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Sleep modifications in a Drosophila melanogaster model of Fragile X syndrome

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Abstract

Fragile X syndrome (FXS) is a neurodevelopmental disorder characterized by intellectual disabilities, disruptions in sleep, and autism in humans. Mutations in Fragile X Mental Retardation gene 1 (FMR1), which codes for a protein that modifies the expression of many target proteins, are primarily responsible for this disorder. Genetic modifications of FMR1 can increase or decrease the overall amount of sleep in humans. A potential pharmaceutical target of FXS is dopamine, a critical neurotransmitter in the regulation of sleep and wakefulness. In fruit flies (*Drosophila melanogaster*) dopamine has been shown to alter sleep. The mushroom body, a structure in the Drosophila brain that regulates sleep and memory, is innervated by dopaminergic neurons and is heavily impacted by low expression of dFMR1. In this study, a Drosophila Activity Monitoring system (DAMs) was used to measure sleep and locomotor activity in Drosophila models of FXS. We found that fruit flies with underexpression of dFMR1 slept more than their control but in shorter periods, which may lead to impaired memory consolidation. Furthermore, the activity of these flies when they are awake is reduced, leaving them with fewer chances to feed, court, and mate. In addition, the mushroom body specific overexpression of dFMR1 in a null background was able to rescue sleep deficits. Further studies on dFMR1 in Drosophila will help us understand how sleep is disrupted and how these changes lead to modifications in behavior.

Keywords: Fragile X syndrome, mushroom body, dopamine, FMR1, sleep

Introduction

Fragile X syndrome (FXS) is a neurodevelopmental disorder and is the most common form of intellectual disability in humans (Specchia et al., 2019). FXS includes but is not limited to hyperactivity, speech impairments, epilepsy, and changes in sleep. In humans, fragile X mental retardation protein (FMRP), the protein translated from the Fragile X Mental Retardation gene 1 (FMR1), expression of this protein is nearly ubiquitous, but it is expressed the most in the nervous system and testes (Dockendorff et al., 2002). The specific brain areas that FMRP is highly expressed in are the cortex, hippocampus, and purkinje cells (Specchia et al., 2019).

One of the genes strongly implicated in this disorder is fragile X mental retardation gene 1 (FMR1). FMRP is a highly conserved RNA binding protein that generally negatively regulates RNA binding proteins (Reeve et al., 2005). However, this protein has the potential to bind to DNA and other proteins (Figure 14). FMRP plays an essential role and helps determine when proteins are translated and when they are not. The FMR1 mutation that generally results in Fragile X syndrome is a trinucleotide repeat of CGG over 200 times when the normal trinucleotide repeat is 10-40 codons ("FMR1 gene," 2010). This makes the gene long and unstable, and the neuron is able to turn off the gene or heavily restrict expression of FMR1. Generally speaking, Fragile X syndrome is attributed to the underexpression of FMR1.

Human FMRP and dFMR1 are highly conserved proteins, they are nearly identical to each other but have slight differences in domain location (Figure 14). FMR1 binds to potentially 4% of all brain messenger RNAs (mRNAs) and then transports these mRNAs to dendrites and regulates local protein synthesis (Tessier & Broadie, 2008). If FMR1 function is lost, then proteins at the local level are made in excess due to the loss of negative translational regulation and this alters synaptic plasticity (Darnell & Klann, 2013). These alterations include changes in synaptic morphology and increases in neuronal branching which is involved in important

functions like learning and memory. In mammals, FMR1 is in a small gene family that contains FMR1, FXR1, and FXR2. Although this gene family is small, their protein products all bind to themselves and to each other, and these proteins can go on to affect other RNA molecules (Zhang, 1995). In addition, there are more mutations that cause FXS than just the CGG trinucleotide expansion. These mutations include large gene deletions, 5'UTR or exon 1 deletions which is sometimes associated with a CGG expansion, and single nucleotide variants (SNVs) in FMR1 coding regions (Quarter et al., 2017). The multiple genetic modifications that lead to FXS caused this experiment to use multiple genotypes of Drosophila hypomorphs to elucidate if certain genetic changes caused different phenotypes in sleep. FMR1 is either inhibited or lost depending on the genotype (Table 1). This leads to little to no protein product from dFMR1 gene and overexpression of many proteins within a cell that are regulated by dFMR1.

In the Drosophila model of FXS, the fruit flies that have reduced expression of dFMR1 have increases in sleep (hypomorph) while the fruit flies with an overexpression of dFMR1 demonstrate decreases in sleep (hypermorph). In Drosophila, FXS causes changes in neuronal plasticity, synapse development, and neural architecture which lead to changes in sleep, learning, and behavior. An important characteristic is that Drosophila exhibit the characteristics of vertebrate sleep with distinct stages (Sitaraman et al., 2015). Drosophila FMR1 (dFMR1) is simpler to study than FMR1 because Drosophila only has one FXS gene, dFMR1, meaning that there is no interaction with other FXS genes. In addition, 60% of the *Drosophila melanogaster* genome is homologous to humans and 75% of the genes of human diseases have a homolog in flies (Mirzoyan et al., 2019). In Drosophila, genes can be inserted and removed from the genome, a procedure that would be impossible to conduct in humans. Drosophila also have a short life cycle compared to humans and they produce many progeny, making it easy to

understand the effects of diseases throughout a Drosophila's lifetime. Drosophila are a short-lived, easy to manipulate organism that produces many progeny that can be used to study many human diseases (Bellen, Tong, & Tsuda, 2010).

One of the regions involved in sleep in Drosophila is the mushroom body (MB) (Figure 15). The mushroom body is an associative learning region in Drosophila and is implicated in changes in memory in FXS (Sitaraman et al., 2015). The mushroom body has also been shown to regulate sleep through the usage of wake-promoting dopaminergic neurons (DANs) and these neurons innervate the mushroom body (Sitaraman et al., 2015). In addition, Overexpression of dFMR1 in hypomorphs in the mushroom body has been shown to reduce sleep, rescuing deficits in sleep (Bushey, Tononi, & Cirelli, 2009). In this study, we targeted dopaminergic neurons in the mushroom body and increased dFMR1 in these neurons to elucidate which neurons are affected by dFMR1 and can rescue sleep defects. We hypothesize that mutations in dFMR1 hypomorphs cause increases in sleep in *Drosophila melanogaster*. In addition to changes in sleep, previous studies have observed immature synapses, impairments in learning and memory, and changes in behavior. Understanding how these changes in sleep occur may lead to a better understanding of the underlying neuronal mechanisms and synaptic morphology changes of FXS. This study aims to come closer to a potential dopaminergic target of treatment for FXS as a result of these sleep changes.

Methods

Fly Strains/Stocks

A 12 h:12 h light-dark cycle was used at 65% relative humidity, at 25°C. To compare the sleep phenotypes of dFMR1 hypomorph lines, wild-type control 5905 and hypomorphs 6928, 67486, 67406, and 6930 of FMR1 flies were collected 1-3 days after eclosion. 5905 was chosen as the control genetic line because it is isogenic for Drosophila chromosomes 1-3 and exhibits

normal memory and circadian rhythm. 6928 is a hypomorphic line that creates a truncated product of dFMR1, making the protein product function limited. Another genotype that has a reduction of expression is 67486, and it also truncates dFMR1 and creates a protein that is unable to bind to other proteins. 6930, is an amophic genotype that makes very little functional protein product. 67403, also creates very little functional protein because it disrupts translation and/or prevents transcription. Male progeny were collected from the control 5905 along with the hypomorphs, 6928, 67486, 67406, and 6930 and continuous collection was performed for 20 days of these genotypes. This allowed the flies to age and then be tested in different age groups (1-5 days, 6-10 days, 11-15 days, and 16-20 days) and see if their sleep changed with age. For mushroom body-specific knockdown, male progeny were collected and tested 3-7 days after eclosion.

Molecular and Genetic Methods

Genomic enhancers in Gal4 and split Gal4 lines were selected based on expression patterns of Gal4 lines using those same enhancers. To determine which neuronal subpopulations were responsible for dFMR1-induced sleep changes, 6928 and 5905 were crossed with mushroom body lines C155, 58E02, and 434B. C155 is a pan-neuronal elav-Gal4 driver and its expression begins in the embryonic nervous system of Drosophila at stage 12 ("Bloomington Drosophila Stock Center Search," n.d.). 58E02 increases dFMR1 expression in dopamine regulating neurons in the mushroom body, these neurons are known as PAM neurons. 434B regulates a different subset of dopaminergic neurons of the mushroom body, these neurons are known as PPL neurons. All GAL4 fly stocks can be acquired from the Indiana University Bloomington Drosophila Stock Center using https://bdsc.indiana.edu/Home/Search.

Sleep Assays

The Drosophila Activity Monitoring system (Trikinetics) was used to measure locomotor activity which was collected in 1 min bins and was also used to measure sleep. This system uses lasers and a computer to count the number of times a fly crosses the beam which is an indicator of waking activity. Male progeny were placed into 65mm x 5mm transparent polycarbonate tubes with standard cornmeal dextrose agar media. Sleep is defined as five minutes or more of behavioral quiescence, or behavioral inactivity and in this experiment, sleep was monitored for three days. We measured total sleep, latency, day sleep, night sleep, waking activity, bout number and bout length. Hypomorphs with the genotypes 6928, 67403, 67486, and 6930 in addition to a control 5905 were grouped based on age (1-5 days, 6-10 days, 11-15 days, 16-20 days).

Once it was determined that 6928 had the largest changes in sleep, we increased dFMR1 expression in the drosophila brain and in specific parts of the mushroom body. The DAM system was used to measure sleep in hypomorph 6928 crossed with different mushroom body neuron lines (C155, 58E02, 434B) and in a control 5905 with the same mushroom body lines. This genetic modification increased the expression of dFMR1 in a subset of mushroom body neurons in an attempt to rescue sleep changes and determine which subsets of mushroom body neurons are involved in FXS. Sleep was monitored for three days.

Statistical Analysis

Prism 8.4.0 (GraphPage Inc, CA) was used for all statistical analyses and graph preparation. For each age group total sleep, day sleep, night sleep, waking activity, bout number, and bout length were compared between wild type and each hypomorph line with a One-Way ANOVA and a Dunnett's test to compare the control to each hypomorph. For each neuronal expression pattern, the same sleep parameters were compared between control and hypomorph line 6928 with a One-Way Independent t-test.

Results

Mutations in dFMR1 increase total sleep in hypomorph 6928

Increases in total sleep were seen in hypomorph 6928 (Figure 1a. $P = 0.0012$; 1b. $P < 0.0001$; 1c. $P = 0.0058$; 1d. $P = 0.0020$) consistently across its lifetime compared to the control 5905 (Figure 1). Hypomorphs 67486 (Figure 1a. $P = 0.0002$; 1b. $P = 0.0020$; 1c. $P = 0.0004$) and 6930 (Figure 1d. $P = 0.0041$) slept for significantly shorter amounts of time than the control, perhaps these hypomorphs were able to compensate for the loss of dFMR1 and decrease their sleep (Figure 1). Determining whether the increases in sleep are attributed to day or night sleep is important to understanding which neuronal mechanisms are involved.

Latency was consistently shorter in 6928 hypomorph than the control 5905

Decreases in latency to sleep in 6928 compared to the control were significantly shorter (Figure 2a. $P = 0.0039$; 2b. $P < 0.0001$; 2c. $P < 0.0001$; 2d. $P < 0.0001$). Latency to sleep in 6928 was about 5 minutes, which is extremely quick. In hypomorphs 67486 (Figure 2a. $P < 0.0001$; 2b. $P = 0.0446$) and 6930 (Figure 2b. $P < 0.0001$; 2d. $P < 0.0001$) increases in latency to sleep were observed. Increases and decreases in latency may be achieved through a variety of neural mechanisms.

Mutations in dFMR1 cause changes in day and night sleep

In an initial series of experiments, we tested multiple hypomorph lines to determine which hypomorph line showed the greatest change in sleep compared to wild type. The purpose of both experiments was to see if day sleep increased because generally, there is a ceiling effect from measuring sleep at night. Measuring an increase in night sleep is difficult because the flies are already sleeping the maximum amount they can. This is called a ceiling effect. Day sleep is a better way to measure changes in sleep because the ceiling is higher. In the experiment where we

screened different hypomorphic lines, the hypomorphs demonstrated statistically significant increased levels of sleep at night, defying the ceiling effect (Figure 4). In addition, there were few differences in day sleep between the hypomorphs and the control (Figure 3).

However, in the experiment where different mushroom body neurons were tested, the 6928 hypomorphs crossed with mushroom body lines slept more than their 5905 controls during the day and did have significant increases in night sleep (Figure 9&10; $P < 0.0001$). The control and hypomorph flies slept for on average 30 minutes per bout, but the hypomorphs had significantly more sleep bouts during the day, resulting in an increased amount of day sleep. What may explain these contradictory results is that the sample sizes were not large enough. A retesting of these assays would yield more conclusive results.

Activity in hypomorph flies is less than their controls

Activity in this experiment was quantified by the number of times a fly crossed the laser beam per minute. Our study found that the activity in 6928 hypomorph flies was significantly lower than the control, 5905 (Figure 5 & 11). 6928 had the lowest OA mean out of all of the hypomorphs which means that they were the least active out of all of the genotypes. However, activity in hypomorphs was able to be rescued. C155, a pan-neuronal Gal4 line, was able to rescue waking activity in 6928 because C155 causes the expression of dFMR1 in all neurons instead of the loss of dFMR1 (Figure 11). In addition, 58E02, a Gal-4 line that targets dopaminergic neurons in the mushroom body called PAM, was able to increase waking activity but not completely rescue it.

Bout number increased while bout length decreased in dFMR1 hypomorphs

Bout number was significantly higher in 6928 hypomorph than the 5905 control (Figure 6a. $P = 0.009$; 6c. $P = 0.0012$). In addition, hypomorphs 67486 (6b. $P = 0.0002$) and 6930 (6d. P

= 0.002) expressed higher bout numbers than the control. When screening hypomorphs (Figure 7) hypomorph 6928 had significantly higher bout lengths than the control 5905. However, when 6928 was crossed with other mushroom body lines its bout length was significantly shorter and was significantly shorter when it was not crossed with any mushroom body lines (Figure 13). In addition, Figure 12 showed significantly higher bout numbers for hypomorph 6928 compared to the control 5905. Sample sizes and age of the flies when testing hypomorphs against the 5905 control may have significantly affected bout length, resulting in an increase in bout length.

Deficits in night sleep were rescued by multiple mushroom body lines

Although total sleep and day sleep were not rescued by mushroom body lines, perhaps these times of sleep use a subset of dopaminergic neurons that was not tested or uses a different neurotransmitter to regulate sleep (Figures 8&9). Increased night sleep in dFMR1 hypomorph 6928 was rescued by C155, 58E02, and 434B mushroom body lines (Figure 10).

Deficits in bout number and bout length can be rescued

In Figure 12, bout number in 6928 was significantly higher than its control 5905 ($P < 0.0001$). However, when 6928 was bred with C155 ($P = 0.0153$) and 58E02 ($P = 0.0029$) bout numbers were significantly reduced but not completely rescued. In Figure 13, bout length in 6928 was significantly higher than its control 5905 ($P = 0.0005$). Bout length was rescued by a Gal-4 line C155, a pan-neuronal line that increases expression of dFMR1 in all neurons.

Discussion

The most common genetic mutation in FXS is a CGG repeat but many others have been reported (Quartier et al., 2017). This study demonstrated that mutations in dFMR1 lead to increases in day and night sleep. Furthermore, decreases in dFMR1 increased the number but not the length of sleep bouts, causing these organisms to wake up frequently throughout the day and

night. When the dFMR1 Drosophila hypomorphs are awake, their activity is less than their controls. They not only have less time to learn novel behaviors than their controls but also take more time to learn these behaviors. Disrupted sleep in *Drosophila melanogaster* is likely to impair memory consolidation, an important process in many organisms. Because the loss of FMR1 decreases generalized synaptic pruning, all neural networks become less efficient in transmitting synaptic signals. The aberrant signaling can lead to impairments in memory consolidation. FMR1 hypomorphs may sleep frequently during the day in order to consolidate their memories. FMR1 hypomorphs may be constantly trying to catch up and consolidate all of their memories efficiently but they cannot because there has not been enough synaptic pruning. This leads to known disruptions in the circadian rhythm (Xu et al., 2012) which further impairs memory consolidation.

How sleep affects memory consolidation

In humans, it is hypothesized that several stages of sleep play a vital role in memory consolidation (Walker & Stickgold, 2014). In mammals, sleep deprivation suppresses memory consolidation (Joiner et al., 2016). A paper by Joiner et al. (2016) demonstrated that sleep in Drosophila is regulated by the mushroom body. In addition, Joiner et al. (2016) described another major function of the mushroom body as the regulator of plasticity in the Drosophila brain, which is strongly implicated in memory consolidation and sleep. The mushroom body is also responsible for consolidating and retrieving memories that are associated with olfactory cues and it uses cAMP signaling to do this (Joiner et al., 2016). Furthermore, specific regions of the mushroom body have been shown to affect specific forms of memory (Joiner et al., 2016). Sleep and memory consolidation are likely to have similar mechanisms in the mushroom body and

dysregulation of synaptic morphology in the mushroom body, sleep or memory formation are likely to affect each other.

Generalized mechanism

Despite the prevalence of this disorder, the exact mechanisms of this disorder are poorly understood. A generalized mechanism for FXS in hypomorphic Drosophila would begin with deficits in synaptic pruning, leaving too many neurons and dendrites in the brain. Blocking metabotropic glutamatergic signaling in dFMR1 amorphs rescues courtship behavior but does not account for the changes in circadian rest/activity rhythms. Blocking this signaling does not explain the defects in mushroom body morphology (Bushey et al., 2009). Abnormalities in multiple pathways are more likely to underlie this complex syndrome than deficits in one pathway (Bushey et al., 2009).

Rac1 and dFMR1 mediate dendritic branching in dopaminergic neurons

A study by Lee et al. (2003) found that dFMR1 binds to GTPase Rac1 and promotes dendritic branching of dopaminergic neurons. This study used point mutations instead of the CGG trinucleotide expansion to study dFMR1's role in dendritic arborization. Rac1 is required for proper dendritic branching in the larval stage within the mushroom body, when higher-order branching in dopaminergic neurons occurs. It is not required in the embryonic stages because that is when the initial growth of dopaminergic neurons occurs. dFMR1 negatively regulates Rac1 and when dFMR1 is lost, increases in the number of higher-order branches is observed. Increases in dopaminergic dendritic branching is likely to lead to changes in learning, memory, and sleep, especially if increases in dopaminergic branches occur within the mushroom body. Because dFMR1 is dose-dependent, different mutations of dFMR1 will cause variable changes in

dendritic branching and with varying severities. This is one way that dFMR1 affects synaptic morphology, sleep, and memory consolidation; there are many other potential pathways.

Synaptic pruning in dFMR1 hypomorphs is decreased

Deficits in synaptic pruning occur in Drosophila with over and underexpression of dFMR1 (Tessier & Broadie, 2008). Decreases in synaptic pruning and increases in dopaminergic dendritic branching may be the foundation to why hypomorphs experience significant alterations in sleep. Many genes are involved in synaptic pruning and dendritic branching, and multiple genetic products cause variability in sleep changes between different FMR1 hypomorphs. dFMRP may regulate the structure of neuronal circuits during development and regulate neuronal plasticity once the organism has matured (Tessier & Broadie, 2008). Furthermore, Tessier and Broadie (2008) stated that the pruning of axon branches during development requires neuronal activity and dFMRP function. This means that axon pruning is activity-dependent and it is regulated by FMR1. If FMR1 is not present or created little protein product, then pruning is not regulated, and little pruning occurs because FMR1 is not activated.

Potential molecular pathway

A molecular pathway in Fragile X syndrome is difficult to elucidate because FMR1 is involved in many physical and neural symptoms. Furthermore, there is FXR1 and FXR2 in humans and their interactions with FMR1. Potential pathways for dFMR1 are histone modifications involved in gene silencing, binding to mRNAs, opening and closing ion channels, binding to proteins like actin and tubulin that regulate cytoskeleton formation and binding to FXR1 and/or FXR2 to regulate protein expression. dFMRP negatively regulates Profilin, a protein that helps insure normal neurons fasciculation and extension (Reeve, 2005). This means that dFMRP is involved in target innervation. In addition, it is believed that Profilin responds in a

dose-dependent manner to dFMRP levels which would lead dFMRP to have an important role in actin dynamics.

If multiple proteins respond to dFMRP levels in a dose-dependent manner, then the severity of dFMRP over or underexpression will have different phenotypes. This demonstrates the importance of the kind of genetic mutation that the researcher is studying. Different mutations could have very different phenotypes depending on how dFMRP levels are affected. Because FMR1 is likely to affect multiple pathways and binds to 4% of brain mRNAs, this protein creates a tree-like pathway for all possible mechanisms. In order to study this disorder, one branch at a time would need to be elucidated.

Implications in behavior

Deficits in sleep in shift workers leads to increased risk in diseases such as heart disease and gastrointestinal disorders (Åkerstedt, 2003). Consistently sleeping too long or too little affects daytime performance. There is an optimal time of sleep for each individual depending on which mechanism their brain uses to regulate sleep. In dFMR1 hypomorphic Drosophila, their activity is lower than their controls, indicating that they not only have less time to eat, socialize, court, and mate but also perform these behaviors slowly.

***dfmr1* expression is required for courtship activity**

Male-specific courtship behavior in drosophila includes but is not limited to following a female, tapping her with his forelegs, wing extension, licking her genitalia, and attempting copulation. In a courtship assay performed by (Dockendorf et al., 2002), nearly 80% of dmfr1 males failed to pass the initial stages of courtship. They only performed following and tapping behaviors. Furthermore, dFMR1 mutant males had significantly less courtship activity compared

to their controls; mutant courtship bouts were consistently short. When male drosophila are active, they do not actively court females and do not do it effectively.

However, in a paper by McBride et al. (2005), they were able to rescue courtship behavior through the mushroom body by using a mGluR antagonist in addition to rescuing short-term memory and morphology deficits within the mushroom body. This study supports the theory that the absence of FMRP leads to the dysregulation of protein synthesis at the synapse. An addition to this theory is that regulation of mGluR activity with antagonists should reverse the phenotypes that are credited to the loss of FMRP function.

Future Directions

Some future directions of this research would be to see if Drosophila with Fragile X syndrome develop an ultradian rhythm. Perhaps it is not that they lose all sense of a circadian rhythm, but it becomes a cycle that is within 24 hours. This could be achieved by using mice and the amount of time they spend wheel running as a measure of activity. Additional studies will need to be conducted to determine which neurons in the mushroom body attribute to changes in sleep in Drosophila. This can be done using sleep assays to determine the neurons most likely involved and then using calcium imaging to see if the neuron is actually involved in sleep.

Limitations

There are potential limitations due to a ceiling effect in overall sleep time. The average amount of sleep for male drosophila is 800-1100 minutes per day while females sleep from about 400-800 minutes per day (Cirelli, 2003). This may result in a ceiling effect when studying increases sleep in male Drosophila. During their lifetime, male Drosophila were socialized with other males. A way to potentially mediate the ceiling effect would be to create social stress by isolating FMR1 flies and seeing if they sleep less due to this stress.

A limitation of the DAMs assay is that it is dependent on the fly crossing the beam every time there is activity. However, Drosophila could still be moving in the glass tube without crossing the beam and this activity is unmeasured in the current DAM system. A novel activity monitoring system that tracks the fly's movements rather than relying on it to cross the beam could be developed and would give a more reliable measurement of activity. This would of course imply that there is software that is able to process video quickly without having to watch hours footage of a fly moving and have to quantify its movements. Furthermore, this study was restricted by small and uneven sample sizes. Generating more data will allow for more definitive conclusions.

Conflict of Interest

The author declares no conflict of interest.

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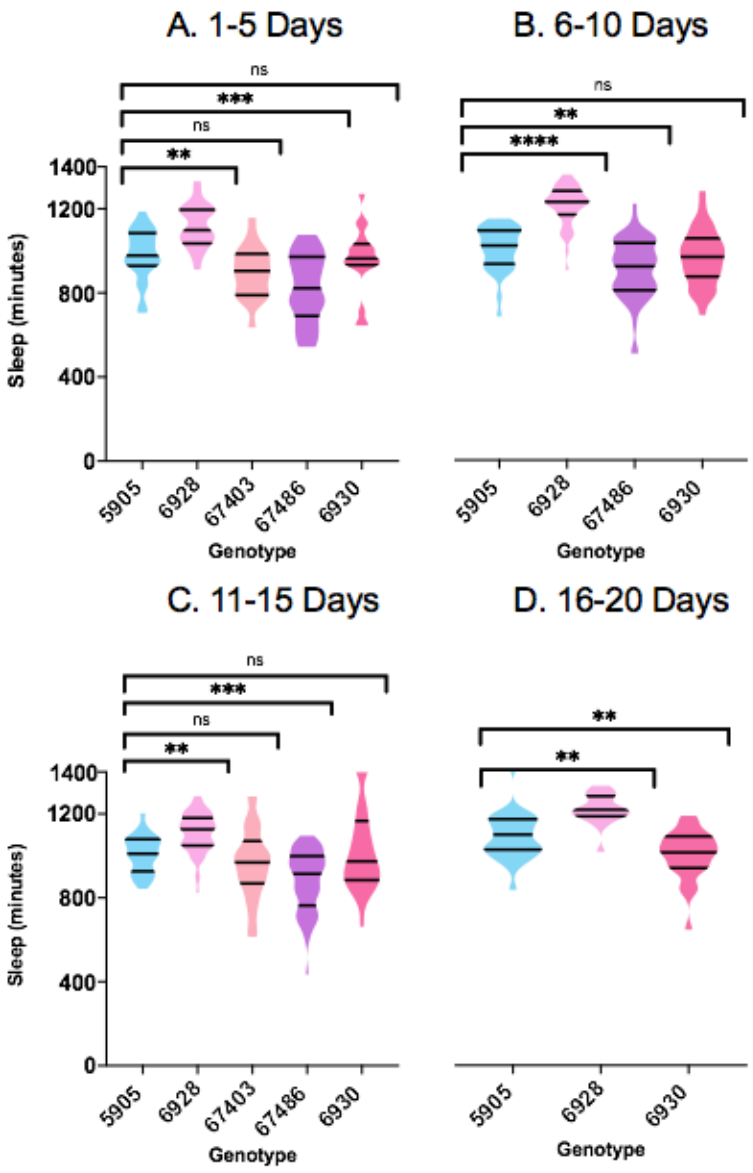

Figure 1. Total sleep: 6928 hypomorphs slept significantly longer than the 5905 control throughout their lifetimes. At 1-5 days (A), 6-10 days (B), and 11-15 days (C) post-eclosion, dFMR1 hypomorphs 6928 and 67486 slept significantly longer than the control 5905. At 16-20 days (D) post-eclosion, 6928 and 6930 hypomorphs slept significantly longer than the control 5905. Significance: **p $\leq$ 0.001, ***p $\leq$ 0.0001, ****p $\leq$ 0.00001.

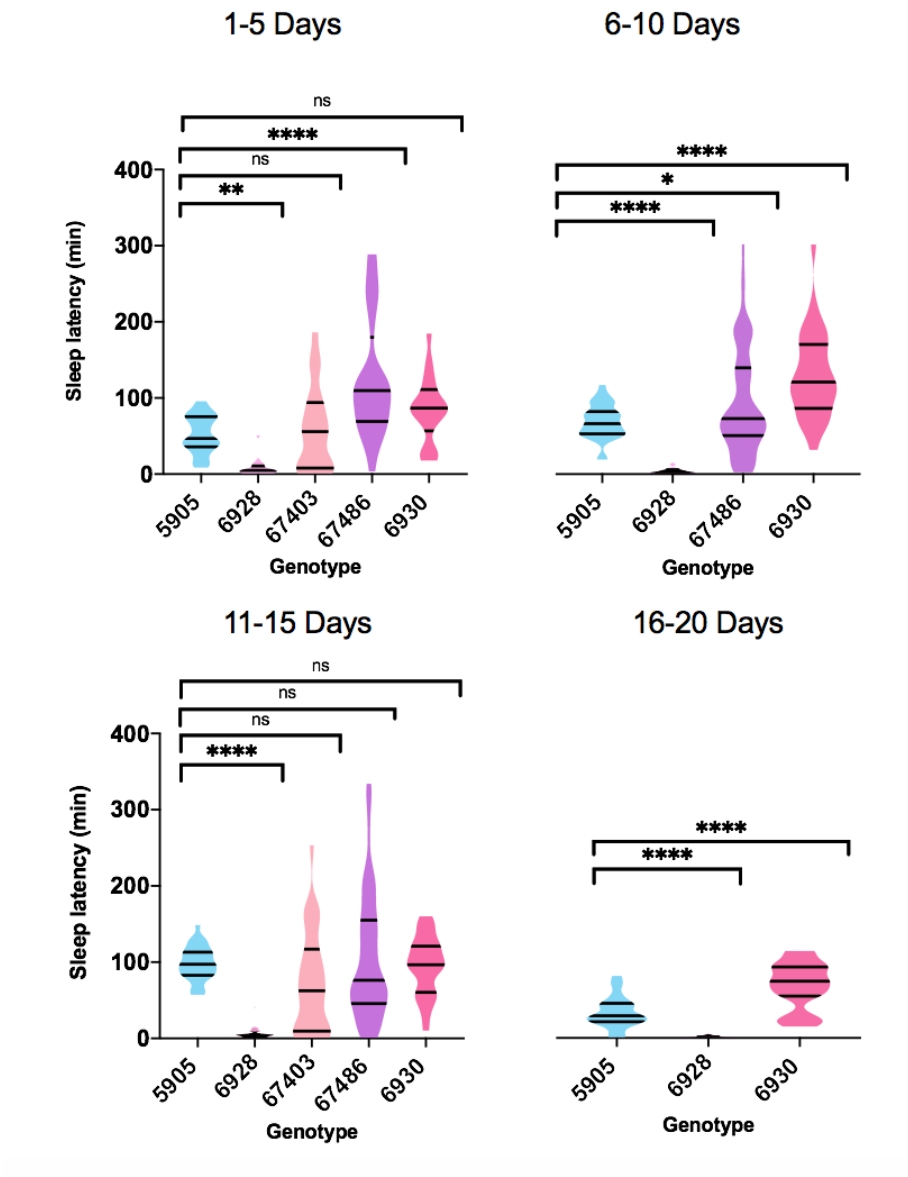

Figure 2. Latency in 6928 flies is significantly lower than other hypomorphs and the control. At 1-5 days (A) post-eclosion dFMR1 hypomorph 6928 had significantly shorter latency and dFMR1 hypomorph 67486 had significantly longer sleep latency than the control 5905. At 6-10 days (B) post-eclosion dFMR1 hypomorphs 6928 had significantly shorter sleep latency and dFMR1 hypomorphs 67486 and 6930 had significantly longer sleep latency than the control 5905. At 11-15 days (C) post-eclosion dFMR1 hypomorph 6928 had significantly shorter sleep latency than the control 5905. At 16-20 days (D) post-eclosion dFMR1 hypomorph 6929 had significantly shorter sleep latency and dFMR hypomorph 6930 had significantly longer sleep latency than the control 5905. Significance: *$p \leq 0.05$, **$p \leq 0.001$, ****$p \leq 0.00001$.

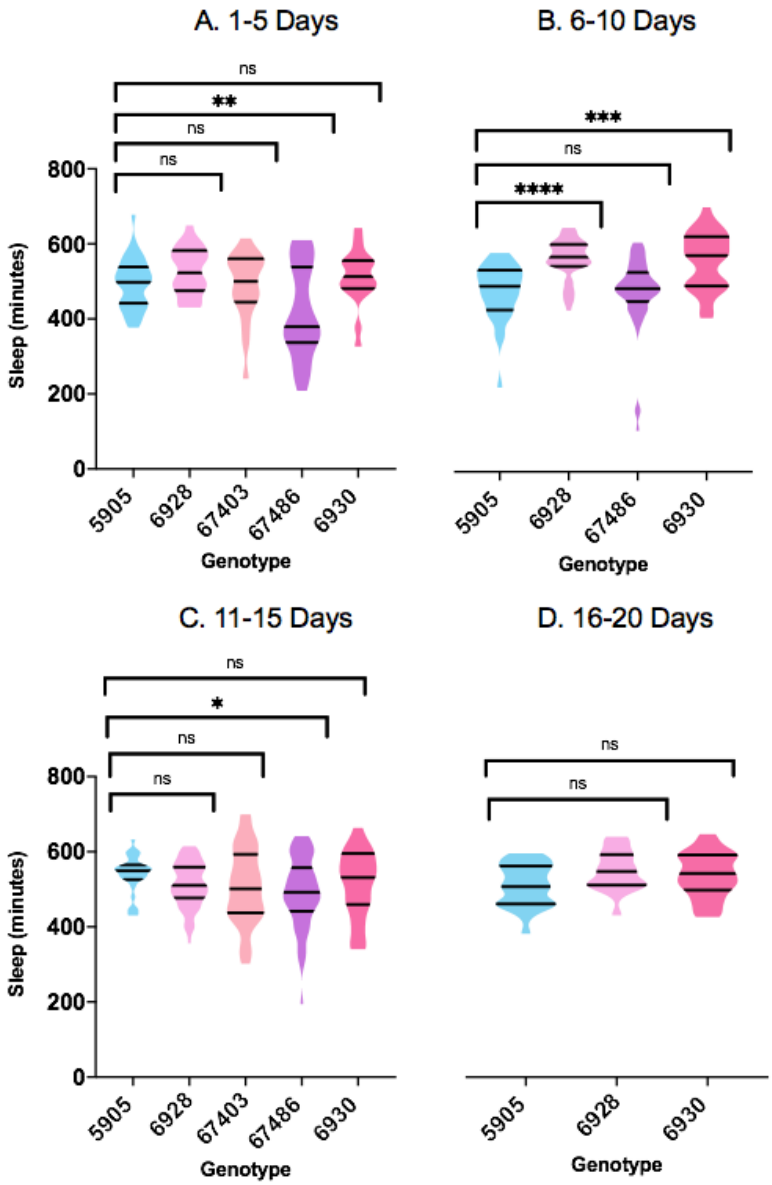

Figure 3. Day sleep has not significantly increased or decreased across genotypes. At 1-5 days (A) post-eclosion, dFMR1 hypomorph 67486 slept significantly shorter than the control, 5905. At 6-10 days (B) post-eclosion dFMR1 hypomorphs 6928 and 6930 slept significantly more than the control 5905. At 11-15 days (C) post-eclosion dFMR1 hypomorph 67486 slept significantly less than the control 5905. At 16-20 days (D) post-eclosion day sleep was not statistically different between the hypomorphs 6928 and 6930 and the control group. Significance: *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.0001$, ****$p \leq 0.00001$.

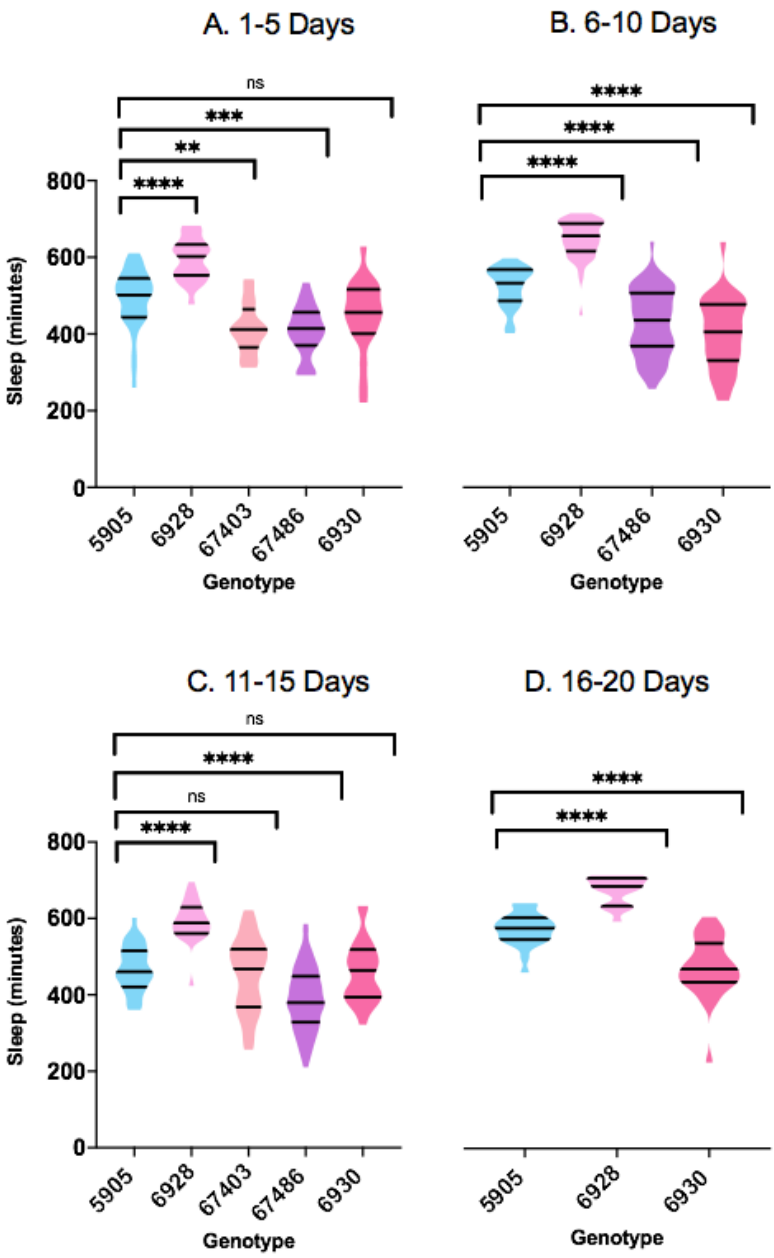

Figure 4. Night sleep significantly increased in 6928 across its lifetime. At 1-5 days (A) post-eclosion night sleep was significantly increased in dFMR1 hypomorph 6928 and significantly decreased in dFMR1 hypomorphs 67403 and 67486 compared to the control 5905. At 6-10 days (B) post-eclosion night sleep was significantly increased in dFMR1 hypomorph 6928 and significantly decreased in dFMR1 hypomorphs 67486 and 6930 compared to the control 5905. At 11-15 days (C) post-eclosion night sleep is significantly increased in dFMR1 hypomorph 6928 and significantly decreased in dFMR1 hypomorph 67486 compared to the control 5905. At 16-20 days (D) post-eclosion night sleep was significantly increased in dFMR1 hypomorph 6928 and significantly decreased in dFMR1 hypomorph 6930 compared to the control 5905. Significance: **$p \leq 0.001$, ***$p \leq 0.0001$, ****$p \leq 0.00001$.

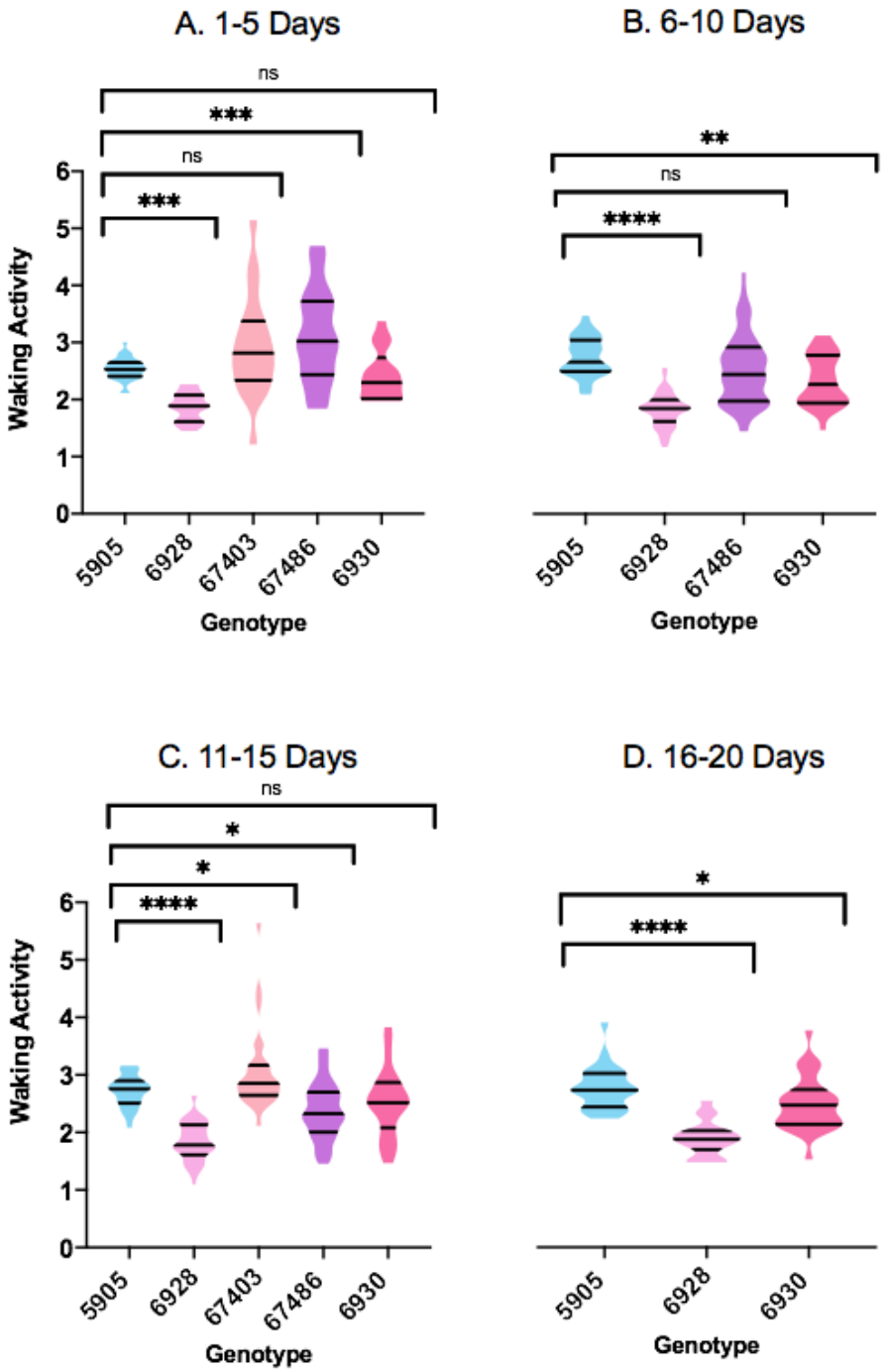

Figure 5. Waking activity was significantly lower in 6928 compared to its control and other hypomorphs. Waking activity is defined as the number of beam crossings per minute. At 1-5 days (A) post-eclosion, waking activity was significantly decreased in dFMR1 hypomorph 6928 and significantly increased in dFMR1 hypomorph 67486 compared to the control 5905. At 6-10 days (B) post-eclosion, waking activity was significantly decreased in dFMR1 hypomorphs 6928 and 6930 compared to the control 5905. At 11-15 days (C) post-eclosion, waking activity was significantly decreased in dFMR1 hypomorphs 6928 and 67486 and significantly increased in dFMR1 hypomorph 67403 compared to the control 5905. At 16-20 days (D) post-eclosion,

waking activity was significantly decreased in dFMR1 hypomorphs 6928 and 6930 compared to the control 5905. Significance: *p ≤ 0.05, **p ≤ 0.001, ***p ≤ 0.0001, ****p ≤ 0.00001.

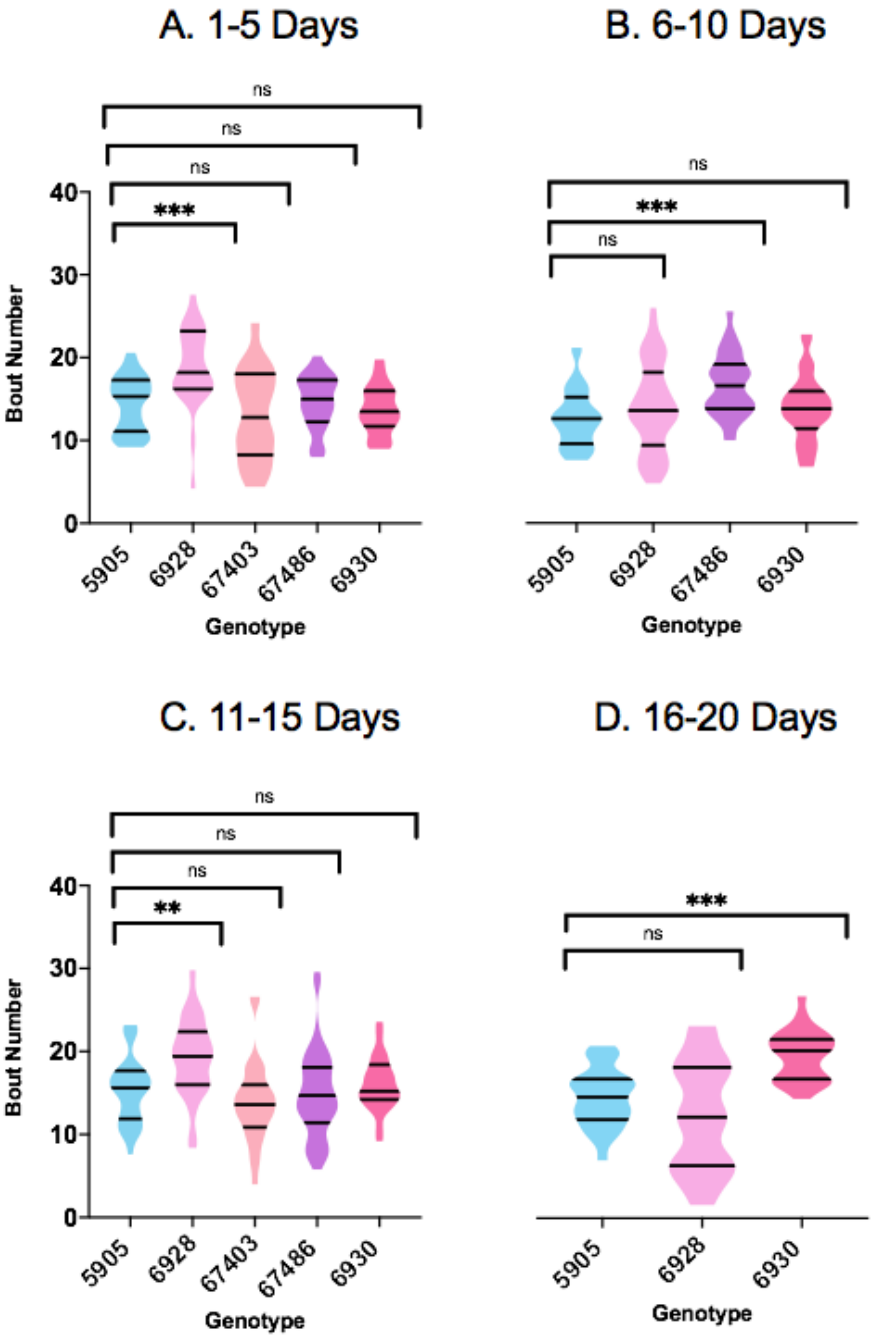

Figure 6. Bout number for 6928 was higher than 5905 at some ages. At 1-5 days (A) and 11-15 days (C) post-eclosion bout number was significantly higher in dFMR1 hypomorph 6928 than the control 5905. At 6-10 days (B) post-eclosion, bout number was significantly higher in dFMR1 hypomorph 67486 than the control 5905. At 16-20 days (D) post-eclosion, bout number was significantly higher in dFMR1 hypomorph 6930 than the control 5905 16-20. Significance: **p ≤ 0.001, ****p ≤ 0.00001.

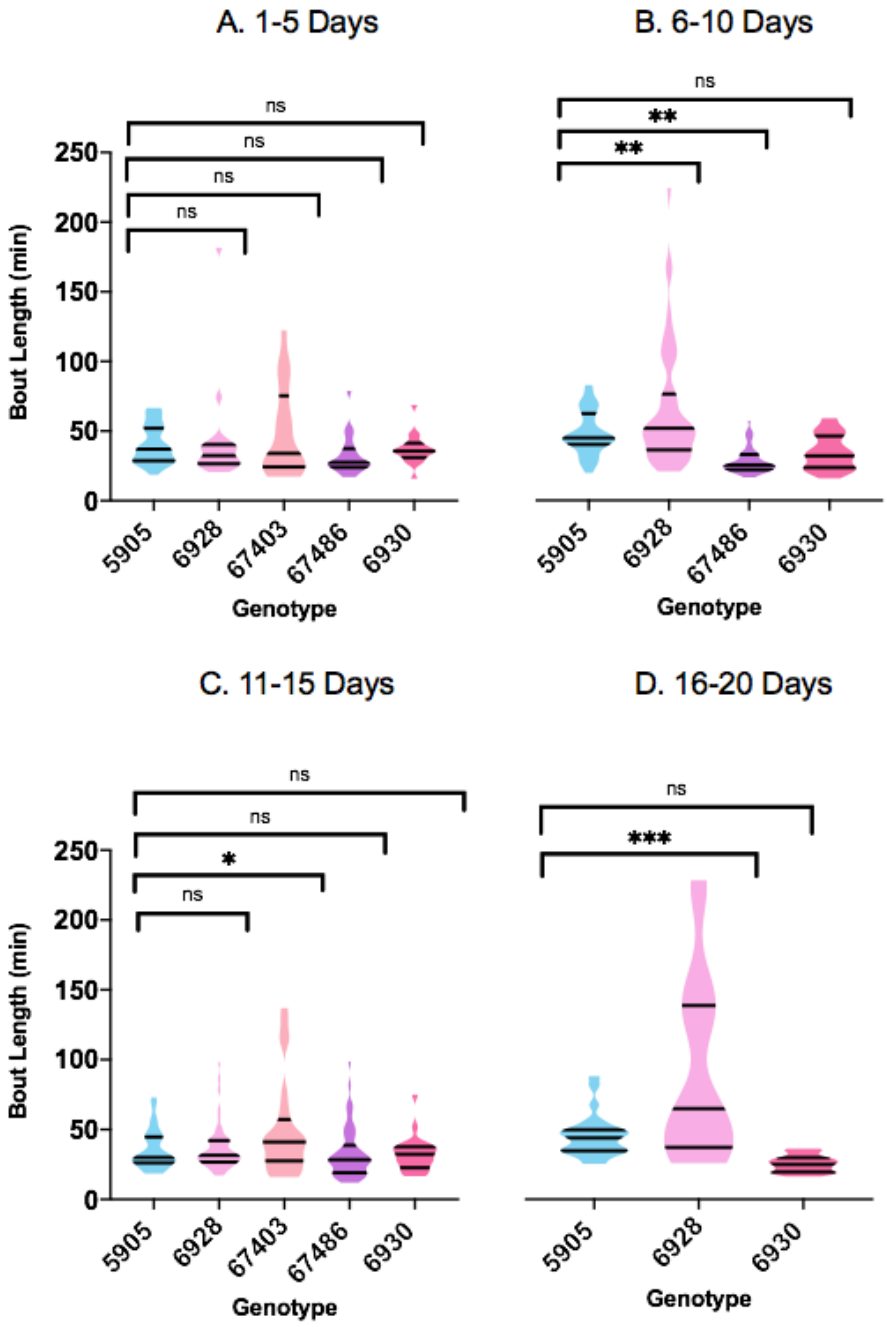

Figure 7. Bout length is significantly higher than control in some hypomorph lines. At 1-5 days (A) post-eclosion, none of the bout lengths were statistically significant when compared to the control 5905. At 6-10 days (B) post-eclosion bout lengths were significantly longer in dFMR1 hypomorph 6928 and significantly shorter in dFMR1 hypomorph 67486 compared to the control 5905. At 11-15 days (C) post-eclosion, bout length was significantly longer in dFMR1 hypomorph 67403 than the control, 5905. At 16-20 days (D) post-eclosion, bout length was significantly longer in dFMR1 hypomorph 6928 than the control, 5905. Significance: *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.0001$.

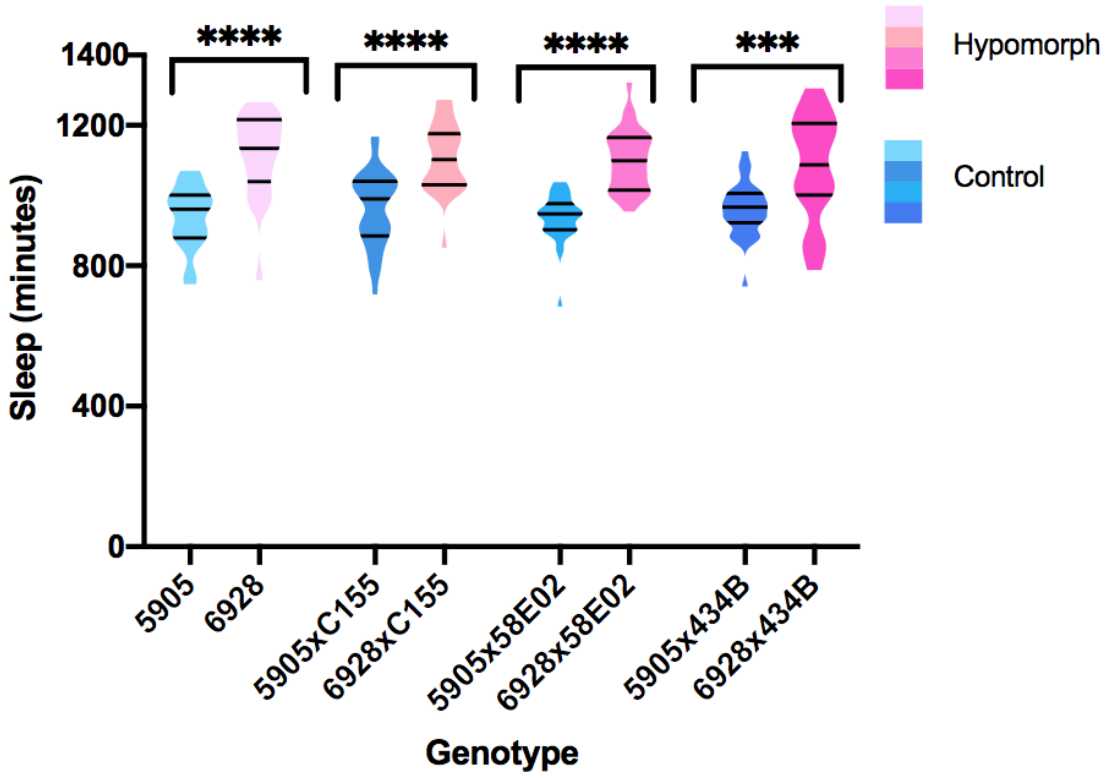

Figure 8. Increased total sleep in the 6928 hypomorph is not rescued by increased expression of dFMR1. Increased dFMR1 expression in mushroom body neurons and pan-neuronally using the UAS-Gal4 system did not rescue increases in total sleep. Significance: ***$p \leq 0.0005$, ****$p \leq 0.0001$.

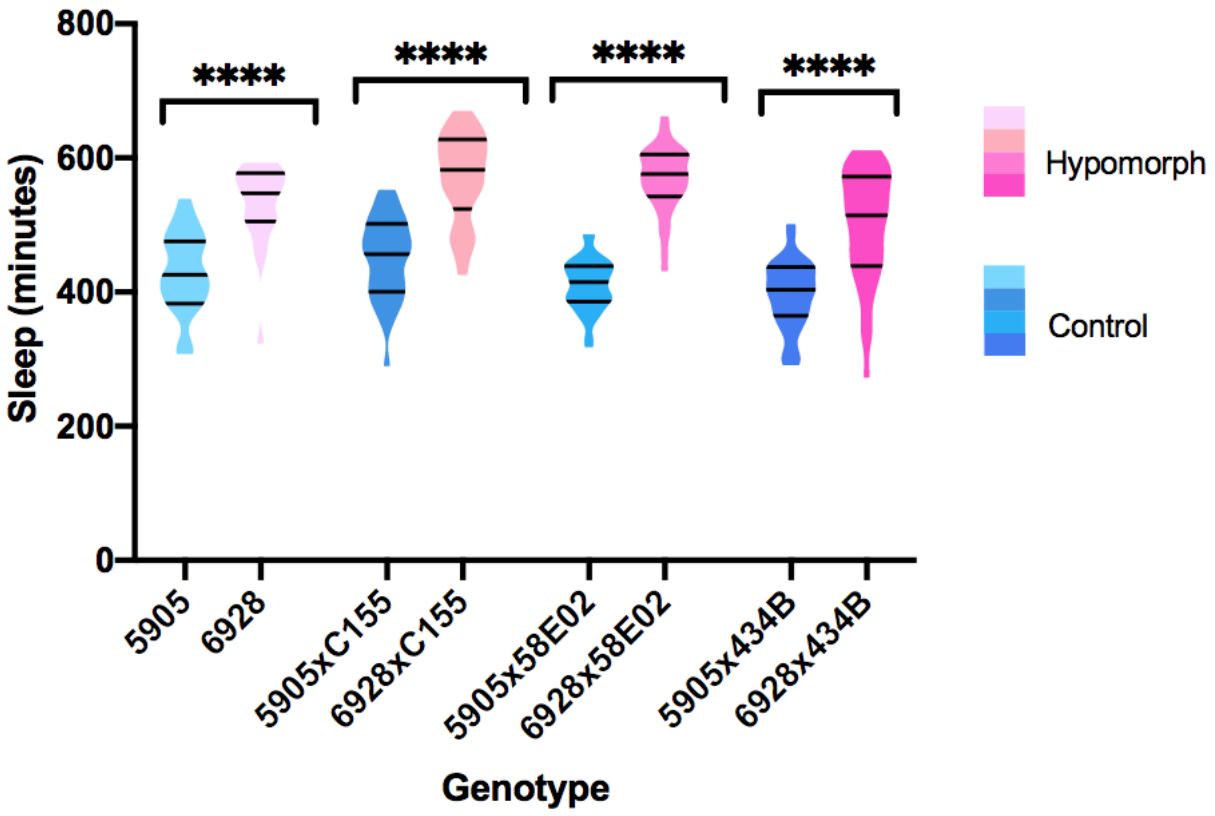

Figure 9. Day sleep is significantly increased in dFMR1 hypomorphs and increased day sleep is not rescued by increased dFMR1 expression. The amount of day sleep was significantly longer for the hypomorphs than the controls. Increased expression of dFMR1 using the UAS-Gal4 system in mushroom body neurons did not rescue these increases in day sleep. Significance: ****$p \leq 0.0001$.

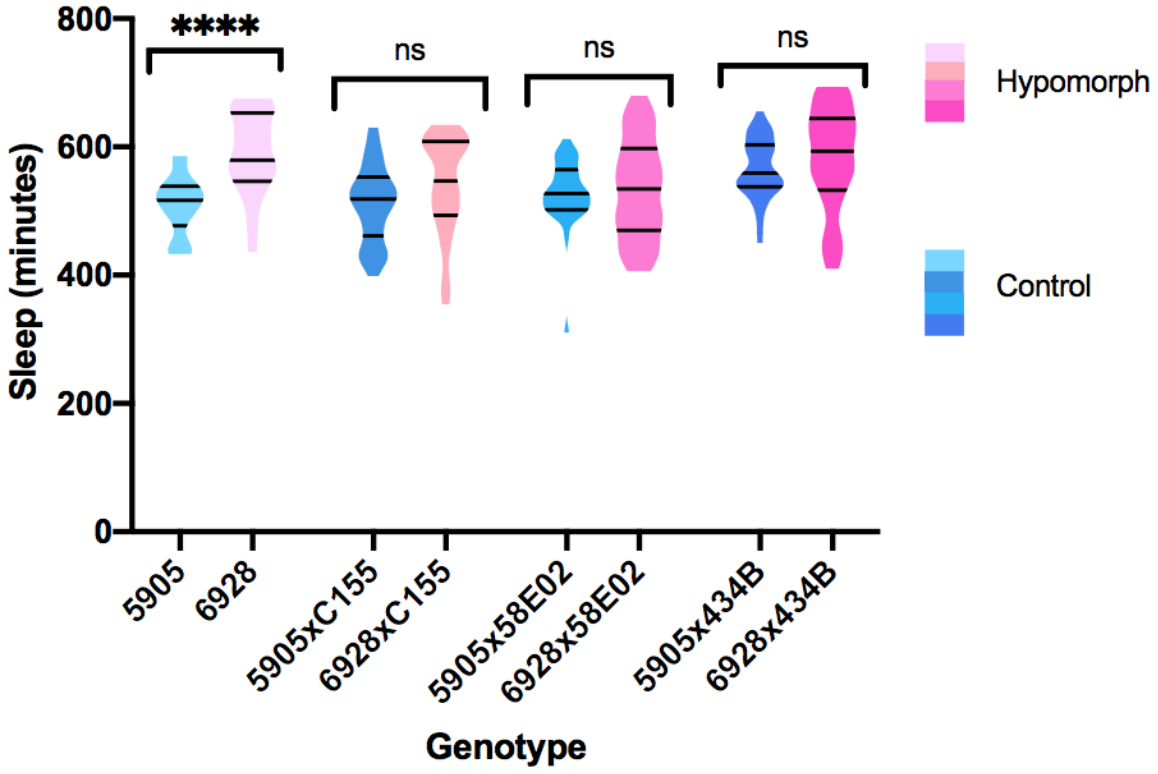

Figure 10. Increased night sleep in 6928 hypomorphs is rescued by increased expression of dFMR1 in mushroom body neurons and pan-neuronally. The amount of night sleep was significantly longer in 6928 hypomorphs compared to their control 5905 when expression was not restricted to specific mushroom body neurons. Increases in sleep were rescued by UAS-Gal4 expression of dFMR1 in C155, 58E02 and 434B neurons. Significance: ****$p \leq 0.0001$.

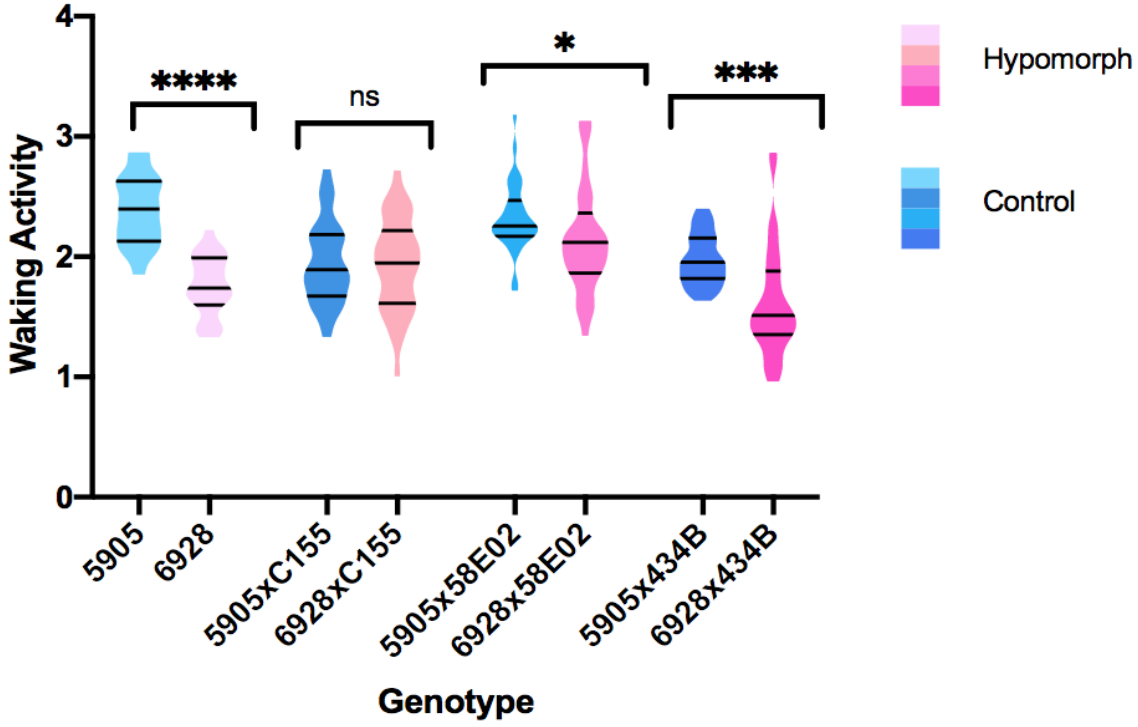

Figure 11. **Decreased waking activity in dFMR1 hypomorphs was rescued by increased expression of dFMR1 pan-neuronally.** Waking activity is defined as the number of beam crossings per minute. Waking activity is significantly lower in 6928 hypomorphs compared to their control 5905. Waking activity was rescued when dFMR1 was expressed pan-neuronally in mushroom body line C155 using the UAS-Gal4 system. Significance: *$p \leq 0.05$, ***$p \leq 0.0005$, ****$p \leq 0.0001$.

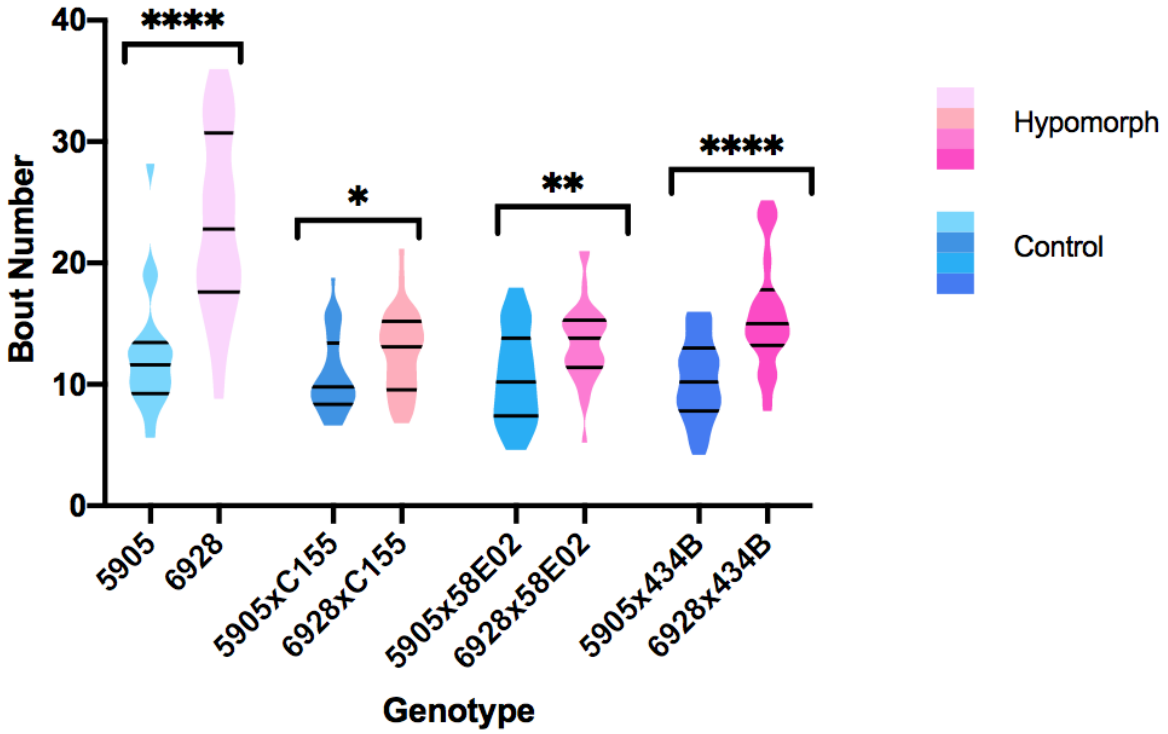

Figure 12. Bout number significantly increased for 6928. Bout number is significantly higher in 6928 hypomorphs compared to their control 5905. When dFMR1 was expressed pan-neuronally using the UAS-Gal4 system, bout number was significantly reduced but not completely rescued by pan-neuronal dFMR1 expression in C133. Significance *$p \leq 0.05$, **$p \leq 0.005$, ****$p \leq 0.0001$.

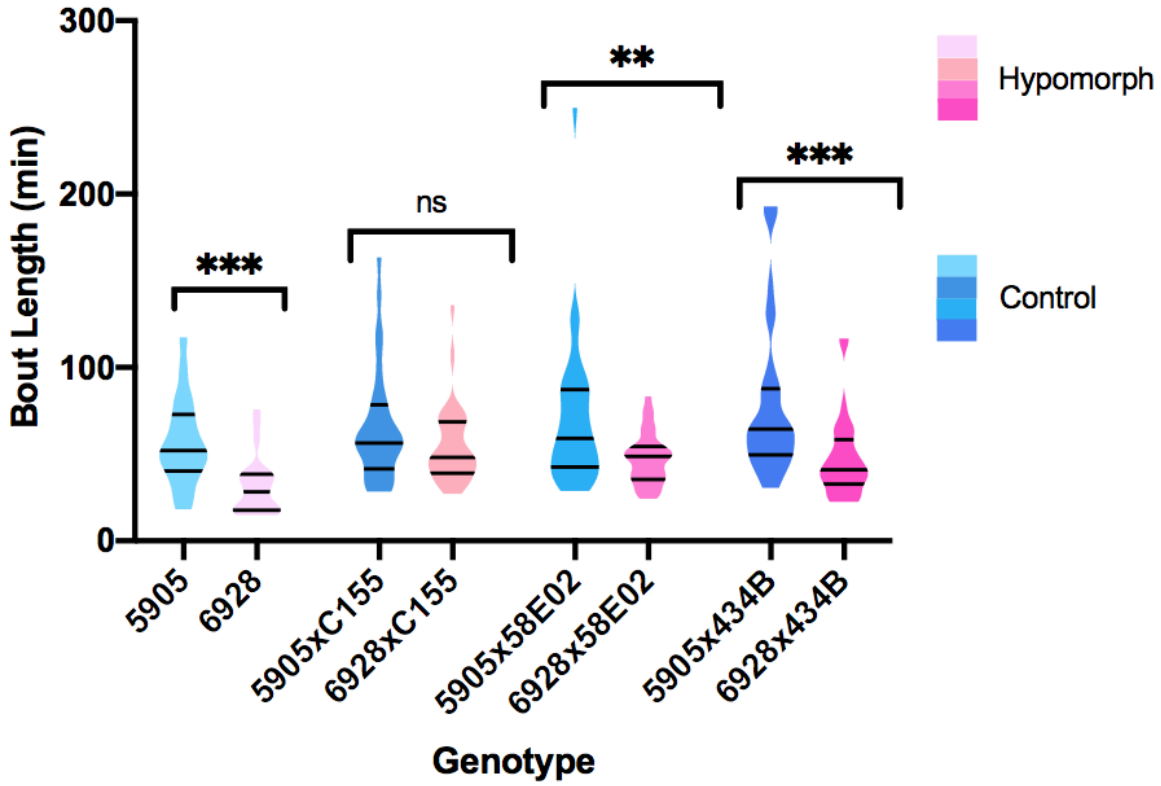

Figure 13. Bout length is significantly decreased for 6928 and this decrease is rescued by pan-neuronal expression of dFMR1. Bout length is significantly lower in 6928 hypomorphs compared to their control 5905. The decreases in bout length were rescued by expression of dFMR1 pan-neuronally using the UAS-Gal4 system. Significance **$p \leq 0.005$, ***$p \leq 0.0005$.

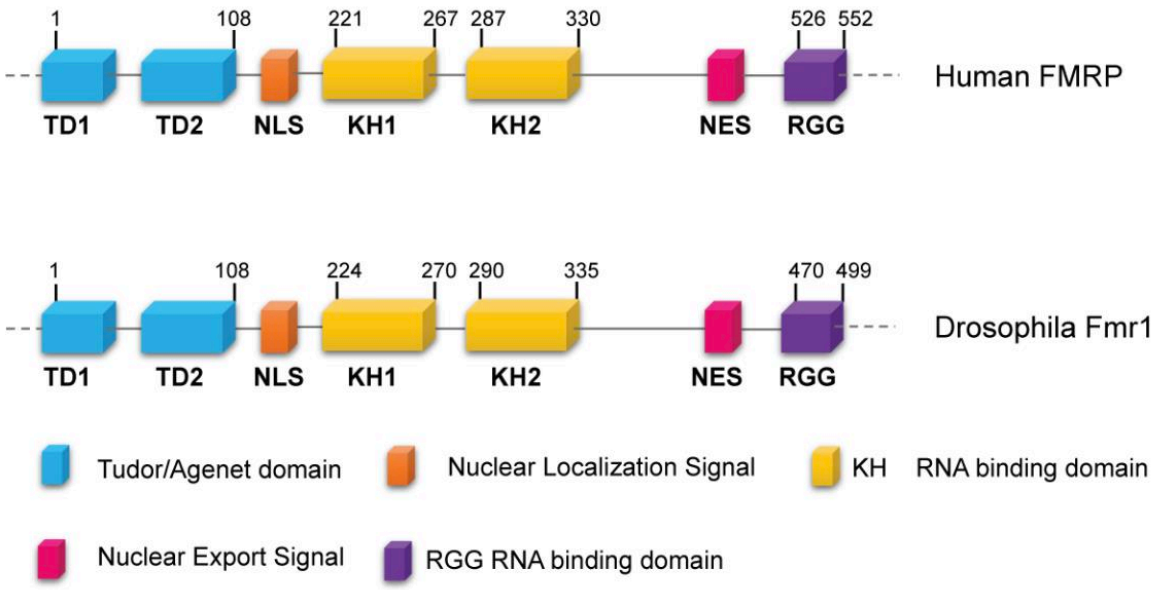

Figure 14. Comparison of human FMRP to Drosophila FMR1. This diagram demonstrates the differences between human FMRP and dFMR1. This figure describes the conserved binding domains of the two proteins. Both proteins contain a nuclear localization signal (NLS) and a nuclear export signal (NES). The two Tudor domains next to each other are likely to be involved in protein-protein interactions and DNA binding. The three RNA-binding domains are the two KH domains and the Arg-Gly-Gly box (RGG). Diagram adapted from Specchia et al. (2019).

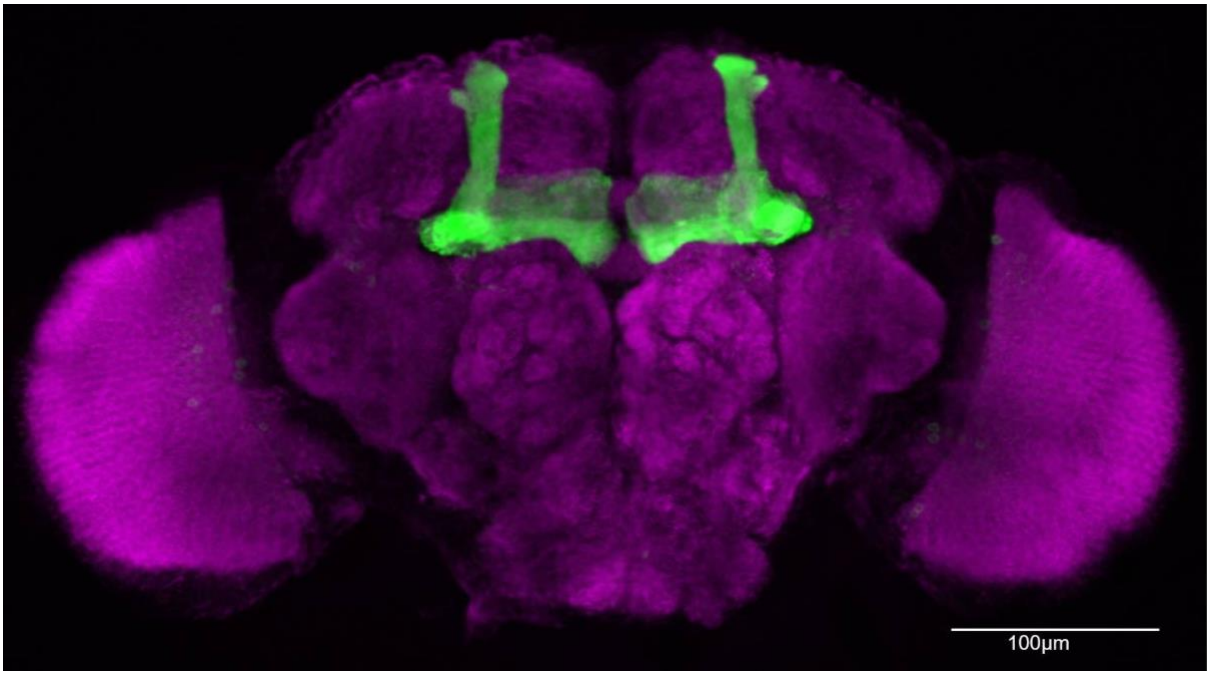

Figure 15. The mushroom body (green) of a Drosophila melanogaster brain. This image was created through the usage of the MB010B Gal-4 line. Diagram adapted from Vogt et al. (2014)

	ID #	Bloomington Stock Number	Genetic Modifications Made	Link to Stock Source
FMR Lines	6928	6928	w[1118]; P{w[+mC]=EP}EP3517 Fmr1[EP3517	https://flybase.org/reports/FBal0131029.html
	5905	5905	w[1118]	https://flybase.org/reports/FBal0018186.html
	67403	67403	w[*]; Fmr1[Delta113M]/TM6B, Tb[1]	https://flybase.org/reports/FBal0131035.html
	67486	67486	y[1] w[*]; Mi{Trojan-Gal4.0}Fmr1{MI09043-TG4.0]/TM3, SB[1] Ser[1]	https://flybase.org/reports/FBti0186273.html
	6930	6930	w[1118]; Fmr1[Delta50M]/TM6B, Tb[+]	https://flybase.org/reports/FBal0131033.html
Mushroom Body Lines	C155	458	P{w[+mW.hs]=GawB}elav[C155]	https://flybase.org/reports/FBti0002575.html
	58E02	41347	w[1118];P{y[+t7.7]w[+mC]=GMR58E02-GAL4}attP2	https://flybase.org/reports/FBti0137105.html
	434B	68325	w[1118]; P{y[+t7.7] w[+mC]=R30E08-p65.AD}attP40/CyO; P{y[+t7.7] w[+mC]=R53C10-GAL4.DBD}attP2	https://flybase.org/reports/FBti0187104.html

Table 1. Genotypes used in this experiment with their genetic modifications. These stocks can be acquired from the Indiana University Bloomington Drosophila Stock Center.

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