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Characterizing Drosophila melanogaster sleep models using an optogenetic engineered caspase and channelrhodopsins

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Characterizing *Drosophila melanogaster* sleep models using an optogenetic engineered caspase and channelrhodopsins

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Abstract

The conservation of sleep suggests it maintains vital functions for survival; however, roughly one-third of the adult population experiences significant sleep loss (Bonnet & Arand, 1995; Hendricks et al., 2000; Ferrara & Gennaro, 2001; Joiner et al., 2006; Buboltz et al., 2010; Dubowy & Sehgal, 2017). Both oversleeping and deprived sleep has been shown to have negative physiological consequences, such as impaired cognition, motor coordination, metabolic activity, and increased risk for all-cause mortality (Gallicchio & Kalesan, 2009; Xu et al., 2011; Xie et al., 2013). In the present study, we explored the effectiveness of novel optogenetic ablation, a light-activated caspase, and neuronal polarization tools, channelrhodopsins (CHR) and anion channelrhodopsins (ACR), using genetic binary expression systems to control sleep behaviors in *Drosophila melanogaster*. We found that depolarizing rhodopsins functioned as expected when paired with the sleep neuron promoter 72G06, increasing total sleep of the fly model by a max of 26%. Depolarizing rhodopsins demonstrated no significance in sleep behavior when paired with the sleep neuron promoter 23E10. Contrarily, hyperpolarizing rhodopsins had opposite results. For hyperpolarizing rhodopsins, total sleep was expectedly decreased with 23E10 by a max of 24%, but no significance in sleep behavior was detected when paired with 72G06. Furthermore, the novel light-activated caspase was effective with both sleep neuron promoters, reducing total sleep by a max of 31% with 72G06 and 18% with 23E10. In summary, we introduced light-sensitive optogentic tools, bypassing previous shortcomings, and characterized a novel sleep model that reduces sleep with high effectiveness over current optogenetic methodologies. We are now looking to use this novel sleep model to test whether enhancing lysosome function can offset the negative effects of sleep deprivation on longevity.

Introduction

Sleep is a conserved behavior among all animals, but the biological function of sleep still remains a fundamental mystery (Hendricks et al., 2000; Joiner et al., 2006, Dubowy & Sehgal, 2017). Studies in animal models indicate that sleep maintains vital functions for survival; oversleep and sleep deprived animals exhibit impaired cognition, metabolic activity and decreased lifespan (Gallicchio & Kalesan, 2009; Xu et al., 2011; Xie et al., 2013). Given that roughly one-third of the adult population suffer from partial sleep deprivation and college students suffer from frequent sleep disturbances (Bonnet & Arand, 1995; Ferrara & Gennaro, 2001; Buboltz et al., 2010), finding ways to offset negative effects of sleep deprivation is a critical topic of interest in our fast-paced modern culture. On a cellular level, sleep deprivation causes an accumulation of cytoplasmic protein aggregates in the brain, which are also a major hallmarks of ageing (López-Otín et al., 2013) and neurodegenerative diseases (Hara et al., 2006). Enhanced removal of neurotoxic waste products from the brain during sleep via protein clearance pathways is thought to be one of the major mechanisms that provides restorative effects (Ellenbogen, 2005; Hara et al., 2006; Xle et al., 2013). To explore the restorative function of sleep in more depth, we are developing various models to control sleep behaviors in flies.

In the present study, we explored the effectiveness of novel optogenetic ablation and neuronal polarization tools to control sleep behaviors in *Drosophila melanogaster*. In mammals, sleep is characterized by inactivity and reduced sensory responsiveness (Hendricks et al., 2000). The genetically tractable model organism *D. melanogaster* exhibits hallmarks of vertebrate sleep (Hendricks et al., 2000; Shaw et al., 2000; Nitz et al., 2002; Swinderen et al., 2003; Joiner et al.,

2006; Dubowy & Sehgal, 2017), making it the ideal organism for sleep behavioral genetic research. In this study, sleep fly models, both for oversleeping and sleep deprivation, were characterized through the use of optogenetics. Optogenetics allow for versatile modulation of cell activity with high spatiotemporal resolution using light. Over the past decade, the optogenetic tool kit has expanded rapidly (Bergs et al., 2018). To alter sleep behavior in flies, we utilized the GAL4/UAS (upstream activation sequence) binary expression system (Brand & Perrimon, 1993; Rodriguez et al., 2011) and the LexA-*lexaop* binary expression system (Diegelman et al., 2008; Rodriguez et al., 2011). These binary expression systems were coupled with *Drosophila* sleep neuron promoter expression lines, 72G06 and 23E10, to specifically express optogenetic transgenes in *Drosophila* sleep neurons (Ting et al., 2011; Ferguson et al., 2017; Smart et al., 2017). This system allows us to control *Drosophila* sleep behaviors by exposing flies to light.

Using the LexA-*lexaop* binary system, we expressed light-driven rhodopsins, channelrhodopsin (CHR) and anion channelrhodopsin (ACR), to manipulate neuronal activity of targeted sleep neurons. Rhodopsin optogenetic tools have previously been used in model organisms, including mice, flies and *C. elegans* (Nagel et al., 2005; Erbguth et al., 2012; Husson et al., 2012; Mohammad et al. 2017). Activation of rhodopsins by light temporally hyperpolarizes or depolarizes sleep neurons causing optogenetic silencing or excitation of those neurons, respectively. CHRs enable prolonged states of depolarization of excitable cells leading to photo-triggered increased excitation of those cells. This is due to an influx of Cl^- into the excitable cell through temporal activation of light-gated channelrhodopsin (Bergs et al., 2018). Contrarily, ACRs have been found in both *C. elegans* (Bergs et al., 2018) and *Drosophila* (Mohammad et al.

2017) to mediate strong hyperpolarization of excitable cells causing photoinhibition of those cells. Long-term depolarization or hyperpolarization using CHRs or ACRs require ongoing supplementation of all-*trans* retinal (ATR) in invertebrates such as *Drosophila* (Bergs et al., 2018). With ongoing supplementations of ATR, we found that depolarizing rhodopsins worked effectively with the sleep neuron promoter 72G06 while hyperpolarizing rhodopsins worked effectively with sleep neuron promoter 23E10.

Another optogenetic tool that has been developed recently is a light-activated caspase that can induce apoptotic cell death upon exposure to blue wavelengths of lights (Smart et.al, 2017). Using the UAS/GAL4 binary system, we expressed the light-activated caspase specifically in sleep neurons in the *Drosophila* adult mushroom bodies to test whether we could induce flies into a permanent state of sleep deprivation by genetically ablating sleep neurons. We found that that although the flies did not exhibit complete sleep deprivation, their sleep was reduced to levels that negatively impacted animal health.

In sum, we have characterized several new optogenetic tools to study sleep in a genetically tractable organism. In the future, we are interested using these sleep models to identify mechanisms that can offset the negative consequences of sleep deprivation.

Methods

Fly Husbandry:

The methods used for maintenance of *D. melanogaster* were consistent with standard practice for the species as described in A Rough Guide to *Drosophila* Mating Schemes (Roote & Prokop, 2017). In this study, four fly lines (*lexaop*-CHR-HD, *lexaop*-ACR1, *lexaop*-ACR1-C102D, and UAS-Caspase-LOV2) were crossed in combination with two sleep neuron promoter expression lines, 72G06 and 23E10, using the transgenic binary expression systems Gal4-*UAS* or LexA-*lexaop* (Rodriguez et al., 2011). Stock fly lines were incubated at 25°C and continuously flipped to new vials every few days for continual propagation of each line.

Fly Food Preparation:

For line maintenance, base fly food was prepared using the following recipe: water (24,900 mL), agar (324 gm), cornmeal (1,800 gm), yeast (449 gm), tegosept (240 mL), propionic acid (72 mL), phosphoric acid (8.5 mL), and molasses (2,400 mL). For experimental fly lines of CHR-HD, ACR1, and ACR1-C102D, the base fly food was prepared in the same manner except supplemented with 10µM of all-*trans*-retinol (ATR), a cofactor required for the function of the optogenetic transgenes (Wang et al., 2010). For caspase fly lines, only base fly food was used.

Genetic crosses:

Crosses performed in this study were consistent with standard practice as described in A Rough Guide to *Drosophila* Mating Schemes (Roote & Prokop, 2017). For each cross. virgin female flies were selected and combined with male flies to generate cross progeny. For sorting purposes of each cross, flies were anesthetized using a porous CO2 pad and individually examined under a dissection microscope. All lines were first balanced prior to crossing using balancer chromosomes. Balancer chromosomes carry multiple inversions that which suppress genetic recombination with wildtype chromosomes are homozygous lethal and produce visible phenotypes for tracking (Roote & Prokop, 2017). The balancer chromosomes used for this study were: cyo (curly wings), IF (irregular facets eyes), MKRS (stubble bristles) and TM6B (tubby).

Drosophila sleep analysis:

To monitor and quantify fly sleep data, we utilized the *Drosophila* Activity Monitor system from TriKinetics. The specific monitor used for this study was the DAM2 Activity Monitor and the set up for each experiment followed the monitor set up as suggested by TriKinetics. The DAM2 system measures locomotor activity of 32 individual flies, each in separate 5mm diameter transparent tubes that are inserted into array slots on the monitor (TriKinetics, n.d.). Each slot on the monitor contains infrared beams that records activity of flies by recording each time a fly crosses and breaks the beam path. In flies, sleep is characterized by 5 min of inactivity. The 5mm tubes housing flies contained the same base fly food (or ATR+ fly food). In all experiments, the DAM2 monitor itself and the flies within the system were housed for 7 days within an incubator set at 25°C and followed a 12-hr. circadian Light-Dark cycle, allowing flies

to acclimate for 2 days and record data for 5 days. For caspase fly lines, prior to monitoring with the DAM2 system, flies were exposed to 3 days of blue light to activate the Caspase-LOV2 enzyme and induce apoptosis-dependent degeneration of sleep neurons (Smart et al., 2017). Conditions were tested over 32 flies for each experiment, 16 control and 16 experimental. Data automatically collected by the DAM2 system was transferred to a host computer in the form of a matrix on monitor files. Sleep analysis of collected locomotor activity data were statistically analyzed using the web application ShinyR-DAM (Cichewicz & Hirsh, 2018).

Lifespan Assay and Analysis:

Preparation for a lifespan assay of caspase flies began with obtaining synchronous populations of flies through means of crossing schemes as described in A Rough Guide to *Drosophila* Mating Schemes (Roote & Prokop, 2017). Mature male and female flies were allowed to mate for 2 days prior to isolation of male flies in vials; 120 male flies separated into 6 vials for each tested condition. During incubation, tested flies were stored at room temperature in a light box with continuous blue light. Blue light is required to activate the fly's engineered caspase and induce apoptotic cell death of sleep neurons (Smart et al., 2017). Flies were transferred to fresh food every 2-3 days and deaths were scored at the time of transfer. Lifespan data were statistically analyzed using the Online Application for the Survival Analysis of Lifespan Assays Performed in Ageing Research (OASIS) (Yang et al., 2011).

Results

To express optogenetic transgenes specifically in neurons, I used the LexA-*lexaop* binary expression system. Each optogenetic transgene is fused with a *lexaop* activating sequence, which only induces expression of the transgene when coupled with LexA. Two LexA drivers (72G06-LexA or 23E10-LexA) that contain a sleep neuron specific promoter fused to LexA were crossed to each *lexaop* transgenic line to achieve specific expression of the optogenes in sleep neurons. Activation of the optogenes also requires ATR, which was administered to flies directly by feeding. Food without ATR (ATR-) was used as a control.

Sleep Model CHR-HD:

I first tested *lexaop*-CHR-HD with either 72G06-LexA or 23E10-LexA . The control group (ATR-) of 72G06; CHR-HD was found to have an average total sleep of 0.464 ± 0.03. 72G06; CHR-HD flies exposed to ATR+ food had an average total sleep of 0.545 ± 0.04, significantly more sleep than control flies (**Fig. 1b**). Observing the fly sleep bouts, it was found that 72G06; CHR-HD flies also demonstrated a significant increase in bout lengths over the control group by at least 2.11 minutes. The control's sleep bout length was 24.08 ± 2.25 while the experimental's sleep bout length was 32.6 ± 4.16 (**Fig. 1c**).

The control group (ATR-) of 23E10; CHR-HD was found to have an average total sleep of 0.355 ± 0.03. The experimental group (ATR+) of 23E10; CHR-HD had an average total sleep of 0.414 ± 0.03, showing no significant difference in total sleep for 23E10; CHR-HD flies (**Fig. 2b**). The sleep bout length of the experimental group however was found to be significantly longer than

the control group's sleep bout length by at least 2.32 minutes. The control group's sleep bout length was found to be 14.75 ± 1.26 and the experimental group's sleep bout length was found to be 20.54 ± 2.21 (**Fig. 2c**). Collectively, 72G06; CHR-HD increased sleep more effectively than 23E10; CHR-HD.

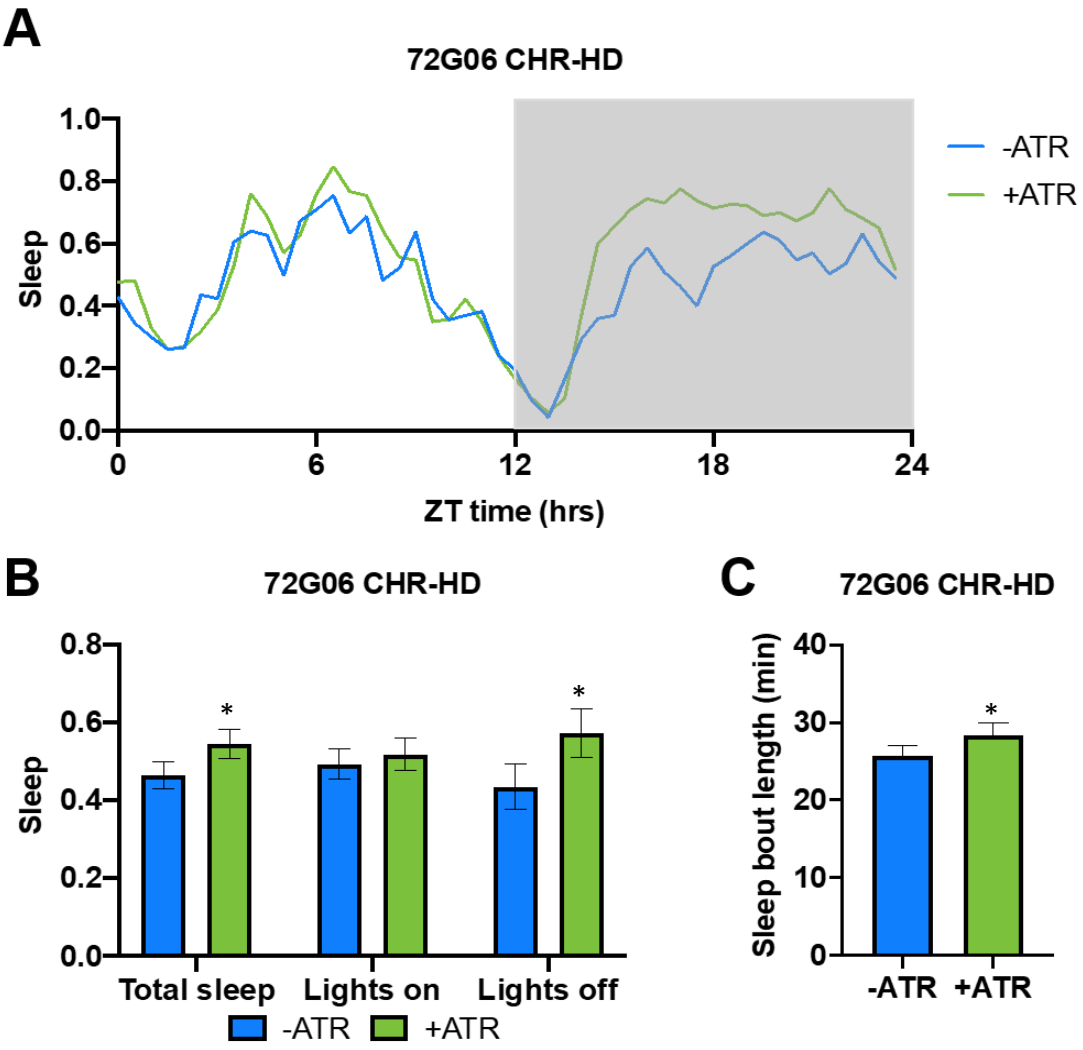

Figure 1. *Testing sleep model 72G06 CHR-HD.* ***A.*** *Average sleep profile generated from a 7-day monitoring period (n=16). Flies were fed food with +ATR for activating the expression of CHR-HD prior to and during the monitoring of sleep activity. The control group were fed food without ATR (-ATR). Flies were incubated in a 12-hr. circadian Light-Dark cycle during the sleep analysis.* ***B.*** *Average total sleep, Day-time sleep (lights on), and Night-time sleep (lights off). Significant difference was found in average total sleep and night-time sleep (*p<0.05) .* ***C.*** *Average sleep bout length. Experimental flies were found to significantly sleep longer than control flie (*p<0.05).*

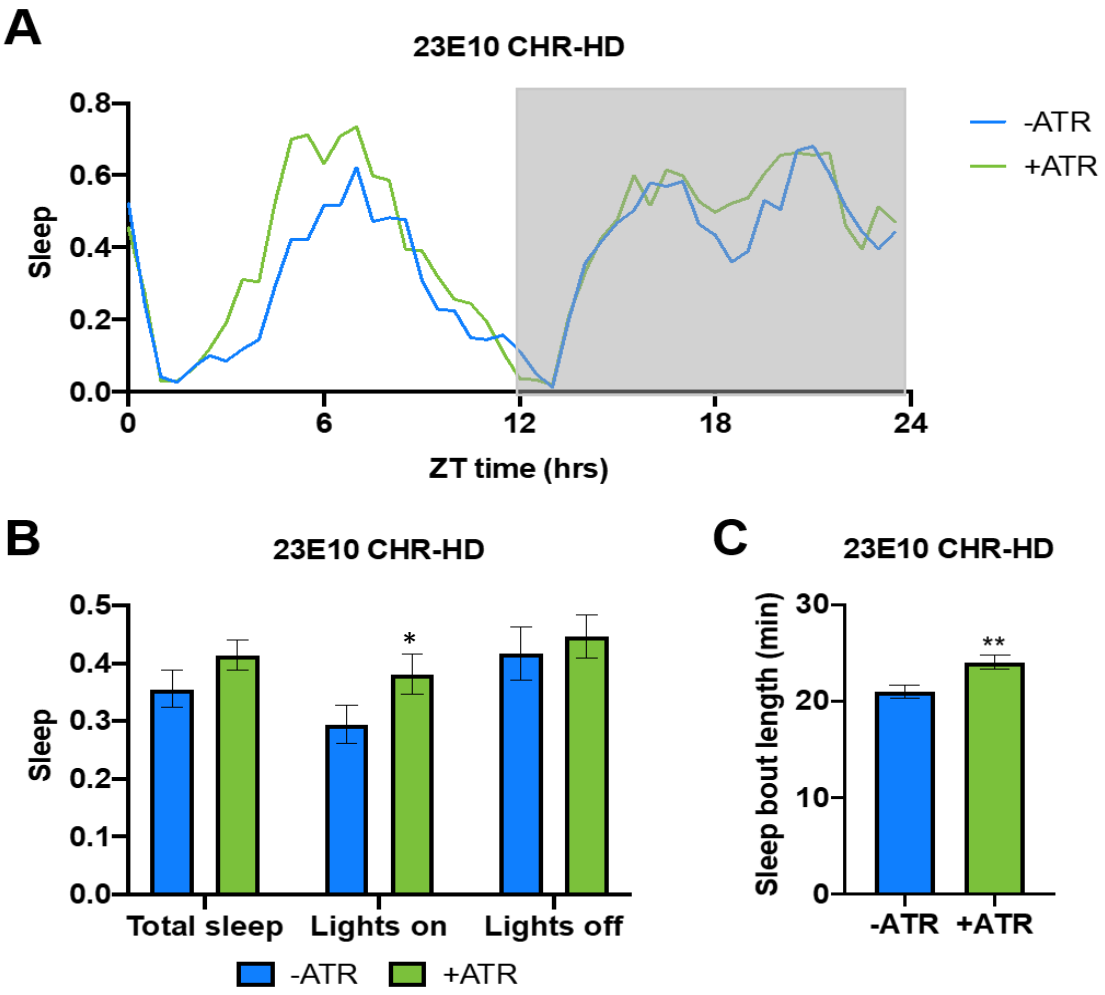

Figure 2. *Testing sleep model 23E10 CHR-HD.* ***A.*** *Average sleep profile generated from a 7-day monitoring period (n=16). Flies were exposed to food with +ATR for activating the expression of CHR-HD prior to and during the monitoring of sleep activity. The control group were fed food without ATR (-ATR). Flies were incubated in a 12-hr. circadian Light-Dark cycle during the sleep analysis.* ***B.*** *Average total sleep, Day-time sleep (lights on), and Night-time sleep (lights off). Significant difference was found in average day-time sleep (*p<0.05).* ***C.*** *Average sleep bout length. Experimental flies were found to significantly sleep longer than control flies (**p<0.01).*

Sleep Model ACR1-C102D:

The sleep model ACR1-C102D was tested using the sleep neuron drivers 72G06 and 23E10 in combination with the LexA-*lexaop* binary expression system. Mean total sleep examined of 72G06 ACR1-C102D flies with ATR- food had a total sleep of 0.491 ± 0.03 while flies with ATR+ food demonstrated a significantly increased total sleep of 0.576 ± 0.04 (**Fig. 3b**). Likewise, the mean sleep bout length of 72G06 ACR1-C102D showed a significant difference between control conditions and the ATR+ food treatment. 72G06 ACR1-C102D control flies with ATR- food had a sleep bout length of 23.41 ± 2.84 and experimental flies with ATR+ food had a significantly increased sleep bout length of 39.56 ± 6.13 (**Fig. 3c**).

The control group (ATR-) of 23E10 ACR1-C102D was found to have a mean total sleep of 0.426 ± 0.03 and the experimental group (ATR+) had an average total sleep of 0.416 ± 0.03. No significant difference was found in total sleep for 23E10 ACR1-C102D flies (**Fig. 4b**). The sleep bout length of the experimental and control groups also was found to not be significantly different. The control group's sleep bout length was found to be 27.68 ± 2.24 and the experimental group's sleep bout length was found to be 29.37 ± 4.73 (**Fig. 4c**). Collectively, 72G06; ACR1-C102D increased sleep more effectively than 23E10; ACR1-C102D.

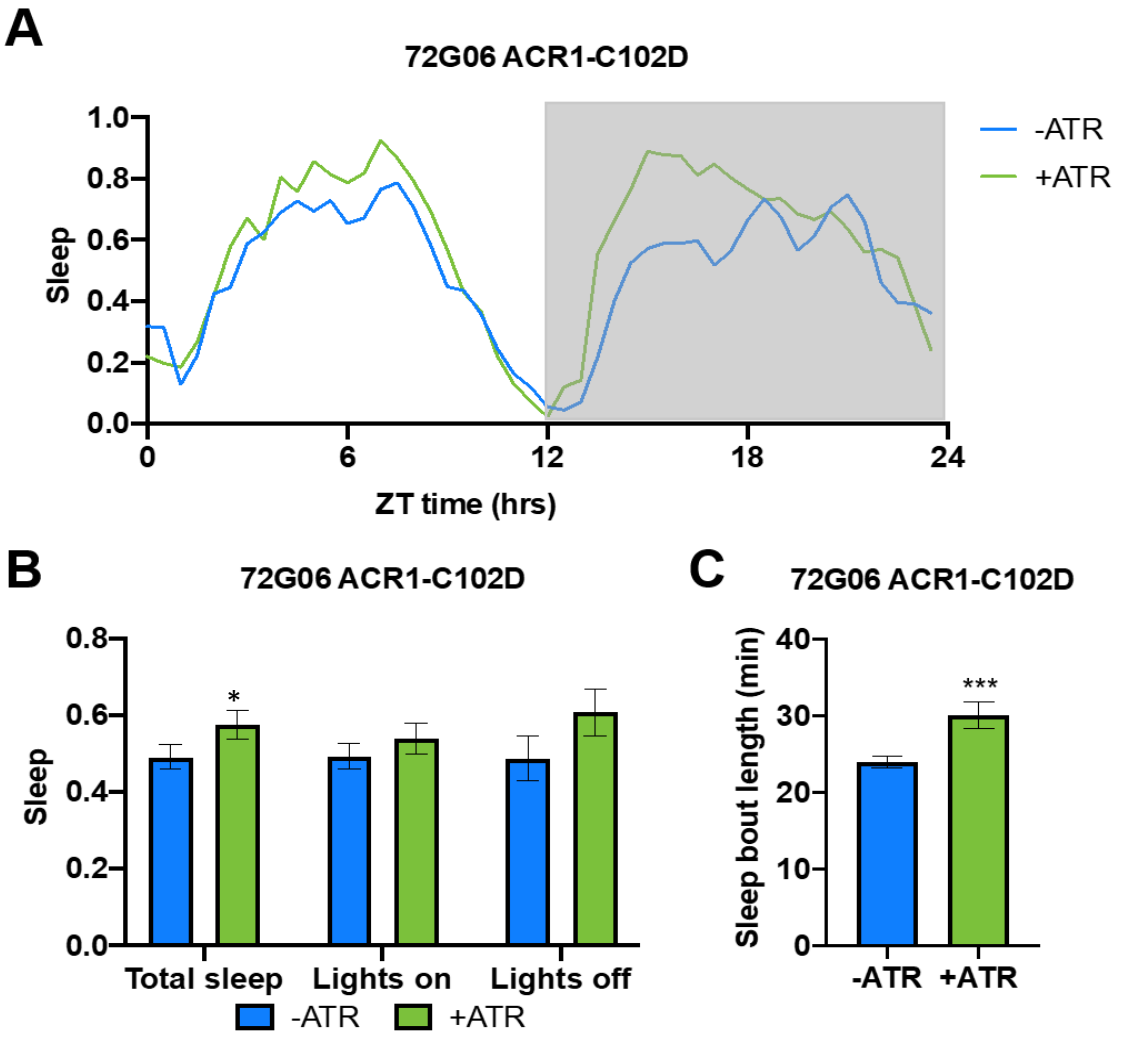

Figure 3. *Validation of sleep model 72G06 ACR1-C102D.* ***A.*** *Average sleep profile generated from a 7-day monitoring period (n=16). Flies were exposed to food with +ATR for activating the expression of ACR1-C102D prior to and during the monitoring of sleep activity. Flies were incubated in a 12-hr. circadian Light-Dark cycle during the sleep analysis.* ***B.*** *Average total sleep, Day-time sleep (lights on), and Night-time sleep (lights off). Significant difference was found in total sleep (*p<0.05).* ***C.*** *Average sleep bout length. Experimental flies had significantly longer sleep bouts than control flies (***p<0.001).*

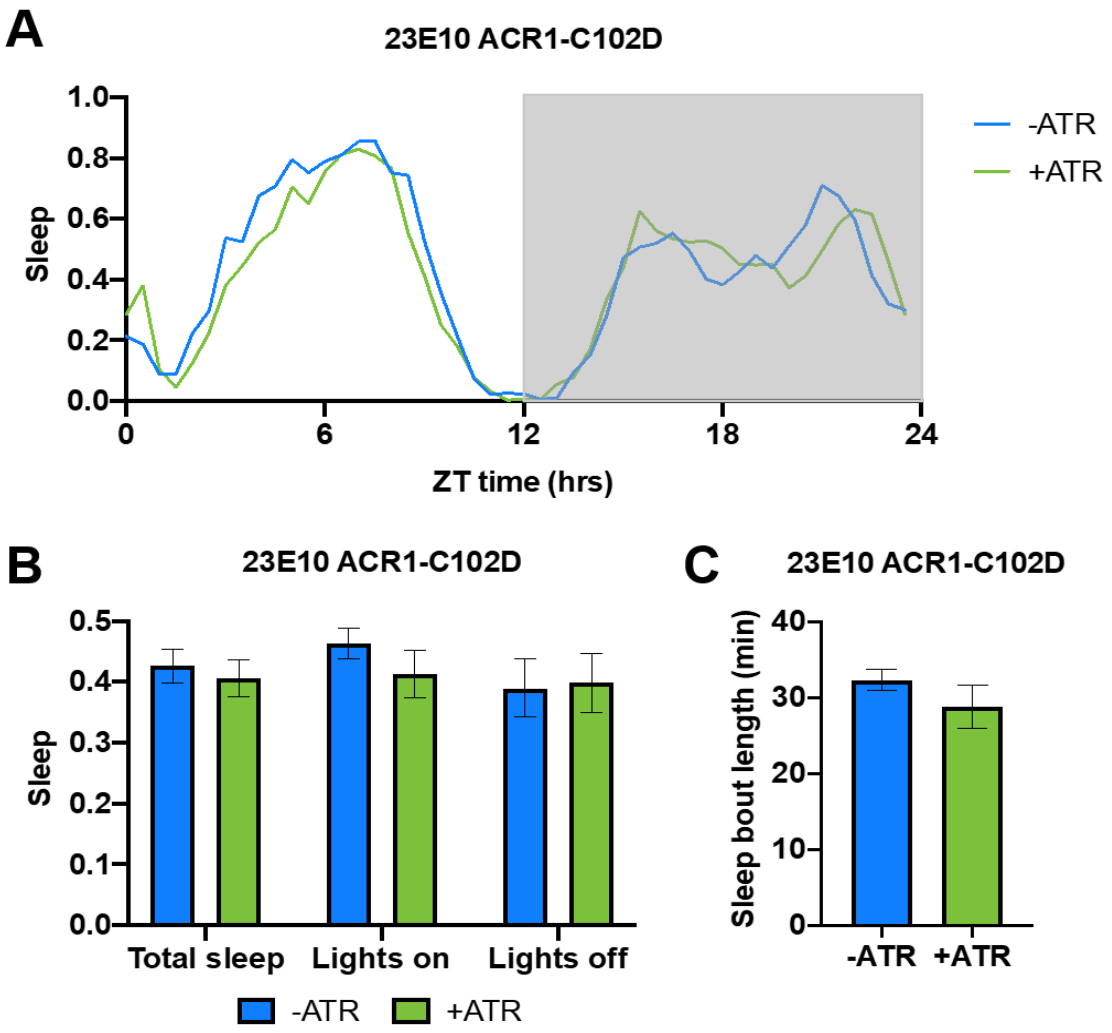

Figure 4. *Validation of sleep model 23E10 ACR1-C102D.* ***A.*** *Average sleep profile generated from a 7-day monitoring period (n=16). Flies were exposed to food with +ATR for activating the expression of ACR1-C102D prior to and during the monitoring of sleep activity. Flies were incubated in a 12-hr. circadian Light-Dark cycle during the sleep analysis.* ***B.*** *Average total sleep, Day-time sleep (lights on), and Night-time sleep (lights off). No significant difference was found in average total sleep, day-time sleep, and night-time sleep.* ***C.*** *Average sleep bout length. No significance was found between experimental and control groups.*

Sleep Model ACR1:

The sleep model ACR1 was tested using the sleep neuron drivers 72G06 and 23E10 in combination with the LexA-*lexaop* binary expression system. The control group (ATR-) of 72G06 ACR1 was found to have an average total sleep of 0.499 ± 0.04. 72G06 ACR1 flies exposed to ATR+ food had an average total sleep of 0.502 ± 0.04. No significant difference was found between the control and experimental groups (**Fig. 5b**). Furthermore, no significant difference was found in sleep bout lengths between 72G06 ACR1 flies exposed to ATR+ food and control flies. The control's sleep bout length was 20.97 ± 2.3 while the experimental's sleep bout length was 20.32 ± 2.75 (**Fig. 5c**).

Treatment of ATR+ food with 23E10 ACR1 caused a significant decrease in total sleep from a mean control value of 0.643 ± 0.04 down to 0.557 ± 0.04 (**Fig. 6b**). Also, the sleep bout length of the experimental group was not found to be significantly different than the control group's sleep bout length. The control group's sleep bout length was found to be 37.61 ± 3.9 and the experimental group's sleep bout length was found to be 30.31 ± 3.4 (**Fig. 6c**). Collectively, 23E10; ACR1 reduced sleep more effectively than 72G06; ACR1.

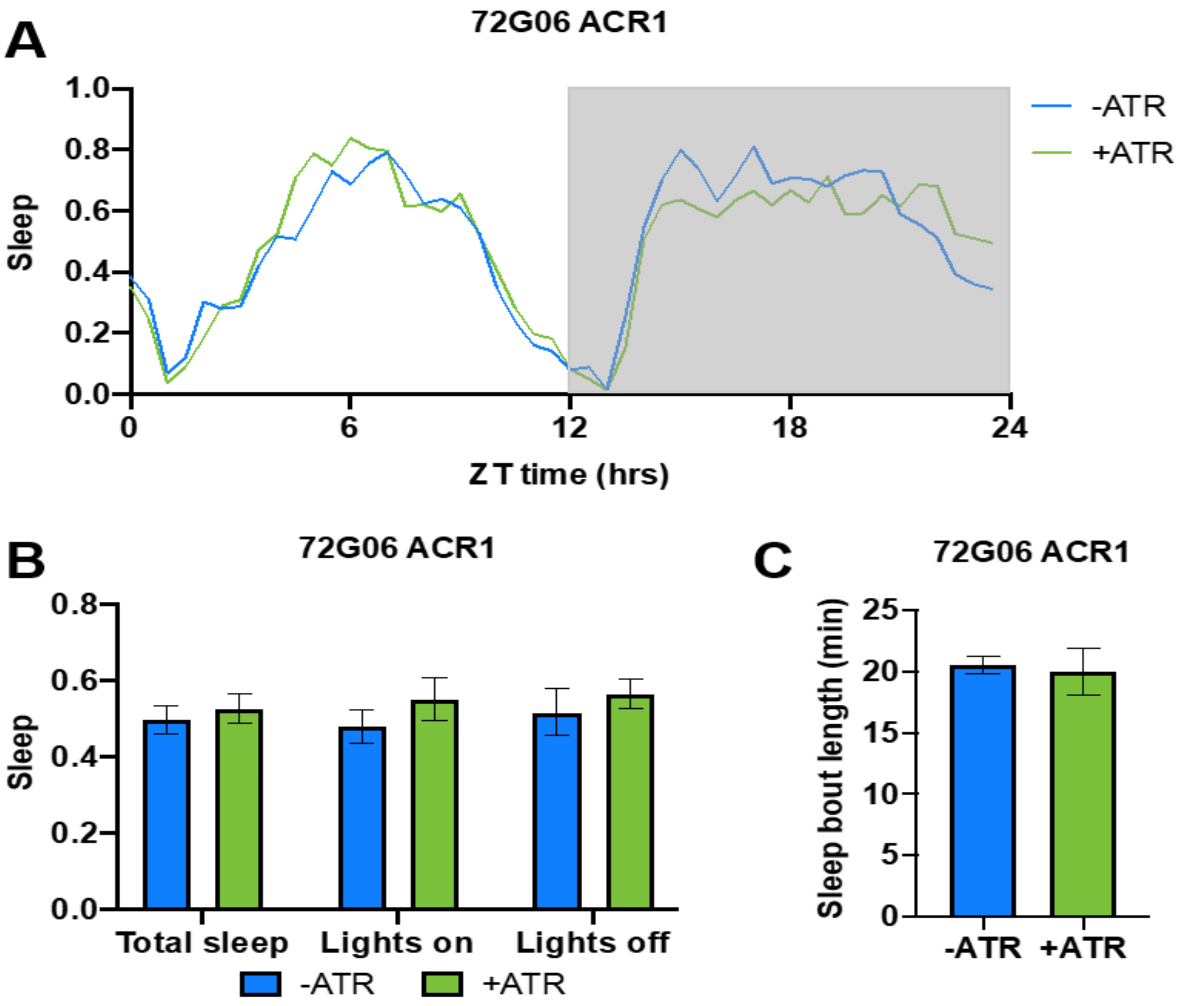

Figure 5. *Validation of sleep model 72G06 ACR1.* ***A.*** *Average sleep profile generated from a 7-day monitoring period (n=16). Flies were exposed to food with +ATR for activating the expression of ACR1 prior to and during the monitoring of sleep activity. Flies were incubated in a 12-hr. circadian Light-Dark cycle during the sleep analysis.* ***B.*** *Average total sleep, Day-time sleep (lights on), and Night-time sleep (lights off). No significant difference was found in average total sleep, day-time sleep, and night-time sleep.* ***C.*** *Average sleep bout length. No significance was found between experimental and control groups.*

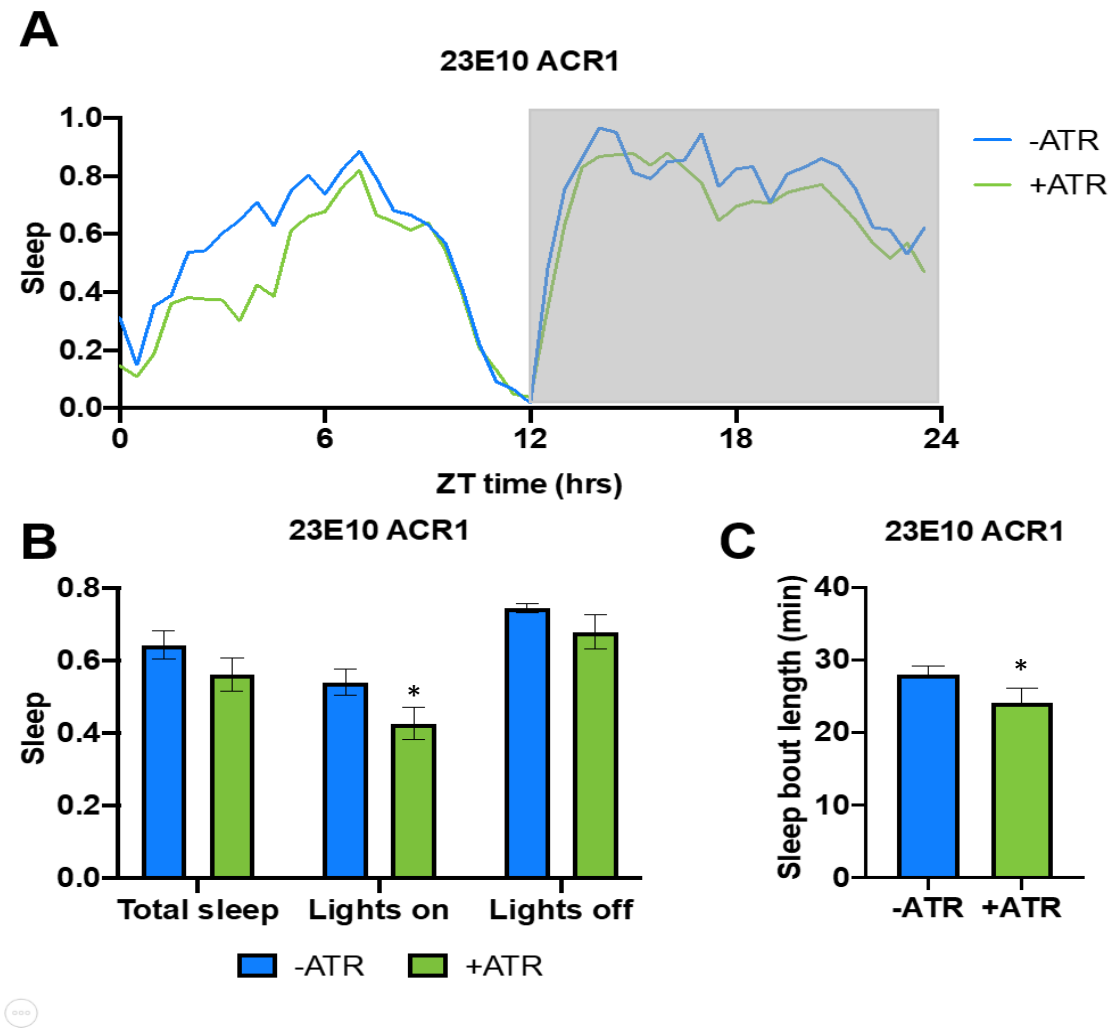

Figure 6. *Validation of sleep model 23E10 ACR1.* ***A.*** *Average sleep profile generated from a 7-day monitoring period (n=16). Flies were exposed to food with +ATR for activating the expression of ACR1 prior to and during the monitoring of sleep activity. Flies were incubated in a 12-hr. circadian Light-Dark cycle during the sleep analysis.* ***B.*** *Average total sleep, Day-time sleep (lights on), and Night-time sleep (lights off). Significant difference was found in average total sleep, day-time sleep, and night-time sleep (*p<0.05).* ***C.*** *Average sleep bout length. No significance was found between experimental and control groups (*p<0.05).*

Caspase Sleep Model:

The caspase sleep model was tested using the sleep neuron drivers 72G06 and 23E10 in combination with the Gal4-*UAS* binary expression system. All caspase conditions were exposed to 3 days of blue light prior to monitoring under the DAM2 system and subsequent sleep analysis. The control group, 72G06, was found to have a mean total sleep of 0.638 ± 0.06. 72G06 caspase flies had a mean total sleep of 0.502 ± 0.02, significantly less sleep than control flies (**Fig. 7b**). For sleep bout length, it was found that 72G06; caspase flies also demonstrated a significant decrease in bout lengths over the control group by at least 4.82 minutes. The control's sleep bout length was 30.66 ± 4.81 while the experimental's sleep bout length was 19.78 ± 1.25 (**Fig. 7c**).

The control group, 23E10, was found to have a mean total sleep of 0.649 ± 0.02. The experimental group, 23E10; caspase, had an average total sleep of 0.557 ± 0.01, demonstrating a significant decrease in total sleep from 23E10 flies (**Fig. 8b**). Also, the sleep bout length of the 23E10 caspase was found to be significantly shorter than the control group's sleep bout length by at least 3.42 minutes. The control group's sleep bout length was found to be 38.65 ± 3.68 and the experimental group's sleep bout length was found to be 29.44 ± 2.11 (**Fig. 8c**). Collectively, 72G06; caspase reduced sleep more effectively than 23E10; caspase.

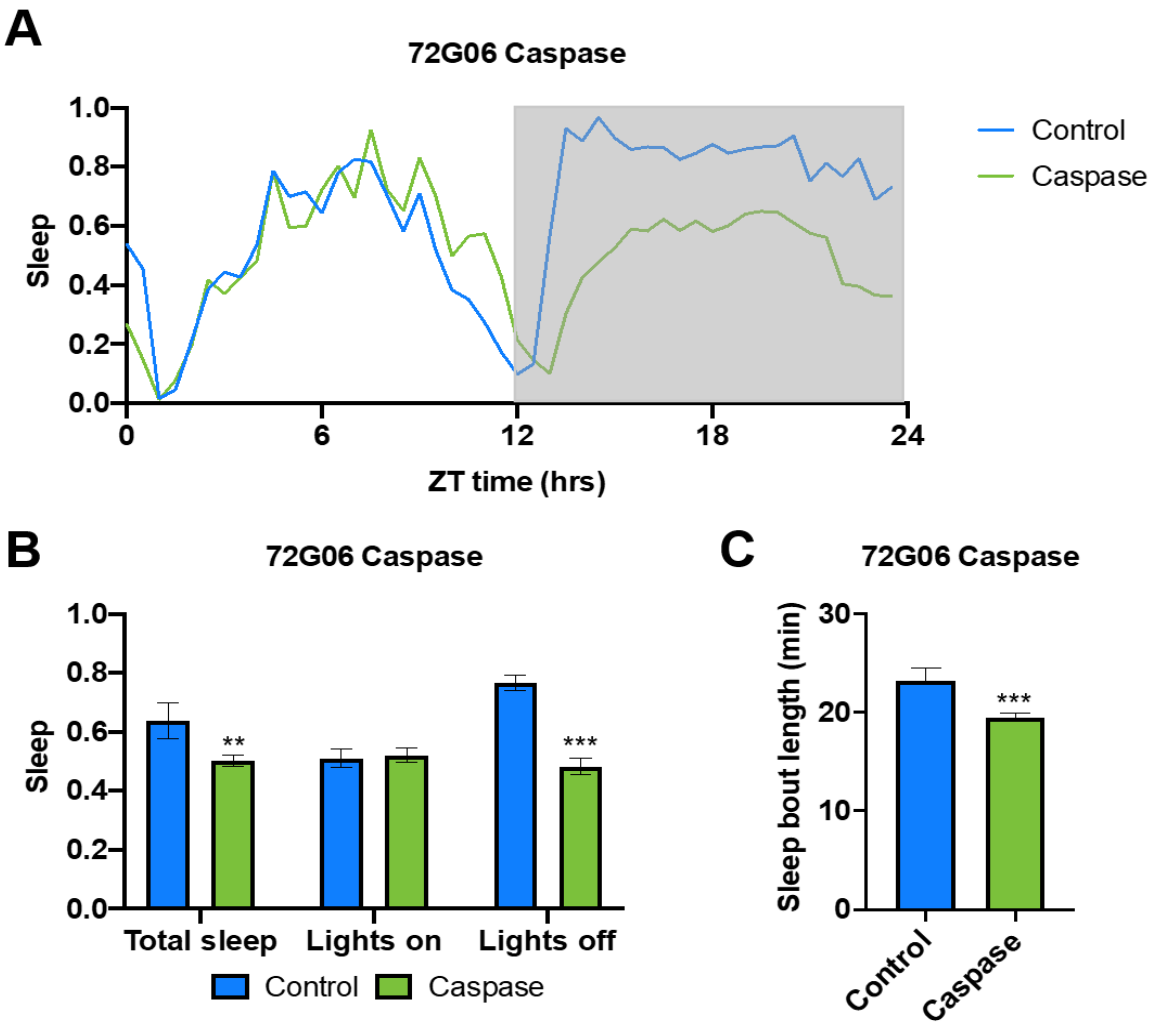

Figure 7. *Validation of sleep model 72G06 Caspase.* ***A.*** *Average sleep profile generated from a 7-day monitoring period (n=16). Flies were exposed to 3 days of blue light prior to the monitoring of sleep activity. Flies were incubated in a 12-hr. circadian Light-Dark cycle during the sleep analysis.* ***B.*** *Average total sleep, Day-time sleep (lights on), and Night-time sleep (lights off). Significant difference was found in total sleep and night-time sleep (**p<0.01, ***p<0.001).* ***C.*** *Average sleep bout length. Experimental flies had significantly shorter sleep bouts than control flies (***p<0.001).*

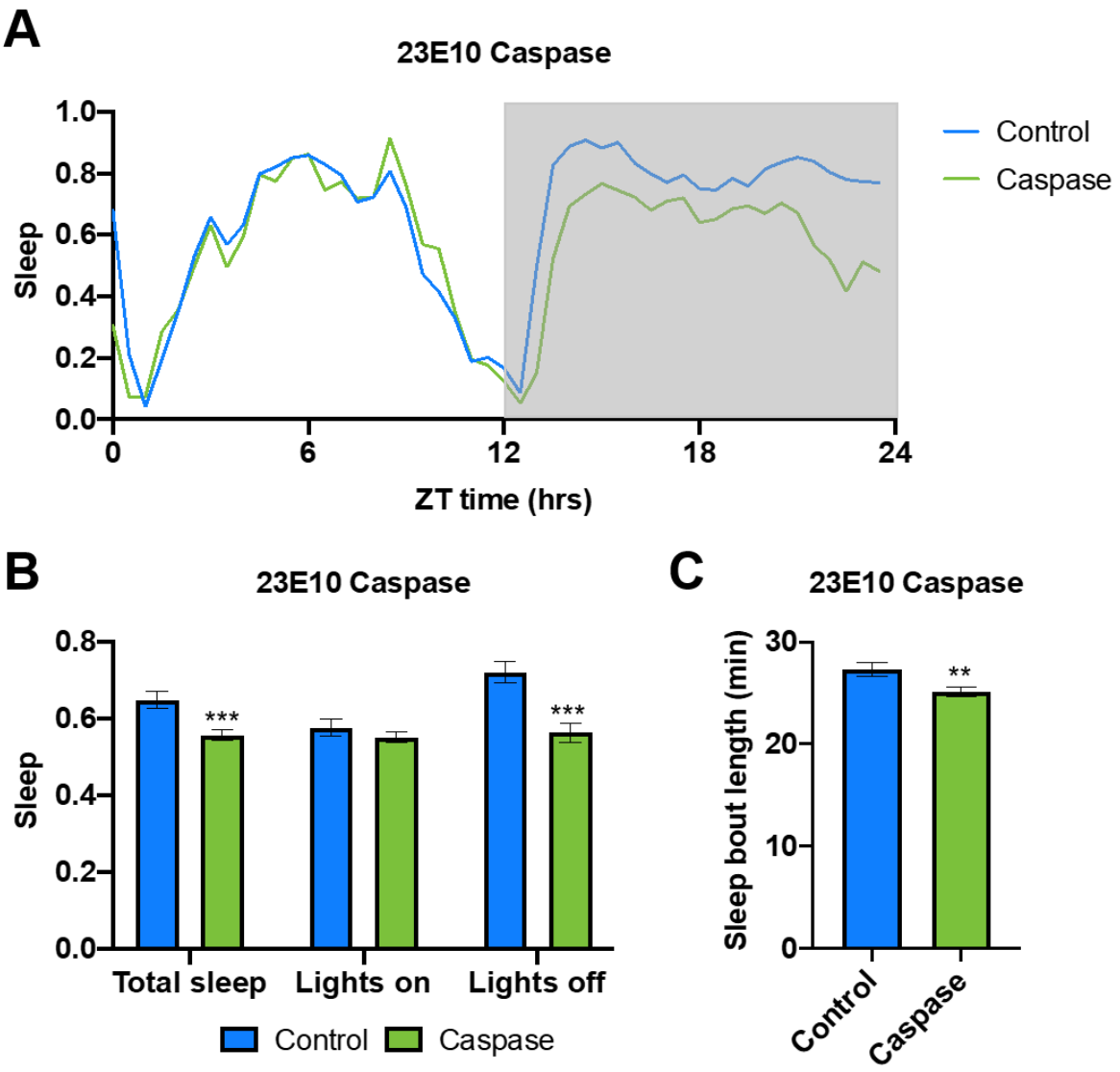

Figure 8. *Validation of sleep model 23E10 Caspase.* ***A.*** *Average sleep profile generated from a 7-day monitoring period (n=16). Flies were exposed to 3 days of blue light prior to the monitoring of sleep activity. Flies were incubated in a 12-hr. circadian Light-Dark cycle during the sleep analysis.* ***B.*** *Average total sleep, Day-time sleep (lights on), and Night-time sleep (lights off). Significant difference was found in total sleep and night-time sleep (***p<0.001).* ***C.*** *Average sleep bout length. Experimental flies had significantly shorter sleep bouts than control flies (**p<0.01).*

Lifespan Assay of 72G06; Caspase Sleep Model:

Immediately following our validation of the caspase sleep model, the 72G06 caspase sleep model was chosen to undergo a lifespan assay to determine that the model was effective over a lifespan. During incubation, tested flies were stored at room temperature in a light box with continuous blue light. The control group, wild-type flies, had an average lifespan of 40.2 days while the experimental's average lifespan was only 24.3 days (n=100) **(Fig. 9).** Consistent with a role for sleep in maintaining health, reduction in overall sleep from partial sleep deprivation caused a significant decrease in the length of the organism's lifespan.

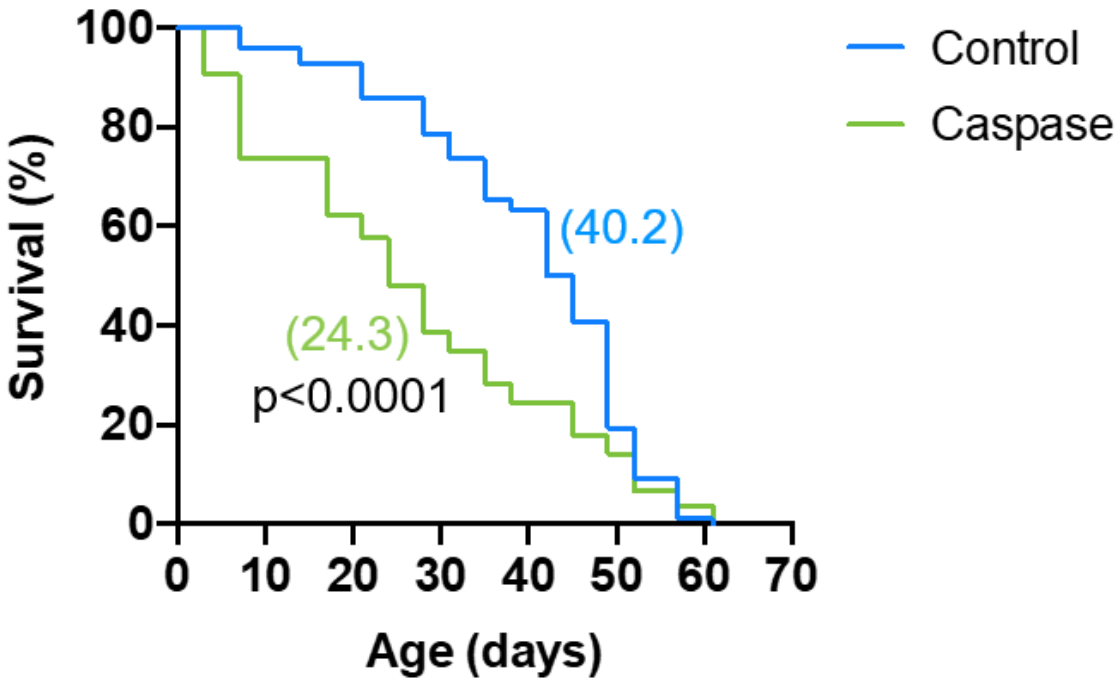

Figure 9. *Survival curve for Control and Light-activated caspase (sleep deprived) flies (n=100 for each group). Mean lifespan for Control=40.2 days; mean lifespan for Caspase flies=24.3 days (p<0.0001).*

Discussion

Sleep Model CHR-HD:

The exploration into the effectiveness of channelrhodopsin modifying sleep behavior revealed an interesting difference between modulation of CHR-HD with the sleep neuron promoter expression lines 72G06 and 23E10. 72G06; CHR-HD flies with continual access to ATR+ food were found to have significantly more sleep than control flies (**Fig. 1b**) and the bout length of these experimental flies were also at least 2.11 minutes longer on average (**Fig. 1c**). This observation is characteristic of our current understanding of the channelrhodopsin's depolarization effect on excitable cells, in this case sleep neurons. CHRs have operational light sensitivity and cause increased depolarization of excitable cells due to delayed closing kinetics (Bergs et al., 2018). This delay allows for an increased influx of depolarizing ions into the cell which would subsequently trigger action potentials that carry signals throughout the body, engaging sleep functions within the fly. This delayed kinetics of the CHRs explains why we see the increased total sleep and sleep bout length in the 72G06; CHR-HD fly model (**Fig. 1b**; Fig 1c). However, this does not hold true for the sleep neuron promotor 23E10. Though sleep bout lengths were longer (**Fig. 2c**), no significant difference was found in total sleep of experimental 23E10; CHR-HD flies exposed to ATR+ food (**Fig. 2b**). Comparing the two data sets together, 72G06; CHR-HD flies demonstrated a 2.2% - 26% increase in sleep. This may be due to insufficient expression of the CHR-HD transgene with this particular GAL4 driver. Further evaluation of the sleep promoter 23E10 is needed to conclude this observation. With that said, 72G06; CHR-HD has shown to increase sleep more effectively than 23E10; CHR-HD.

Sleep Model ACR1-C102D:

ACRs have been used previously in *C. elegans* to inhibit neuronal function (Bergs et al., 2018). As an inhibitor, ACRs cause hyperpolarization of excitable cells when activated by light which effectively leads to silencing of those cells and inhibition of neuronal activity. This understood characterization of ACRs in *C. elegans* was not observed for ACR1-C102D. When tested with 72G06, ACR1-C102D flies exposed to ATR+ food experienced significantly increased total sleep (**Fig. 3b**) and had longer sleep bout lengths (**Fig. 3c**). In sum, 72G06; ACR1-C102D flies demonstrated a 2.8% - 25% increase in sleep. This observed behavior is characteristic of a depolarizing CHR, such as 72G06; CHR-HD, rather than a hyperpolarizing ACR. A possible explanation for this seemingly mismatched observation is that the point mutation of ACR1 may have altered the ion channel in such a way to increase affinity for cations rather than anions. This could then make the ACR functionally similar to a CHR, causing depolarization of the *Drosophila* sleep neuron rather than hyperpolarization. Consequentially, this would then increase fly sleep and sleep bout length, just as we observe in the recorded data. Also similar to CHR-HD, no significant difference was found in the total sleep of 23E10; ACR1-C102D flies exposed to ATR+ food (**Fig. 4b**) in addition to observing no significant difference in fly sleep bout length (**Fig. 4c**). In conclusion, 72G06; ACR1-C102D has shown to increase sleep more effectively than 23E10; ACR1-C102D.

Sleep Model ACR1:

Testing of ACR1 fly sleep models revealed an interesting observation regarding the sleep neuron promotors. The 72G06 ACR1 flies exposed to ATR+ food were found to have no significant

change in their total sleep (**Fig. 5b**) or sleep bout lengths (**Fig. 5c**) as compared to control groups. However, the 23E10 ACR1 flies exposed to ATR+ food saw a significant reduction in total sleep by 1.0% - 24% (**Fig. 6b**), although their sleep bout length remained unchanged (**Fig. 6c**). The expected result of ACR1 was that fly sleep would decrease in models exposed to ATR+ food, which is what was observed in 23E10; ACR1. This is due to the hyperpolarization effect that ACRs have on excitable cells, causing inhibition of neuronal activity (Bergs et al., 2018). What is unexpected though is that ACR1 demonstrated no significant effect on fly sleep activity when paired with 72G06; completely polar to the previously observed sleep models. A possible biological explanation for this data may be that the sleep neuron promoters that are directing the transgenes to sleep neurons might be selecting for different types of sleep neurons. As such, it may be possible that sleep neurons selected for by 72G06 may be more susceptible to depolarization by CHRs and sleep neurons selected for by 23E10 may be more susceptible to hyperpolarization by ACRs. This of course would require further testing for confirmation, but the data of this study suggests some relationship is present between the sleep neuron promotor used and the type of channelrhodopsin used.

Caspase Sleep Model:

Unlike the previous sleep models, the caspase sleep model found significant reductions in fly sleep using both sleep neuron promoters. This is likely due to the function of the caspase within the cell rather than relying on channels and cell polarizations. The caspase functions by inducing cell-mediated apoptosis within the target cell, which in this case would be sleep neurons (Smart et al., 2017). As such, it would be expected to see caspase sleep model flies to have a reduced ability to sleep as shown by the total sleep data for both sleep neuron promotors 72G06 and

23E10 (**Fig. 7b**; **Fig. 8b**). Likewise, the experimental fly groups of both sleep neuron promotors experienced shorter sleep bout lengths, indicative of a reduced ability to sleep (**Fig. 7c**; **Fig. 8c**). Comparing the two data sets together, 72G06 caspase flies demonstrated a 10% - 31% reduction in sleep while 23E10 caspase flies demonstrated a 10% - 18% reduction in sleep. Also, the 72G06 caspase flies had a significantly lower total sleep average of 0.502 ± 0.02 as opposed to 23E10s total sleep average of 0.557 ± 0.01. Considering that 72G06 caspase flies had a larger range of sleep reduction and a significantly lower total sleep than 23E10 caspase flies, I conclude that the sleep neuron promoter 72G06 was a more effective promoter to use for reducing sleep with the light-activated caspase.

Lifespan Assay of 72G06; Caspase Sleep Model:

Immediately following our validation of the caspase sleep model, the 72G06 caspase sleep model was chosen to undergo a lifespan assay to determine that the model was effective over a lifespan. Through this lifespan assay of our 72G06; caspase sleep model, we found that a 10 – 31% reduction in total acquired sleep causes a significant decrease in organismal longevity (**Fig. 7b, Fig. 9**). This is consistent with a role for sleep in maintaining organismal health and validates the effectiveness of the caspase sleep model over extended durations (Gallicchio & Kalesan, 2009; Xu et al., 2011; Xie et al., 2013). Using this caspase sleep model, we are now looking to test whether enhancing lysosome function can offset the negative consequences of sleep deprivation on organismal longevity as it is thought that enhanced removal of neurotoxic waste products from the brain during sleep via protein clearance pathways is one of the major mechanisms that provides restorative effects from sleep (Ellenbogen, 2005; Hara et al., 2006; Xle et al., 2013).

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