A screen for sleep and starvation resistance identifies a wake-promoting role for the auxiliary channel unc79

Kazuma Murakami1, Justin Palermo1, Bethany A. Stanhope1, Allen G. Gibbs2 and Alex C. Keene1#

1. Department of Biological Sciences, Florida Atlantic University, Jupiter, FL 33458, United States.
2. Department of Biological Sciences, University of Nevada, Las Vegas, Las Vegas, NV 89154, United States.

# Address correspondence to KeeneA@FAU.edu

Abstract

The regulation of sleep and metabolism are highly interconnected, and dysregulation of sleep is linked to metabolic diseases that include obesity, diabetes, and heart disease. Further, both acute and long-term changes in diet potently impact sleep duration and quality. To identify novel factors that modulate interactions between sleep and metabolic state, we performed a genetic screen for their roles in regulating sleep duration, starvation resistance, and starvation-dependent modulation of sleep. This screen identified a number of genes with potential roles in regulating sleep, metabolism or both processes. One such gene encodes the auxiliary ion channel UNC79, which was implicated in both the regulation of sleep and starvation resistance. Genetic knockdown or mutation of unc79 results in flies with increased sleep duration, as well as increased starvation resistance. Previous findings have shown that unc79 is required in pacemaker for 24-hour circadian rhythms. Here, we find that unc79 functions in the mushroom body, but not pacemaker neurons, to regulate sleep duration and starvation.
resistance. Together, these findings reveal spatially localized separable functions of
unc79 in the regulation of circadian behavior, sleep, and metabolic function.

Keywords: Sleep; metabolic rate; Drosophila; feeding; energy stores; mushroom
body

Running title: Regulation of sleep and metabolism
Introduction

Sleep acutely regulates metabolic function, and growing evidence suggests that these processes interact to regulate many biological functions including cognition, physiology, and longevity (Hartmann 1974; Siegel 2005; Joiner 2016; Beckwith and French 2019). At the clinical level, many diseases related to metabolic dysfunction including diabetes, heart disease and obesity are associated with chronic sleep loss (Taheri et al. 2004; Arble et al. 2015; Reutrakul and Van Cauter 2018). In addition, diet potently influences sleep duration and quality, indicating that neural systems regulating sleep are sensitive to internal nutrient stores and food availability (Catterson et al. 2010; Grandner et al. 2010, 2014; Linford et al. 2012). Identifying how sleep, diet, and metabolic regulation are interconnected is critical to understanding the fundamental functions of sleep.

Interactions between sleep, feeding, and metabolic regulation are highly conserved between the fruit fly and mammals (Griffith 2013; Yurgel et al. 2015; Beckwith and French 2019; Shafer and Keene 2021). Experimental evolution and artificial selection approaches have revealed a relationship between sleep, feeding, and starvation resistance (Masek et al. 2014; Slocumb et al. 2015; Brown et al. 2019). For example, selection for short-sleeping flies results in reduced energy stores and sensitivity to starvation, while selecting for starvation resistance increases sleep duration (Seugnet et al. 2009; Masek et al. 2014; Slocumb et al. 2015). Further, examining naturally occurring genetic variation in sleep and starvation resistance in Drosophila melanogaster from different geographic localities suggests sleep and starvation resistance are inversely related (Brown et al. 2018; Sarikaya et al. 2020). In
addition, shared neural circuits appear to regulate both feeding and starvation resistance, including a role for Insulin-like peptides and the Leucokinin Receptor, and naturally occurring variation in levels of the foraging gene impact feeding and starvation resistance (Thimgan et al. 2012; Allen et al. 2017; Zandawala et al. 2018). The interactions between these traits under conditions of experimental evolution raise the possibility that shared genetic factors underlie sleep and starvation resistance.

Energy conservation has long been proposed to be primary function of sleep (Hartmann 1973; Berger and Phillips 1995). Drosophila live for only a few days in the absence of food, providing an excellent model to examine the effects of sleep on metabolic regulation and energy conservation (Yurgel et al. 2015; Ly et al. 2018). Quantifying longevity under starvation conditions provides a readout of overall energy stores and metabolic rate (Baldal et al. 2006; Schwasinger-Schmidt et al. 2012). In addition, flies acutely suppress sleep and increase activity in response to starvation, providing a system to investigate acute modulation of sleep and metabolic function (Lee and Park 2004; Keene et al. 2010). Genetic screens and genomic analyses have identified many regulators of sleep, metabolic regulation, and starvation resistance, establishing flies as a model for studying the interactions between these processes (Harbison et al. 2005; Jumbo-Lucioni et al. 2010; Murakami et al. 2016; Sonn et al. 2018). Many of the genes initially identified through screening for short-sleeping mutants have reduced life spans or increased sensitivity to stressors, though the relationship with starvation resistance is less clear (Koh et al. 2008; Bushey et al. 2010; Hill et al. 2018). A complete understanding of how these processes are integrated
requires the localization of genes and neurons that regulate sleep and metabolic processes.

The study of sleep in flies has predominantly focused on the role of genes and neurons under fed conditions, leading to the identification of many distinct circuits that promote sleep and wakefulness (Allada and Siegel 2008; Sehgal and Mignot 2011; Ly et al. 2018). There is growing evidence that additional cell types are critical regulators of sleep including multiple classes of glia, endocrine cells, and the fat body (Artiushin et al. 2018; Stahl et al. 2018; Vanderheyden et al. 2018; Yurgel et al. 2018; Ertekin et al. 2020). Further, the genes and neurons regulating sleep can differ based on environmental context (Griffith 2013; Beckwith and French 2019; Shafer and Keene 2021). These studies highlight brain-periphery interactions that are change in response numerous environmental contexts including food availability. Therefore, identifying genetic regulators that impact both sleep and metabolic function requires investigating both neuronal and non-neuronal cell-types.

Here, we have performed a genetic screen to identify genetic regulators of sleep and metabolic function, targeting genes ubiquitously to identify factors that function both within the brain and the periphery. Flies were tested in a pipeline that measured sleep parameters under fed and starved conditions, followed by assessment of starvation resistance. This screen identified several candidate genes regulating sleep and metabolic function including the sodium leak channel NALCN accessory subunit uncoordinated 79 (unc79). The gene was first identified in the nematode C. elegans in a screen that identified numerous regulators behavior and morphology (Brenner 1974). Both unc79 and its partner unc80, are auxiliary subunits of r the drosophila Narrow
Abdomen (na), that is a critical regulator of the circadian clock in Drosophila (Lear et al. 2005, 2013; Flourakis et al. 2015). Flies mutant for na mutants have altered response to anesthetics in this behavioral assays, deficits in circadian locomotor rhythms, and altered social clustering (Humphrey et al. 2007; Burg et al. 2013). Here, we find additional roles for unc79 in regulating sleep and metabolic regulation. Further, we provide evidence that unc79 regulates these processes through independent neural circuit and molecular mechanism from those that govern circadian rhythms.

Methods

Fly husbandry: Flies for behavioral experiments were maintained and tested in humidified incubators at 25°C and under 65% humidity (Powers Scientific). Flies were reared on a 12 h:12 h light–dark cycle for experiments prior to behavioral analysis. All flies were maintained on Nutri-fly Drosophila food (Genesee Scientific). All RNAi lines tested were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA) (Ni et al. 2008; Perkins et al. 2015) and the Vienna Drosophila Resource Center stock (# 45780) was used to validate RNAi phenotypes from the initial screen (Vienna, Austria) (Dietzl et al. 2007) (See table 1). Bridget Lear (Northwestern) generously provided unc79 F01615 and unc79 F01615 lines (Lear et al. 2013).

Behavioral Analysis: Drosophila Activity Monitors (DAM; Trikinetics, Waltham, MA) were used for all behavioral analyses. The DAM system detects activity by monitoring infrared beam crossings for each animal (Pfeiffenberger et al. 2010a). These data were used to calculate sleep information by extracting immobility bouts of 5 minutes using the Drosophila Sleep Counting Macro (Pfeiffenberger et al. 2010b). All behavioral
experiments used 5-7 day old mated female flies unless otherwise noted. We have previously found that females display more robust starvation-induced sleep suppression, and have used mated females in a prior genetic screen (Keene et al. 2010; Murakami et al. 2016). For experiments examining the effects of starvation on sleep, activity was then measured for 24 hours on food, prior to transferring flies into tubes containing 1% agar (Fisher Scientific) at ZT0 and activity was recorded for an additional 48 hours.

To measure starvation resistance, flies were starved on experimental day 2 by transferring them from food 1% agar (Fisher Scientific) individual DAM tubes containing 1% agar. Activity across the first 48 hours on agar was used to measure starvation-induced sleep suppression as previously described (Masek et al. 2014). While previous studies measured starvation-induced sleep suppression on day 1 of starvation, we measured this process for multiple days (Keene et al. 2010). To measure starvation resistance, activity was then recorded until death. Death was manually determined as the last activity time point from the final recorded activity bout for each individual fly.

For experiments quantifying circadian rhythm analysis, locomotor activity under free-running conditions was measured using the DAM system as previously described (Chiu et al. 2010). Individual flies were housed in 10% sucrose DAM tubes instead of standard fly food to prevent larval development that interferes that the circadian assay. Five day old adult flies were entrained to light-dark (LD) 12 hour: 12 hour (12:12) cycles for three days, then transferred to constant darkness (DD) for 7-8 days. Locomotor activity data were analyzed using Clocklab software (ActiMetrics, Version 2.72). Individual periods
were calculated from 7-8 days activity data during DD using chi-square periodogram. Rhythm strength was determined by Fast Fourier Transform (FFT) analysis as previously described (Chiu et al. 2010).

**Statistical Analysis**

Statistical analyses were performed using InStat software (GraphPad Software 6.0). For analysis of sleep, we employed a one- or two-way ANOVA followed by a Tukey’s post hoc test. For starvation resistance, we applied Kaplan–Meier analysis by grouping each genotype.
Results

We developed a pipeline that measured sleep, starvation-induced sleep suppression, and starvation resistance in individual flies. Sleep was measured for 24 hours on standard food, after which, flies were transferred to agar where they were maintained until death to measure starvation-induced sleep suppression and starvation resistance (Fig. 1A). We first validated the pipeline in w^{1118} flies, and found that flies robustly reduce sleep the first 24hrs of starvation and live an average of 48hrs without food (Fig. 1C).

To screen for novel regulators of sleep and starvation resistance we ubiquitously knocked down genes by expressing RNAi transgenes from the TRiP Collection under control of the Actin5C-GAL4 driver (Perkins et al. 2015). Control flies harboring Actin5C-GAL4 driving UAS-luciferase-RNAi (Act5c>Luc RNAi), a control with no endogenous targets, flies suppressed sleep during the first day of starvation, and an even greater suppression was observed on day two of starvation. To enrich for genes that may be involved in sleep or metabolic function, candidate genes were selected from a genome-wide analysis of polymorphisms and genomic markers of selection in flies selectively bred for starvation resistance (Hardy et al. 2018). Of the 1429 significant genes from this analysis, we identified 914 genes that TRiP RNAi stocks available (Perkins et al. 2015; Hardy et al. 2018). Of these, 299 lines (32.7%) were lethal with ubiquitous knockdown and, therefore, were not screened. In total, we screened 616 lines for sleep, starvation-induced sleep suppression (Fig. 1D and Table S1). Ubiquitous knockdown of the previously identified Ly-6 transmembrane protein, qvr/sleepless, resulted in the shortest sleep duration, confirming the ability of the screening procedure to effectively
identify genetic regulators of sleep (Koh et al. 2008). To examine the relationship
between sleep and starvation resistance we plotted the average for each trait. There
was no association between these traits ($r^2<0.001$, $p>0.772$), suggesting sleep and
starvation resistance independently regulated (Fig. 1D). However, we identified a
number of genes where ubiquitous knockdown resulted in increased sleep on food and
greater starvation resistance (Fig. 1D). We also examined the correlation between
genes screened for different sleep parameters. For example, daytime sleep duration is
correlated with nighttime sleep duration, suggesting shared genes regulate both
processes (Fig S1A). We found average bout length was inversely correlated with sleep
bout number, suggesting these traits are functionally related (Fig. S1B). However, no
correlation was observed between waking activity and total sleep (Fig. S1C) suggesting
independent regulation of these traits. We chose to focus on the gene encoding for the
NALCN auxiliary protein, *unc79* because of the robustness of each phenotype and its
role as an essential regulator of circadian rhythms sleep regulation (Lear et al. 2005;
Joiner et al. 2013).

To validate the sleep and starvation phenotypes associated with *unc79* we repeated
experiments and examined the sleep profile. Flies with ubiquitous knockdown of *unc79*
(*Act5c>*unc79*RNAi) slept significantly more than control flies (Fig. 2A,B). Further, while
both control groups suppressed sleep during day 1 and 2 of starvation, *Act5c>*unc79*RNAi
flies did not suppress sleep, suggesting that *unc79* is required for metabolic regulation
of sleep (Fig. 2A,B). In addition, starvation resistance was significantly increased in
*Act5c>*unc79*RNAi flies compared to *Act5c>*Luc*RNAi controls (Fig 2C). In agreement with
our previous findings, waking activity in female control flies increased starvation, but
was unchanged in Act5C>Unc79RNAi flies (Fig S2A) (Keene et al. 2010). Further, the overall waking activity was elevated in Act5C>Unc79RNAi flies compared to Act5c>LucRNAi controls under fed conditions suggesting that the increased sleep in flies deficient for unc79 is not due to general lethargy.

To confirm that the observed phenotypes are not due to RNAi off targets we tested flies with a genetic mutation in the unc79 locus. The independent Pbac element insertions in the unc79 locus slept longer on food and failed to suppress sleep when starved, phenocopying RNAi knockdown (Fig. 2D,E) (Lear et al. 2013). Further, unc79 mutants (unc79F03453 and unc79F01615) survived significantly longer on agar than respective controls (Fig. 2F). Analysis of flies heterozygous for the mutation revealed the long sleeping phenotype is semi-dominant (Fig. 2D-F).

We also assessed male flies to determine whether these phenotypes generalize across sexes. Male unc79 mutants (unc79F03453 and unc79F01615) flies slept longer on food and failed to suppress sleep when starved (Fig S2A) and survived longer on agar than respective controls (Fig S2B), but the response was attenuated compared to female flies. Waking activity in male flies did not differ between fed and starved groups of unc79 mutants, while controls increase waking activity (Fig S2C). Therefore, ubiquitous RNAi knockdown or genetic mutation of unc79 results in increased sleep and starvation resistance, and impaired metabolic regulation of sleep.

To localize unc79 function in metabolism and sleep we first targeted unc79RNAi to all neurons using the driver n-synaptobrevin-GAL4 (nsyb-GAL4) (Riabinina et al. 2015).
Knockdown in neurons led to flies that slept significantly more than background controls harboring expressing RNAi to luciferase under fed conditions (Fig. 3A). Further, flies with pan-neuronal knockdown of unc79 (nSyb-GAL4>unc79RNAi) also did not significantly reduce sleep during starvation and survived significant longer, suggesting unc79 functions in neurons to regulate sleep and starvation resistance (Fig. 3 A,B). To further localize the function of unc79 we targeted RNAi to six types of neurons known to modulate sleep. Knockdown in the circadian neurons using Pdf-GAL4 or Tim-GAL4 did not affect sleep or starvation resistance, suggesting the effects on sleep and metabolic function are independent of its role in circadian activity (Fig. S3A). Further no effect was observed knocking down unc79 in the sleep promoting central complex (23E10-GAL4) or broad classes of peptidergic cells (C929-GAL4) (Fig. S3A-D). Knockdown of unc79 selectively in the mushroom bodies (OK107-GAL4) increased total sleep, specifically during the day, and resulted in increased starvation resistance (Fig. 3C-F). Therefore, selective knockdown with OK107-GAL4 largely phenocopies ubiquitous knockdown, raising the possibility that unc79 functions in the mushroom body to regulate sleep and starvation resistance.

The driver OK107-GAL4 expresses in some neurons that are extrinsic to the mushroom bodies including the Pars Intercerebralis (Aso et al. 2009). To determine whether the phenotypes observed localize to the mushroom body we expressed unc79RNAi using R13F02-GAL4, a highly selective driver for the mushroom body (Jenett et al. 2012). These flies also slept longer than controls, failed to suppress sleep and survived longer under starvation conditions (Fig. 3G-H). Similar to findings with OK107-GAL4, knockdown of unc79 in the mushroom bodies body with R13F02 increased daytime, but
not nighttime sleep (Fig. S3E-F). Therefore, these findings confirm that \textit{unc79} functions in the mushroom bodies to regulate sleep and starvation resistance.

To determine whether the phenotypes observed are specific lobes of the mushroom body, we tested the effects of \textit{unc79} knockdown in the \(\alpha/\beta\) lobes (\textit{c739-GAL4}), \(\alpha'/\beta'\) lobes (\textit{c305a-GAL4}), and the \(\gamma\) lobes (1471 GAL4 drivers) (Krashes \textit{et al.} 2007; Aso \textit{et al.} 2009). Flies with \textit{unc79} knockdown in \(\alpha/\beta\) lobes and \(\alpha'/\beta'\) lobes fail to suppress sleep compared to \textit{luc} control, while knockdown in the \(\gamma\) lobes increased total sleep duration compared to \textit{luc} control and fail to suppress sleep when starved (Fig. 4A). Starvation resistance is increased in \textit{unc79} knockdown in each mushroom body subtype compared to their respective controls expressing luciferase-RNAi (Fig. 4B). Therefore, loss of \textit{unc79} function in each subset of mushroom body neurons impacts sleep and metabolic regulation, while selective loss in the \(\gamma\) lobes largely recapitulates the full extent of ubiquitous knockdown. These findings reveal that \textit{unc79} is required in all lobes of the mushroom body for proper sleep and metabolic regulation.

To verify that the increase in sleep and starvation resistance is specific to the mushroom bodies, we examined whether including of the \textit{MB-GAL80} transgene reverses the effects of \textit{unc79} knockdown in 1471-GAL4 positive neurons. Expression of GAL80 in the mushroom bodies restores sleep and starvation-induced sleep suppression to control levels to flies with \textit{unc79} knocked down in the \(\gamma\) lobes (Fig. 4C). Starvation resistance increased in flies with \textit{unc79} selectively knocked down in the (1471-GAL4>\textit{unc79}\textit{RNAi}). Blocking expression of GAL4 within the mushroom body restored starvation resistance, and partially restored sleep duration on food, confirming
that loss of *unc79* in the mushroom body leads to dysregulated sleep (Fig. 4D). In addition, the expression of MB-GAL80 restored normal starvation resistance to 1471-GAL4>*unc79* RNAi flies. These findings validate that the results obtained with *unc79* knockdown using 1471-GAL4 are specific to loss of *unc79* function within the γ-lobe of mushroom bodies.

Previous work has revealed that *unc79* functions within the circadian neurons in association with the *unc80* accessory protein and the ion channel narrow abdomen (*na*) to maintain locomotor rhythms during constant darkness (Lear *et al*. 2005, 2013; Moose *et al*. 2017). To further investigate whether the sleep and circadian phenotypes are controlled by shared or distinct neural circuits, we knocked down additional components of the *unc79* complex in the mushroom bodies and measured the effects on sleep and starvation resistance. We used RNAi lines from the Vienna *Drosophila* Stock Center, for all lines tested. Knockdown of *unc79* in the mushroom bodies phenocopied the effects on sleep and starvation resistance that were observed with the RNAi line used for screening and genetic mutants, confirming these phenotypes. Knockdown of *na* or *unc80* throughout the mushroom bodies did not increase sleep duration on food or disrupt starvation-induced sleep suppression (Fig 5A). In addition, knockdown of *unc80* and *na* in the mushroom body had little impact on starvation resistance (Fig 5B). These findings raise the possibility that *unc79* functions independently of its canonical complex with *unc80* and *na* to regulate sleep and starvation resistances.

Expression of *unc79* is required within pacemaker neurons to circadian rhythms. Therefore, it is possible that *unc79* functions in distinct populations of neurons to regulate circadian rhythms, from those regulating sleep and metabolic phenotypes. To
directly test this possibility, we measured the effects of mushroom body-specific knockdown of unc79 on free-running activity in entrained animals. As expected, flies with pan-neuronal knockdown are arrhythmic under conditions of constant darkness, while control flies show robust rhythms (Fig 5C-F). Conversely, knockdown in OK107-expressing cells does not impact circadian activity (Fig. S4; Fig 5G-H). Therefore, unc79 function to regulate circadian rhythms through distinct neural mechanisms that regulate sleep and starvation resistance.

Discussion

Here, we screened by targeting gene function ubiquitously to identify regulators of sleep and metabolic function. Growing evidence suggests sleep is regulated by complex interactions between the brain and periphery, including the findings that mutants impacting fat storage, and communication from the fat body to the brain significantly impact sleep (Thimgan et al. 2010; Slocumb et al. 2015; Ertekin et al. 2020).

We have identified numerous candidate regulators of sleep, including a novel role for unc79 in the regulation of sleep and metabolic function. Unc79 and unc80 are auxiliary subunits of the sodium leak channel na, an ortholog of mammalian NALCN family of ion channels (Swayne et al. 2009). A number of functions have been identified for this complex including a role in the regulation of circadian rhythms, and anesthesia sensitivity (Lear et al. 2005; Humphrey et al. 2007). Previous work found that loss of narrow abdomen or unc79 increased sensitivity to the anesthetics, halothane and isofluorane, and increases sleep (Humphrey et al. 2007; Joiner et al. 2013) consistent
with our findings of increased quiescence in \textit{unc79} mutants. Mutation of \textit{unc79} also facilitates the emergence from anesthesia, raising the possibility that loss of \textit{unc79} promotes state transitions, rather than directly impacting isofluorane sensitivity (Joiner \textit{et al.} 2013). Therefore, suppression of arousal may be involved in anesthesia and sleep (Joiner \textit{et al.} 2013). Mutation of \textit{na} also impacts a number of complex behaviors including social clustering (the distance maintained between individual flies) (Burg \textit{et al.} 2013), and light-mediated locomotor activity (Nash \textit{et al.} 2002). These findings suggest a complex role for \textit{na} and associated \textit{unc79} genes in regulating brain function.

Multiple lines of evidence suggest the role of \textit{unc79} in the regulation of sleep, metabolic regulation of sleep, and starvation resistance is separate from its essential role in regulating circadian rhythms. First, we localize function to the mushroom body, a region that is critical for regulation of sleep and modulation of behavior in accordance with feeding state (Joiner \textit{et al.} 2006; Pitman \textit{et al.} 2006; Sitaraman \textit{et al.} 2015a; Tsao \textit{et al.} 2018). Selective knockdown of \textit{unc79} in the gamma-lobes alone phenocopies \textit{unc79} mutants. While there is little evidence to date for direct involvement of gamma-lobe neurons in sleep regulation, mushroom body output neurons from the gamma-lobes have been found to regulate sleep (Aso \textit{et al.} 2014a; Sitaraman \textit{et al.} 2015b). We previously reported that the mushroom bodies are dispensable for starvation-induced sleep suppression, however the manipulations that led to this conclusion involved pharmacological ablation or acute genetic silencing of the mushroom bodies (Keene \textit{et al.} 2010). Therefore, it is possible that loss of \textit{unc79} function impacts sleep circuitry through a mechanism that would not be detected in flies with the previously applied genetic manipulations. Second, \textit{na} and \textit{unc80}, two components of a complex that
interacts with *unc79* to regulate circadian rhythms, are dispensable for regulation of sleep and starvation resistance in the mushroom bodies. These findings raise the possibility that *unc79* may function independently of its canonical complex with *unc80* and *na*. However, it is important to note that we are unable to validate the efficacy of the RNAi lines tested, and therefore cannot rule out a possible role for *unc79* functioning through *na*. Further work is needed to address how *unc79* functions may modulate mushroom body physiology and sleep circuitry. Studies examining the role of *unc79* in circadian function and anesthesia sensitivity suggests it functions by regulating *na* activity to modulate neural activity (Moose *et al.* 2017), and it is possible that *unc79* modulates the function of a different ion channel within the mushroom bodies. The structure and function of *na/NALCN* appear to be highly conserved with the channel enhancing neural excitability (Chua *et al.* 2020). Therefore, imaging activity of neurons within the mushroom bodies of flies lacking *unc79* may be informative.

We identify three independent phenotypes to the mushroom bodies. First, we find knockdown of *unc79* in the mushroom bodies promotes sleep suggesting a wake-promoting role for mushroom bodies. The mushroom bodies contain both wake and sleep-promoting neurons, and genetic ablation or silencing of the mushroom body increases wakefulness (Joiner *et al.* 2006; Pitman *et al.* 2006; Sitaraman *et al.* 2015a). It is possible that loss of *unc79* is functioning in either sleep promoting or wake-promoting neurons to elicit this phenotype. We also identify two independent metabolic phenotypes to the mushroom bodies. In *Drosophila*, the mushroom circuits have been well-defined including the identification of modulatory neurons, and output neurons that modulate sleep (Aso *et al.* 2014b; Haynes *et al.* 2015; Sitaraman *et al.* 2015b).
Therefore, the identification of \textit{unc79} as a regulator of sleep provides the opportunity to examine how gamma lobe output neurons are regulated by input neurons and impact the physiology of output neurons.

In addition to the sleep phenotypes, we find \textit{unc79} mutants are resistant to starvation. This finding is particularly interesting because the list of genes chosen for the screen derived from those identified in a Genome Wide Analysis Study for factors associated with starvation resistance (Harbison \textit{et al.} 2004; Hardy \textit{et al.} 2018). Many different factors contribute to starvation resistance including energy stores, basal metabolic rate, and changes in metabolic rate upon starvation. Animals selected for starvation resistance have elevated sleep and do not suppress sleep when starved (Masek \textit{et al.} 2014). Therefore, future work studying starvation selected lines, or other populations of outbred fly lines have potential to identify whether variable expression of \textit{unc79} is associated with naturally occurring differences in sleep and metabolic regulation.

We find that \textit{unc79} most potently impacts sleep and starvation resistance in the gamma lobes, suggesting this population is critical for both sleep metabolic regulation. Output neurons from the gamma lobes have been directly implicated in feeding and fat storage supporting the notion that this region is critical for metabolic regulation (Al-Anzi and Zinn 2018). Future work examining the effects of \textit{unc79} deficiency on the physiology and function of mushroom body output neurons may help identify the role of \textit{unc79} in regulating mushroom body circuits that ultimately regulate behavior and metabolic function. Taken together, these findings add to growing evidence that sleep and metabolic function are integrated. The identification of additional genetic factors that regulate the relationship between sleep and nutritional state through behavioral studies...
will improve our understanding of the strong associations between sleep loss and metabolism-related diseases. The ubiquitous screen has identified numerous candidate genes that impact sleep, starvation-induced sleep suppression, and starvation resistance, providing candidates that function within and outside of the nervous system. Future study of these genes, such as unc79, has potential to advance our understanding of sleep-metabolism interactions and brain-periphery communication.

Acknowledgements: This work was supported by National Institute of Health awards R01HL143790 and R01DC01790 to ACK. The authors are grateful to Peter Lewis (FAU) for technical support.

Data Availability: Fully analyzed data and statistics are available as supplemental files. Supplemental Material available at figshare: https://www.doi.org/10.25387/g3.14738895. Raw data will be made available upon request.
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Figures

Figure 1. Screening for sleep and starvation resistance
A. Schematic of TRiP Screen. Ubiquitous Act5c GAL4 driver is crossed to TRiP lines, F1 flies were transferred to Drosophila Activity Monitor tubes. Sleep was measured for 24 hours on standard food, after which, flies were transferred to agar where they remained until death to quantify starvation-induced sleep suppression and starvation resistance. B. Control w^{1118} flies suppressed sleep during the first day of starvation, and an even greater suppression was observed on day two of starvation. C. w^{1118} flies survived approximately three days on agar, providing a robust readout sleep and starvation resistance. Total sleep (mins) is measured over 24 hours period and starvation resistance is measured in hours. D. Scatter plot for fed total sleep on x-axis plotted (mins) to starvation resistance on y-axis (hrs.) (Simple Linear Regression: F (1, 609) equals 0.1265, R^2 value equal 0.0001, P-value > 0.7223, N = 616 fly lines). Control flies with no endogenous targets Act5c GAL4 drive luc^{RNAi} (yellow), lines tested in (grey), qvr has lowest sleep and SR (blue), and unc79 highest sleep and SR (red).

Figure 2. unc79 RNAi and mutants have increased sleep and starvation resistance
A. Sleep profiles depicting the average sleep each hour over a 72 hour experiment for Act5c>luc^{RNAi} (grey) and Act5c>unc79^{RNAi} (red). Flies were on food for day 1, then transferred to agar for days 2 and 3. B. Act5c>unc79^{RNAi} (red) during fed (Two-way ANOVA: F (2, 261) = 8.551, P<0.0001 N>39), starved day 1 (Two-way ANOVA: F (2, 261) = 8.551, P<0.0001, N>39) and starved day 2 (Two-way ANOVA: F (2, 261) = 8.551, P<0.0001 N>39) flies slept significantly longer compared to Act5c>luc^{RNAi} (grey) controls.
C. Starvation resistance of Act5c>unc79RNAi (red) is significantly higher than Act5c>lucRNAi (black) control (Gehan-Breslow-Wilcoxon test: Chi $^2 = 94.42$, df = 1, P-value < 0.0001). D. Sleep profile for hourly sleep averages over a 72 hour experiment for w$^{1118}$, unc79$^{F01615}$ (red) and unc79$^{F03453}$ (maroon) flies are on food for day 1, then transferred to agar for day 2 and 3. E. Total sleep is greater in unc79$^{F03453}$ mutant under fed (maroon, $F_{(2,651)} = 71.46$, P<0.0001, N≥39), starved day 1 (P<0.001; N≥39) and starved day 2 (P<0.001; N≥39) conditions compared to control (grey). Total sleep is greater in unc79$^{F01615}$ mutant (red) under fed ($F_{(2,651)} = 71.46$, P<0.0001, N≥39), starved day 1 (P<0.001; N≥39) and starved day 2 (P<0.001; N≥39) conditions compared to w$^{1118}$ control (grey). F. Starvation resistance is greater in unc79$^{F03453}$ (maroon, Gehan-Breslow-Wilcoxon test: Chi $^2 = 29.1$, df = 1, P-value < 0.0001) and unc79$^{F01615}$ (red, Gehan-Breslow-Wilcoxon test: Chi $^2 = 18.6$, df = 1, P-value < 0.0001) flies compared to w$^{1118}$ control (grey). All sleep data are violin plots and SR data are survival curves. ****p < 0.0001.

Figure 3. Localization of sleep and starvation resistance phenotype to mushroom body

A. Pan-neuronal knockdown of unc79 (nSyb>unc79RNAi, red) is significantly increased in sleep during fed (Two-Way ANOVA: $F_{(2,300)} = 57.82$, P<0.0001, N>30), starved day 1 (P<0.0001, N>30), and starved day 2 (P<0.0001, N>30); while nSyb>attp2 (light grey) and nSyb>luc RNAi controls shows starvation-induced sleep suppression. B. Starvation resistance for pan-neuronal knockdown of unc79 nSyb>unc79RNAi (red) is significantly increased compared to nSyb>attp2 (grey, Gehan-Breslow-Wilcoxon test: Chi $^2$ equal 42, df equals 1, P-value<0.0001, N>30) and nSyb>luc RNAi (light grey, Gehan-Breslow-
Wilcoxon test: Chi $^2$ equals 64.6, df equals 1, P-value <0.0001, N>37) control flies. C. Sleep profile hourly sleep averages over a 72-hour experiment for mushroom body knockdown of unc79. Flies are on food for day 1, then transferred to agar for day 2 and 3. D. Mushroom body knockdown of unc79 (OK107>unc79 RNAi, red) is significantly increased in sleep during fed (Two-Way ANOVA: $F_{(2, 300)} = 57.82$, P<0.0001, N>30) starved day 1 (P<0.0001, N>31), and starved day 2 (P<0.0001, N>31); while Ok107>attp2 (light grey) and Ok107>luc RNAi controls shows starvation-induced sleep suppression. E. Daytime sleep in flies with mushroom body knockdown of unc79 (OK107>unc79 RNAi, red) is significantly increased under fed conditions (Two-Way ANOVA: $F_{(2, 348)} = 43.42$, P<0.0001, N>30), starved day 1 (P<0.0001, N>31), and starved day 2 (P<0.0001, N>31); while OK107>attp2 (grey) and OK107>luc RNAi (light grey) controls maintain normal daytime sleep. F. Starvation resistance is increased in OK107>unc79RNAi flies compared to Ok107>attp2 (grey, Gehan-Breslow-Wilcoxon test: Chi $^2$ equals 47.6, df equals 1, P-value <0.0001, N>31) and OK107>luc RNAi (light grey, Gehan-Breslow-Wilcoxon test: Chi $^2$ equals 50.5, df equals 1, P-value <0.0001, N>31) control flies. All sleep data represent violin plots and SR data are survival curves. ****p < 0.0001.

Figure 4. unc79 function in the mushroom body γ-lobe to regulate sleep and starvation resistance
A. Flies with unc79 knocked down in the αβ lobes (c739>unc79 RNAi, pink) fail to suppress starvation during starved day 1 (Two-way ANOVA $F_{(10, 1075)} = 28.56$, P<0.0001, N>53) and starved day 2 (P<0.0001) compared to control (c739>luc RNAi, light grey); while fed day 1 did not differ (P>0.1587). Mushroom body α′β′ knockdown of
unc79 (c305a>unc79 RNAi, red) fails to suppress starvation during starved day 1 (Two-way ANOVA $F_{(10, 1075)} = 28.56, P<0.0001, N>51$) and starved day 2 (P<0.0001) compared to control (c305a>luc RNAi, light grey); while fed day 1 did not differ (P>0.999). Mushroom body γ knockdown of unc79 (1471>unc79 RNAi, maroon) significantly increase total sleep during fed day (Two-way ANOVA $F_{(10, 1075)} = 28.56, P<0.0001, N>53$) and fails to suppress sleep on starved day 1(P< 0.0001), and starved day 2 (P<0.0001) compared to control (1471>luc RNAi, dark grey). B. Starvation resistance increased when mushroom body αβ knockdown of unc79 (c739>unc79 RNAi, pink) is significant (Gehan-Breslow-Wilcoxon test: Chi $^2$ equals 71.36, df equals 1, P-value<0.0001, N>63) compared to control (c739>luc RNAi, light grey). Starvation resistance increased when mushroom body α'β' knockdown of unc79 (c305a>unc79 RNAi, red) is significant (Gehan-Breslow-Wilcoxon test: Chi $^2$ equals 90.45, Df equals 1, P-value <0.0001, N>51) compared to control (c305a>luc RNAi, grey). Mushroom body γ knockdown of unc79 (1471>unc79 RNAi, maroon) is significant (Gehan-Breslow-Wilcoxon test: Chi $^2$ equals 124.6, df equals 1, P-value<0.0001, N>53) compared to control (1471>luc RNAi, dark grey). C. Mushroom body GAL80 rescue γ knockdown of unc79 RNAi (1471>MB Gal80; unc79 RNAi) significantly rescues total sleep compared to γ knockdown of unc79 (1471>unc79 RNAi, P-value <0.0432); while, total sleep for γ knockdown of unc79 (1471>unc79 RNAi) is high compares to other control groups (MB Gal80;luc RNAi/+, P-value <0.0001; unc79 RNAi/+, P-value <0.0001; 1471/+, P-value <0.0001; MB Gal80;luc RNAi/+, P-value <0.0001; 1471>MB Gal80;luc RNAi, P-value <0.0001; and, MB Gal80;unc79 RNAi/+, P-value <0.0001). Mushroom body Gal80 rescue γ knockdown of unc79 RNAi (1471>MB Gal80; unc79 RNAi) restored total sleep to controls (unc79 RNAi/+,...
P-value equal 0.3715; MB\textsuperscript{Gal80,unc79RNAi/+}, P-value equals 0.7292; and 1471>MBGal80; lucRNAi, P-value equals 0.8177). However, 1471>MB\textsuperscript{Gal80}, unc79 RNAi vs. 1471/+ remained significant (P-value <0.0001). D. Starvation resistance of mushroom body GAL80 rescue γ knockdown of unc79 RNAi (1471>MB\textsuperscript{Gal80}, unc79 RNAi) significantly lower (Gehan-Breslow-Wilcoxon test: Chi \textsuperscript{2} equals 11.13, Df equals 1, P-value <0.0009, N>37) compared to mushroom body γ knockdown of unc79 (1471>unc79 RNAi, maroon). All sleep data are violin plots and SR data are survival curves. ***p < 0.001; ****p < 0.0001.

**Figure 5. MB knockdown of unc79 RNAi has normal circadian rhythm.**

A. Mushroom body knockdown of unc79 (OK107>unc79\textsuperscript{RNAi}) significantly increased sleep (Two-way ANOVA: F \textsuperscript{(2, 711)} = 169, P<0.0029, N>40) compared to control (unc79\textsuperscript{RNAi/+}). Mushroom body knockdown of narrow abdomen (OK107>na\textsuperscript{RNAi}) did not differ (P>0.9999, N>35) compared to control (na\textsuperscript{RNAi/+}). Mushroom body knockdown of unc80 (OK107>unc80\textsuperscript{RNAi}) did not differ (P>0.9964, N>42) compared to control (unc80\textsuperscript{RNAi/+}). B. Starvation resistance mushroom body knockdown of unc79 (OK107>unc79\textsuperscript{RNAi}) is increased (Gehan-Breslow-Wilcoxon test: Chi \textsuperscript{2} equal 25.95, df equal 1, P-value <0.0001, N>40) compared to control (unc79\textsuperscript{RNAi/+}). Starvation resistance mushroom body knockdown of narrow abdomen (OK107>na\textsuperscript{RNAi}) is increased (Gehan-Breslow-Wilcoxon test: Chi \textsuperscript{2} equal 33.54, df equal 1, P-value <0.0001, N>42) compared to control (na\textsuperscript{RNAi/+}). Starvation resistance mushroom body knockdown of narrow abdomen (OK107>unc80\textsuperscript{RNAi}) is no different (Gehan-Breslow-Wilcoxon test: Chi \textsuperscript{2} equal 2.486, df equal 1, P-value <0.1148, N>43) compared to control (unc80\textsuperscript{RNAi/+}). C.-H. Actogram double plot. Female flies were entrained in light-dark cycle for days 1-3 and...
dark-dark cycles 4-10 days. A, B, C, E and F have normal rhythm. Circadian rhythm is disrupted when unc79 is knocked down pan-neuronally, while unc79 knockdown in mushroom body has restored rhythm. All sleep data are violin plots and SR data are survival curves. ***p < 0.001; ****p < 0.0001.
### Table 1.

| Name                  | Power | +/-   | Period (hrs) | +/-   | N  |
|-----------------------|-------|-------|--------------|-------|----|
| w^{1118} (+)          | 145.5 | 18.38 | 24.78        | 0.1326| 32 |
| luc^{RNAi} /+         | 418   | 38.99 | 24.24        | 0.09269| 32 |
| unc79^{RNAi} /+       | 207.7 | 22.14 | 23.84        | 0.04861| 32 |
| nSyb^{GAL4}>luc^{RNAi}| 420.4 | 56.79 | 25.58        | 0.02795| 32 |
| nSyb^{GAL4}>unc79^{RNAi}| 45.4  | 7.049 | 26.32        | 0.9583 | 31 |
| OK107^{GAL4}>luc^{RNAi}| 309.8 | 38.95 | 23.54        | 0.09379| 32 |
| OK107^{GAL4}>unc79^{RNAi}| 129   | 13.16 | 23.58        | 0.1117 | 30 |
Figure 1.

A. 

![Diagram showing the experimental setup with Act5C-GAL4 and UAS-RNAi}

B. 

![Bar graph showing total sleep (mins/24 hrs.) across different days]

C. 

![Survival plot depicting the survival percentage over different starvation resistance hours]

D. 

![Scatter plot illustrating the relationship between total sleep (mins/24 hrs.) and starvation resistance (hrs.)]
Figure 2.

A. 

B. 

C. 

D. 

E. 

F. 

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Figure 3.

A.

B.

C.

D.

E.

F.
Figure 5.

A. Bar graph showing total lifespan (in min) for flies expressing various RNAi constructs. 

B. Survival curve of flies expressing different RNAi constructs over starvation resistance (hrs.).

C. Graph showing survival data for flies expressing nSybGAL4>luc RNAi.

D. Graph showing survival data for flies expressing unc79 RNAi/+. 

E. Graph showing survival data for flies expressing nSybGAL4>luc RNAi.

F. Graph showing survival data for flies expressing unc79 RNAi/+. 

G. Graph showing survival data for flies expressing OK107GAL4>luc RNAi.

H. Graph showing survival data for flies expressing OK107GAL4>unc79 RNAi.
Table 1.

| Name                  | Power | +/- | Period (hrs) | +/- | N  |
|-----------------------|-------|-----|--------------|-----|----|
| w^{1118} (+)          | 145.5 | 18.38 | 24.78       | 0.1326 | 32 |
| luc^{RNAi}/+          | 418   | 38.99 | 24.24       | 0.09269 | 32 |
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