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Article

Dantrolene-induced ER stress alters sleep in *Drosophila melanogaster*

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SUMMARY

Sleep is a well-conserved behavior that, despite our limited understanding of it, has been shown to provide a number of neurological and neuroprotective benefits such as immune function and neurogenesis. Sleep-deprived animals have been found to exhibit higher levels of cellular stress, especially endoplasmic reticulum (ER) stress. In this work, we aimed to determine if an inverse relationship occurs between sleep and ER stress. We hypothesized that an increase in ER stress would cause a disruption in sleep patterns, specifically causing short intermittent sleep rather than continuous sleep. To test this, we introduced an ER-stress inducing drug, dantrolene, to one-week-old White Canton-S 10 (WCS10) Drosophila melanogaster through their diet. We then measured their sleep using video recording for three full days to determine sleep duration, the number of sleep bouts, and sleep bout length during the day and at night. After three days, we measured the concentration of ER stress-specific markers, binding immunoglobulin protein (BiP) and phosphorylated eukaryotic translation initiation factor 2 alpha (p-eIF2α), to determine the amount of ER stress. Flies that ingested dantrolene had more sleep especially at night with an increase in the sleep bout number during the day. The increase in sleep correlated with the increase in BiP and p-eIF2α in dantrolene-fed flies. Our findings provide new insight into the connections and relationships of sleep with the body's functions, suggesting a correlation between ER stress levels and sleep.

INTRODUCTION

Sleep is extremely important to the body's functions such as restoration and healing (1). During sleep, increased neuronal activity of sleep-active neuronal populations allows for the clearing of toxins in the brain (1). But this state can be disrupted by a number of factors including stress, medicine, caffeine, and traveling (2). When the body is unable to get proper sleep, its disease resistance and immune function deplete rapidly, which cause additional problems such as high blood pressure, diabetes, and obesity (1).

On a cellular level, the inability to get proper sleep can cause stress within the endoplasmic reticulum (ER) (3). The ER is a membrane organelle found in every eukaryotic cell that folds proteins, synthesizes proteins and lipids, and transports proteins (8). ER stress can arise from disruption in the glycosylation process or protein dysregulation (6). Glycosylation is a post-translational modification that occurs in the ER or in the Golgi apparatus wherein a carbohydrate is covalently attached to a target macromolecule, typically a protein or lipid (7,8). Approximately 30% of all proteins are glycosylated, and this modification serves various functions including cell signaling and protein folding (8). Proteins that are not properly glycosylated get misfolded, leading to cellular dysfunction, diseases, or in extreme cases, cell death (8). To mitigate production of unfolded proteins, cells activate a pathway known as the unfolded protein response (UPR) (6,9). The UPR serves to reinstate homeostasis and eliminate ER stress by increasing folding capacity through protein-folding chaperones and degradation of misfolded proteins (6,9). The UPR targets ER stress through three simultaneous pathways: inositol requiring protein-1 (IRE1), activating transcription factor-6 (ATF6), and protein kinase RNA-like ER kinase (PERK) (9). Activation of these pathways requires modulation by the HSP70 molecular chaperone or immunoglobulin-binding protein (BiP), which acts as a marker for misfolded proteins (10). Once the stress is recognized, BiP binds to the misfolded proteins, resulting in the activation of the IRE1, ATF6, and PERK pathways (3,9). When PERK is activated, it phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α), which reduces general protein translation in order to relieve the ER from an overload of protein folding stress (11). The increase in BiP chaperone and reduction in protein translation are adaptive and protect the cell (3).

Decreasing ER stress in aged animals has been determined to improve previously short and fragmented sleep to more consolidated and continuous sleep (4,5). Therefore, we hypothesized that an increase in ER stress would disrupt the sleep-wake cycle and that there would be an increase in the sleep bout number meaning a reduced amount of continuous sleep. To test our hypothesis, we utilized dantrolene, a drug known to induce ER stress, to observe its effects on the sleeping patterns of *Drosophila melanogaster*. Dantrolene causes ER stress by inhibiting the release of calcium ions from the sarcoplasmic reticulum. This change in calcium ion concentration within the ER stimulates the UPR (12). We found that dantrolene altered the sleep and wake patterns of *D. melanogaster* and observed a direct correlation between dantrolene dosage, sleep patterns, and concentration of ER stress markers BiP and p-eIF2α.

These findings provide a greater understanding of the bidirectional relationship between sleep and ER stress. In particular, our study examines the relationship between calcium signaling, ER stress, and sleep. Further, our data suggests that modulating ER calcium can be therapeutic in addressing sleep disturbances, such as insomnia or drug-related sleep disorders. This research provides a framework

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for future studies to explore the impact of ER stress across different tissues and the specific brain circuits affected by ER stress.

RESULTS

We first carried out dose-response experiments to determine the appropriate dantrolene concentration for our sleep studies. We conducted six trials using four different doses of dantrolene: 0 (vehicle), 1, 10, and 20 μM provided in food. Towards the end of three days, the flies that ingested 20 μM of dantrolene exhibited a marked increase in total sleep time, particularly during the daytime, when flies are active and baseline sleep levels are typically lower, suggesting that they could be sick (13,14). Based on this, we determined that the ideal doses to use in our experiments were 0 μM, 1 μM, and 10 μM.

To test the effects of ER stress on sleep, flies were treated with three different doses of dantrolene (0μM, 1μM, 10μM) and kept in an incubator for three days. A video camera recorded their movements, and sleep patterns were analyzed before the fly lysates were used to determine the concentration of ER stress response markers BiP and p-eIF2α via western blotting (**Figure 1**). Sleep in Drosophila is behaviorally defined as immobility for five minutes or more (15-16). A sleep bout is a continuous, uninterrupted period of sleep, that makes up a segment of total sleep time.

Dantrolene increases overall sleep

We recorded sleep and wake time under 12:12 light-dark conditions over three days in the flies fed dantrolene or vehicle (0 μM) (**Figure 2**). After quantifying the total sleep amount across the day and night, we found that the dantrolene-treated flies had more sleep during the night compared to the control vehicle-treated flies (**Figure 3**). Although there was not a significant difference during the day, the flies treated with 1 μM slept for an average of 530 minutes at night, which was significantly more than the control flies, which slept for an average of 412 minutes at night ($p<0.01$ one-way ANOVA; Welch's t-test $p<0.001$, n=45). Flies treated with 10 μM dantrolene slept for 477 minutes at night, which was also significantly more at night than control flies ($p<0.01$ one-way ANOVA; Welch's t-test, $p<0.05$, n=45).

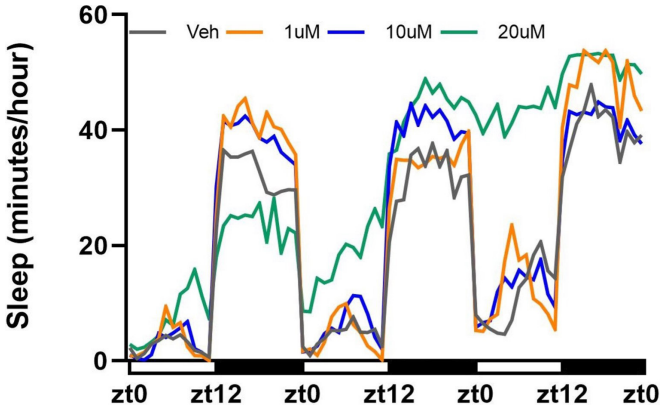

Figure 2: *Drosophila melanogaster* sleep over time with dantrolene treatment. Sleep profile of WCS10 flies treated with 0, 1, 10 and 20 μM dantrolene across 3 days. White and black bars indicate day and night periods. Vehicle treated (n=42), 1 μM (n=45), 10 μM (n=45), 20 μM (n=8).

Dantrolene increases number of sleep bouts

We also determined the number of sleep bouts and the length of the sleep bouts during the day and night. The number of sleep bouts at night were not different between the drug-treated flies and the control flies. However, there were significantly more sleep bouts in the 1 μM dantrolene flies, which had 11.3, compared to the control flies, which had 7.9, ($p<0.05$ one-way ANOVA, Welch's t-test, $p=0.028$; n=39) during the day (**Figure 4**). The average number of sleep bouts was higher in the 10 μM dantrolene-treated flies (9.3), compared to the control group, though this difference was not statistically significant.

A similar trend was observed in bout length, which had a longer, insignificant result in the dantrolene treated flies (**Figure 5**). The control flies had an average nighttime sleep bout length of 26 minutes while the bout length in 1 μM dantrolene treated flies was 36 minutes (t-test, $p=0.6$; n=45) and 60 minutes (t-test, $p=0.06$; n=45) in the 10μM dantrolene flies during the night (**Figure 5**). Daytime sleep bout lengths also trended longer in the 10 μM drug-treated flies (15.1 minutes) when compared to the control flies (10.2

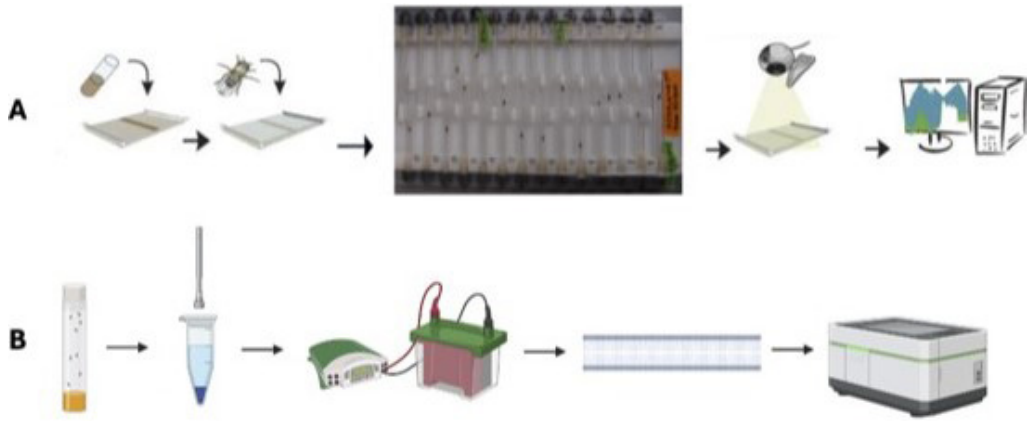

Figure 1: Experiment overview. A) WCS10 flies were placed in tubes in an incubator for 3 days with video cameras monitoring their movement. Three different doses (0 μM, 1 μM, and 10 μM) of dantrolene were given with food. **B)** The flies were then turned into a lysate using 10x PIC and homogenization buffer, and western blotting was performed for protein analyses. Image was created in BioRender (20).

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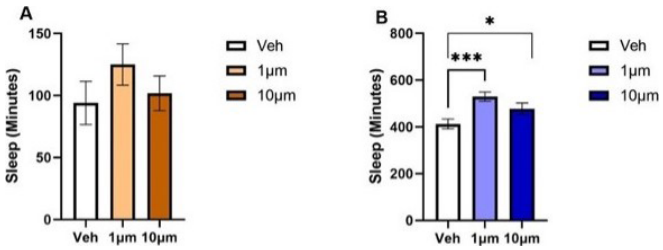

Figure 3: Dantrolene increases sleep at night. Quantification of **A)** daytime and **B)** nighttime sleep in flies treated with dantrolene. Nighttime sleep was significantly increased with dantrolene compared to vehicle (p<0.01 one-way ANOVA; ***p<0.001; *p<0.05; Welch's T-Test); Veh (n=42), 1 μM (n=45), 10 μM (n=45).

minutes) but the difference was not significant (t-test, p=0.07; n=45). The sleep bout length in the 1 μM treated flies was 9.7 minutes and not significantly different from that of the vehicle-treated flies (t-test, p=0.38, n=45).

Dantrolene induces ER stress markers BiP and p-eIF2α

To determine if dantrolene was able to induce ER stress in flies fed the drug, we prepared lysates of fly heads and bodies. We used heads and bodies separately to distinguish effects that may occur in the brain as opposed to the periphery of the body. We hypothesized that levels of BiP, eIF2α, and p-eIF2α would be higher in the bodies versus the heads due to ingestion of the drug via food. Using our lysates, we performed western blotting, to look at the levels of BiP, eIF2α, and p-eIF2α in each. The ratio of p-eIF2α to eIF2α was used to indicate how much of the total eIF2α in the lysate was phosphorylated and this is indicative of the activity of the enzyme PERK that phosphorylates eIF2α.

The amount of BiP found in the heads and bodies of flies treated with dantrolene was compared to that in the vehicle-fed control flies (**Figure 6**). We found significantly more BiP in the heads of flies treated with dantrolene compared to the heads of the controls (p<0.01 one-way ANOVA); BiP levels were not increased in the bodies of flies fed dantrolene (p=0.2 one-way ANOVA, n=8).

We also measured the levels of p-eIF2α and eIF2α as an indicator of PERK activation. Similarly to our findings for BiP, the amount of phosphorylated eIF2α was higher in the heads of dantrolene-fed flies (**Figure 7**). We saw that p-eIF2α was increased in heads of dantrolene-treated flies compared to control flies (p<0.01 one-way ANOVA). There were no significant differences in p-eIF2α levels in the bodies of dantrolene-treated flies compared to vehicle-treated flies.

DISCUSSION

Our study focused on establishing a potential two-way relationship between ER stress and the quality of sleep as well as discovering any alteration of the sleep/wake cycle caused by ER stress. Past experiments have determined that, with a decrease in sleep, ER stress is more likely to occur (4,5). We decided to reverse the question and investigate how ER affects the quality of sleep. First, we saw that feeding flies dantrolene altered sleep. We found that dantrolene increased sleep at both 1μM and 10μM concentrations compared to the control flies. Nighttime sleep was significantly increased in the dantrolene-fed flies and, as *D. melanogaster* sleep more at night, our data suggests that dantrolene could be useful to increase nighttime sleep. While we did not observe significant changes in sleep bout duration we did however find that the number of daytime sleep bouts was increased in the drug-treated flies indicating a fragmentation of daytime sleep.

We also saw that dantrolene induced ER stress within the heads of treated flies. Our data also showed that, at a cellular level, both BiP and p-eIF2α increased. Both BiP and p-eIF2α have been associated with an increase in sleep in both Drosophila and mice. Our lab has shown that increasing PERK expression and p-eIF2α increases sleep in Drosophila (17). Another study also showed that increasing p-eIF2α increases slow wave sleep in rats (18). It has also been shown that low levels of BiP are associated with poor quality sleep and overexpressing BiP increases recovery sleep in sleep-deprived flies and in aged mice (5,19). Together, these findings suggest that activation of the UPR and ER stress signaling, particularly through the PERK–eIF2α pathway, is broadly associated with increased sleep across species. Our results are consistent with this pattern: we observed that flies treated with dantrolene exhibited increased total sleep.

In line with our hypothesis, we found that there is a connection between ER stress and sleep, but our specific hypothesis that sleep would be disrupted by dantrolene was not entirely correct. Daytime sleep was fragmented but nighttime sleep was unaffected. Our findings are consistent with several studies showing that activation of ER stress pathways, such as increased p-eIF2α or overexpression of BiP, can promote sleep or improve sleep recovery (17-19). However, other work has reported that chronic ER stress, particularly in the context of neurodegenerative disease or aging, is associated with poor sleep quality (5). These differing outcomes may be explained by the duration and severity of ER stress: in our study, the treatment window was relatively short, and flies were likely not exposed to prolonged ER stress. This may have limited the stress to an adaptive range, avoiding the effects seen with chronic exposure. Our data suggest that dantrolene increases sleep through the adaptive ER stress response by increasing BiP and inhibiting protein translation through p-eIF2α.

These results also come with limitations. One limitation of this study is the acute nature of the dantrolene treatment. Because ER stress pathways and their downstream behavioral effects may take longer to manifest, a three-day treatment

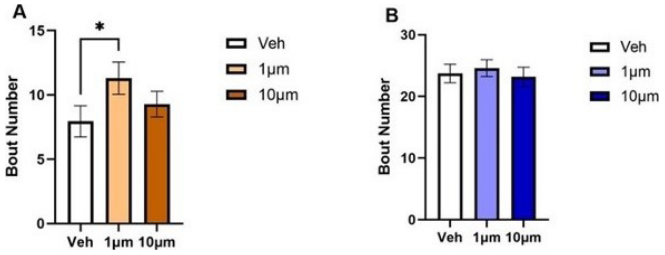

Figure 4: Dantrolene increases daytime sleep bouts. The number of sleep bouts during the **A)** daytime and **B)** nighttime for flies treated with 1 or 10 μM dantrolene. The number of daytime sleep bouts increased in flies treated with 1μM dantrolene, (p<0.05 one-way ANOVA, Welch's T-test *p<0.05); vehicle treated (n=42), 1 μM (n=45), 10 μM (n=45).

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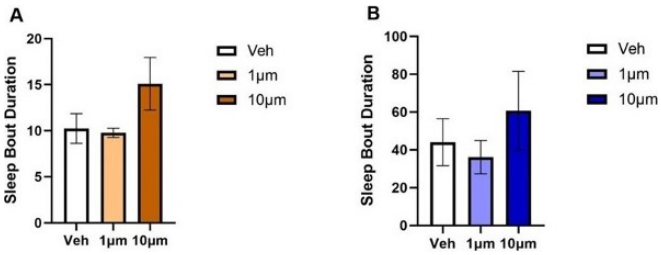

Figure 5: Sleep bout length in flies treated with dantrolene. Sleep bout duration was measured in *D. melanogaster* over 3 days during the **A)** day and **B)** night while being treated with 1 or 10 µM dantrolene. Daytime and nighttime sleep bout lengths trend higher in the 10 µM treated flies, but the increase is not significant. Vehicle treated (n=42), 1 µM (n=45), 10 µM (n=45).

window may have been temporally insufficient for dantrolene to exert its full effects. As such, longer-term treatment may be necessary to observe more sustained outcomes as dantrolene's effects on ER stress and sleep behavior may require prolonged modulation of calcium signaling and proteostasis pathways to fully emerge. We also note that our observations with respect to sleep behavior and BiP and p-eIF2α levels in bodies did not scale with the drug dose. The ER stress response in heads indicates as expected that molecular changes in the brain drives sleep behavior. Future work should assess ER stress within the fly over time, such as with age. Additionally, we could tease apart the pathways in the brain that are affected by dantrolene-induced ER stress.

We aimed to explore the impact of ER stress on sleep quality by administering dantrolene, a drug that modulates ER calcium and is known to induce ER stress, to *D. melanogaster*. We hypothesized that increased ER stress would disrupt the sleep cycle and lead to fragmented sleep; instead, our results revealed that dantrolene enhanced sleep. Our findings indicated that inducing the adaptive ER stress response, which is characterized by elevated levels of BiP and phosphorylated eIF2α, may promote longer and more consolidated sleep. This suggests that a moderate level of ER stress can trigger protective mechanisms that enhance sleep, offering new insights into the complex relationship between cellular stress and sleep regulation.

MATERIALS AND METHODS

Sleep studies under dantrolene-induced ER stress

Wildtype *Drosophila melanogaster* used in this study are in the White Canton-S 10 genetic (wCS10) background strain, which was originally a gift from Ronald Davis (Scripps Research Institute, Jupiter, FL). We selected newly eclosed female WCS10 flies and let them age for one week. Each fly was placed in its own tube that was enclosed with yarn on one side, to keep the fly from escaping the tube, and the other with a cap that was filled with food. The flies were then split into four different groups, control, 1 µM dantrolene, 10 µM dantrolene, and 20 µM dantrolene. Each of the groups were administered drug (dantrolene sodium salt, Thermo-Scientific, No. AAJ60887MC) or vehicle (DMSO) through their food. The flies stayed in an incubator that was on a 12:12-hour light cycle for three days. During this time, a video camera recorded them, taking photos every five seconds to determine movement within the tube and sleep the flies achieved. Through these photos, we were able to determine when the flies were asleep and awake by using a custom program called CSRNAPP.exe. If a fly had not changed position for more than five minutes, it was deemed asleep. Drosophila sleep data was collected from six independent experiments with the following fly numbers: n=42 (vehicle), n=45 (1µM), n=45 (10µM), and n=8 (20 µM).

Western blotting

Whole *D. melanogaster* bodies were placed on dry ice before being transferred to 5 mL snap-tubes. Each tube held 2–4 flies and was mechanically agitated on a table to break the cervical connective tissue, which maintains the connection between the head and thorax. The head and bodies were then manually separated into the two respective groups.

Flies were homogenized using a protease inhibitor cocktail (PIC), phosphatase inhibitor, and homogenization buffer (20 mM Tris-HCl, 1 mM EDTA, 10% Glycerol, 1% Triton-X, 150 mM NaCl). Following a BCA protein assay, a sample containing 30 µg protein was centrifuged at 12,000 rpm for one minute with β-mercaptoethanol and 2x Laemmli sample buffer and heated for five minutes at 95°C. Protein lysates were loaded on sodium dodecyl sulfate (SDS) polyacrylamide gels (10% Tris-HCl) and then transferred to nitrocellulose membranes (Bio-Rad) and blocked in Odyssey® TBS Blocking Buffer (LI-COR). Membranes were incubated in primary antibodies overnight at 4°C. Following washing (3 x 5 minutes), the membranes were subsequently incubated with secondary antibodies. Protein expression was detected and analyzed using the Odyssey® Infrared Scanner (LI-COR). Images were quantified using the software package on the scanner.

Primary antibodies used were: rat anti-BiP (1:1000, Abcam, cat# ab50571), rabbit anti-phospho-eIF2α (Ser51) polyclonal antibody (1:1000, Cell Signaling, cat# 3597), mouse anti-eIF2α (1:1000, Cell Signaling, cat# 2103), and mouse anti-β-Actin (1:2000 Cell Signaling, cat# 3700). The

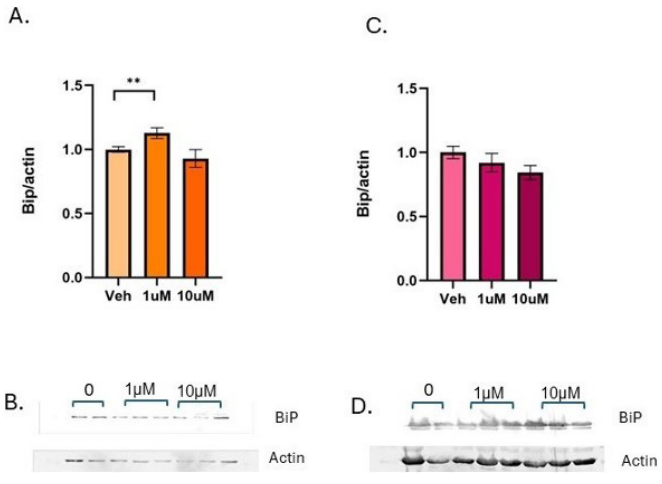

Figure 6: Amount of BiP in *D. melanogaster* treated with dantrolene. BiP normalized to actin in fly **A)** heads and **C)** bodies. BiP is increased in heads treated with 1µM drug, ($p<0.01$. one-way ANOVA, $^{**}p<0.01$ t-test. Representative Western blot showing BiP and actin in fly **B)** heads and **D)** bodies. Heads (n=8), bodies (n=8).

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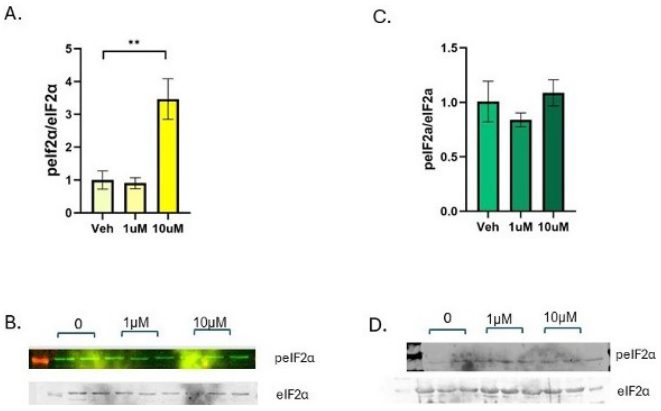

Figure 7: The ratio of p-eIF2α to eIF2α in *D. melanogaster* treated with dantrolene. p-eIF2α in **A-B)** heads and **C-D)** bodies. p-eIF2α is increased in heads, ($p<0.01$, one-way ANOVA; $^{**}p<0.01$, t-test). Note that the original IR fluorescent image is shown for p-eIF2α for clarity; all other western blot images have been converted to black and white. Heads (n=8), bodies (n=8).

following secondary antibodies were used: Goat anti-Mouse (1:10,000, LiCor IRDye, 680RD), Goat anti-Mouse (1:10,000, LiCor IRDye, 800RD), Goat anti-Rabbit (1:10,000, LiCor IRDye, 800RD), Goat anti-Rabbit (1:10,000, Odyssey IRDye, 680), and Goat anti Rat (1:10000, LiCor IRDye, 800RD).

Statistical analyses

One way ANOVA and Welch's T-test were conducted to compare drug and vehicle groups in the Drosophila sleep assays and IR-quantified protein concentrations. Statistical significance was set at $p<0.05$.

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