Restless Legs Syndrome Model Drosophila melanogaster Show Successful Olfactory Learning and 1-day Retention of the Acquired Memory

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Restless Legs Syndrome (RLS) is a prevalent but poorly understood disorder that is characterized by uncontrollable movements during sleep, resulting in sleep disturbance. Olfactory memory in Drosophila melanogaster has proven to be a useful tool for the study of cognitive deficits caused by sleep disturbances, such as those seen in RLS. A recently generated Drosophila model of RLS exhibited disturbed sleep patterns similar to those seen in humans with RLS. This research seeks to improve understanding of the relationship between cognitive functioning and sleep disturbances in a new model for RLS. Here, we tested learning and memory in wild type and dBTBD9 mutant flies by Pavlovian olfactory conditioning, during which a shock was paired with one of two odors. Flies were then placed in a T-maze with one odor on either side, and successful associative learning was recorded when the flies chose the side with the unpaired odor. We hypothesized that due to disrupted sleep patterns, dBTBD9 mutant flies would be unable to learn the shock-odor association. However, the current study reports that the recently generated Drosophila model of RLS shows successful olfactory learning, despite disturbed sleep patterns, with learning performance levels matching or better than wild type flies.

Abbreviations: LTM - long-term memory, MCH - 4-methylcyclohexanol, OCT - 3-octanol, REM – rapid eye movement, RLS - Restless Legs Syndrome, WT - wild type

Keywords: sleep disorder; olfactory conditioning

Introduction

Sleep is known to play an important role in learning and memory (Stickgold, 2005). Sleep deprivation after learning a task negatively impacts the consolidation of newly formed memories in humans (Stickgold and Walker, 2007), specifically in working memory (Chee et al., 2006), spatial memory (Graves et al., 2003), declarative memory (Backhaus et al., 2006), and visuomotor memory (Maquet et al., 2003) tasks. REM sleep has been strongly implicated in the consolidation of procedural motor learning (Nitsche et al., 2010). This pattern is proposed to reflect the offline processing of newly formed memories and leads to improved performance on the task the following day (Maquet et al., 2003). Compared to sleep-deprived patients, typically-sleeping controls showed both improved task performance and increased functional connectivity between brain regions that support consolidation, such as the superior temporal sulcus and the cerebellum (Maquet et al., 2003). Thus it is apparent that sleep enhances learning and memory consolidation, while sleep deprivation negatively affects consolidation in humans.

A disorder for which the effects of chronic sleep loss on cognitive functioning can be studied is Restless Legs Syndrome. Restless Legs Syndrome (RLS) is a sensorimotor neurological disorder characterized by an urge to move the legs, motor restlessness, worsened symptoms or exclusively present at rest, and worsened symptoms at night (Allen et al., 2003;
Symptoms can be partially or temporarily relieved with movement (Byrne et al., 2006). Sleep fragmentation, disrupted non-REM/REM sleep cycles, is often characteristic of RLS (Trenkwalder and Paulus, 2010). However, the pathophysiology of RLS is still poorly understood. Trenkwalder and Paulus (2010) report that the expression of RLS could involve dopamine neuromodulation, but did not specify where dopaminergic dysregulation is observed in the brain. They found that dopaminergic agents are effective at reducing symptoms in low to moderate doses (Trenkwalder and Paulus, 2010). Unfortunately, it is difficult to dissociate the effects of sleep deprivation from the effects of neurotransmitter dysregulation on cognition. Future research should localize changes in dopamine levels to specific brain regions to understand the relationships between the dopaminergic system, sleep disturbances, and cognition.

Given the observed chronic sleep disruption in RLS patients, past research has identified differences in cognitive performance between patients with RLS and typically-sleeping controls (Pearson et al., 2006) and sleep-restricted controls (Gamaldo et al., 2008). According to Pearson et al. (2006), participants with RLS did significantly worse on two cognitive tasks, a letter fluency test, and trail-making test, than typically sleeping controls. The trail-making test required the subject to connect a set of 25 numbered dots, providing information about visual search speed, mental flexibility, and executive functioning. These tasks were chosen in order to activate the prefrontal cortex, which is particularly sensitive to sleep loss (Pearson et al., 2006). However, Gamaldo et al. (2008) found that RLS subjects performed significantly better on an array of cognitive tasks, such as a letter fluency and category fluency task, when compared to age-matched sleep-restricted controls. The discrepancy between these two studies is likely due to the population that RLS patients are compared to—typically sleeping controls vs. sleep-restricted subjects. Thus, it has been proposed that RLS may be associated with an enhanced physiological level of alertness that compensates for long-term sleep loss (Gamaldo et al., 2008).

Genome-wide association studies have reported that a polymorphism in an intron of the BTBD9 gene of chromosome 6 is associated with an increased risk for RLS (Freeman et al., 2012). The Drosophila gene CG1826 is the homolog to the human gene dBTBD9. Freeman et al. (2012) found that the deletion of CG1826 in Drosophila disturbs sleep similarly in flies as RLS does in human patients (Freeman et al., 2012). Prior to the dBTBD9 mutants, there was no Drosophila model for Restless Legs Syndrome (Freeman et al., 2012). The flies display disrupted sleep and increased motor activity (Freeman et al., 2012). This motor restlessness is associated with sleep disturbances in a similar fashion to what is observed in humans (Freeman et al., 2012).

More generally, Drosophila is a promising model species in which to study sleep, as they exhibit rest/wake patterns similar to those of humans (Cirelli and Bushey, 2008). Sleep in Drosophila has been defined as behavioral immobility for at least five minutes (Li et al., 2009). This sleep state is regulated by both circadian and homeostatic processes (Cirelli and Bushey, 2008). Flies are active during the day and at night, displaying periods of immobility and increased arousal threshold (Cirelli and Bushey, 2008). An increased arousal threshold means that higher stimulus intensities are necessary to produce motor responses; this characteristic distinguishes sleep from quiet wakefulness (Cirelli and Bushey, 2008). Thus, Drosophila dBTBD9 is a potentially useful model for RLS because they display motor restlessness and sleep fragmentation similar to humans.

In this study, we compared the performance of Drosophila dBTBD9 mutant flies to wild type (WT) flies, Canton-S strain, in an olfactory conditioning task. Sleep fragmentation and sleep deficits in humans have resulted in impaired performance on cognitive tasks compared to subjects with more typical sleeping patterns (Pearson et al., 2006). We hypothesized that fragmented sleep loss impairs associative learning and memory retention in the dBTBD9 mutant when compared to typically-sleeping controls. While there has been evidence of higher cognitive performance in RLS patients when compared to sleep-restricted controls
(Gamaldo et al., 2008), this study used typically sleeping controls. Here, we extend previous research on the effects of sleep disturbances on cognitive functioning in a new model of RLS.

**Material and Methods**

*Drosophila* were cultured on a standard medium (Bloomington Fly Stock, 66-112, Bloomington, IN)) at 25°C and 60% relative humidity in a 12h light/dark cycle. Approximately 30 flies were reared in one food vial and transferred to fresh vials once every three days. The *dBTBD9* mutant flies were provided by Dr. A. Freeman of Emory University. The Canton-S strain (WT) was obtained from Bloomington Stock Center (DBst0000001).

*Drosophila* Activity Measurements

Activity of 24 WT and 20 *dBTBD9* mutant flies was measured with the TriKinetics Drosophila Activity Monitor (Waltham, MA), using DAMSystem3038. Activity was recorded between 7 and 9 pm. The light was set to turn off at 8 pm so that flies were subjected to 1 hour of light and 1 hour of darkness.

Odor Discrimination Test

Flies were tested for initial odor discrimination to ensure that both odors were aversive. Both groups of flies, WT and *dBTBD9* mutants, were kept in the same conditions and transferred at three days. Flies were put in an initial chamber of a T-maze and then allowed to stay in the chamber or move to the choice point and choose between a neutral odor and one aversive odor (either 3-octanol, OCT or 4-methylcyclohexanol, MCH) (Sigma-Aldrich; 0.5 μL) (Tully and Quinn, 1985). Flies were kept in the T-maze for three to four minutes. The choice point was defined to be the place where the T-maze split into the two sides. In addition, a test was performed in which flies had to choose between the two aversive odors, OCT and MCH.

Pavlovian Olfactory Conditioning

Behavioral tests were conducted in a room at 25°C temperature and 60% humidity under red light in order to remove visual input (Ejima and Griffith, 2007). Training was performed using the protocol described by Tully and Quinn (1985), which has successfully been used in many *Drosophila* olfactory conditioning paradigms (Li et al., 2009). Flies were exposed to two consecutive aversive odors (3-octanol, OCT and 4-methylcyclohexanol, MCH) for 60 seconds with a 45 second rest between the presentations of each odor. During exposure to the first odor (either OCT or MCH: trials were counterbalanced), the odor was paired with a mechanical shock produced by vortex at 2000rpm in 1.5s pulses for 60 seconds (Lagasse et al., 2012). After 60s of rest, the flies were presented with the other odor. The flies rested for 2 minutes after which the conditioning was repeated two more times. After the conditioning procedure, flies were transferred into the initial chamber of the T-maze where they were allowed to choose between the side with the shock-paired odor, the side with the unpaired odor, or not choose a side by staying in the initial chamber. Flies that learned the task and avoided the paired odor went through a retention test the following day, similar to what has been used previously (Tully and Quinn, 1985). The retention test consisted of the same T-maze as on the training day with the paired odor on one side and the unpaired odor on the other side. Flies that avoided the paired odor and instead chose to go to the unpaired odor were counted as having successful long-term memory (LTM) retention for the paradigm.

Data Analysis

Activity measurements are presented as 24 data points per fly, with each data point indicating the activity for a 5-minute bin. Any activity in a 5-minute period would indicate wakefulness for that time and, thus, no activity would be considered as a period of sleep. (Schaper et al., 2012). The sleep ratio analyzed was the number of sleep bouts within the total number of bins for each phase. Actograms were made with ActogramJ (Schmid et al., 2011). Activity data were analyzed with a repeated-measures ANOVA (SPSS).

Data are presented as mean ± standard deviation (SD) for odor testing. For odor discrimination, preference ratios were calculated per test. Each test was performed with 4-14
flies. In the neutral-aversive test, each fly was given a score of 1 for neutral, 0 for aversive, and 0.5 for no choice. For choosing between the two aversive odors, each fly was given a score of 1 for OCT, 0 for MCH, and 0.5 for no choice. These ratings were converted into a percentage of the total number of subjects per test, and tests were counterbalanced for the left and right side of the T-maze. To determine if flies showed a significant preference for the neutral odor, preference scores were tested using a one sample t-test against 0.5 (not having a preference for one of the odors). Preferences for choosing between the two aversive stimuli was tested using Student’s t-test. For the olfactory conditioning task, chi-squared tests were performed for WT and dBTBD9 mutant flies. Flies that did not choose the paired or unpaired side (i.e., stayed in the initial chamber) were excluded from analysis for the olfactory conditioning task tests.

Statistics
Repeated-measures analyses on sleep ratios were performed in SPSS (Version 19). To compare preference ratios, analyses were performed in Excel. Conditioning tasks were evaluated by calculating the chi-squared values. All statistical analyses utilized an alpha level of 0.05.

Results
Actograms of Drosophila
To compare sleep patterns between WT and dBTBD9 mutants, flies were subjected to both light and dark treatments, and activity was measured at a resolution of 5 minutes. Sleep is defined as 5-minute periods of inactivity (Schaper et al., 2012) (Figure 1). On average, the sleep ratios during the light phase were 0.27 ± 0.06 for WT and 0.30 ± 0.08 for dBTBD9 mutant flies. For the dark phase, the ratios were 0.52 ± 0.07 for WT and 0.39 ± 0.09 for dBTBD9 mutant flies (Figure 2). There was a significant effect of ‘Treatment’ (Light versus Dark; repeated-measures ANOVA, $F(1,42) = 12.780$, $p = 0.001$). There was a trend that approached significance for the interaction effect between Group*Treatment (repeated-measures ANOVA, $F(1,42) = 3.077$, $p = 0.087$), indicating that while both groups behaved similarly during the light phase, dBTBD9 mutant flies sleep less than WT flies in the dark phase.

![Figure 1: Actograms show that RLS D. melanogaster sleep less. Representative examples of actograms from one WT (a, b) and one dBTBD9 mutant (c, d) fly are shown. The gray bar on top indicates the first hour when activity was measured during the light phase; the black bar on top indicates the second hour, when activity was measured during the dark phase.](image1)

![Figure 2: dBTBD9 mutant flies (light grey), but not WT (dark grey), continue to be active in the dark. Mean sleep ratios (± SEM) for WT flies (N = 24) and dBTBD9 mutant flies (N = 20) are shown during the light phase (left two bars) and the dark phase (right two bars).](image2)
WT and dBTBD9 mutant flies are able to discriminate odors

We tested flies on their ability to discriminate odors. When choosing between a neutral odor and an aversive odor, dBTBD9 mutant flies as well as WT flies had a significant preference for the neutral odor compared to the aversive odor (WT: t(2) = 10.661, \( p = 0.009 \); N = 30; dBTBD9 mutant: t(2) = 6.638, \( p = 0.022 \); N= 24). There were no significant differences between WT and dBTBD9 mutant flies (Figure 3).

When choosing between the two aversive odors, both strains exhibited no preference for either odor (WT: t(1) = 1, \( p > 0.05 \); N = 20; dBTBD9 mutant: t(1) = 1, \( p > 0.05 \); N = 21). There were no significant differences in preference ratio between WT and dBTBD9 mutant flies (Figure 4).

dBTBD9 mutant flies are able to learn shock association

We examined learning immediately after flies were trained using Pavlovian olfactory conditioning. Significant differences in learning were found between the WT group (Canton-S) and the dBTBD9 mutant fly group. A chi-squared test was performed to examine the observed effects after training. Flies that did not choose a side were excluded from the analysis. WT flies did not choose the unpaired odor significantly more often than the paired odor, although a trend towards significance was found, \( X^2 (1, N = 62) = 3.630, \ p = 0.0568 \) (Figure 5). However, dBTBD9 mutant flies did show a learned association and avoided the paired odor significantly more often, \( X^2 (1, N = 36) = 13.444, \ p = 0.0002 \) (Figure 5).
Long-Term Memory Tests

We also tested the LTM of the flies after 1 day. Many flies, both WT and dBTBD9 mutants, died overnight and were thus excluded from our analysis. Flies that learned the task went through the same T-maze the following day without any additional training. A chi-squared test was performed to examine retention of the learned association. The result showed that the shock association was retained for the dBTBD9 mutant flies, $X^2 (1, N = 12) = 7.143, p = 0.0075$ (Figure 6). Although all WT flies chose the unpaired odor side (see Figure 6), the number of flies used ($N = 3$) for this task was not large enough to detect a significant preference.

Discussion

The newly developed dBTBD9 mutant of Drosophila has recently become a model for the study of the cognitive functioning in human Restless Legs Syndrome (RLS) patients. The mutant was created by disruption of the gene BTBD9, which is hypothesized to play a role in the regulation of sleep. Mutation of this gene in flies caused phenotypic sleep disruption similar to human expression of RLS (Freeman et al. 2012). The current study shows that dBTBD9 mutant flies are capable of learning a Pavlovian olfactory association with an aversive odor (Figure 5), and that this memory persists after one day (Figure 6). This study provides clues to cognition in RLS model flies, extending upon what is already known about RLS in humans.

As expected, both WT and dBTBD9 mutant flies were more active during the light-phase. Furthermore, dBTBD9 mutant flies may not sleep as much as WT flies during the dark-phase. Although this trend was not significant, a bigger sample size or better techniques to prolong the period of observation may change this result. Many of the flies did not survive for more than several hours during the night. It is
possible that the hyperactivity of dBTBD9 mutant flies caused fatalities due to exhaustion (four out of the original 24 dBTBD9 mutant died during experimentation, versus none in the WT group). In our experiment, none of our flies survived through the entire night, whereas previous experiments have measured sleep activity over 2-3 days (Gilestro, 2012). Although sleep is defined as 5-minute periods of inactivity, the current analysis only includes one possible timeframe of this bin length (Schaper et al., 2012). Bins of shorter durations might represent more accurate sleep bouts.

Interestingly, our findings suggest that dBTBD9 mutant flies are able to learn an odor-shock association and retain this learned material at a higher rate than WT flies despite their fragmented sleep (Freeman et al., 2012). This result is particularly interesting because it is in contrast to predictions that sleep fragmentation limits an animal’s ability to learn and retain associations. It is possible that the ability to learn an odor-shock association in dBTBD9 mutant flies may be a result of the physical characteristics of the strain: specifically, physical weakness in comparison to WT flies (Freeman et al., 2012). One explanation of these findings is that the dBTBD9 mutant flies may have been more affected by the shock, resulting in a stronger association between the shock and odor. In human subjects, Gamaldo et al. (2008) found that patients with RLS performed significantly better on several cognitive tasks than sleep-restricted controls. This suggests that the physiological alertness level may be higher in RLS patients in order to compensate for sleep fragmentation. The results from this study suggest that dBTBD9 mutant flies might show stronger associative learning through increased physical and physiological responses from a shock.

The dBTBD9 mutant flies’ success on the associative task in this study is in strong contrast to past research done with other sleep-disturbed flies. Similar to dBTBD9 mutant flies, hyperkinetic Drosophila display decreased duration of sleep episodes as compared to WT flies (Bushey, 2007). Additionally, related studies in a Drosophila model of Parkinson’s disease (aS) found that sleep deprived aS flies at an intermediate stage of pathology exhibited short-term memory deficits (Seugent et al., 2009). Similar to the aS fly model, decreased cognitive performance has been seen in human patients with Parkinson’s (Hietanen and Teräväinen, 1986). Current theories propose that the sleep disturbances seen in Hyperkinetic and Shaker flies are a main component of the cognitive deficits seen in each mutant. While dBTBD9 mutant flies may exhibit shorter durations of sleep (Freeman et al., 2012), the present study does not report cognitive deficits in associative learning in dBTBD9 mutant flies. Future research should compare associative learning in dBTBD9 mutant flies with Hyperkinetic and Shaker flies to determine specifically which aspects of each disease affect cognitive abilities. Our study was limited by the small number of flies that survived in our task (Fig. 6). Further research should focus on characterizing performance of WT flies in similar cognitive batteries in order to draw more solid conclusions.

We should also consider the cellular mechanisms related to RLS in order to understand how sleep fragmentation may affect cognitive functioning. Dysfunction of the dopaminergic system has been implicated in the symptoms of RLS (Montplaisir et al., 1991; Rye, 2004), and has been studied in Drosophila models of Parkinson’s Disease (Seugent et al., 2009). Fly strains in which dopamine was genetically reduced showed age-dependent loss of DA neurons characteristic of the progression of Parkinson’s disease in humans (Seugent et al., 2009). dBTBD9 mutant flies treated with the drug Pramipexole, which targets D2-like receptors, show improvements in sleep fragmentation (Freeman et al., 2012). Taken together, these results implicate dopamine as a main component of RLS symptoms.

Restless Legs Syndrome affects nearly 10% of adults in the United States. Unfortunately, the molecular pathways underlying development of the disorder remain unclear (Trotti and Rye, 2011). We found that dBTBD9 mutant flies are able to learn using the olfactory conditioning paradigm and retain the association the next day. Our results suggest the RLS fly model can now be used to gain a better understanding of how RLS-related sleep disturbances affect cognitive functioning, as
well as the molecular underpinnings, of the disorder.

Acknowledgements

We thank Dr. A. Freeman of Emory University for graciously allowing use of the recently developed dBTBD9 mutant and for providing these flies for our study. We thank Dr. Gobes for her support and guidance in this project.

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