type flies did not survive on cysteine food as well (Proportion alive  $\pm$  SD:  $0.03 \pm 0.0004$ ). We suggest that our mutants could not properly catabolize tryptophan or isoleucine into glutamate: tryptophan is high hydrophobic and so potentially did not dissolve in sucrose solution. While the branched chain amino acids (isoleucine, leucine, and valine) are also hydrophobic, our data indicate that flies can ingest them because leucine and valine rescue the *gabat<sup>f</sup>* metabolic phenotype (fig. 5b, table 1). We therefore suspect the branched-chained aminotransferase (EC 2.6.1.2) does not work as effectively using isoleucine as a substrate.

While this study establishes that GABAT is functionally important for regulating energy homeostasis through glutamate, we found that glutamate had no effect on the long sleeping phenotype observed previously in *gabat<sup>f</sup>* mutants (Chen, et al. 2014). This is surprising given that glutamate reduces total sleep in wild-type controls by ~200 minutes compared to sucrose alone ( $t(6.81)$ ;  $p < 0.0001$ ). It is possible that the energy phenotype of *gabat<sup>f</sup>* is so severe that most exogenously provided glutamate is incorporated into other metabolic processes such as the TCA cycle (McKenna 2007). In this scenario, the remaining glutamate levels are not sufficiently high to counteract the effects of high GABA: in fact we found using HPLC that GABA is 6X higher in the brains of *gabat<sup>f</sup>* mutants maintained on sucrose (fig. 4b). As previously reported (Agosto, et al.

2008; Chung, et al. 2009; Parisky, et al. 2008; Saper, et al. 2010), GABA is the major sleep-promoting neurotransmitter in flies and in mammals.

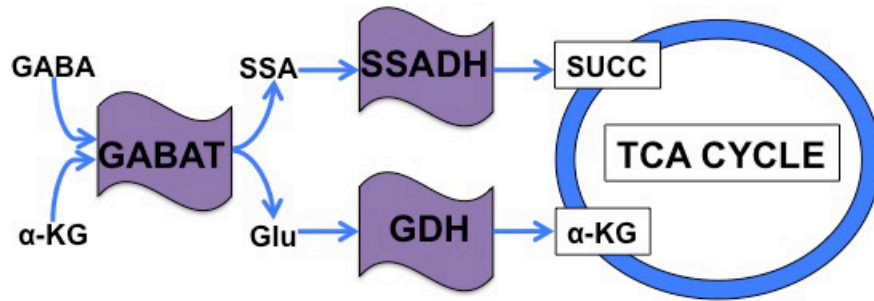
To our knowledge, this is the first study to report the dual role of GABAT in regulating two fundamental processes: energy and sleep homeostasis. We found that mutants of *gabab* exhibit overt signals of metabolic stress (fig. 1) and a long-sleeping phenotype (Chen, et al. 2014). Our data support the idea that GABAT regulates energy homeostasis by breaking down GABA into glutamate (fig. 5), which can be used to generate energy via the TCA cycle, and also promotes wake. Previous studies have identified a link between an individual's behavioral state and cellular function, particularly with metabolism. For example, using genome-wide expression profiling, Cirelli *et al.* 2004 (Cirelli, et al. 2004) showed that transcripts associated with energy metabolism – including those involved in glutamate synthesis – are upregulated during wake, a phenomenon that likely supports the elevated brain metabolic requirements during this state (Steriade and Timofeev 2003).

In humans, GABAT deficiency is a rare but fatal disease. Clinicians have described the neuropathic conditions comorbid with the disorder including psychomotor retardation, convulsions, and hyperreflexia (Jaeken, et al. 1984; Medina-Kauwe, et al. 1999) but have not performed metabolic analysis of energy substrates. Our data suggest that a disruption in GABA and glutamate metabolism could be one mechanism by which a deficiency in GABAT produces neuropathic effects.

## **Acknowledgements**

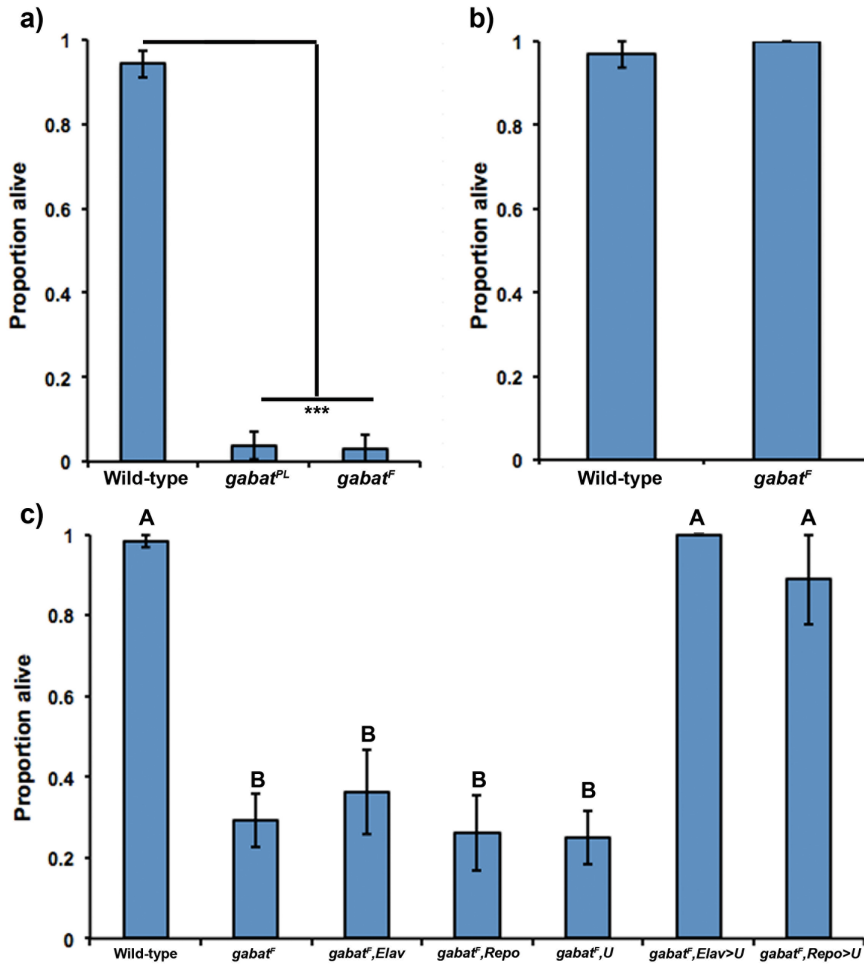
The authors would like to thank Wen-Feng Chen for invaluable advice on the early development of this project. HPLC analysis was performed by Raymond Johnson (Neurochemistry Core at Vanderbilt University). SEM was supported by an NIH predoctoral training grant (HL07953). AS was supported by a grant from the NIH (RO1 NS048471).

Figure 1



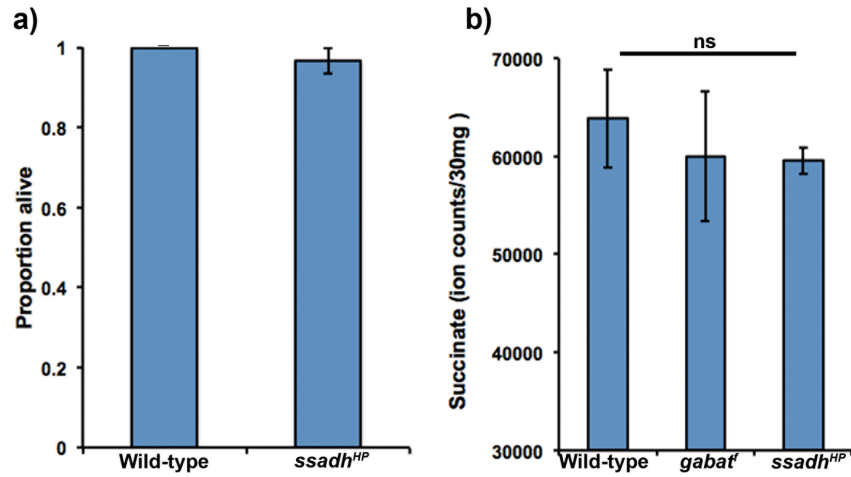
**Schema of the metabolic connections between GABAT and the TCA cycle.** Once transported into mitochondria, GABA is converted by GABAT into succinic semialdehyde (SSA) and glutamate (Glu) using alpha-ketoglutarate ( $\alpha$ -KG) as an amino acceptor. SSA is then reduced by succinic semialdehyde dehydrogenase (SSADH) to form succinate (SUCC), which then enters the TCA cycle. If retained in the mitochondria, GABAT-derived GLU is catabolized by glutamate dehydrogenase (GDH) into  $\alpha$ -KG, which also enters the TCA cycle.

Figure 2



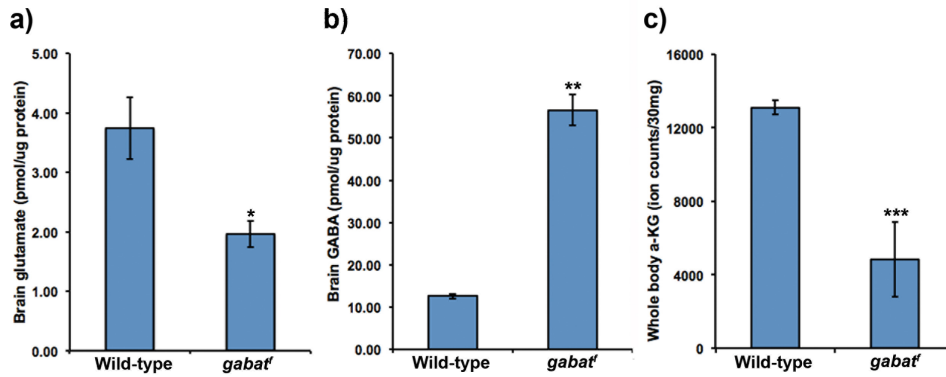
**Fly mutants of *gabat* exhibit overt signals of metabolic stress. (a) Mutants of *gabat* cannot survive on sucrose food.** When fed a sucrose-only diet, males of our *gabat<sup>PL</sup>* and *gabat<sup>F</sup>* strains die after 8 days. Fisher's exact test: \*\*\* $p < 0.0001$  **(b) The GABAT metabolic phenotype is rescued on regular food.** When males of the *gabat<sup>F</sup>* strain are transferred to a regular food diet, they no longer exhibit overt signals of metabolic stress. Fisher's exact test:  $p = 0.24$ . **(c) The metabolic phenotype of *gabat* is rescued by driving genomic *gabat* expression in either neurons or glia.** A one-way between subjects ANOVA was conducted to compare the effect of genotype on lethality in *gabat<sup>F</sup>* flies carrying UAS-GABATvH (*U*) driven by GAL4 drivers in glial cells using Repo-GAL4 (*Repo*) or neurons using ELAV-GAL4 (*Elav*). The following lines were used as a negative controls: *gabat<sup>F</sup>*; *gabat<sup>F</sup>,Elav*; *gabat<sup>F</sup>,Repo*; and *gabat<sup>F</sup>,U*. There was a significant effect of genotype on lethality at the  $p < 0.05$  level for the 4 groups [ $F(6) = 30.8$ ,  $p < 0.0001$ ]. Lines with different letter values (A, B) are statistically different as determined by the Tukey post-hoc HSD test.

Figure 3



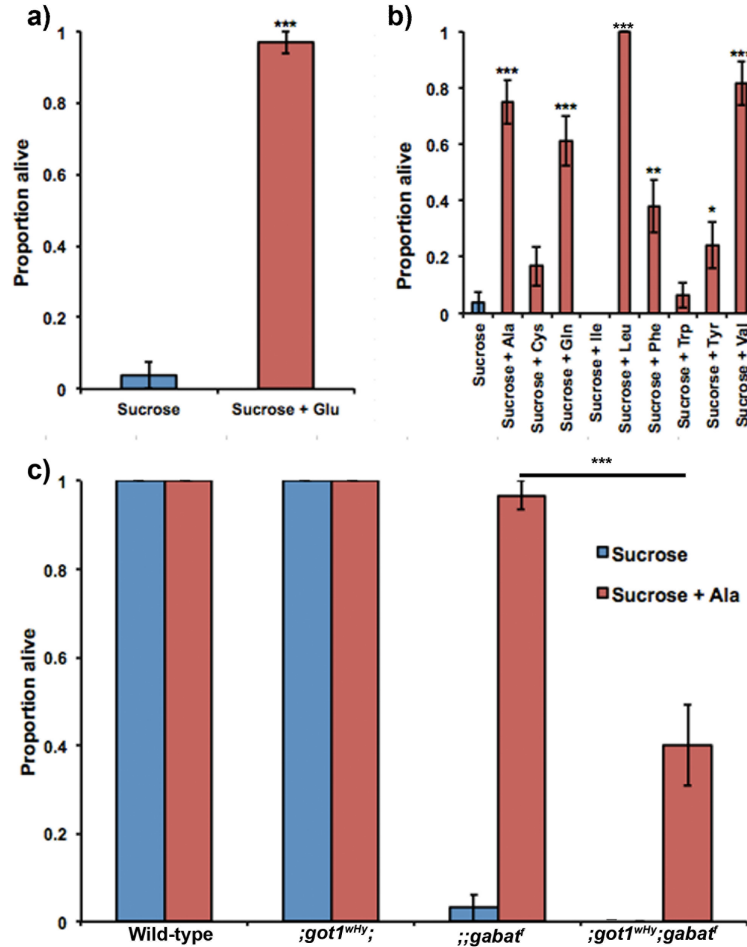
The biochemical pathway linking GABAT-derived succinic semialdehyde (SSA) to the TCA cycle is normal in *gabat*<sup>f</sup> flies. (a) Mutants of SSADH do not exhibit a metabolic phenotype on sucrose food. Fisher's exact test:  $p=1.0$ . (b) Succinate concentrations are equivalent among wild-type, *gabat*<sup>f</sup>, and *ssadh*<sup>HP</sup> strains. A one-way between subjects ANOVA was conducted to compare the effect of genotype on succinate concentrations among wild-type, *gabat*<sup>f</sup>, *ssadh*<sup>HP</sup> strains. There was no significant effect of genotype on succinate concentrations for the 3 groups [ $F(3)=0.19$ ,  $p=0.84$ ].

Figure 4



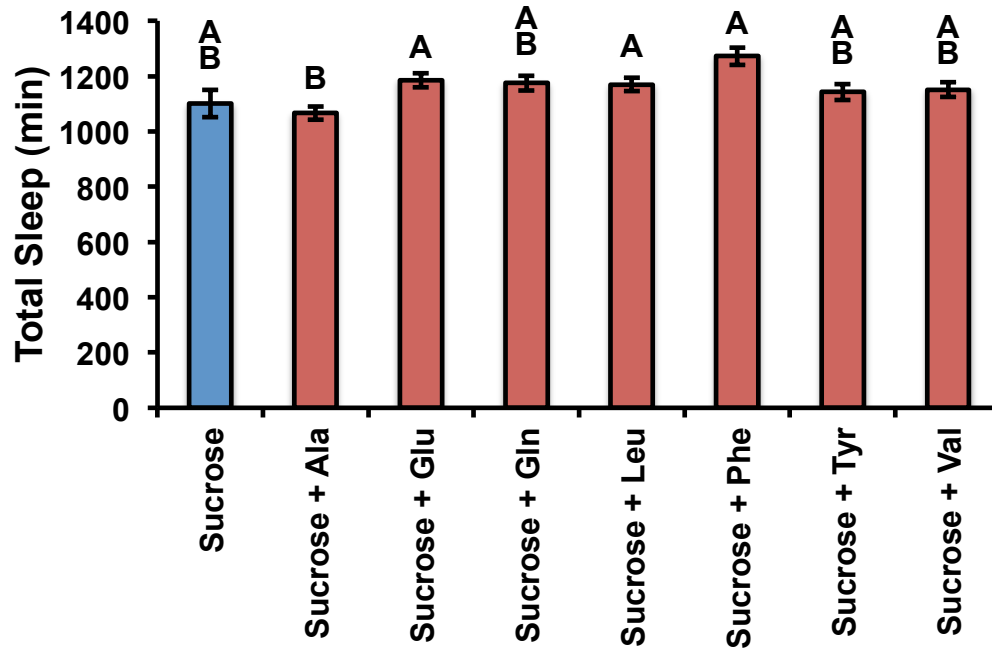
**The biochemical pathway linking GABAT-derived glutamate to the TCA cycle is impaired in *gaba1* flies. (a) Brain glutamate levels are reduced in *gaba1* mutants.** Using HPLC, we compared brain glutamate levels between *gaba1* and wild-type strains after one day of sucrose-food treatment. Independent sample t-tests  $*p < 0.05$ . **(b) Brain GABA levels are increased in *gaba1* mutants.** Using HPLC, we compared brain GABA levels between *gaba1* and wild-type after one day of sucrose-food treatment. Independent sample t-tests  $**p < 0.005$  **(c) Whole body  $\alpha$ -ketoglutarate ( $\alpha$ -KG) levels are lower in *gaba1*.** Using liquid chromatography-mass spectrometry, we compared whole body  $\alpha$ -KG levels between wild-type and *gaba1* flies after a four-day treatment of sucrose food. Independent sample t-tests  $***p < 0.0001$ .

Figure 5



**A decrease in glutamate causes the metabolic phenotype of *gabat<sup>f</sup>* (a) Glutamate rescues the metabolic phenotype of *gabat<sup>f</sup>*.** When supplementing sucrose food with glutamate (final concentration = 0.05M), the metabolic phenotype of *gabat<sup>f</sup>* mutant males is rescued. Fisher's exact test: \*\*\* $p < 0.0005$ . **(b) Specific amino acids rescue the metabolic phenotype of *gabat<sup>f</sup>* males.** When we supplement the sucrose diet with the 9 amino acids that can be metabolized into glutamate by specific aminotransferases, we found that the following amino acids rescue the metabolic phenotype: alanine, glutamine, leucine, phenylalanine, tyrosine, valine. Fisher's exact test: \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ . **(c) GOT1 mediates the metabolic rescue of *gabat<sup>f</sup>* by alanine.** We placed males of our wild-type, *gabat<sup>f</sup>*, ;*got1<sup>wh/y</sup>*, and ;*got1<sup>wh/y</sup>;gabat<sup>f</sup>* strain on alanine and sucrose food. When combined with ;*got1<sup>wh/y</sup>*, the metabolic phenotype of *gabat<sup>f</sup>* cannot be rescued by alanine. Fisher's exact test: \*\*\* $p < 0.0001$ .

Figure 6



**GABAT regulates sleep independently of glutamate.** When we fed *gabat*<sup>-</sup> mutants a sucrose diet supplemented with the amino acids that can be metabolized into glutamate and can rescue the metabolic phenotype (fig. 5b, table 1), we found that the sleep phenotype of *gabat*<sup>-</sup> cannot be rescued, suggesting that GABAT regulates sleep independently of metabolic homeostasis. We used a one-way between-subjects ANOVA to compare total sleeptime (minutes) of *gabat*<sup>-</sup> mutants fed alanine, glutamate, glutamine, leucine, phenylalanine, tyrosine, and valine. There was a significant effect of the food treatment on total sleep at the  $p < 0.05$  level for the 8 groups  $F(7)=4.53$ ,  $p < 0.00001$ . We used Tukey post-hoc comparisons to compute the groups that were statistically significant: different levels (A, B) indicate statistical significance. None of the amino acids reduced sleep relative to the negative control.



Table 1

Treatment	Aminotransferase	Gene ( <i>Dmel</i> )	Proportion Alive + SEM
Alanine	EC 2.6.1.2	cg1640	0.75+0.08 <sup>a</sup>
Cysteine	EC 2.6.1.1	<i>got1, got2</i>	0.17+0.07
Glutamine	EC 2.6.1.16	<i>gfat1, gfat2</i>	0.61+0.09 <sup>a</sup>
Isoleucine	EC 2.6.1.42	cg1673	0.00+0.00
Leucine	EC 2.6.1.42	cg1673	1.00+0.00 <sup>a</sup>
Phenylalanine	EC 2.6.1.1 EC 2.6.1.5	<i>got1, got2</i> cg1461	0.38+0.09 <sup>b</sup>
Tryptophan	EC 2.6.1.1	<i>got1, got2</i>	0.06+0.04
Tyrosine	EC 2.6.1.1 EC 2.6.1.5	<i>got1, got2</i> cg1461	0.24+0.08 <sup>c</sup>
Valine	EC 2.6.1.42	cg1673	0.81+0.08 <sup>a</sup>
Sucrose	n/a		0.04+0.04

<sup>a</sup> $p < 0.0005$

<sup>b</sup> $p < 0.005$

<sup>c</sup> $p < 0.05$

**Amino acids that can be metabolized to glutamate by specific aminotransferases in *Drosophila melanogaster*.** Results of figure 5b are listed in the 'proportion alive' column. Data on the aminotransferase and gene were compiled from Kanehisa *et al.* 2014 (Kanehisa, et al. 2014).

**CHAPTER 3: Natural populations of *Drosophila melanogaster* reveal features of an uncharacterized circadian property: the lower temperature limit of rhythmicity**

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**Published in the *Journal of Biological Rhythms*:**

**Vol. 29(3): 167-180 June, 2014**

## Abstract

Most cyclic biological processes are under control of a circadian molecular timing system that synchronizes these phenomena to the 24hr day. One generic property of circadian-controlled processes is that they operate within a specific temperature range, below which the manifestation of rhythm ceases. Little is known about the evolutionary relevance of the lower temperature limit of rhythmicity or about the mechanism underlying the loss of overt circadian behavior below this lower-limit, especially in one model organism of chronobiology, *Drosophila melanogaster*. Natural populations of *Drosophila* are evolving under divergent selection pressures and so provide a source of diversity necessary to address these issues. Using lines derived from African populations, we find that there is natural variation in the expression of rhythmic behavior under low temperature conditions. We found evidence that this variability is evolutionarily relevant at *extremely* low temperature (12°C) because high altitude populations exhibit selection for locally adapted genomes that contribute to rhythmic behavior. Lines resistant to 15°C show an additional layer of diversity in their response to temperature extremes because some lines are resistant to low temperature (15°C) only whereas others are cross-resistant to high and low temperature (15°C and 30°C). Genetic analysis of one cold-resistant circadian line at 15°C reveals that the phenotype maps to the X-chromosome but not to the core clock genes, *per* and *sgg*. Analysis of the central clock cells of this line reveals that maintenance of rhythm is associated with robust clock function, which is compromised in a standard laboratory strain. These data indicate that the cold-resistant circadian phenotype is clock-based. This study highlights the importance of using natural populations to inform us of the basic features of circadian traits, especially those that might be under temperature-based selection.

## Introduction

Most biochemical, physiological, and behavioral phenomena exhibit daily cycles that are under control of an internal circadian timing system that serves to synchronize these processes to

peak at critical times of the 24hr day. The ability to anticipate and coordinate biological processes according to light:dark transitions of the environment confers a fitness advantage in several organisms (Dodd, et al. 2005; Ouyang, et al. 1998; Woelfe, et al. 2004), which is presumably why the internal timing mechanism is evolutionarily conserved from bacteria to mammals. In flies (*Drosophila melanogaster*), the molecular clock is characterized by a well-established negative feedback loop in which core clock proteins – PERIOD (PER) and TIMELESS (TIM) – inhibit the transcription of their own mRNAs. The ~24hr cycle begins when two transcription factors (CLK and CYC) bind to E-boxes upstream of the *period* (*per*) and *timeless* (*tim*) genes and transcribe *per* and *tim* mRNA. These mRNAs are translated into proteins which accumulate in the cytosol, form a heterodimer complex, and translocate into the nucleus. Phosphorylation plays a central role in the accumulation of the proteins as well as their nuclear transfer. Once the complex translocates into the nucleus, PER-TIM bind to CLK-CYC, inhibiting their binding affinity for the *per* and *tim* E-boxes, thereby negatively regulating the transcription of their own mRNAs. The negative inhibition is removed once other proteins degrade the PER-TIM complex, allowing the cycle to begin again. For a detailed review of the molecular clock, see Zheng and Sehgal 2012 (Zheng and Sehgal 2012).

In the *Drosophila* brain, several neuronal clusters exhibit oscillations of the molecular clock and these 'clock cells' coordinate the timing of circadian-controlled processes. Within the environment, there are a number of stimuli such as light, chemicals, and temperature that affect the timing and efficacy of circadian outputs. Of these, temperature is especially interesting because it influences overt rhythms in several ways, one of which is that low temperature blocks rhythm, which resumes once returned to permissive temperatures. Thus, the overt manifestation of circadian rhythms occurs within specific temperature limits (Francis and Sargent 1979; Martino-Catt and Ort 1992; Njus, et al. 1977). With the exception of one study in the fungus *Neurospora crassa* (Liu, et al. 1997), little is known about the biological significance of this circadian property nor why species lose their ability to maintain rhythm below the lower limit of rhythmicity. Although *Drosophila* has been the focus of intense research on the evolutionary and molecular biology of

circadian rhythms and some studies have addressed mechanisms and selection pressure for temperature-dependent changes in activity patterns (Low, et al. 2008; Majercak, et al. 1999) – the lower temperature limit property of circadian rhythm has not been investigated.

Natural populations of *Drosophila melanogaster* are evolving in diverse environments across the world (Kyriacou, et al. 2008) and their molecular clocks have been shown to be under selection pressure based on how well they resonate with the ~24hr cycle of the environment (Low, et al. 2008; Sawyer, et al. 1997; Sawyer, et al. 2006). In addition, cold tolerance and correlated phenotypes (reproductive diapause expression, longevity, starvation tolerance, lipid content, fecundity profiles (Schmidt, et al. 2005)) are directly relevant to *Drosophila* populations in the wild and are affected by selection pressures that vary spatially and temporally (Mitrovski and Hoffman 2001; Schmidt and Conde 2006; Schmidt and Paaby 2008). This suggests that the maintenance of fundamental biological processes (including circadian rhythmicity) during exposure to cold temperatures is directly relevant to performance and fitness of *Drosophila* evolving in temperate environments. Here we tested the response of wild-derived populations of *Drosophila* to *extremely* low (12°C) and low-temperature (15°C) stress to determine whether this trait is evolutionarily relevant. We then use lines rhythmic at 15°C to address the mechanism underlying the maintenance of rhythm under this condition.

## Methods

**Isofemale lines.** Isofemale lines are strains derived from a single, multiply mated, wild-caught female and are often used to investigate the genetic architecture of natural populations (David, et al. 2005). In this study, 46 Isofemale lines derived from six natural populations in Africa [9 from CO: Oku, Cameroon (6.25, 10.43; altitude: 2169m); 3 from ED: Dodola, Ethiopia (6.98, 39.18; altitude: 2492m); 11 from GA: Franceville, Gabon (-1.65, 13.6; altitude: 350); 9 from NG: Maiduguri, Nigeria (11.85, 13.16; 295m); 9 from RG: Gikongoro, Rwanda (-2.49, 28.92; 1927); SP: 5 from Phalaborwa, South Africa (-23.94, 31.14; 350m)] were used to investigate the evolutionary relevance and mechanism of this circadian property. All 46 lines were used in the

12°C dataset and a random subset of these lines was used for analyses at 15°C. J. Pool collected lines from CO and ED in 2004 as well as RG in 2008; lines from GA were collected by B. Ballard and S. Charlat in 2002; lines from NG were collected by D. Gwary and B. Sastawa in 2004; and SP lines were collected by R. Corbett-Detig in 2010 (Pool, et al. 2012). Cosmopolitan admixture data for these lines are available in (Pool, et al. 2012). All *Drosophila* lines used in this study were maintained according to the principles and practices of the US National Institute of Health's (NIH) *Guide for the Care and Use of Laboratory Animals*.

**Behavioral analysis.** The following behavioral assays were conducted under *extremely* low (12°C), low (15°C), standard (25°C), or high (30°C) temperature. Adult males between 1 to 5 days old were entrained in TriKinetics Activity Monitors (TriKinetics, Waltham, MA) under a 12hr:12hr light:dark schedule for three days in a temperature-regulated Precision incubator model 818 (Thermo Electron Corporation; Marietta, Ohio). Flies were then placed in constant darkness (DD) and allowed to free-run for 4 days, after which locomotor activity – a commonly used metric of circadian behavior in *Drosophila* (Frenkel and Ceriani 2011) – was scanned into 5 minute bins using the DAM File Scan (v.1.1.06, TriKinetics). We used ClockLab software (v.2.61; Actimetrics, IL) to score an individual fly as either rhythmic or arrhythmic, which we characterized based on the strength of the rhythm (FFT). Flies were scored as rhythmic only if they achieved an FFT value of  $\geq 0.01$ . For each fly, we reported its behavioral state (rhythmic/arrhythmic); and, if it achieved rhythmicity, the strength of its rhythm and its corresponding period length.

**Mapping the NG7 cold-resistant circadian behavior.** *Establishing chromosomally isolated genetic lines.* We mapped the chromosome responsible for the NG7 cold-resistant circadian behavior by establishing chromosomally isolated genetic lines of the NG7 isofemale line. To do this, we first crossed a group of NG7 males that exhibited rhythm (FFT $\geq 0.01$ ) at 15°C to virgins balanced for each of the three fly chromosomes. Balancer lines were obtained from Bloomington Drosophila Stock Center (<http://flystocks.bio.indiana.edu/>) and each was initially outcrossed to our isogenized  $w^{118}$  background (Iso31) for 7 generations.

To isolate the NG7 X-chromosome, we used the attached X line XX/Y (Bloomington stock #988); to isolate the 2<sup>nd</sup> chromosome, we used the balancer line, Sco/Cyo (Bloomington stock #5907); and to isolate the 3<sup>rd</sup> chromosome, we used the balancer line TM2/TM6b (Bloomington stock #5906). F1 males were sorted by the presence of the chromosomal balancer and then crossed to virgin females with the appropriate chromosomal balancer line used in the P1 cross. This process was repeated for a total of three generations. To achieve homozygosity of each chromosome after the third generation (F3), we sorted and then crossed males and virgin females based on the absence of the chromosomal balancer, thereby generating three outcrossed genetic lines of X, 2<sup>nd</sup>, and 3<sup>rd</sup> chromosomal isolates for NG7. We then phenotyped each chromosomally isolated stock at 15°C to see which could maintain rhythmicity (see 'behavioral analysis' section above).

*Generating recombinants.* To test whether the NG7 *per* or *sgg* locus accounts for its cold-resistant circadian behavior, we created recombinant lines of the NG7 and Iso31 X-chromosome. To do this, we first crossed a male from the chromosomally isolated X NG7 line positive for the behavior to a female Iso31 virgin negative for the behavior. We then selected female virgins from the F1 generation (which are all heterozygous for the NG7 and Iso31 X-chromosome) and crossed them to a male from the Iso31 strain. Because recombination occurs in the female sex of this species, the resulting males of this F2 progeny carry an X-chromosome that underwent one round of meiosis and was therefore recombinant for the Iso31 and NG7 X-chromosome. We used eye color as a marker for *per* and *sgg* allelic identity because the genes are linked. We then assayed behavior of 64 white and 62 red-eyed males from the cross at 15°C.

**Immunohistochemistry (IHC) and confocal microscopy.** For the 15°C and 25°C IHC experiments, we entrained Iso31 and X-chromosome isolated NG7 males according to the behavioral analysis section above. Once we characterized the behavior of the two genotypes, we then re-entrained these animals for one day on a 12hr:12hr light:dark schedule, followed by a return to constant darkness (DD1). All samples were collected on DD1. IHC preparation and quantification protocols for PER in the Pigment Dispersing Factor (PDF)-positive small ventral-

lateral neurons (s-LNVs) have been described previously (Benito, et al. 2007; Blanchard, et al. 2010; Zhang, et al. 2013). We quantified PER in this cell cluster because it is essential for robust circadian behavior in DD (Zheng and Sehgal 2012). Briefly, to compare PER intensity in the s-LNVs, NG7 and Iso31 were sacrificed at 4-hour intervals starting at CT0 (CT0, 4, 8, 12, 16, and 20). For the 15°C experiment, all Iso31 males (which were arrhythmic) were sampled for immunohistochemistry, whereas both arrhythmic and rhythmic NG7 males were sacrificed (NG7A and NG7R respectively). For the 25°C experiment, males from both genotypes were rhythmic.

After dissection in chilled PBS-T (PBS with 0.1% Triton), brains were fixed in 4% paraformaldehyde diluted in PBS-T for 25 minutes at room temperature. Subsequently, the brains were rinsed and washed with PBS-T three times (5 minutes each wash) and then incubated with 5% normal donkey serum in PBS-T for 30 minutes and then incubated with primary antibodies at 4°C overnight. To stain for PER, we used rat anti-PER (1:1000). Because the PDF-containing s-LNVs control circadian behavior in DD (Renn, et al. 1999; Stoleru, et al. 2005), we co-stained the brains for PDF expression using rabbit anti-PDF (1:250). The following day, brains were washed 3 times with PBS-T (5 minutes each wash) then incubated in the following secondaries at room temperature for 1.5hrs: Alexa 488-conjugated donkey anti-rat (2:1000) and Alexa 555-conjugated donkey anti-rabbit (LifeTechnologies. 1:500 dilution). Brains were mounted on a glass slide using Vectashield (Vector Laboratories, Burlingame, CA).

Images of the s-LNVs were acquired on a Leica TCS SP5 confocal microscope with a 40X oil immersion lens by sequentially collecting z-sections at 1.5uM for both Alexa fluorophores. To avoid oversaturating the image, we calibrated the gain and offset for the PER detector to the s-LNV with the maximal amount of PER expression for each experiment, therefore the scales in figures 4b, d and 5b are not comparable with each other. The calibration of the detector remained constant throughout the given experiment for all groups. Once collected, the images were digitally projected using ImageJ v1.42q (<http://rsb.info.nih.gov/ij>). Mean PER fluorescence was taken for each s-LNV and normalized to the field adjacent to the imaged neuron. PDF (magenta channel)



was used as a marker for the neuron. For each time point, signal intensity was measured bilaterally in up to 4 neurons from at least 5 brains.

**Statistical analyses.** All statistical analyses were performed using JMP PRO v.10 (SAS Institute, Cary, NC). We used two nominal logistic regression models to examine the relationship between a fly's altitude of origin (high/low), the proportion of a fly's genomic admixture, and a fly's likelihood of maintaining rhythm under *extremely* cold (12°C) and cold temperature stress (15°C) respectively. We classified a fly as originating from a 'high altitude' source if it derives from a location that exceeds 1900m [Cameroon (CO), Ethiopia (ED), Rwanda (RG)] and originating from a 'low altitude' source if the location is below or equal to 350m [Gabon (GA), Nigeria (NG), South Africa (SP)]. Data on a fly's proportion of genomic admixture from cosmopolitan sources was taken from genomic analysis of its isofemale line (Pool, et al. 2012). When a fly line's admixture data was not available, we used the average population admixture as an input (Pool, et al. 2012). In all models, we controlled for the nested structure of the data (e.g. we included each population nested within a high or low altitude). In our 12°C (but not 15°C) dataset, we conducted three assays in which we characterized circadian behavior. In this dataset, we controlled for the effects of each assay (assay 1, 2, and 3) and reported the effects when statistically significant.

To compare the differences among the proportion of rhythmic individuals between two or more independent groups, we used a Chi-square test. When the proportions between the independent groups consisted of sparse data (cells<5), we used a Fisher's exact test. To compare differences in means between two groups, we used an independent sample t-test; to compare differences in means in groups that exceeded two, we used a one-way between subjects ANOVA followed by a post-hoc test. To take into account the nested structure of the immunohistochemistry data (e.g. we analyzed mean PER intensity in cells nested within fly brains) we used a linear mixed effects model to analyze differences in PER intensity at each timepoint for all groups.

## Results

**Rhythmicity at low temperatures varies in naturally occurring *Drosophila* populations.** To determine if natural populations of *Drosophila* display variation in the manifestation of circadian behavior under *extremely* low temperature stress, we evaluated the locomotor rhythm of isofemale strains derived from ancestral African populations at 12°C. We phenotyped between 10-128 individuals for each of the 46 isofemale lines, recording the individual's behavioral state (rhythmic/arrhythmic). We found that the proportion of rhythmicity in these lines varies at 12°C (fig. 1a).

As the lines we tested derived from different altitudes, and higher altitudes are typically associated with lower temperatures, we wondered whether higher altitude strains show selection for low temperature-adapted traits. Previous work has demonstrated that altitude is functionally important for the selection of fitness related traits (e.g. desiccation, heat knock-down and starvation resistance; developmental time; (Sambucetti, et al. 2006; Sørensen, et al. 2004)), but it is unknown whether it is relevant for the circadian clock as well. Because the circadian clock is known to be under selection based on its ability to maintain a ~24hr period regardless of the temperature variance (Sawyer, et al. 1997; Sawyer, et al. 2006), we predicted that extreme environmental flux associated with high altitude would select for cold-resistant circadian traits at 12°C.

To test the effects of altitude on a fly's likelihood of maintaining rhythm under *extremely* low temperature stress, we performed a nominal logistic regression model. We found a significant association between a fly's altitude of origin and its behavioral response to 12°C, where flies from high altitude sources are more rhythmic than those sampled from low altitude (table 1a).

Pool et al. 2012 (Pool, et al. 2012) recently showed that introgression from non-native sources (e.g. populations outside of sub-Saharan Africa) varies among the lines evaluated in this study. When previously isolated populations meet and mix, the resulting admixed population can benefit from several fitness-related advantages (Keller and Taylor 2010) including increased genetic variation, the creation of novel genotypes, and the masking of deleterious mutations. However, in populations that are locally adapted for maintaining rhythm under *extremely* low

temperature stress, admixture can also be selected against if the costs of diluting this local adaptation outweigh the benefit of reducing an inbreeding depression (Verhoeven, et al. 2004). To determine if cosmopolitan admixture was relevant for the response of a line to *extremely* low temperature stress, we added information on a fly's percent admixture as an input in our model (data from (Pool, et al. 2012)). We found a significant association between a fly's proportion of cosmopolitan admixture and its circadian response to *extremely* low temperature stress because as admixture increases, flies are less likely to maintain rhythmicity (fig. 1b, table 1b). Interestingly, we also found that the association between altitude and a fly's circadian response becomes non-significant (table 1b), indicating that genomic admixture drives the relationship between altitude and rhythmicity.

Next we sought to determine the extent to which natural populations display variation in their ability to maintain rhythm at a slightly higher temperature (15°C) and so we phenotyped a random subset of our original pool under this less-stressful cold condition. Similarly to the 12°C dataset, we found that natural populations of *Drosophila* display variation in the manifestation of circadian behavior (fig. 1c). However, unlike the 12°C dataset, we did not find that altitude is a predictor of a fly's behavioral response (table 2a). We also found that cosmopolitan admixture is not a significant predictor of a fly's likelihood of rhythmicity when included in the model (fig. 1d, table 2b).

For the remainder of the study in which we investigate the mechanistic aspects of the lower-limit trait, we chose to phenotype the isofemale lines under 15°C, as this temperature yielded more penetrant and robust rhythms.

**In natural populations, cold-buffered circadian behavior at 15°C is resistant only to low temperature stress or cross-resistant to high and low temperature extremes (15°C and 30°C).** *Drosophila* respond adaptively to selection for heat (Hoffman, et al. 1997) or cold stress (Watson and Hoffman 1996), resulting in a change of the resistance to high or low temperature respectively. Less understood in insect thermal physiology is the concept of 'cross-resistance,' in

which an adaptation to one stressor (e.g. low temperature) confers an advantage in counteracting the deleterious effects of another (e.g. high temperature). This property may be due to selection of genes that curb the effects of stressors that induce similar physiological responses, predicting positive genetic correlations between the traits of interest. In this experiment, we tested whether cold-buffered circadian behavior at 15°C (fig. 1c) is resistant to low temperature stress only or is 'cross-resistant' to high and low temperature extremes. If the cold-buffered rhythm is resistant to low temperature only, we predicted that any of the following behaviors related to rhythm – a line's proportion of rhythmicity, average rhythm strength (FFT), and/or ability to temperature compensate – would degrade from low (15°C) to high (30°C) temperature stress. In contrast, if the cold-buffered rhythm is cross-resistant to high and low temperature stress, we predicted that there would be no change in the three behaviors between 15°C and 30°C. From the isofemale lines we phenotyped in fig.1c, we identified 4 —CO1, NG8, NG10, and NG7— that were highly rhythmic at 15°C, meaning that the proportion of rhythmic individuals in the line exceeded 70%. We consider these lines as resistant to low temperature stress because they have robust, cold-buffered rhythm.

For the first resistance assay, we compared the difference in each line's proportion of rhythmic individuals between low and high temperature stress. Of the 4 lines assayed, NG8 shows a significant decrease in rhythmicity from low to high temperature and so we categorize this line as cold-resistant (fig. 2a, *note*: comparisons between high and low temperature phenotypes for the same line are shown with black asterisks). Next, we used rhythm strength as an output measure of a line's circadian response to temperature stress. We found that the rhythms of NG8 and NG7 show a decrease in strength when placed under high temperature stress, indicating that their rhythms are cold-resistant (fig. 2b).

Temperature compensation (Hastings and Sweeney 1957; Pittendrigh 1993) is a generic circadian property in which the period length of the rhythm does not change within the temperature limits of rhythmicity. To determine whether temperature compensation is maintained in the lines that display rhythms a low temperature, we assessed the circadian period of CO1,

NG10, NG8, and NG7 at low and high temperatures (15°C and 30°C). We found that NG10 and NG8 show a significant lengthening of their period as a function of temperature, indicating that their rhythm is not temperature compensated (fig. 2c).

The results of the resistance assays in figs. 2a-c suggest that rhythms of the NG lines are resistant to cold temperature stress only because their rhythmic behavior degrades with high temperature in at least one of the three resistance assays: NG7 – rhythm strength; NG8 – proportion rhythmicity, rhythm strength, period length; NG10 – period length. In contrast, our data suggest that the rhythm of CO1 is cross-resistant to high and low temperature extremes (15°C and 30°C) because its circadian behavior does not vary as a function of temperature. Taken together, these results suggest that in nature, cold-buffered circadian lines at 15°C can be either resistant to cold temperature stress or cross-resistant to both high and low temperature extremes (30°C and 15°C).

**Cold-buffered rhythmic lines withstand low temperature stress better than a generic wild-type laboratory line.** Using the same data, we next determined whether the cold-buffered rhythmic lines withstand cold temperature stress better than Iso31. We predicted that if an isofemale line is better able to withstand cold temperature stress, then either its proportion of rhythmic individuals or the rhythm strength would be higher than Iso31 at 15°C. Our data are consistent with this prediction: cold-buffered rhythmic lines have a higher proportion of rhythmic animals (fig. 2a) and two cold-buffered rhythmic lines have a higher rhythm strength (fig. 2b) under low temperature stress than Iso31 (*note*: comparisons made between Iso31 and an isofemale line are shown in blue for low temperature). Next we determined whether the rhythm of Iso31 is better buffered against high temperature stress than our cold-buffered lines. If so, then either the proportion of rhythmic individuals or the rhythm strength would be higher in Iso31 than in any of the cold-buffered rhythmic lines at high temperature. Figure 2a is consistent with this prediction because at 30°C, Iso31 is more rhythmic than two cold-buffered rhythmic lines (NG10 and NG8, *note*: comparisons made between Iso31 and an isofemale line are shown in red for

high temperature). The rhythm strength was not different among the lines at this temperature (fig. 2b). These data suggest that our laboratory line can buffer high temperature extremes better than some of our cold-resistant (but not cross-resistant) isofemale lines.

**Mapping the genetic locus of a 15°C cold-resistant rhythmic line (NG7).** To determine whether the cold-resistant circadian behavior of one line (NG7) could be mapped to a single chromosome or if it represented a quantitative output of any combination of the three major fly chromosomes (X, 2<sup>nd</sup>, or 3<sup>rd</sup>), we isolated each NG7 chromosome into three independent lines in an Iso31 background (see Methods). As we determined that Iso31 is negative for the cold-resistant rhythmic phenotype (fig. 2a, b), isolating each NG7 chromosome in an Iso31 background would reveal the source of the behavior. We found that the X-chromosome of NG7 contributes to the phenotype while the other isolated chromosomes do not (fig. 3a).

Next we tested whether the *period* and *shaggy* loci are functionally important for the phenotype because these are two important clock genes on the X-chromosome and they are implicated in interactions of the clock with temperature (Cheng, et al. 1998; Low, et al. 2008; Majercak, et al. 1999). Because *per* and *sgg* are linked to the eye color gene, *white* (*w*) – meaning that they are located so positionally close together that they do not recombine – *w* can be used as a marker of *per* and *sgg* allelic identity: NG7 contains a wild-type copy of the allele (*w*<sup>+</sup>), which produces red-eyes and Iso31 contains the mutant allele *w*<sup>118</sup>, which results in a white-eyed phenotype. Therefore red-eyed recombinants of the NG7 and Iso31 X-chromosome are expected to have NG7 *per* and *sgg* alleles. If the cold-resistant rhythmic phenotype maps to *per* or *sgg*, then recombination of the NG7 X-chromosome with the Iso31 X-chromosome (see Methods) should yield only red (and no white) eyed recombinants that are positive for the behavior. However, we were able to recombine the *w*<sup>118</sup> gene of Iso31 with the NG7, cold-resistant circadian behavior (fig. 3b), suggesting that the phenotype is not linked to NG7 *per* or *sgg*.

**A more robust circadian clock underlies the 15°C cold-resistant behavioral rhythm of NG7.**

Stronger behavioral rhythm at lower temperature could result from enhanced clock function or from better transmission of output signals from the clock. To distinguish between these two possibilities we assayed molecular cycling of the clock protein, PER, in the small ventral-lateral neurons (s-LNVs), which are central clock cells of the *Drosophila* brain. As not all NG7 flies are rhythmic at lower temperature, we first assayed all flies for behavior to separate them into rhythmic and arrhythmic groups. Briefly, we entrained flies from the NG7 X-chromosome cold-resistant circadian strain (generated in fig. 3a) as well as Iso31 to 3 days of 12hr:12hr light:dark cycles. On the fourth day, we moved the flies into constant darkness and let them free-run for four days, after which we scored the behavior of the flies as either rhythmic or arrhythmic based on the strength of the rhythm. All Iso31 flies were arrhythmic in this experiment. Once we characterized the behavior of individual flies, we re-entrained them for one day to resynchronize molecular oscillations across flies and then returned them to constant darkness. On the first day of constant darkness (DD1), we dissected the brains of rhythmic and arrhythmic NG7 as well as Iso31 flies at four-hour intervals. We then examined PER expression by immunohistochemistry in the s-LNVs, which are central clock cells of the *Drosophila* brain that maintain free-running rhythm (Grima, et al. 2004) and can be identified through their expression of a neuropeptide, Pigment Dispersing Factor (PDF). Representative images of the immunohistochemistry are shown in fig. 4a. We found that rhythmic and arrhythmic NG7 flies have stronger PER oscillations in central clock cells at 15°C than Iso31 (fig. 4b), indicating that the strong behavioral rhythm of NG7 is due to stabilization of the central clock as well as the clock output under cold-temperature stress. Table 3 illustrates the linear mixed-effects model regression weights and significance levels for each treatment group in predicting mean PER expression for each timepoint.

In comparison with NG7, PER expression appeared low and noncyclic in Iso31 at 15°C (fig. 4a,b). To determine whether PER oscillations in Iso31 are indeed disrupted or merely reduced in amplitude, we enhanced the detection of PER expression in Iso31 using a calibration specific to an Iso31 s-LNV cell with maximal PER expression. A disrupted molecular profile would

produce a flat protein expression profile throughout the circadian time course. Representative images of this re-quantification are shown in fig. 4c. The group means of these samples show that PER cycling is not disrupted but considerably dampened, due to an overall reduction in levels relative to expression in NG7.

**Enhanced PER expression of the NG7 cold-resistant clock is specific to cold temperature stress.** To determine whether enhanced PER expression of the NG7 cold-resistant clock relative to Iso31 (fig. 4) is specific to low temperature stress, we performed IHCs on NG7 and Iso31 s-LNVs at room temperature (25°C) using the same methods as above. Both genotypes were rhythmic in this experiment. Representative images of NG7 and Iso31 flies are shown in fig. 5a. Group means of the two genotypes show that there is no difference in PER expression at room temperature (fig. 5b). Table 4 illustrates the linear mixed-effects model weights and significance levels for each treatment group in predicting mean PER expression for each timepoint.

## Discussion

Little is known about the evolutionary significance and mechanistic features of the lower temperature limit property of circadian rhythm, especially in *Drosophila*, in which there has been no research effort. In this study, we used 46 isofemale lines isolated from 6 different natural populations of *Drosophila melanogaster* to address these issues. Previous work has shown that physiological traits influenced by temperature (such as the temperature compensation property of the clock, cold-tolerance, and reproductive diapause) are under predictable selection pressures of the environment so natural populations offer a unique medium to understand the biological (e.g. whether the trait is evolutionarily relevant) and mechanistic (why *Drosophila* experience overt loss of behavior rhythm) features of this trait.

Our study is the first to capture the diversity in how the *Drosophila* circadian system responds to low temperature stress: under both *extremely* low (12°C) and low (15°C) temperatures, some isofemale lines are better at maintaining behavioral rhythm than others (fig.



1a, c). Interestingly, we found evidence that this variability is evolutionarily relevant at 12°C because high altitude populations exhibit selection for locally adapted genomes that contribute to rhythmic behavior (table 1). However, our data show that the maintenance of rhythmicity at 15°C is not functionally significant because we did not find evidence of selection for rhythmic behavior as a function of altitude or cosmopolitan admixture (table 2). While the evolutionary significance of differential patterns of rhythmicity at 12°C and 15°C is unknown, one possible explanation for this difference is that both native and cosmopolitan populations may experience temperatures of 15°C or higher. In this scenario, both high and low altitude populations would experience equal selection pressures of native and non-native genomes. However, high altitude populations might experience cold temperature extremes (<15°C), where a local adaptation to the environment would be selectively advantageous. This condition might then cause a differential selection pattern for native genomes that contain circadian allele(s) specialized to buffer extreme cold in the high population group.

For the lines that withstand cold temperature stress under 15°C, we found additional diversity in how they respond to temperature stress in general. Some of these cold-buffered lines display rhythms that are resistant only to low temperature stress (NG7, NG8, NG10; fig. 2) whereas others are cross-resistant to both high and low temperature extremes (15°C and 30°C; CO1, fig. 2). Extension of this work to natural populations on other continents and from other habitats would be required to further evaluate the significance of resistance to a specific temperature extreme and cross-resistance to temperature extremes.

Our research also highlights the advantage of using lines derived from natural populations to identify novel loci relevant to temperature-influenced circadian traits. Although the locus responsible for the phenotype of one cold-resistant strain (NG7) maps to the X chromosome (fig. 3a), it does not appear to be the canonical clock gene, *period* (*per*) (fig. 3b). This is distinct from findings in *Neurospora crassa*, where the determinants of the lower limit are linked to the central clock gene, *frequency* (*frq*) (Liu, et al. 1997). In this study, we have shown that the rhythm of at least one isofemale line is resistant to low temperature stress due to a locus

unrelated to *per* (fig. 3b), which is surprising given that this gene is important for other ways the clock interacts with temperature. For example, the structure of *per* is fundamentally important for temperature-dependent changes in the distribution of daily activity patterns (Low, et al. 2008; Majercak, et al. 1999) whereby temperature-sensitive splicing of *per* (Cheng, et al. 1998) facilitates the temperature-mediated advance and delay in behavior.

Although *per* is not the allelic source of NG7, we believe that the enhanced PER expression we observed in NG7 at low temperature (fig. 4a,b; table 3) is functionally important for maintaining rhythmicity. The cold-resistant circadian behavior of NG7 is most likely due to more robust oscillations of core clock genes. As shown from our immunohistochemistry data, PER expression is significantly higher in the s-LNVs of NG7 than Iso31 during times at which it is typically elevated, from CT20-4 (fig. 4a,b; table 3). As shown previously (Lim and Allada 2013; Liu, et al. 1997), a threshold amount of PER is required for the circadian rhythm to persist, and this level changes as a function of temperature. Our wild-type line is likely arrhythmic under cold temperature stress because their s-LNVs do not reach sufficient PER expression to achieve rhythmicity. We note that we observed oscillations (fig. 4c,d) when imaging the circadian cells under a lower calibration for PER.

Interestingly, our data also suggest that while enhanced clock function is necessary for overt rhythm to persist under low temperature stress, it is not sufficient because there is no difference in PER levels between rhythmic and arrhythmic NG7 flies throughout the circadian cycle (fig. 4a,b; table 3). Thus, mechanisms downstream of the clock also need to be resistant to low temperature to produce rhythmic behavior. Given that the overall behavior can be recombined with *w* (and, therefore, is not linked to *per/sgg*), we suggest that the NG7 line contains component(s) distinct from *per* and *sgg* on the X-chromosome (fig. 3b) that enhance PER oscillations in the s-LNVs at low temperature and stabilize output circuits necessary for rhythmic behavior. Furthermore, our data also suggest that these component(s) are active in response to cold-temperature stress only, since we did not observe a difference in PER cycling between NG7 and Iso31 under standard temperature conditions (fig 5a,b; Table 4).

What accounts for the molecular genetic clock's ability to buffer cold temperature stress? It is possible that transcriptional and/or translational modifications within its molecular clock circuit enhance clock oscillations. Under low temperature stress, numerous insults could interfere with the clock circuit's transcriptional machinery, which must be maintained for precise spatiotemporal control of clock genes. For example, under low temperature, single enhancers sometimes fail to drive the complete expression of genes, and studies have shown that there are several regulatory mechanisms that counteract these insults (Lagha, et al. 2012), two of which include intra-enhancer redundancy (Ludwig, et al. 2011) and shadow enhancers (Frankel, et al. 2010; Perry, et al. 2010). In *Drosophila*, shadow enhancers are good candidates for a cold-resistant circadian phenotype because some have been shown to stabilize gene expression of certain loci specifically at 15°C (Frankel, et al. 2010), which is the temperature regime under which this study was conducted.

Once clock genes are transcribed, clock RNA structures might also encounter problems unique to cold stress. Under low temperature conditions, misfolded single stranded RNA structures become overstabilized and certain transcript variants do not always produce optimally behaving proteins for the given abiotic condition. Therefore post-transcriptional modification mechanisms might play a key role in modulating clock gene expression by generating different mRNA variants with altered stability, translation efficiency, and/or distribution. Some candidates include alternative splicing (which is already known to be relevant to the *Drosophila* circadian clock (Majercak, et al. 1999)) and RNA editing (Savva, et al. 2012). Similarly, tight translational control mechanisms might play a key role in optimizing clock gene expression. Cold-temperature stress in general reduces protein synthesis and so tight translational control mechanisms of NG7 clock genes might guarantee that a sufficient amount of clock expression is produced to maintain circadian oscillations.

Low temperatures also result in abnormally high levels of misfolded or damaged proteins, which could affect the efficacy of the molecular clock. Molecular chaperones can assist with protein folding and protein degradation by capturing damaged proteins and refolding them back

into their native confirmation. If this re-folding fails, molecular chaperons, with the help of other co-factors can target such proteins for degradation. In the plant *Arabidopsis thaliana*, targeted degradation of certain proteins installs new regulatory mechanisms that upregulates the expression of cold-responsive genes, mediating tolerance to cold conditions (Lyzenga and Stone 2012). To our knowledge, it is unknown whether post-translational modifications due to molecular chaperones or other cofactors are involved with the *Drosophila* response to low temperature stress.

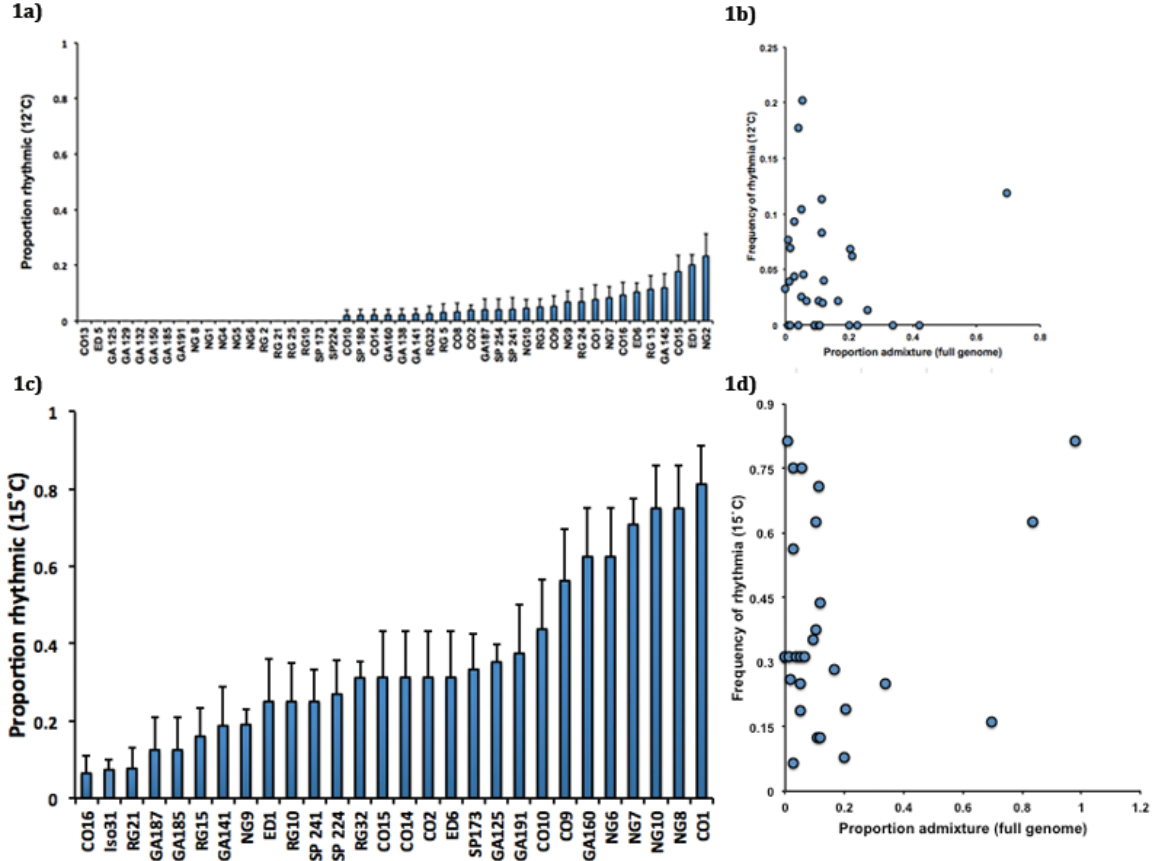
The data presented here also suggest that stability of the output pathway is functionally important for cold-resistant clock behavior (fig. 4a,b; table 3). Downstream of the molecular clock, circadian pacemaker neurons form a functional circuit necessary for the ~24hr output of circadian behavior and our data suggest that the NG7 line also regulates its output circuit during temperature stress. Temperature stress is generally associated with massive changes in the *Drosophila* transcriptome, which include upregulation of several physiological relevant genes (Colinet and Hoffman 2010; Kelty, et al. 2002). For example, the senescence marker protein *smp-30* – which is important for  $Ca^{2+}$  ion homeostasis and neuron signaling – is thought to be relevant to cold tolerance in *Drosophila* because *smp-30* mRNA accumulates after cold acclimation to 15°C (Goto 2000). In addition, there is an association between genetic variation at *smp-30* and with variation in chill-coma recovery (Clowers, et al. 2010). These or other relevant mechanisms might be important for how the NG7 clock circuit and/or circadian output maintains physiological homeostasis during low temperature stress.

## **Acknowledgements**

We thank David Garbe and Mi Shi for their help on the IHC experiments and mapping aspect of the project respectively. Chris Reece provided invaluable support for the statistical work. This manuscript benefited from the comments of anonymous reviewers. SEM was funded by an NIH pre-doctoral training grant (HL07953). PS was supported by a grant from the NIH

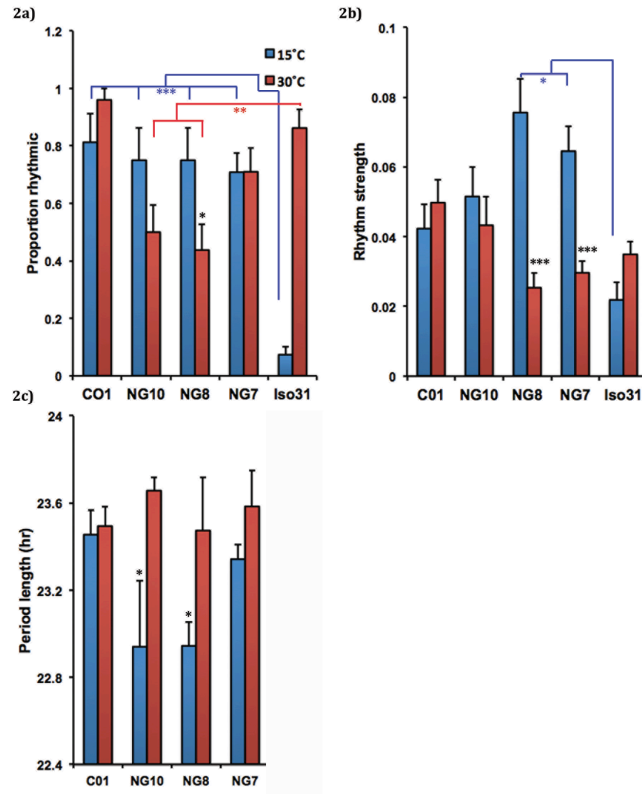
(RO1GM100366). AS was supported by a grant from the NIH (RO1NS048471). Data included in this manuscript will be made available upon request.

Figure 1



**Rhythmicity at lower temperatures varies in naturally occurring *Drosophila* populations. (a) 12°C.** Using a Chi-square test, we found that the proportion of rhythmic individuals differs among 46 isofemale lines at 12°C  $\chi^2(df=45, N=1937)=145, P<0.001$ . We computed the standard error of the proportion using the formula:  $SE = \sqrt{(p(1-p)/n)}$ , where  $p$  = the proportion of rhythmic individuals in our sample;  $n$  = the number of individuals in the sample; and  $\sqrt{\phantom{x}}$  = square root. **(b) Isofemale lines with a lower percentage of genomic admixture are more likely to maintain rhythm under extreme temperature stress.** Results of the logistic regression are found in table 1b. **(c) At 15°C, rhythmicity varies in naturally occurring *Drosophila* populations** Using a Chi-square test, we found that the proportion of rhythmic individuals differs under low temperature stress among a random 27 line subset of our isofemale strains  $\chi^2(df=28, N=862)=145, P<0.001$ . We computed the standard error of the proportion using the formula:  $SE = \sqrt{(p(1-p)/n)}$ , where  $p$  = the proportion of rhythmic individuals in our sample;  $n$  = the number of individuals in the sample; and  $\sqrt{\phantom{x}}$  = square root. **(d) There is no significant association between a line's percentage of cosmopolitan admixture and a fly's likelihood of maintaining rhythm under low temperature stress.** Results of the logistic regression can be found in table 2.

Figure 2

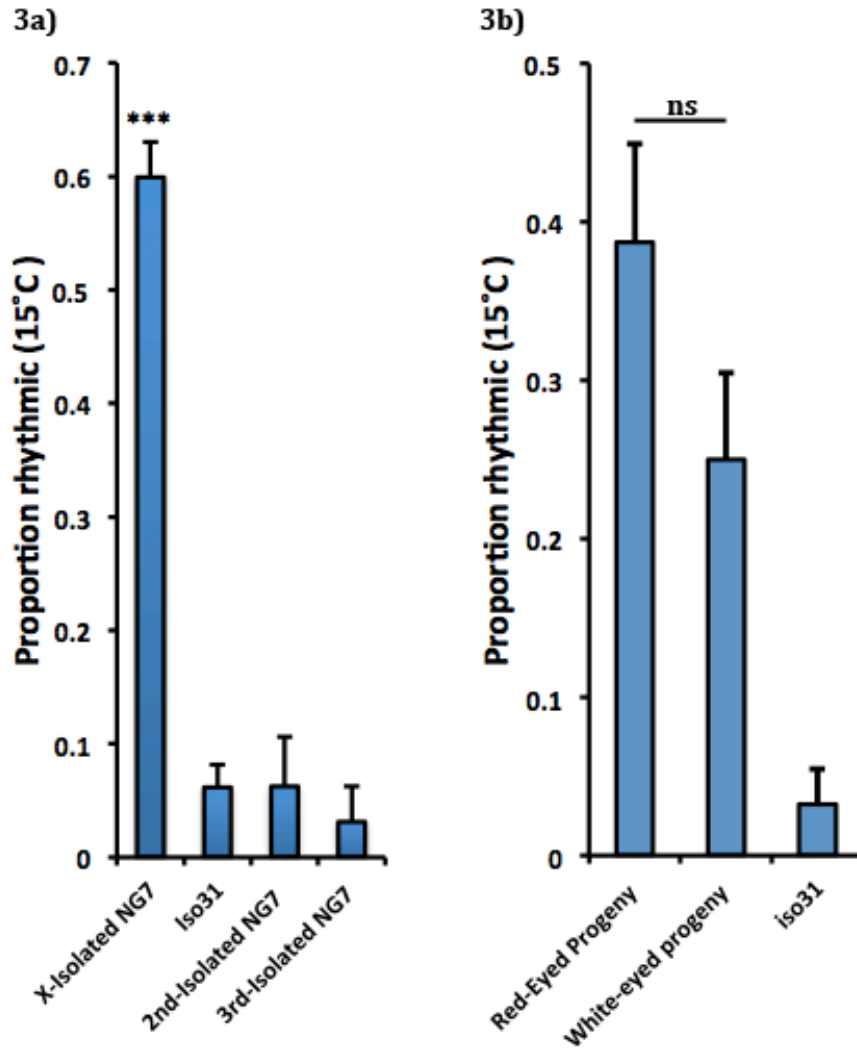


In natural populations, cold-buffered circadian behavior at 15°C is resistant only to low temperature stress or cross-resistant to high and low temperature extremes (15°C and 30°C). Note: Comparisons between high and low temperature phenotypes for the same line are shown in blue for low temperature and red for high temperature. **(a) Resistance assay for the proportion of isofemale rhythmicity.** We used a Fisher's exact test to compare differences in the proportion of rhythmic individuals between low and high temperature stress for CO1, NG8, NG10, and NG7. \* $P < 0.05$ . There is a non-significant trend for the proportion of rhythmic individuals in NG10 to decrease from 15 to 30°C ( $P = 0.09$ ). **Cold-buffered lines are more rhythmic than Iso31 at 15°C.** We used Fisher's exact tests to compare differences in the proportion of rhythmic individuals between Iso31 and CO1, NG8, NG10, and NG7 respectively. \*\*\* $P < 0.0005$ . **Iso31 is more rhythmic than some cold-buffered isofemale lines at 30°C.** We used Fisher's exact tests measuring the proportion of rhythmic individuals between Iso31 and CO1, NG10, NG8, and NG7 respectively under high temperature stress. \*\* $P < 0.005$ . **(b) Resistance assay for rhythm strength.** Independent sample t-tests were conducted to compare the rhythm strength (FFT) between an isofemale line at 15°C and 30°C. \*\*\* $P < 0.0005$ . NG7 and NG8 show degradation of rhythm strength as a function of temperature. **Two cold-resistant lines have stronger rhythm strength than that of Iso31 at 15°C.** We used a one-way between subjects ANOVA to compare the rhythm strength of Iso31, CO1, NG8, NG10, and NG7. There was a significant effect of low temperature on rhythm strength at the  $p < 0.05$  level for the 5 groups [ $F(4) = 3.64$ ,  $P < 0.01$ ]. Post hoc

comparisons using the Dunnett's Method indicate that the FFT of Iso31 is significantly lower than that of NG8 and NG7;  $*P<0.05$ . There was no significant difference in mean FFT values among the isofemale lines. **There is no difference in rhythm strength between Iso31 the isofemale lines at 30°C.** We used a one-way between subjects ANOVA to compare the rhythm strength of Iso31, CO1, NG8, NG10, and NG7. There was a significant effect of high temperature on rhythm strength at the  $p<0.05$  level for the 5 groups [ $F(4)=3.35$ ,  $P=0.01$ ]. However, post hoc comparisons using the Dunnett's Method indicate that the FFT of Iso31 is not significantly different from any of the isofemale lines. Instead, we found that the rhythm strength of CO1 is higher than that of NG8 ( $p<0.03$ ) and NG7 ( $p<0.05$ ). **(c) Resistance assay for temperature compensation.** Independent sample t-tests were conducted to compare the period length between an isofemale line at 15°C and high 30°C.  $*P<0.05$ .

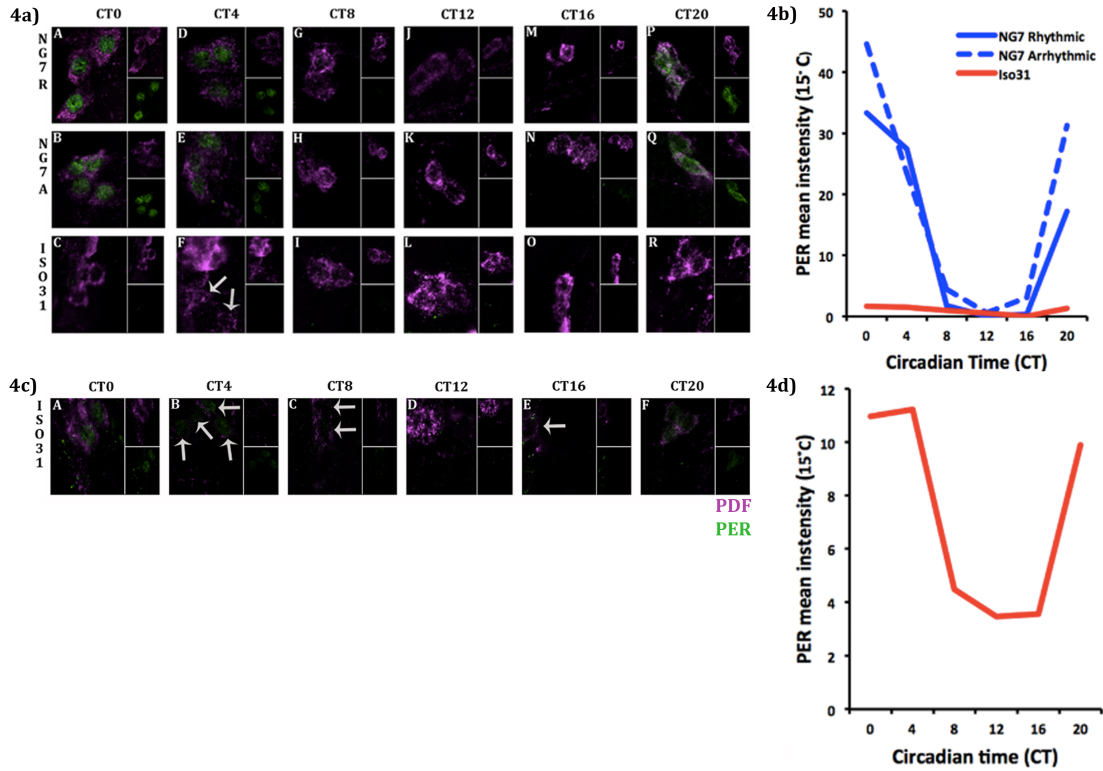


Figure 3



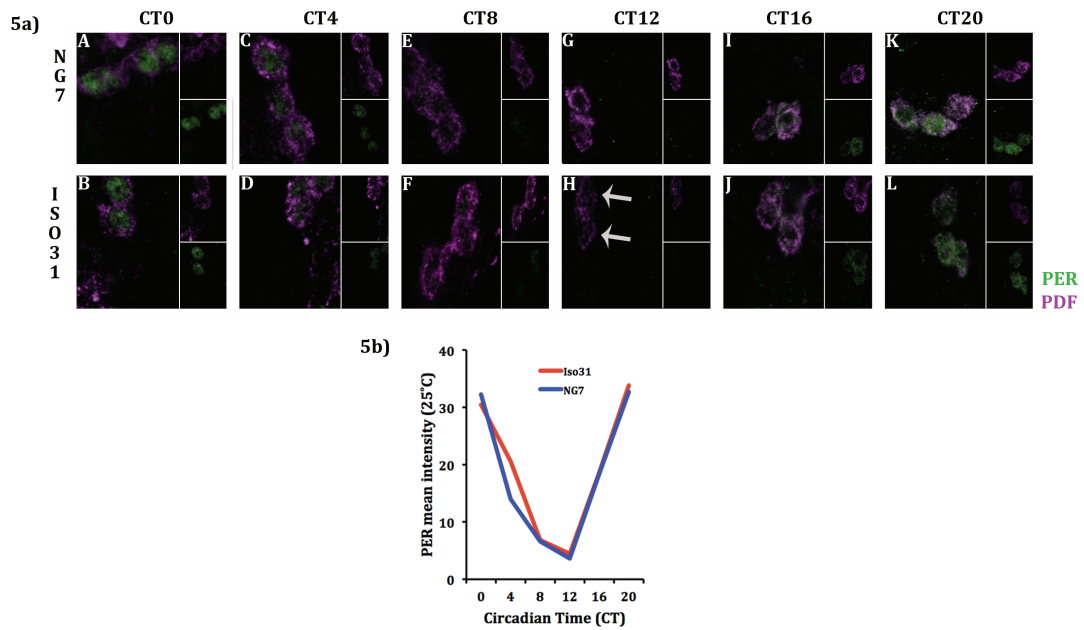
**Mapping the genetic locus of a 15°C cold-resistant circadian line (NG7) (a) The X-chromosome of NG7 underlies cold-resistant circadian behavior.** A one-way between subjects ANOVA was conducted to compare the effect of low temperature (15°C) on rhythmicity for the 4 groups (X-isolated NG7, Iso31, 2<sup>nd</sup>-isolated NG7, and 3<sup>rd</sup>-isolated NG7). There was a significant effect of low temperature on rhythmicity at the  $p < 0.05$  level for the 4 groups [ $F(3) = 71.1$ ,  $P < 0.0001$ ]. Post hoc comparisons using the Tukey HSD test indicate that the mean proportion of rhythmic individuals for the X-isolated NG7 line ( $M = 0.60$ ,  $SD = 0.49$ ) was significantly higher than that of Iso31 ( $M = 0.06$ ,  $SD = 0.24$ ), the 2<sup>nd</sup>-isolated NG7 line ( $M = 0.06$ ,  $SD = 0.25$ ) and the 3<sup>rd</sup>-isolated NG7 line ( $M = 0.03$ ,  $SD = 0.18$ );  $***P < 0.0001$ . There is no difference in the proportion of rhythmic individuals at cold temperature among Iso31, the 2<sup>nd</sup>-isolated NG7 line, and the 3<sup>rd</sup>-isolated NG7 line. **(b) The NG7 clock genes *per* and *sgg* do not underlie the cold-resistant rhythmic behavior.** Fisher's exact test,  $p = 0.07$ .

Figure 4



**A more robust circadian clock underlies the 15°C cold-resistant behavioral rhythm of NG7. (a) Representative images of PER (green) and PDF (magenta) staining in the s-LNVs of rhythmic and arrhythmic NG7 flies (NG7R and NG7A respectively) as well as Iso31.** Rhythmic and arrhythmic NG7 flies express classic cycling of PER protein in the s-LNVs throughout the circadian cycle: PER is primarily nuclear between CT0 and CT4 (panels A, B, and D, E), absent in the middle of the subjective day at CT8-16 (panels G, J, M and H, K, N) and then cytoplasmic towards the end of the cycle at CT 20 (panels P and Q). In contrast, PER is absent in the s-LNVs in Iso31 throughout the circadian cycle (panels C, F, I, L, O, R) under the same detection level for PER. **(b) NG7 has enhanced PER oscillations relative to Iso31 at cold temperature.** Group means of PER expression in the s-LNVs of rhythmic and arrhythmic NG7 flies as well as Iso31. **(c) Representative images of the Iso31 s-LNVs captured under enhanced detection.** When we captured images of the Iso31 samples using a stronger PER detector than in fig.4a, b we found that PER expression is not disrupted at low temperature. **(d) Iso31 has a dampened (not disrupted) circadian clock at cold temperature.** Group means of the re-quantified Iso31 genotype show that PER oscillations persist, suggesting that Iso31 produces lower levels of PER protein in the s-LNVs than NG7 throughout the circadian cycle. The pattern of PER cycling is the same between Iso31 and NG7 individuals: PER is present at CT0, 4, and 20 but absent at CT 8, 12, and 16. *Note: figures 4a,b and 4c,d were analyzed using a different calibration of mean PER intensity. Therefore scales of 4b and d are not the same and are not comparable.*

Figure 5



Enhanced PER expression of the NG7 cold-resistant clock is specific to low temperature stress (a) Representative images of NG7 and Iso31 PER expression in the s-LNVs under room temperature. (b) The higher level of PER expression in the s-LNVs of NG7 observed in figure 4a, b is a specific response to cold temperature stress. Group means reveal that there is no difference in PER expression between the genotypes throughout the circadian cycle. Both groups exhibit classic patterns of PER nuclear and cytoplasmic localization. Note: figures 4a,b and 4c,d and 5a,b were analyzed using a different calibration of mean PER intensity. Therefore scales of 4b,d, and 5b are not the same and are not comparable.

**Table 1a**

<b>Coefficient</b>	<b>Estimate</b>	<b>Std. Error</b>	<b>ChiSquare</b>	<b>p-value</b>
Intercept	3.82	0.23	275.5	<0.0001*
Altitude(low)	0.75	0.22	12.2	0.0005*
Altitude(low):population(GA)	0.41	0.56	0.53	0.47
Altitude(low):population(NG)	0.16	0.56	0.08	0.78
Altitude(high):population(CO)	0.24	0.25	0.86	0.35
Altitude(high):population(ED)	-0.48	0.26	3.47	0.06
Assay 3	0.67	0.30	4.96	0.03*

**Table 1b**

<b>Figure 1b</b>				
<b>Coefficient</b>	<b>Estimate</b>	<b>Std. Error</b>	<b>ChiSquare</b>	<b>p-value</b>
Intercept	2.91	0.34	72.39	<0.0001*
Altitude(low)	0.42	0.25	2.73	0.10
Altitude(low):population(GA)	0.54	0.57	0.92	0.34
Altitude(low):population(NG)	0.28	0.57	0.23	0.63
Altitude(high):population(CO)	0.41	0.25	2.63	0.11
Altitude(high):population(ED)	-0.83	0.27	9.60	0.002*
Proportion admixed (full genome)	7.96	3.33	5.73	0.02*

**(a) Altitude is a significant predictor of a fly's likelihood of rhythmicity under extremely low temperature stress (12°C).** A nominal logistic regression model of a fly's likelihood of rhythmicity at 12°C in relation to its altitude of origin. We controlled for the population (GA, NG, CO, ED, RG) nested within altitude and the assay in which we phenotyped the flies. In this model, a fly's altitude of origin is a significant predictor its likelihood of rhythmicity under *extremely* low temperature stress. The positive relationship between altitude and a fly's likelihood of rhythmicity indicate that males from high altitude sources are more rhythmic than those from a low altitude source. **(b) Percentage of cosmopolitan admixture drives the relationship between altitude and a fly's circadian response to 12°C.** A nominal logistic regression model of a fly's likelihood of rhythmicity at 12°C in relation to its altitude and cosmopolitan admixture. We controlled for the lines sampled within a population (GA, NG, CO, ED, RG) nested within altitude and the assay in which we phenotyped the flies. In this model, a fly's proportion admixture (full genome) is significantly associated with its likelihood of rhythmicity under *extremely* low temperature stress (fig. 1b). There is no relationship between altitude and a fly's likelihood of rhythmicity. The negative relationship between the ED population nested within altitude indicates that flies from ED are more likely to be rhythmic at 15°C than the remaining populations.

**Table 2a**

<b>Coefficient</b>	<b>Estimate</b>	<b>Std. Error</b>	<b>ChiSquare</b>	<b>p-value</b>
Intercept	0.72	0.09	59.1	<0.0001*
Altitude(low)	-0.14	0.09	2.25	0.13
Altitude(low):population(GA)	-0.02	0.14	0.02	0.90
Altitude(low):population(NG)	-0.33	0.14	5.97	0.02*
Altitude(high):population(CO)	-0.29	0.19	2.47	0.12
Altitude(high):population(ED)	0.08	0.27	0.08	0.77

**Table 2b**

<b>Figure 2b</b>				
<b>Coefficient</b>	<b>Estimate</b>	<b>Std. Error</b>	<b>ChiSquare</b>	<b>p-value</b>
Intercept	0.77	0.11	53.5	<0.0001*
Altitude(low)	-0.12	0.10	1.56	0.21
Altitude(low):population(GA)	-0.02	0.14	0.03	0.87
Altitude(low):population(NG)	-0.32	0.14	5.52	0.02*
Altitude(high):population(CO)	-0.31	0.19	2.71	0.10
Altitude(high):population(ED)	0.07	0.27	0.06	0.80
Proportion admixed (full genome)	-0.41	0.38	1.20	0.27

**(a) Altitude is not a significant predictor of a fly's likelihood of rhythmicity under low temperature stress (15°C).** A nominal logistic regression model of a fly's likelihood of rhythmicity at 15°C in relation to its altitude of origin. Controlling for the sampling population (GA, NG, CO, ED, RG) nested within altitude, we found that altitude is not a significant predictor of a fly's likelihood of rhythmicity under cold temperature stress (15°C). The negative relationship between the NG population nested within altitude indicates that flies from NG are more likely to be rhythmic at 15°C than the remaining populations. **(b) There is no significant association between a line's genomic admixture and its likelihood of maintaining rhythm under low temperature stress (15°C).** A nominal logistic regression model of a fly's likelihood of rhythmicity at 15°C in relation to its altitude and cosmopolitan admixture. Controlling for the sampling population (GA, NG, CO, ED, RG) nested within altitude, we found that there is no significant association between a fly's percentage of cosmopolitan admixture (full genome) and its altitude of origin on its likelihood of being rhythmic under cold temperature stress (15°C).

**Table 3**

<b>Figure 4b</b>		<b>Value</b>	<b>Std. Error</b>	<b>t-value</b>	<b>p-value</b>
<b>CT0</b>	<b>Intercept</b>	1.75	4.10	0.43	0.67
	<b>NG7 Arrhythmic</b>	42.7	5.54	7.71	0.00
	<b>NG7 Rhythmic</b>	31.9	6.00	5.31	0.00
<b>CT4</b>	<b>Intercept</b>	1.84	7.17	0.26	0.80
	<b>NG7 Arrhythmic</b>	21.4	9.05	2.37	0.03
	<b>NG7 Rhythmic</b>	24.3	8.77	2.77	0.02
<b>CT8</b>	<b>Intercept</b>	1.04	1.81	0.57	0.57
	<b>NG7 Arrhythmic</b>	2.71	2.50	1.08	0.30
	<b>NG7 Rhythmic</b>	0.49	2.80	0.17	0.86
<b>CT12</b>	<b>Intercept</b>	0.53	0.40	1.30	0.20
	<b>NG7 Arrhythmic</b>	0.04	0.51	0.08	0.94
	<b>NG7 Rhythmic</b>	-0.53	0.59	-0.90	0.39
<b>CT16</b>	<b>Intercept</b>	0.00	3.23	0.00	1.00
	<b>NG7 Arrhythmic</b>	3.40	3.72	0.92	0.40
	<b>NG7 Rhythmic</b>	0.57	3.60	0.16	0.88
<b>CT20</b>	<b>Intercept</b>	1.34	3.82	0.35	0.72
	<b>NG7 Arrhythmic</b>	29.9	5.23	5.73	0.00
	<b>NG7 Rhythmic</b>	15.9	4.88	3.25	0.01

**Results of the linear mixed-effects model for fig 4b.** PER mean signal intensity is significantly higher in rhythmic and arrhythmic NG7 individuals than in Iso31 animals at CT0, CT4, and CT20: times when PER classically reaches maximal expression in the circadian cycle. Mean PER intensity between arrhythmic and rhythmic NG7 individuals is not significantly different at any point throughout the circadian cycle. There is no difference among the three groups between CT8-16.

**Table 4**

<b>Figure 5b</b>		<b>Value</b>	<b>Std. Error</b>	<b>t-value</b>	<b>p-value</b>
<b>CT0</b>	<b>Intercept</b>	29.2	6.25	4.67	0.00
	<b>NG7</b>	-2.18	10.4	-0.21	0.83
<b>CT4</b>	<b>Intercept</b>	19.3	5.34	3.62	0.00
	<b>NG7</b>	-5.24	8.25	-0.64	0.54
<b>CT8</b>	<b>Intercept</b>	6.83	4.86	1.40	0.19
	<b>NG7</b>	-0.17	5.33	-0.03	0.98
<b>CT12</b>	<b>Intercept</b>	4.35	1.01	4.32	0.00
	<b>NG7</b>	-0.54	1.58	-0.34	0.74
<b>CT16</b>	<b>Intercept</b>	17.0	6.68	2.54	0.01
	<b>NG7</b>	-1.99	8.84	-0.22	0.83
<b>CT20</b>	<b>Intercept</b>	35.8	5.53	6.46	0.00
	<b>NG7</b>	-7.11	5.47	-1.30	0.20

Table 4: **Results of the linear mixed-effects model for fig. 5b.** There is no difference in PER mean intensity between NG7 and Iso31 individuals throughout the circadian timecourse.

## CHAPTER 4: SUMMARY AND PERSPECTIVES

The goal of this thesis is to understand the 'ins and outs' of sleep, or the mechanisms by which one internal metabolic and one external temperature cue regulate sleep behavior. At its most basic level, sleep is a function of two independent processes: one that controls the quality and amount of sleep (Process S) and one that controls the timing, or more specifically the ~24hr rhythm of sleep (Process C) (Borbély 1982) (Chapter 1, fig. 1). While nothing is known about the homeostatic mechanisms that underlie Process S, several molecules have been identified that influence sleep amount and quality, molecules that we refer to here as 'Process S molecules.' In contrast, it is well understood that an internal circadian timing system known as the 'molecular genetic clock' controls the timing of sleep, or Process C.

Because sleep is attributable to two processes, we investigate how internal and external cues affect Process S and Process C respectively in two chapters. In Chapter 2, we investigate the link between Process S molecules and amino acid/energy metabolism. Since the emergence of *Drosophila melanogaster* as a model organism of sleep, several Process S molecules have been identified based on their role in activating either sleep or wake-promoting pathways e.g. GABA (Enell, et al. 2010; Rajan and Perrimon 2012); octopamine (Erion, et al. 2012; Luo, et al. 2014); serotonin (Luo, et al. 2014); and the neuropeptide short neuropeptide 'F' (Lee, et al. 2008). Interestingly, recent studies have revealed an additional function of certain Process S molecules: not only do they act to regulate the duration/quality of sleep but they are also intimately tied to metabolism (reviewed in Chapter 1). This is an intriguing relationship because it suggests a common mechanism linking an individual's behavioral state and metabolic expenditure (Cirelli, et al. 2004), whereby wake-promoting neurotransmitters are also involved in energy-forming reactions. We decided to evaluate whether this relationship exists in the context of the GABA shunt, which according to its biochemical pathway is intimately tied to the sleep-promoting amino acid,  $\gamma$ -Aminobutyric acid (GABA); the wake-promoting amino acid, glutamate; and the energy-forming Tricarboxylic Acid (TCA) cycle through the actions of GABA Transaminase (GABAT), the



catabolic enzyme of the GABA shunt (Chapter 2, fig. 1). We specifically chose to study the relationship between sleep and energy homeostasis using the GABA shunt because, in comparison to how Process S molecules regulate lipid/carbohydrate homeostasis (Enell, et al. 2010; Erion, et al. 2012; Lee, et al. 2008; Luo, et al. 2014; Rajan and Perrimon 2012), it was unknown whether there was a link between Process S molecules and amino acid metabolism/energy homeostasis in *Drosophila*. In chapter 2, we evaluate this relationship by 1) determining whether and how byproducts of GABAT regulate an individual's metabolic homeostasis *in vivo* and 2) determining whether GABAT regulates sleep homeostasis through the same or independent mechanisms. Our work suggests that GABAT does in fact regulate both energy and sleep homeostasis because mutants of GABAT cannot survive on sucrose media and exhibit a long-sleeping phenotype (Chen, et al. 2014). Interestingly, our data support that while GABAT does regulate sleep and energy homeostasis, it does so through different mechanisms, whereby glutamate mediates the link between GABAT and the TCA cycle while the effects of GABAT on sleep do not depend on glutamate.

While the work accomplished in this chapter reports an intriguing metabolic phenotype that likely involves a deficiency in the TCA cycle, future biochemical studies should verify whether the phenotype is in fact caused by a deficiency of carbon/energy metabolism. We suggest in chapter 2 that GABAT regulates energy homeostasis by producing glutamate and that GABAT-derived glutamate is used for energy metabolism via the TCA metabolite  $\alpha$ -KG. However, the carbon skeleton of GABA is not catabolized to glutamate or  $\alpha$ -KG via GABAT. Instead, GABAT transfers the nitrogen of GABA to  $\alpha$ -KG to make glutamate, which can in turn be converted back to  $\alpha$ -KG via GDH or another type of aminotransferase (AMT)<sup>6</sup>. Therefore inherently, this pathway cannot yield any net  $\alpha$ -KG production from GABA (figure 1, chapter 2). In chapter 2, we favor the hypothesis that the disrupted glutamate metabolism we observed in the *gabat*<sup>f</sup> mutant strain causes a reduction  $\alpha$ -KG because under conditions of low glutamate levels, reactions forming

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<sup>6</sup> The reaction catalyzed by GDH or an AMT is also reversible so that  $\alpha$ -KG can be used to synthesize glutamate.

glutamate from  $\alpha$ -KG (via GDH or an AMT) are relatively more favored than reactions producing  $\alpha$ -KG, namely because glutamate is needed for several other fundamental processes, such as direct use for glutamine formation, participation in the purine nucleotide cycle, incorporation into proteins or tripeptide glutathione, cellular signaling, and so forth. Because some of these processes are irreversible, we believe that a limit of glutamate will cause a reduction of  $\alpha$ -KG levels, low energy production, and thus decreased survival on sucrose food. The carbon/energy metabolism hypothesis yields several predictions that one can test biochemically such as: 1) On sucrose food, *gabat*<sup>f</sup> mutants should have a lower ATP/ADP and a lower activity/respiration rate. 2) When inhibiting GABAT acutely with pharmacological inhibitors *in vitro*, there should be a fast energy state change of the TCA cycle (indicating a direct effect of GABAT rather than a secondary effect of another physiological change) 3) Feeding flies with isotopic labeled glutamate should cause considerable labeling of TCA intermediates.

In chapter 3, we investigate the role of temperature on the timing mechanism of the molecular genetic clock. While the molecular circadian clock is an endogenous oscillator – meaning that the internal timing mechanism persists in constant conditions – it can be modulated by external environmental cues, such as light, chemicals and temperature. Of these external factors that affect the timing of the circadian clock, temperature is especially interesting because it can modulate the timing of the circadian clock and thus the overt manifestation of rhythm in three distinct ways (reviewed in Chapter 1), one of which is that extreme high or low temperature conditions abolish the overt manifestation of rhythmic cycles such as sleep. Until recently, nothing was understood about why circadian organisms lose their ability to maintain rhythm under low temperature stress and so in chapter 3, we investigate the molecular mechanism contributing to the overt loss of circadian behavior in *Drosophila*. The challenge inherent in addressing this question is the difficulty in identifying genetic lines capable of maintaining rhythm under low temperature stress that can serve as a basis of comparison. We overcame this challenge by sampling from natural populations of *Drosophila melanogaster* in the wild that experience distinct climatic regimes. In phenotyping these lines under cold-temperature stress, we observed

tremendous variability in the overt manifestation of circadian rhythm, where some lines are able to buffer the effects of temperature whereas others are not. When addressing whether this variability is ecologically relevant, we found evidence to suggest that the maintenance of rhythm is under selection based on the climatic regime of the environment. To address the molecular and genetic mechanism for why flies lose their ability to maintain rhythm under low temperature stress, we analyze the behavioral adaptation of one cold-resistant circadian line. Genetic analysis of this line revealed that the ability to buffer the effects of cold temperature to maintain rhythm is due to the actions of an unidentified gene involved in the circadian network. This is because analysis of the central clock cells in this line reveal that the maintenance of rhythm is associated with robust clock function, which is compromised in a standard laboratory strain. Overall, the work accomplished in Chapter 3 highlights the importance of using natural populations of *Drosophila melanogaster* to identify novel molecular genetic mechanisms of circadian clock function, especially in populations that are under temperature-based selection of the environment. Future directions of this project would be to map and characterize the genes of the cold-resistant circadian line that stabilize the molecular clock under low temperature stress.

Why is it important to understand how internal and external signals affect the sleep process? In humans, disturbances in sleep amount/quality and the timing of sleep either endogenously or exogenously are associated with a wide variety of clinical pathologies including metabolic disorders (Chao, et al. 2011; Kobayaski, et al. 2012), psychiatric disorders (Arey, et al. 2014), substance abuse (Logan, et al. 2014), cognitive impairment (Belenky, et al. 2003), and so forth. Considering the prevalence of these disorders in the United States and the substantial costs to the health care system in treating these disorders, it is a clinical and economic imperative to understand 1) the relationship between sleep disturbance, circadian dysfunction, and disease phenotypes and thus 2) how internal and external cues mediate this relationship. For example, the prevalence of metabolic disorders (such as obesity and type-two diabetes) have nearly tripled in the US population from 13% in 1960-2 to 36% in 2009-10 (May, et al. 2013)! Interestingly, this rise cannot be fully explained by changes in traditional lifestyle factors such as diet and physical

activity. In fact, epidemiological studies have consistently reported that a disruption in sleep amount or quality is highly associated with metabolic risk (for a review, see Knutson and Cauter (Knutson and Cauter 2008)). For instance, results from the Nurses' Health Study found an increased risk of symptomatic incident diabetes over 10 years among those reporting sleep durations of 5hrs or less, even after controlling for many covariates such as BMI, shiftwork, hypertension, exercise, and depression (Ayas, et al. 2003). This observation has been supported experimentally in human studies, which show that when sleep is deprived behaviorally, glucose homeostasis (Spiegel, et al. 2005) and neuroendocrine control of appetite (Spiegel, et al. 2004) are impaired independently of the stress associated with deprivation. In addition, disruption in the timing of sleep caused by a mutation within the molecular clock or through exogenous influences is also associated with metabolic defects: there is considerable epidemiological evidence showing a positive relationship between shift work (Morikawa, et al. 2007) and increased risk for obesity, diabetes, and cardiovascular disease and more recently, experimental work has shown that human subjects with forced circadian misalignment show impaired leptin and glucose homeostasis (Scheer, et al. 2009). Considering the association between sleep loss, circadian misalignment, and metabolic risk, it is alarming that current representative surveys indicate that 35-40% of the US population reports sleeping less than 7 hours on weekday nights (Center for Disease Control 2011), an amount commonly associated with increased metabolic risk (Chao, et al. 2011)!

While the work accomplished in this thesis is not clinical in nature, we hope that it will provide short-term advances in our understanding of the basic biology of clinically relevant pathologies so that it can eventually be used to improve the lives of people suffering from disease. Advances in our understanding of basic, biological mechanisms of sleep will be crucial in developing effective treatments that target the source of the disorder. *Drosophila melanogaster* is an ideal model organism to study the translational applications of sleep research not only because it has a relatively simple genome (consisting of only 3 essential chromosomes) that can be readily manipulated to genetically dissect behavior, but also because it shares conserved

elements important for the homeostatic and circadian process of sleep in mammals. For example, flies and humans show overlap in the neurochemical molecules that promote or inhibit sleep: virtually all neurotransmitters that regulate sleep in humans – e.g. dopamine, serotonin, octopamine (the *Drosophila* equivalent of norepinephrine), and GABA – also do so in flies (Chung, et al. 2009; Crocker and Sehgal 2008; Kume, et al. 2005; Saper, et al. 2010; Yuan, et al. 2006). In addition, the molecular circadian clock in flies and humans also share common elements: their oscillators use loops that close within cells, and these loops consist of positive and negative elements in which transcription of clock genes yields proteins (negative elements) which block the action of positive element(s) and the positive elements, in turn, are regulated through feedback mechanisms (Dunlap 1999). Importantly, virtually all the actual molecules that function in these loops are conserved from flies to humans.

We hope that researchers take away from this thesis the importance of using *Drosophila* to study the basic biology of sleep. Understanding the 'ins and outs' of this sleep process will take us one step closer in advancing medical interventions for treating disorders associated with sleep and circadian dysfunction.

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