ROLES OF THE RNA-BINDING PROTEIN TDP-43 IN SLEEP AND LOCOMOTOR ACITIVITY IN *DROSOPHILA*

By

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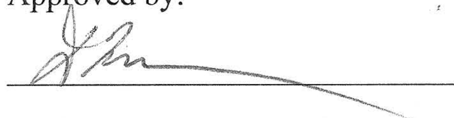

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Abstract

Amyotrophic lateral sclerosis (ALS) is a lethal, progressive neurodegenerative disease that leads to death of motors neurons, loss of voluntary muscles, respiratory failure, progressive muscle paralysis and death within 3-5 years. In the recent past, the RNA binding protein TDP-43 has emerged as a major player in the pathology of this fatal disease due to its linkage to the majority of ALS cases known to date. This discovery has led to an avalanche of research investigating the role of TDP-43 in ALS. Here, we use the *Drosophila* Activity Monitoring (DAM) system to investigate sleep and locomotor behavior in adult flies expressing wild type or mutant variants of TDP-43 in motor neurons or glia. We show that activity is significantly decreased in all variants compared to the control, and that average total sleep is increased in wild-type TDP-43 flies but decreased in mutant flies. Furthermore mutant and wild-type flies exhibit an increased number of sleep bouts and a decreased length of sleep bout during the day and night compared to the control. These newly discovered sleep phenotypes in the Drosophila model resemble restlessness and sleeplessness reported in ALS patients and open up new avenues of study for the role of TDP-43 in motor neuron disease and neurodegeneration.

Introduction

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (also known as Lou Gehrig's disease, motor neuron disease or Charcot's disease) is a lethal, progressive neurodegenerative disorder that is defined by selective death of motor neurons in the brain, spinal cord and motor cortex, and results in loss of control of voluntary muscle movement, respiratory failure, muscle paralysis and death within 3-5 years of diagnosis (Banks et al. 2008, Jefferson et al. 2011, Pasinelli et al. 2006). The disease

was first described by Charcot in the mid-nineteenth century. It is predominantly a sporadic disease (SALS) that affects 1 in 200,000 people by some estimates, though it has been observed that ALS incidence in the Western Pacific is 50-100 times greater than average. Only 10% of ALS cases considered familial (FALS, associated with one or more genes), (Byrne et al. 2011, Boillee et al. 2006, Jefferson et al. 2011, Pasinelli et al. 2006, Banks et al 2008). Onset is usually between 55-65 years of age on average. Because of this, ALS has become a topic of much interest over the last few decades as rising life expectancy and an aging population brings the disease increasingly into the public eye (Jefferson et al. 2011, Kiernan et al. 2011, Pasinelli et al. 2006, Boillee et al. 2006).

Symptoms and behavioral phenotypes of ALS

Onset of ALS is clearly age dependent and accompanied by loss of muscle strength and coordination. Bulbar muscles (those of the mouth and throat responsible for speech and swallowing) are frequently the first affected in ALS patients (Ferguson et al. 1996, Kiernan et al. 2011). This leads to upper airway dysfunction causing respiratory weakness, salivation and swallowing problems and abnormal speech. This respiratory weakness is progressive and death is frequently due to respiratory failure (Ferguson et al. 1996). In addition to these symptoms, patients may experience difficulty turning or changing position, muscle cramps and pain, and also uncontrollable leg movements. These factors in addition to psychological distress contribute to decreased activity, and insomnia during the course of the disease (Blackhall et al. 2011, Kiernan et al. 2011, Lo Coco et al. 2012).

Sleep-related complaints are some of the most commonly reported from patients with ALS. These include insomnia, disturbed sleep, frequent waking, nightmares, headaches and

daytime sleepiness. Typically these symptoms are attributed to problems with respiration, such as hypoventilation, drooling and or choking (Ferguson et al. 1996, Kiernan et al. 2011, Lo Coco et al. 2012). However, there is some evidence to suggest that sleep disturbance may have a component not related to the respiratory failure that is so inevitable in this tragic disease. Lo Coco et al. reports that many patients describe reduced sleep quality and increased sleepiness during the day even in the absence of respiratory dysfunction. Furthermore in a study of fifty nine patients with ALS and 36 controls, they found that forced vital capacity (the maximum air a person can expel after a maximum inhalation), nocturnal ventilation, and altered nocturnal pulse oximetry (a tool used to determine a patient's arterial oxygen saturation and heart rate) were not associated with poor sleep quality, confirming the suggestion that sleep problems in ALS patients do not depend solely on respiratory dysfunction (Lo Coco et al. 2012).

Genetics of ALS-inheritance patterns and associated genes and molecular pathways

A clear cellular pathway has not yet been defined as the cause of ALS, and it is likely that the disease arises due to a complex interaction between several cellular mechanisms. One common factor in the pathology of ALS is the presence of intra-neuronal inclusions, including the extensively described ubiquitinated inclusion bodies found in both SALS and FALS cases and also in SOD1 transgenic mice that provide a model of FALS. Ubiquitin positive inclusions are also seen in TDP-43 transgenic mice (Wegorzewska et al. 2009). These inclusions are observed in 95% of ALS cases and are characterized by a positive ubiquitin stain and RNA binding protein TDP-43 as a major component (Jefferson et al. 2011, Banks et al. 2008, Neumann et al. 2006).

FALS follows a Mendelian pattern of inheritance, and to date, 13 genes and loci have been identified that play a major role in this devastating disease (Kiernan et al. 2011, Jefferson et al 2011). Mutations in *SOD1* (copper/zinc ion-binding superoxide distumatase), *TARDBP* (otherwise known as *TDP-43* and encoding TAR DNA binding protein), *FUS* (encodes Fused in Sarcoma), *ANG* (encodes angiogenin), and *OPTN* (encodes optineurin) are some of the most frequently mentioned in the current literature on ALS, and produce a predictable clinical phenotype (Pasinelli et al. 2006, Valdmanis et al. 2009, Kiernan et al. 2011). Mutations in *SOD1* account for 15% to 20% of cases of FALS, the highest percentage known until recently. For this reason, *SOD1* and its role in ALS has been the target of extensive studies. This research has unearthed a considerable number of proposed factors that may cause ALS. These include excitotoxicity (where excessive stimulation of amino acid modulators like glutamate may cause damage to the neurons), protein aggregation, impaired axonal transport due to axonal strangulation, mitochondrial abnormalities that may lead to caspase-mediated cell death, and oxidative damage—known to contribute to a number of neurodegenerative diseases (Jefferson et al. 2010, Kiernan et al. 2011, Valdmanis et al. 2009, Pasinelli et al. 2006)

It has now been repeatedly shown that unlike SOD1, several mutations in the RNA binding protein TDP-43 are present in cytoplasmic inclusions of not only FALS, but also in the more common and less well-understood cases of SALS. More importantly, a direct link has been established where dominant mutations of TDP-43 protein are the primary cause of ALS (Banks et al. 2008, Da Cruz et al. 2011, Estes et al. 2011, Jefferson et al. 2010). In 2009, Valdmanis et al. concluded that mutation in TDP-43 account for 1% to 3% of ALS cases, both sporadic and familial. The most common cause of FALS to date is a GGGGCCC expansion in the 5' UTR of the *C9ORF72* gene, a highly conserved gene of unknown function. Identified in the majority of

families with ALS of TDP-43 based pathology, it is now considered now the most common genetic abnormality in FALS (23.5%) (De-Jesus-Hernandez et al. 2011). Despite this, TDP-43 has emerged as a common factor in the majority of ALS cases known, and represents an important target for study in the quest to describe the pathology of ALS and other neurodegenerative diseases.

TDP-43

TDP-43 is a 414-residue, 43-kDa, highly conserved nuclear protein originally identified for its role in repression of HIV-1 transcription (Banks et al. 2008). It has now been shown to have multiple roles in transcriptional repression, RNA-splicing and translational regulation, as well as mRNA stability and transportation (Jefferson et al. 2011, Banks et al. 2008). TDP-43 is known to bind both DNA and RNA. It possesses two RNA-recognition motifs, a glycine-rich C terminal sequence thought to act as an interface for interaction between proteins, a nuclear localization signal and a nuclear export signal. The protein is ubiquitously expressed—it is found in heart, lung, liver spleen, kidney, muscle, and brain tissue. Though TDP-43 is normally a nuclear protein, studies indicate that under pathological conditions it is removed form the nuclei of ubiquitinated-inclusion-bearing neurons and is found to accumulate in said inclusions (Jefferson et al. 2011, Banks et al. 2008, Estes et al. 2011, Neumann et al. 2006). Neumann and colleagues suggested that this accumulation could result in loss of normal TDP-43 nuclear function, which presented a possible explanation of TDP-43 mutation as a causative agent of ALS. However, it was not until a couple of years after this study that someone succeeded in showing that TDP-43 played a more active role in neurodegenerative disease and was not simply and consequence of disease (Neumann et al. 2006, Banks et al. 2008, Da Cruz et al. 2011).

TDP 43 is encoded by *TARDBP* in humans, a 6-exon gene on human chromosome 1. *TARDBP* has several evidently pathogenic mutations, mostly on the sixth exon. This pathogenicity was first demonstrated when two research groups showed that the protein was cleaved in such a way that mutations in several highly conserved regions of DNA segregated reliably with the disease. Mutations were never present in controls (Kiernan et al. 2011, Banks et al. 2008, Valdmanis et al. 2009). A summary of novel mutations in the gene that encodes TDP-43 is presented in Figure 1 (Banks et al. 2008). Our study focused on four: D169G, G298S, A315T and N345K, all of which are on the sixth exon, except notably D169G.

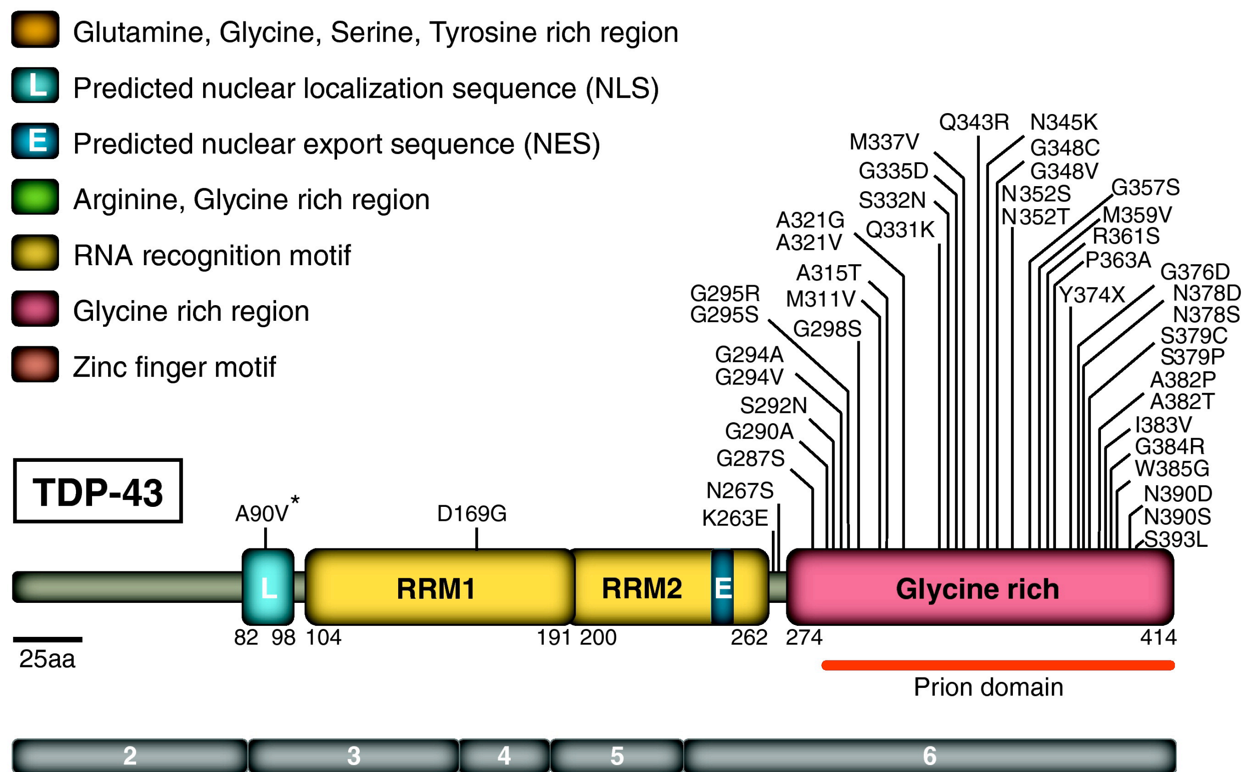

Figure 1: Summary of known mutations in *TDP-43* that are associated with ALS. Forty-four mutations have been identified in TDP-43 in sporadic and familial ALS patients and in rare FTLD patients, with most lying in the C-terminal glycine-rich region. Figure from Cruz et al, 2011.

Models of TDP-43 in animals

Our improved understanding of the genetic components involved in ALS have led to the creation of a large number of ALS models in animals that aim to elucidate ALS pathology. Models of TDP-43 have been created in *Drosophila, C. elegans,* zebrafish*,* mice and rats. Animal models do not reproduce all the clinical or pathological aspects of the disease, but they provide a valuable background in which to study one or more molecular pathways or tissues of interest, as well as recreate ALS symptoms in a way that is reliable and replicable (Rockenstein et al. 2007, Joyce et al. 2011). In this way animal models offer valuable insight into a disease that is harrowingly complex.

Animal models are used to study TDP-43 in two main ways. First is through knockout studies the look at the impact of removing TDP-43 entirely or partially in the animal. Other studies do the exact opposite, and study the effect of increased levels of wild type and mutant TDP-43 on the animal. Perplexingly, loss of function and gain of function phenotypes have been demonstrated in nearly every animal model. Transgenic mice expressing human wild-type TDP-43 or mutant variants show motor neuron degeneration, paralysis, and possess cytoplasmic ubiquitin inclusions and fragmentation and relocalization of TDP-43 (Da Cruz et al. 2011). Knockdown studies in zebrafish cause shorter motor axons, disorganized branching and swimming problems but not lethality (Kabashi et al. 2010, Da Cruz et al. 2011, Joyce et al. 2011). Overexpression of TDP-43 in *C. elegans* causes lack of coordination and abnormal motor neuron synapses. In one study, researchers investigated the effect of specific TDP-43 domains on toxicity in *C. elegans*. They found that deletion of the C-terminal region as well as either RNA recognition domain blocked neurotoxicity. Only overexpression *in vivo* of the full-length protein allowed for proper nuclear localization and neurotoxicity (Ash et al. 2010)

Perhaps one of the best studies to date in rats has shown that overexpression of mutant TDP-43 in neurons and muscles of rats causes paralysis in a progressive manner reminiscent of ALS when the transgene is turned on. In many other gain-of-function and loss-of-function models, animals often fail to show the progressive nature of ALS, so this particular study is encouraging (Huang et al. 2012). Furthermore, the study showed that rats with limited motor neuron loss experience a dramatic recovery of motor function after progression of the disease was halted (i.e. transgene expression was turned off) (Huang et al. 2012). It is not well understood how toxicity via overexpression of TDP-43 in animals relates to the human disease, because no copy number variants of *TARDBP* or increased mRNA levels have been found in human patients. However, the finding by Huang and colleagues is promising because it shows that motor neuron degeneration can be halted and at least partially reversed in a mammal (Huang et al. 2012, Da Cruz et al. 2011).

Mechanistically, the use of animal models is advantageous because scientists have the ability to drive expression of proteins associated commonly with ALS in specific tissues of interest. In the case of *Drosophila* this is often done using the UAS-Gal4 system, heralded as one of the most useful tools in *Drosophila* research (Sanyal et al. 2009). In this system, a responder gene (TDP- 43 in the case of this project) is under the control of an Up-stream Activating Sequence (UAS). Gal4, a yeast transcription factor capable of binding UAS elements in *Drosophila*, is necessary to stimulate transcription of the responder gene that is downstream of UAS element. Thus, Gal4 can be expressed in flies in a specific spacio-temporal domain using endogenous enhancers such that the responder gene that is under the control of the UAS only gets expressed in the pattern of the endogenous enhancer (Sanyal et al. 2009, Duffy et al. 2002). In this way, if the endogenous enhancer of interest is exclusive to motor neurons, that motor

neuron Gal4 driver will drive expression of the UAS-responder gene only in the motor neurons of *Drosophila*. One such Gal4 motor neuron driver in *Drosophila* is D42-Gal4, the driver used for this project. It was chosen because motor neurons are a tissue known to be severely impacted in patients with ALS, and therefore studying the impact of TDP-43 on this tissue specifically is very important.

Drosophila Model of ALS

Loss of function in TDP-43 in *Drosophila* is extremely deleterious in flies, and often lethal. Flies that do make it out of the pupal stage have shortened lifespan, defects in their neuromuscular junctions and locomotive behavior, reduced dendritic branching and axonal loss and neuronal death (Da Cruz et al. 2011, Li et al. 2012, Fenguin et al. 2009, Estes et al. 2011). Some studies have shown that RNAi knockdown of endogenous *Drosophila* TBPH causes expected motor defects, but that this can be rescued by neuronal expression of human TDP-43 (Fenquin et al. 2009, Li et al. 2002). These findings are interesting because of the fact that other studies have shown that expression of human TDP-43 variants in flies causes a distinct deleterious phenotype. As mentioned previously, it is not known why this is the case, though some people have suggested that TDP-43 aggregates have deleterious effects. The studies by Fenquin and colleagues and Li and colleagues propose that results found in overexpression studies are due more to lack of normal TDP-43 function rather than toxicity of aggregates. If true, this would help reconcile the problem of the impact of overexpression of TDP-43 in *Drosophila* with the seeming lack of an analogous scenario in humans.

Different variants of TDP-43 exert different levels of toxicity, a factor we kept in mind during our own study (Estes et al. 2011). Many studies of TDP-43 in *Drosophila* make use of

both larval and adult stages. Our project focused specifically on the adult stage, because ALS is an adult onset disease and therefore validation studies in the adult were preferred. Using the Trikinetics *Drosophila* Activity Monitoring system (DAM), we intended to monitor fly activity and gather conclusions about the differences between TDP-43 variants on fly locomotion. Here we show that a project that originally targeted the locomotor activity of flies expressing D42-Gal4 driven human TDP-43 variants, and was designed to confirm or deny the results of others, yielded some exciting new data on the role of TDP-43 on sleep and possibly even circadian rhythms of *Drosophila.*

Materials and Methods

Fly strains and culture conditions

w^{1118} and transgenic flies expressing human TDP-43 variants wt, D169G, G298S, A315T, and N345K were obtained from the Zarnescu laboratory at the University of Arizona. We used specific lines wt2L, D169G8A1, G298S9, A315T6, and N345K9—generated as described in Estes et al. 2011. In this study the Gal4 motor neuron driver D42 Gal4 was used. D42 Gal4 and w^{1118} stocks were obtained from the Bloomington Stock Center. Wild type and TDP-43 mutant males, D169G8A1, G298S9, A315T6 and N345K were crossed with homozygous D42 virgins. Progeny were reared at 22°C, 50% humidity, under a 12 hour light/12dark cycle, and on a conventional cornmeal/glucose/yeast/agar medium. D42/+ was used as a control.

Sleep analysis

1 to 3-day-old adult flies were monitored individually in autoclavable plastic tubes (22 mm in diameter and 95 mm in length) on the same medium described above under 12-hr-light

and 12-hr-dark cycles at 22°C with 45-60% humidity. Preparation of individual fly vials was performed prior to the experiment by melting pre-prepared fly food to liquidity and filling plastic vial end-caps with the mixture ¾ full. Vials were then inserted into the caps and the food was allowed to set for approximately 30 minutes prior to use. Sleep and locomotor activity data were collected using the Drosophila Activity Monitoring (DAM) system (Trikinetics, Waltham, MA) and analyzed using Pysolo, a multiplatform software for analyzing sleep and locomotor activity in *Drosophila* (Gilestro and Cirelli 2009). Flies were given 32 hours to habituate to the experimental conditions prior to data collection. Approximately 32 flies were gathered and placed in each monitor (with maximum capacity 32) for each genotype for each experimental replicate. Sleep and locomotor activity data were collected at 1 minute intervals for 5 days. Sleep was defined as a 5 minute period of no activity. Three experimental replicates were performed and data from each replicate was compiled.

Statistical analysis

Preprocessing of the Pysolo data was performed in Microsoft Excel. Normality of the data was tested using the Anderson-Darling test using statistical software package R. Based on the fact that at least one group per parameter was not normally distributed when compared to the control, a non-parametric Wilcox test was used in R to calculate all p-values.

Results

Overexpression of human TDP-43 in adult Drosophila causes decreased sleep and problems in rhythmicity and regularity of sleep

The Zarnescu lab has generated transgenic flies expressing wild type and mutant TDP-43 under the control of a UAS promoter. Using the UAS-Gal4 system, expression of wild type and mutant TDP-43 in *Drosophila* can be performed in specific tissues of interest (Sanyal et al. 2009, Duffy et al. 2002). In this study, we used a D42-Gal4 driver to express human mutant and wild-type TDP-43 in the motor neurons of *Drosophila*. This allowed us to study the effects of individual missense mutations found in ALS patients in relation to a control and to human wild-type TDP-43, in the context of motor neurons—a tissue known to be severely affected by this fatal disease.

Little or no work has been done concerning the relationship between various human mutations of TDP-43 known to cause ALS and sleep or circadian rhythms in *Drosophila.* However, all that has been described in previous literature concerning sleep regularity and rhythmicity in ALS patients, the inherent connection between TDP-43 and ALS, and the role of motor neuron structure and function in the progression of this disease set a precedent for studying these three main components together. As such, we set out to examine sleep phenotypes in flies expressing variants of human TDP-43 in motor neurons.

Wild type and mutant TDP-43 flies had an overall greater number of sleep episodes during both the day and night ($p<0.001$) and decreased length of sleep episodes during the day and night when compared to the control ($p<0.001$ in all cases excepting day time average length of sleep episode for wild-type, $p<0.05$, Figs. 3-6). The length of a sleep episode was measured as all additional minutes of inactivity, after the initial five-minute period for which we define sleep, until new movement. Based on this, the number of sleep episodes is averaged over the course of the first or second 12 hours of the day for five days in each fly. Mutants had significantly decreased average total sleep across five days of observation as well as significantly

decreased average nighttime sleep compared to the control (Fig. 10 and Fig. 8 respectively). This is clearly visible in the second half of Figure 2, which shows decreased minutes of sleep per 30 minutes in all TDP-43 variants during the second 12 hours of the day. Wild-type TDP-43 flies slept significantly more on average over a full 24 hours when compared to the control, though their nighttime sleep was not significantly different (Fig. 10). Average daytime sleep was less conclusive—wild type TDP flies slept significantly more during the day than the control, while G28S9 flies slept significantly less. This is strongly reflected in even the subset of data represented in the second half of Figure 2. A315T slept less than the control, while D169G and N345K slept more than the control during the day, but these findings were not statistically supported (Fig. 9).

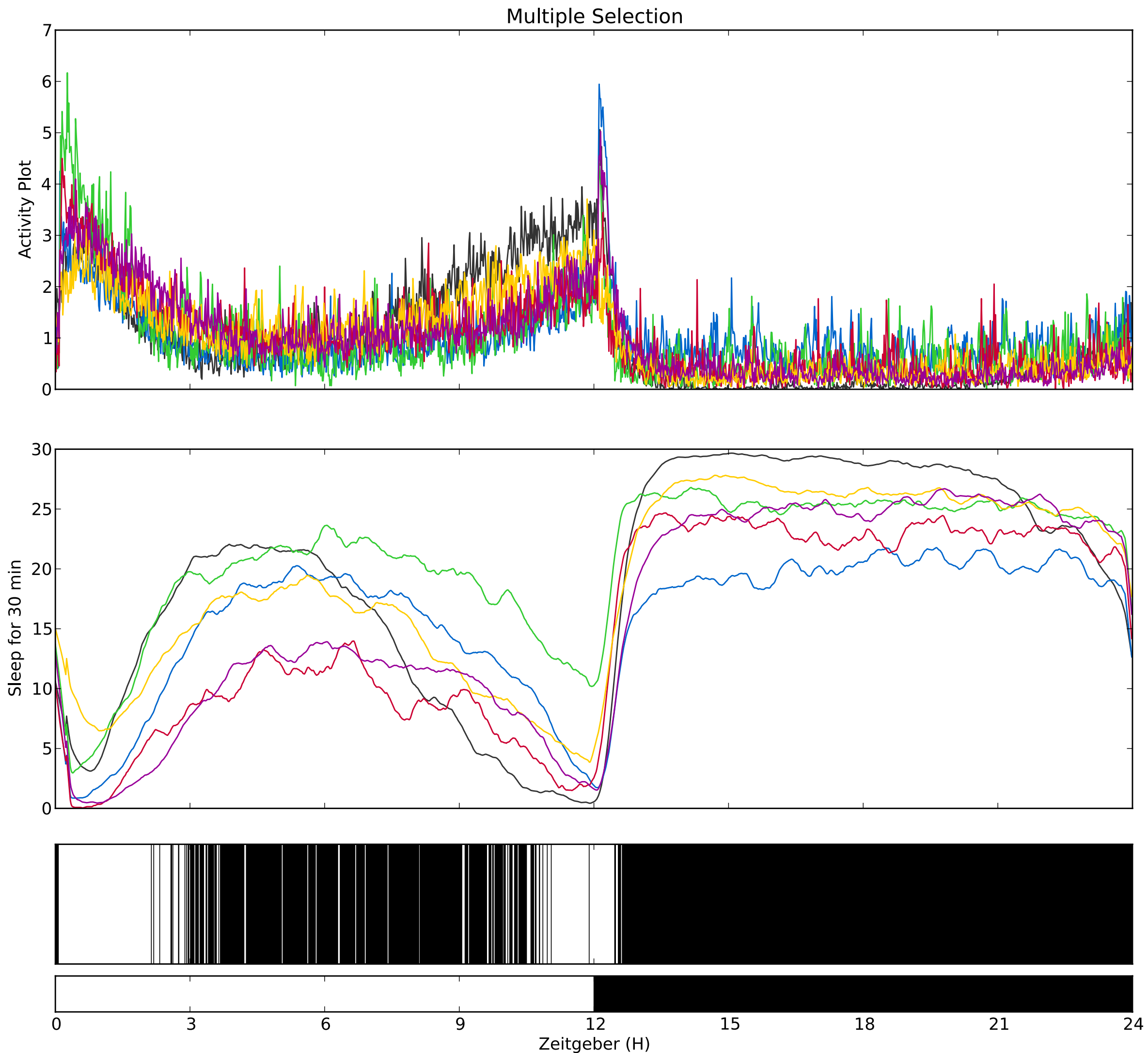

Figure 2. Activity plot and sleep curves for a subset of the data collected on flies expressing D42-Gal4 driven TDP-43 variants. Figure depicts the most recent replicate of data only for better visualization. Plots are generated in Pysolo. Sleep for 30 minutes indicates the number of additional consecutive minutes of sleep past 5 minutes in each 30 minutes of the day. Colors: blue-A315T, green-wt, red-G298S, yellow-N345K, purple-D169G. Zeitgeber (H) refers to the 24-hour light/dark cycle (first 12 hours light, second dark) imposed by the incubator containing the DAM system to synchronize the internal clock of the *Drosophila*.

Overexpression of human TDP-43 in adult Drosophila causes significantly decreased activity levels in both wild type and mutant TDP-43 flies

Previous studies of *Drosophila* locomotor activity and development in the context of human TDP-43 or its *Drosophila* homolog TBPH (or dTDP) have shown a number of interesting things. TBPH null mutants eclose at a significantly decreased rate than wild type controls. Adult locomotor speed is significantly reduced, but this can be rescued through neuronal expression of TBPH or human TDP-43 (Wang et al. 2011). The negative impact of depletion of dTDP on locomotor activity in *Drosophila* was again shown by Lin and colleagues. That same study and others have also shown motor dysfunction caused by over-expression of human TDP-43 in motor neurons in both larvae and adults (Lin et al. 2011, Estes et al. 2011). Overexpression of TBPH is lethal at the larval or pupal stages. Comparable levels of human TDP-43 variants are less toxic than their *Drosophila* homologs—flies reared at 18°C survived to adulthood while flies reared at 25°C did not. These flies exhibited a more severe decline in their ability to climb in climbing assays when compared to the control (Estes et al. 2011).

Given this plethora of research, we sought to use the DAM system to document the effect of overexpression of human TDP-43 on locomotor activity in *Drosophila*. As mentioned, flies expressing D42-Gal4 driven hTDP-43 variants do not survive when reared at 25°C, but they do survive at lower temperatures. In this study, flies reared at 22°C took longer to eclose than the control D42/+ when raised at the same temperature (qualitative observation only). Previous information indicates presumably that this is not only because of the reduced temperature, but also because of the health of the progeny. One to three days old male flies expressing D42-Gal4 driven human wild-type, A135T, D169G, G298S and N345K variants of TDP were housed in individual vials and their locomotor activity and sleep was observed using the DAM system.

After five days of observation, we found that all mutants and wild-type TDP-43 had a decreased activity counts on average compared to the control, consistent with the findings of others (Fig. 7, Fig. 2 part 1).

Note: Figures 3-10 represent compiled data for approximately 92 flies (three biological replicates) observed over the course of 5 days during a specified period of the day. Three, two and one asterisk(s) indicate p<0.001, p<0.01 and p<0.05 respectively.

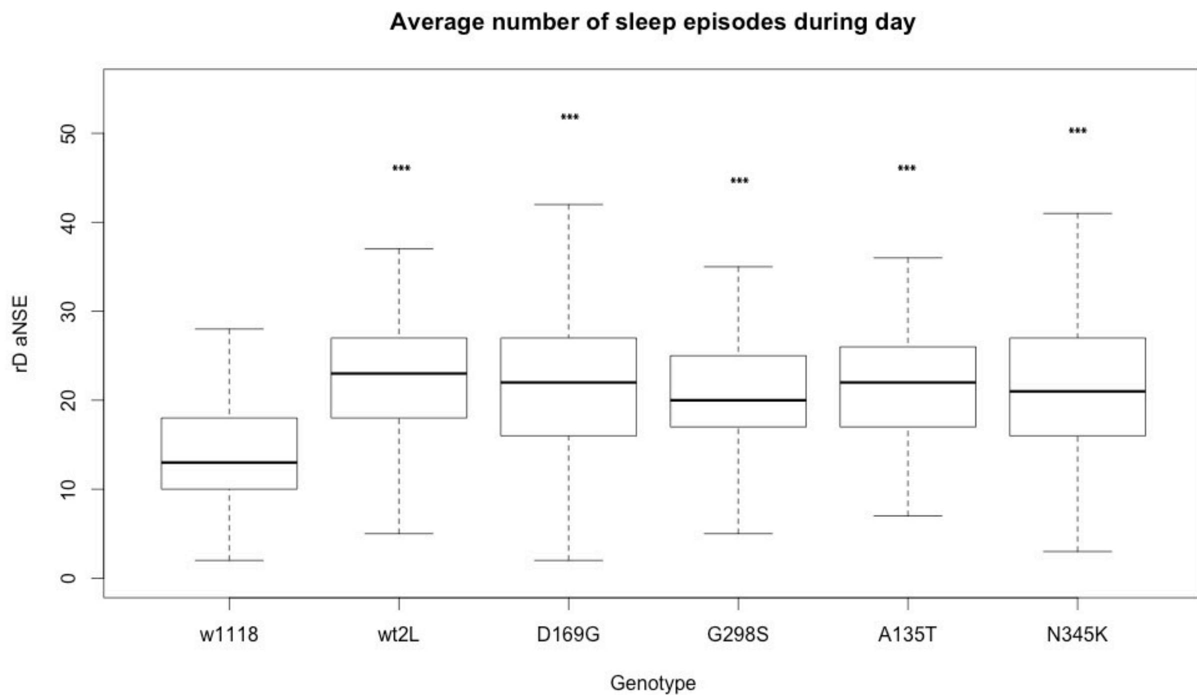

Figure 3. Average number of sleep-episodes during the first 12 hours of the day

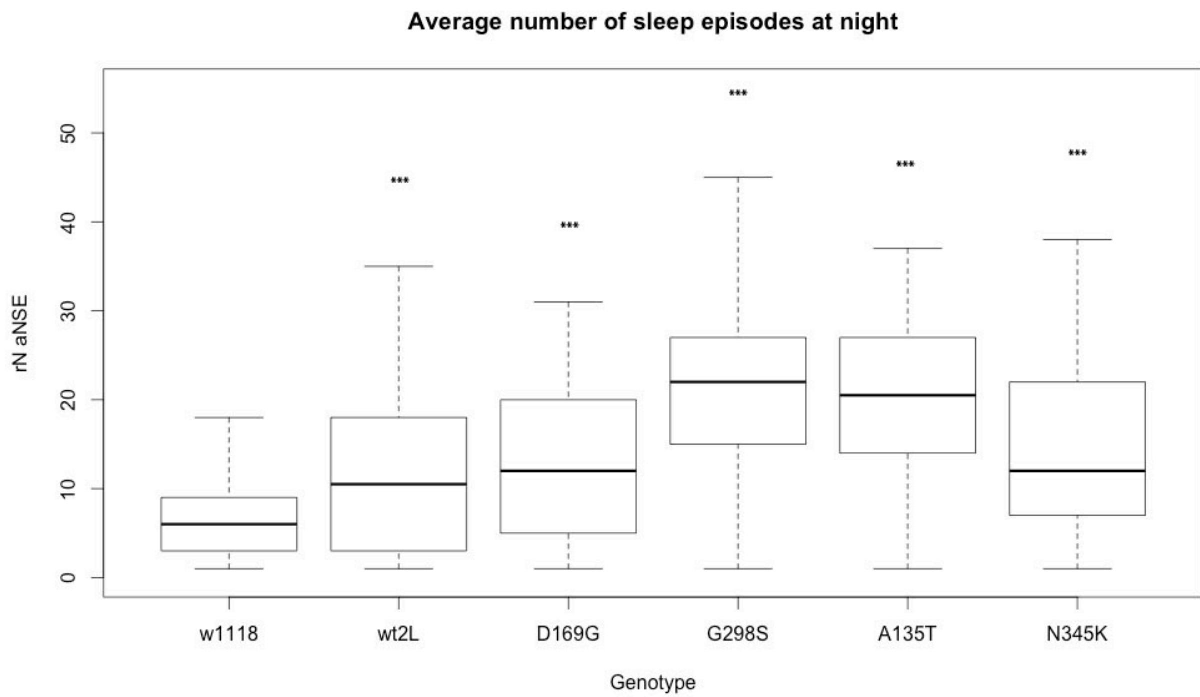

Figure 4. Average number of sleep-episodes during the second 12 hours of the day

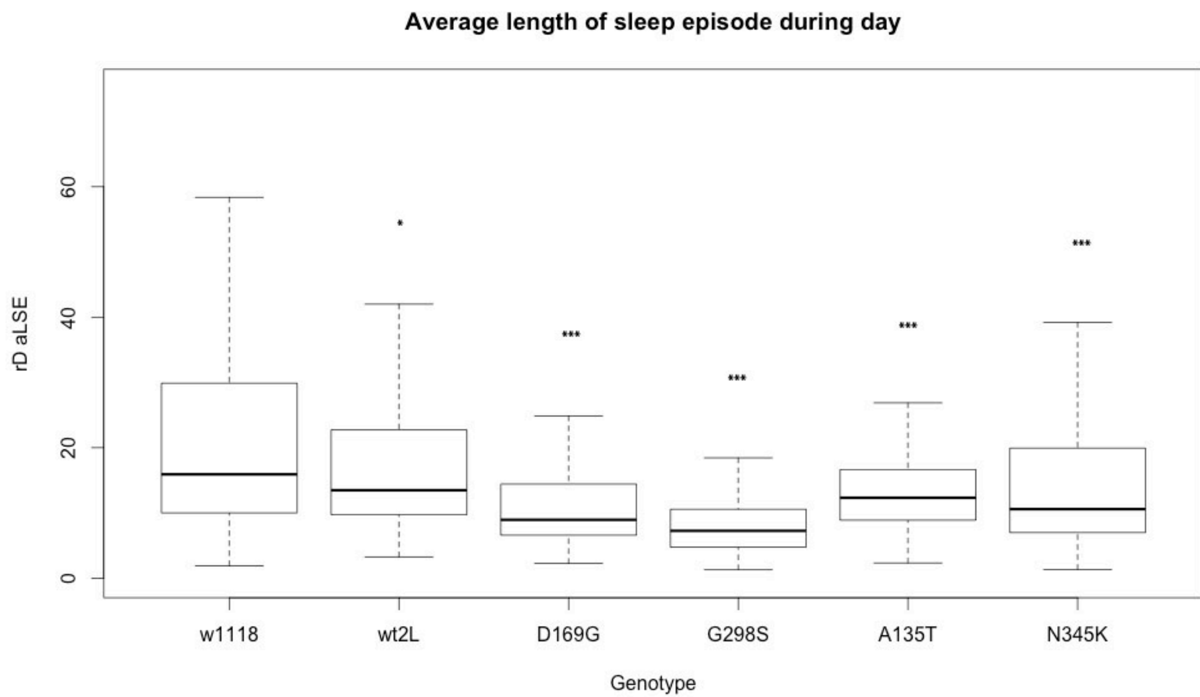

Figure 5. Average length of sleep-episodes during the first 12 hours of the day

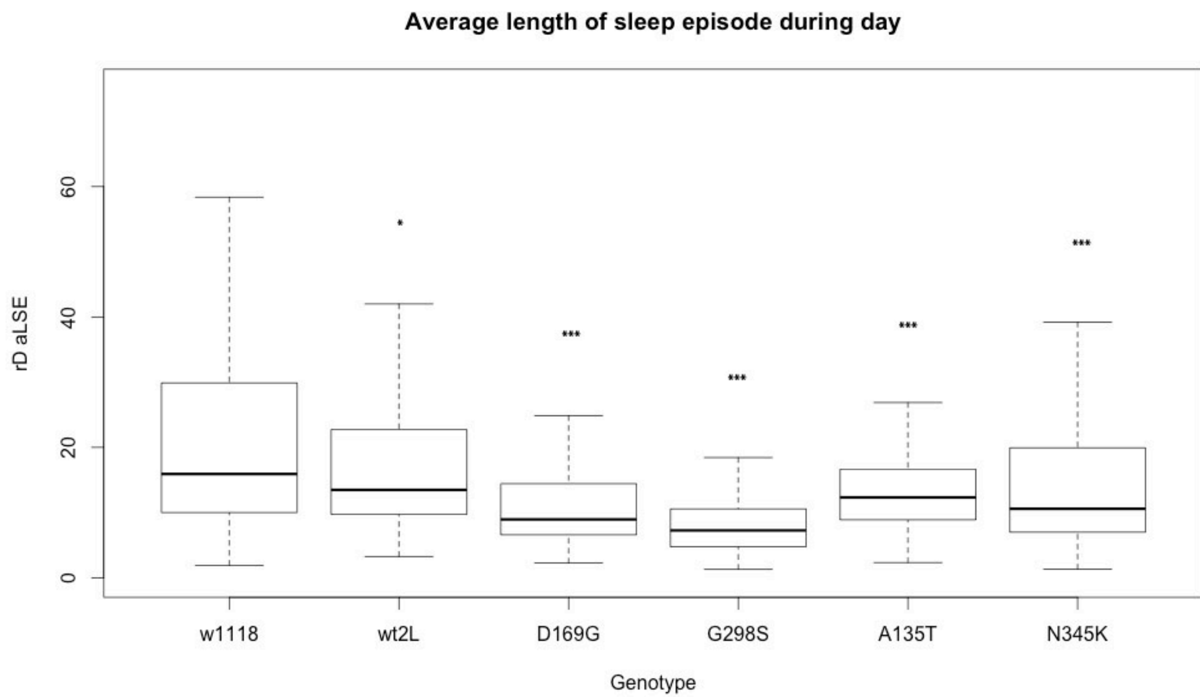

Figure 6. Average length of sleep-episodes during the second 12 hours of the day

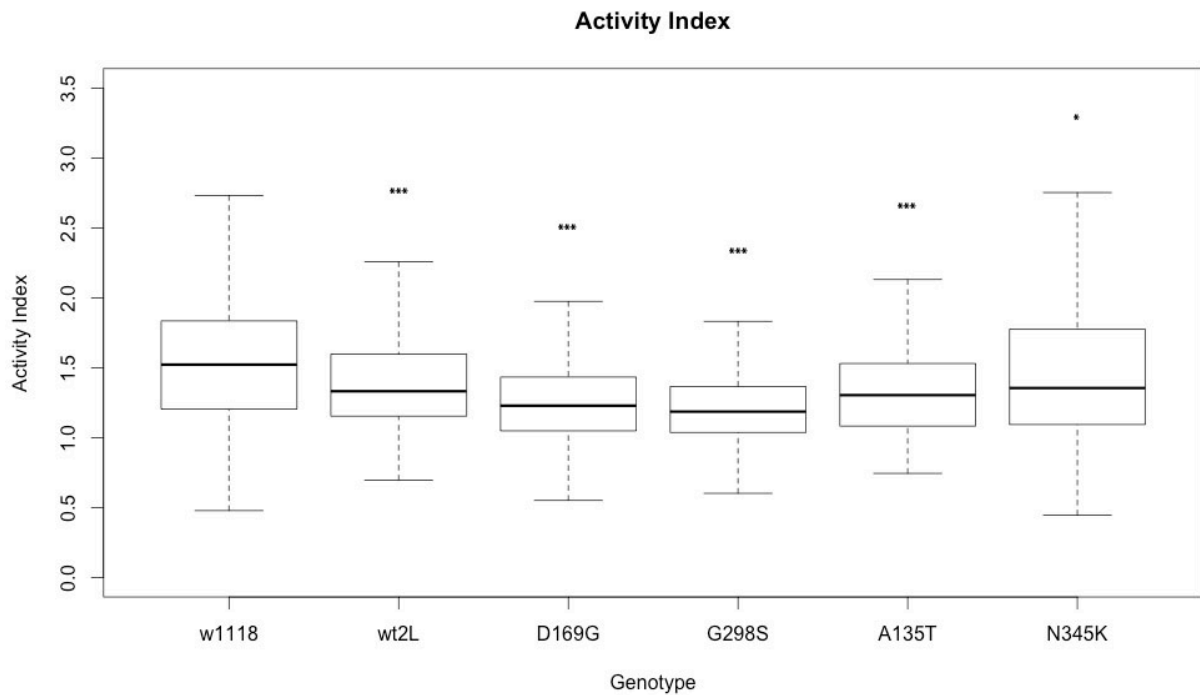

Figure 7. Activity count per minute—number of times on average each fly crosses the infrared beam of a channel in the DAM each minute

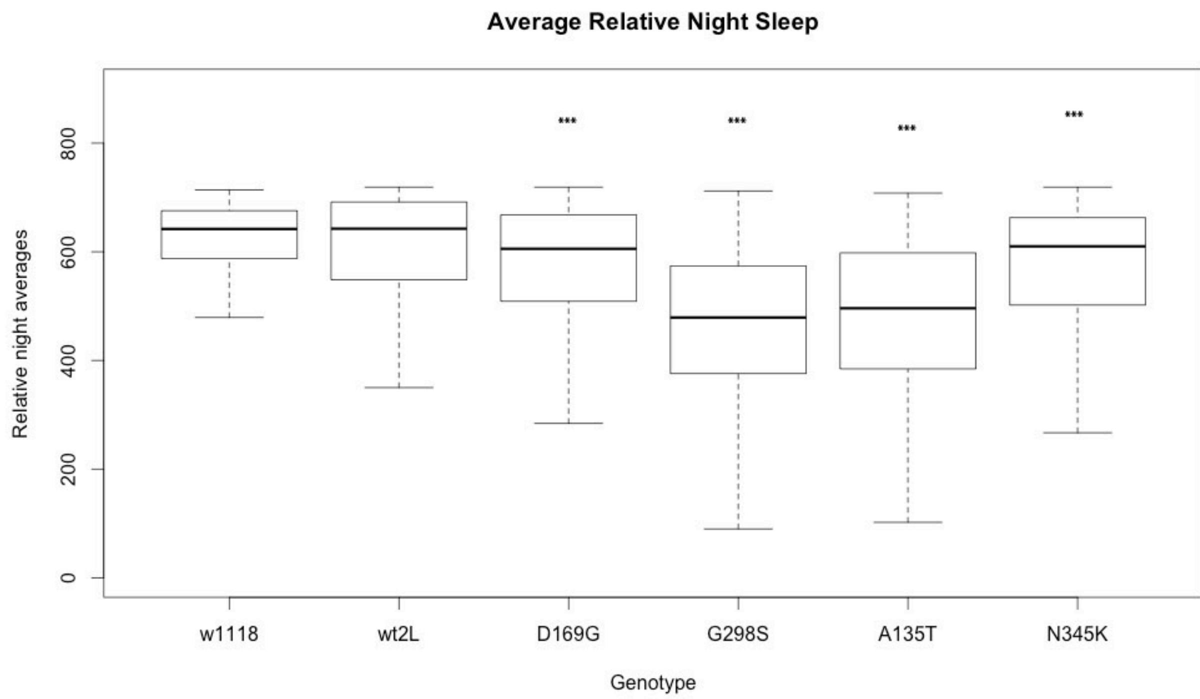

Figure 8. Average minutes of sleep during the second 12 hours of the day

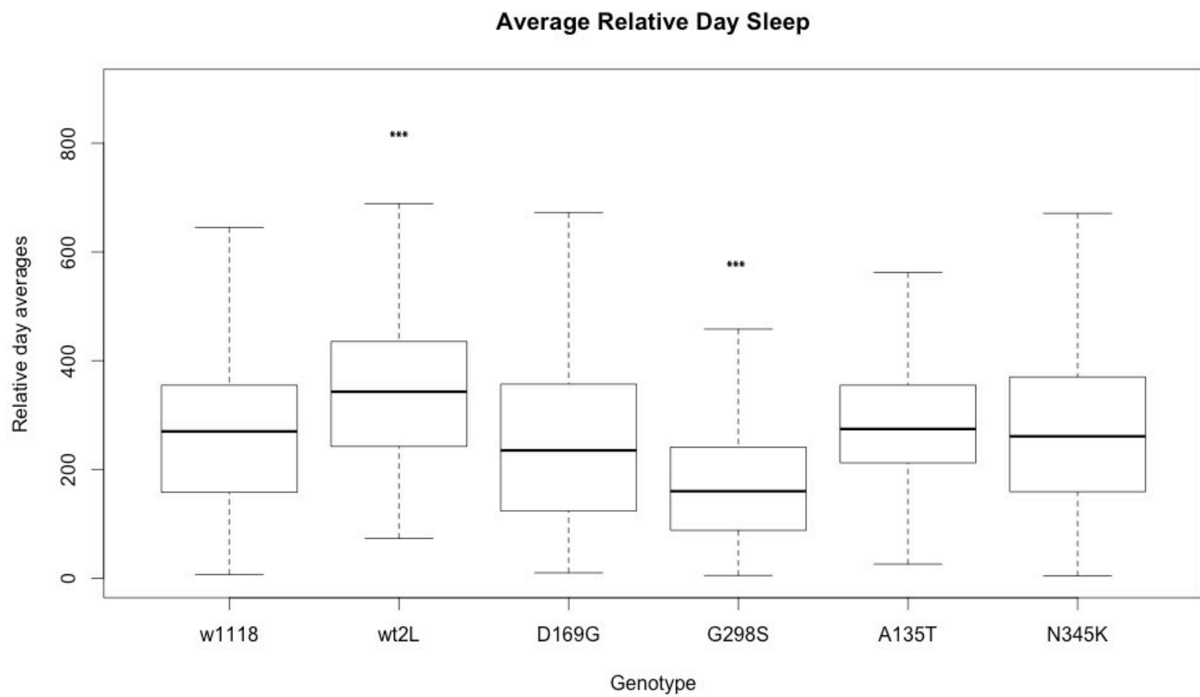

Figure 9. Average minutes of sleep during the first 12 hours of the day

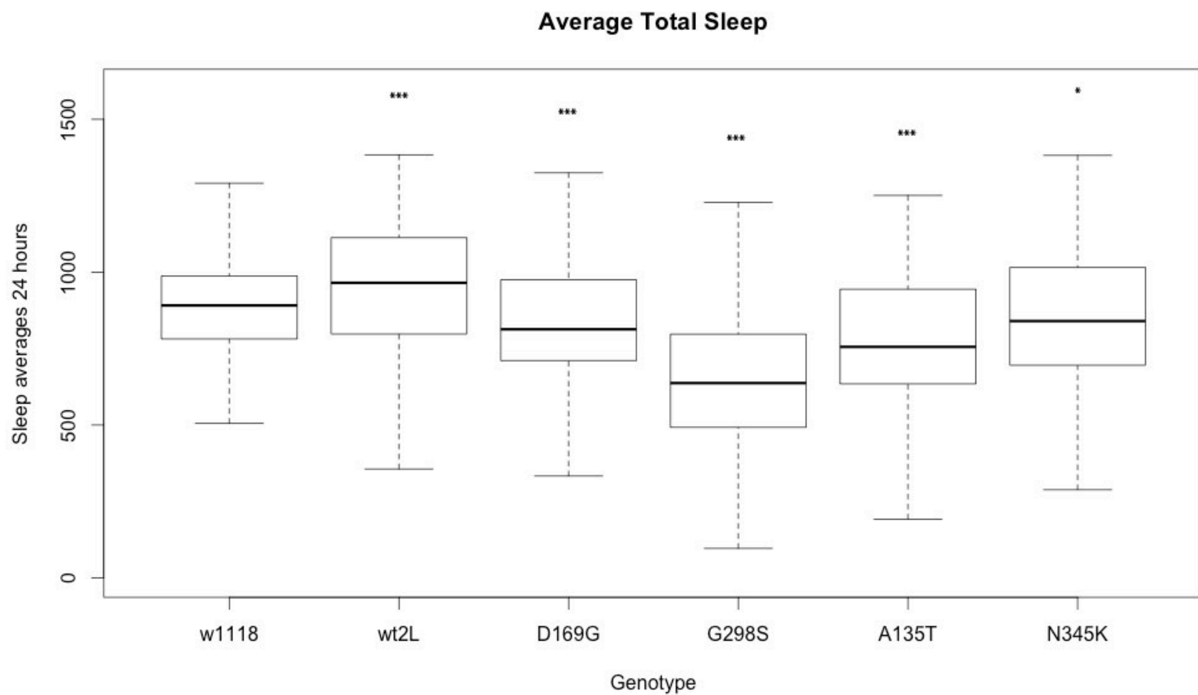

Figure 10. Average minutes of sleep for each genotype during the full 24 hours of a day

Statistics

There was considerable debate concerning the best method to use and analyze the data gathered by the DAM system and subsequently processed in Pysolo and R. Using the Anderson-Darling test for normality we found that the behavioral data was not normally distributed and therefore particularly unsuited for a paired Student's t-test. Instead we employed a Wilcox test, a non-parametric test useful in scenarios where the data distribution is non-normal (Siegal S, 1957). Similarly, skewed data cannot be well represented using a bar-plot because barplots cannot represent the variation of the data effectively. In a boxplot (such as those in Fig. 3-10), the dark banded line represent the median of the data, the bottom and top of the box represent the first and third quartile respectively—taken together the entire box represents the "inter-quartile range". "Whiskers" are drawn by finding the most extreme data point that lies within 1.5 times the inter-quartile range above and below the median. All other data points are labeled as

individual points and are considered outliers. In Figures 3-10, outliers are left out to better visualize the data, though all statistical tests included outliers.

Discussion

Sleep phenotypes

Using the DAM equipment and Pysolo software we were able to quantify not only the locomotor activity of TDP-43 mutants and wild-type flies compared to controls but also their sleep behavior. Sleep has emerged as the most interesting aspect of fly behavior over the course of the experiments. Sleep phenotypes were somewhat unexpected because of the fact that sleep problems experienced by ALS patients are so frequently attributed to respiratory problems (Ferguson et al. 1996, Kiernan et al. 2011). Flies do not have lungs (or bulbar muscles), so an explanation of respiratory dysfunction does not apply directly. A more likely explanation for our findings is one analogous to the problems human ALS patients have with restless movements. It is known that many ALS patients experience aspects of (or are diagnosed with) restless leg syndrome (RLS, Byrne et al. 2011). Some models of ALS suggest a hyperexcitability mechanism. In keeping with this scenario, it is plausible that the expression of TDP-43 in the motor neurons of *Drosophila* impacts the release and re-uptake of neurotransmitters (by a yet to be defined molecular pathway), and this leads to hyperexcitability. This could easily explain the sleep phenotypes observed in this study in the same way that Byrne et al. attributed sleep problems in ALS patients not so much to respiratory dysfunction but to RLS (Byrne et al. 2011)

Worth investigating in the future is the possibility that TDP-43 has a direct impact on actual circadian and sleep function of the brain in *Drosophila* and by extension perhaps in the brain of humans. A possible explanation in our fly model is based on the fact that D42 drives

expression not only in motor neurons, but also in neural stem cells (i.e., neuroblasts, Sanyal et al 2009). Pitman and colleagues found that when certain neuroblasts in *Drosophila* were targeted for chemical ablation using hydroxyurea, flies experienced significantly decreased sleep, and modest increase in sleep bout number (Pitman et al. 2006). Flies expressing D42-Gal4 driven mutant forms of TDP-43 in this project also showed decreased average total sleep and decrease in sleep bout number during both the day and night. Pitman and colleagues assert that their data emphasizes the importance of the mushroom bodies of *Drosophila* on sleep. Though neuroblasts represent only a small fraction of the cells in which D42-Gal4 drives expression, it is possible that the expression of TDP-43 in neuroblasts responsible for generating neurons that control circadian activity may impact the function of the circadian clock and by extension result in the sleep phenotype observed in adult flies.

An important topic of discussion concerning TDP-43 in ALS is the toxicity of TDP-43 aggregates and their impact on cellular processes. Zhang and colleagues showed that overexpression and aggregation of the 25kDa C-terminal fragment generated by caspase cleavage of TDP-43 is detrimental to cells (Zhang et al. 2009). Interestingly, in Drosophila, the overexpression of the C-terminus does not lead to deleterious effects (Li et al. 2010). As discussed, TDP-43 accumulation in inclusions is a common feature of ALS pathology. However, toxicity of TDP-43 aggregates seems like an unlikely explanation for the sleep phenotypes observed in the results of this study specifically because TDP-43 aggregates have not been demonstrated in motor neurons *in vivo,* only in culture (Estes and Zarnescu, unpublished observations).

Differences between TDP-43 variants

A notable result of this project is the fact the wild type TDP flies sleep significantly more on average than controls, whereas mutants sleep less (Fig. 9). Several studies have shown that wild type is toxic in overexpression, but effects vary depending on what is examined in the study. The impact of wild-type TDP-43 may even be opposite to that of other TDP-43 variants in some experimental outputs (Lagier-Tourenne et al. 2010). For example, Estes et al. (2011) found that wild-type TDP-43 has a more severe impact on the structure and viability of neuromuscular junctions and on neuron loss when expressed at similar levels to TDP-43 mutant A315T. However, in terms of locomotion, A315T mutants had a more severe phenotype. As mentioned previously, Ash and colleagues expressed wild type and mutant forms of TDP in *C. elegans*. In part of the study they used a temperature sensitive mutation that allowed them to express varying levels of the TDP transgene through temperature changes. At higher temperatures, transgenic worms exhibited a more uncoordinated phenotype, suggesting that excess TDP results in neurotoxicity (Ash et al. 2010). However, mutant TDP-43 has shown higher toxicity in zebrafish, and rats (Kabashi et al. 2010, Huang et al 2012). Our results show an apparent gain-of-function scenario whereby expression of wild type TDP causes excess sleep whereas mutant TDP causes reduced sleep, consistent with a loss of function phenotype. Our study reiterates a noticeable difference in the effect of wild-type TDP-43 on behavior in *Drosophila* that has now been repeatedly shown. The most likely explanation for these results is that the molecular mechanisms of action of wild-type TDP expression are different from the mutant forms, despite that fact that both wild type and mutants frequently exhibit similar pathology (i.e. toxicity *in vivo*).

Future directions

The differences seen in behavioral phenotypes between flies expressing mutant forms of TDP and flies expressing wild-type TDP open up new avenues of study. Having now shown that wild-type TDP flies sleep more on average than their mutant counterparts, we can begin to ask questions associated with the molecular pathways of sleep in flies expressing TDP-43 and begin to elucidate new molecular mechanisms of this protein. These behavioral studies can also be used as validation assays for future drug screens and other genes of interest. Having a variety of phenotypic assays that tell us different things about ALS pathology and the varying impact of different TDP-43 mutants, we can selectively target aspects of the disease and approach each issue separately.

Similarly, since expression of TDP-43 variants in motor neurons shows sleep phenotypes across the board, we can now begin to ask new questions about how TDP-43 impacts motor neurons (whether it be through toxic aggregation or neurotransmitter release or another undefined pathway) and perhaps if neuroblasts play a more important role than previously considered.

Finally, using the *Drosophila* model of ALS and the procedures developed using the DAM system, we can pursue more avenues of study in *Drosophila* behavior such as aging—a known risk factor in ALS or the effect of expressing TDP-43 in other tissues such as glia. Knowing what we do about sleep and locomotor phenotypes in young *Drosophila*, it is now important to observe how these phenotypes change over the course of a fly's lifetime. Glia are known to take up more than half the space of the human brain, and are altered radically by disease. Almost every aspect of development and function of the brain involves the partnership

of neurons and glia (Barres BA, 2008). This information brings us ever closer to elucidating and possibly developing therapeutic avenues for such a common and devastating disease.

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