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Motor restlessness, sleep disturbances, thermal sensory alterations and elevated serum iron levels in *Btbd9* mutant mice

Mark P. DeAndrade[1], Russell L. Johnson Jr[2], Erica L. Unger[3], Li Zhang[4], Thomas van Groen[5], Karen L. Gamble[2] and Yuqing Li[1,*]

[1]Interdisciplinary Program in Biomedical Sciences and Department of Neurology, College of Medicine, University of Florida, Gainesville, FL 32610, USA, [2]Department of Psychiatry and Behavioral Neurobiology, [3]Department of Nutritional Sciences, Pennsylvania State University, University Park, PA 16802, USA, and [4]Center for Neurodegeneration and Experimental Therapeutics, Department of Neurology, School of Medicine and [5]Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

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Restless legs syndrome (RLS), also known as Willis–Ekbom disease, is a sensory–motor neurological disorder with a circadian component. RLS is characterized by uncomfortable sensations in the extremities, generally at night or during sleep, which often leads to an uncontrollable urge to move them for relief. Recently, genomic studies identified single-nucleotide polymorphisms in *BTBD9*, along with three other genes, as being associated with a higher risk of RLS. Little is known about the function of BTBD9 or its potential role in the pathophysiology of RLS. We therefore examined a line of *Btbd9* mutant mice we recently generated for phenotypes similar to symptoms found in RLS patients. We observed that the *Btbd9* mutant mice had motor restlessness, sensory alterations likely limited to the rest phase, and decreased sleep and increased wake times during the rest phase. Additionally, the *Btbd9* mutant mice had altered serum iron levels and monoamine neurotransmitter systems. Furthermore, the sensory alterations in the *Btbd9* mutant mice were relieved using ropinirole, a dopaminergic agonist widely used for RLS treatment. These results, taken together, suggest that the *Btbd9* mutant mice model several characteristics similar to RLS and would therefore be the first genotypic mouse model of RLS. Furthermore, our data provide further evidence that *BTBD9* is involved in RLS, and future studies of the *Btbd9* mutant mice will help shine light on its role in the pathophysiology of RLS. Finally, our data argue for the utility of *Btbd9* mutant mice to discover and screen novel therapeutics for RLS.

INTRODUCTION

Restless legs syndrome (RLS), also known as Willis–Ekbom disease, is a common neurological disorder that has a motor, sensory and a circadian component. It is characterized by an uncontrollable urge to move the legs for relief, generally accompanied by an unpleasant sensation in the legs, with an increase in symptoms during rest or at night (1–4). RLS affects ~3–10% of the general population, with women generally having higher rates than men (2). The symptoms of RLS often lead to sleep disturbances and can severely affect the patient's daytime function and quality of life (5). The primary treatment for RLS is dopaminergics (6,7), but can also include opioids (8,9), anticonvulsants (10,11) or iron supplementation (12–15).

In ~60% of RLS cases, there is a family history of RLS (16–20). Moreover, during evaluations of 12 identical twin pairs in which one or both members have RLS, a concordance rate of 83.3% was found, suggesting a high genetic component (21). Recently, two genome-wide association studies (GWAS) were performed with the aim of identifying polymorphisms in genes that are highly associated with RLS if any existed. In these two studies, single-nucleotide polymorphisms (SNPs), which are single-nucleotide variations that exist naturally within the human population, in four genes were found to impart varying

*To whom correspondence should be addressed. Tel: +1 3522736546; Fax: +1 3522735989; Email: yuqing.li@neurology.ufl.edu

increased risk of having RLS. The genes identified were *MEIS1*, *MAP2K5*, *PTPRD* and *BTBD9* (22,23). As SNPs in *BTBD9* were found to impart an increased susceptibility to RLS in both studies, it made for an excellent candidate gene to study.

BTBD9 has two highly conserved domains, a BTB/POZ domain and a BACK domain, which have been associated with transcriptional regulation, cytoskeleton dynamics and protein ubiquitination (24,25). Previously, a polymorphism in *BTBD9* that has been associated with an increased risk for RLS was correlated with decreased serum iron levels (23). Furthermore, a quantitative trait loci including *Btbd9* was associated with ventral midbrain iron levels (26). However, little is known about the normal function of BTBD9 and how it could potentially be involved in the pathophysiology of RLS.

Additionally, efforts have been made to generate and characterize animal models of RLS. These have included iron-deficient mice (27–31), lesioning of either the A11 dopaminergic nucleus (32–36) or the spinal cord at the T9 level (37) and D3 dopamine (DA) receptor knockout mice (31,38,39). However, as others have noted, these phenotypic models lack clear etiology or symptomology with RLS, thereby limiting their potential utility (40). For instance, no neurodegeneration or gross abnormalities have been found in the A11 dopaminergic nucleus in RLS patients compared with the control (41). Additionally, no mutations or polymorphisms in *D3DR*, the gene encoding the D3 DA receptor, or systemic loss of the D3 DA receptor have been reported in RLS patients to date. This therefore suggests that the D3 DA receptor knockout mice, though a genetically modified line of mice, are a phenotypic mouse model and not a genotypic mouse model of RLS. In this study, we aimed to examine a line of *Btbd9* mutant mice we recently generated to explore its potential utility as a genotypic mouse model of RLS (42). As direct application of standard diagnostic methods for RLS (e.g. International Restless Legs Syndrome Study Group rating scale) are not feasible, we thoroughly examined the *Btbd9* mutant mice for similar, relevant phenotypes. We found that the *Btbd9* mutant mice had motor restlessness, in both voluntary activity and total activity, thermal sensory alterations likely limited to the rest phase, and decreased sleep time and increased wake time during the rest phase. Furthermore, we have found that the *Btbd9* mutant mice had elevated levels of iron in the serum and alterations in the monoamine neurotransmitter system. Therefore, these results suggest that the loss of Btbd9 in mice results in behavioral and biochemical abnormalities that have particular relevance to RLS, including motor activity, sensory alterations and levels of monoamine neurotransmitters and iron. Furthermore, we have found that the thermal sensory alterations in the *Btbd9* mutant mice can be relieved using the dopaminergic D2 receptor-like agonist ropinirole, which is a common treatment for RLS patients. These results taken together suggest that *BTBD9* is involved in RLS, and further studies of the *Btbd9* mutant mice are warranted to examine its role in RLS pathophysiology.

RESULTS

Motor restlessness in *Btbd9* mutant mice

A cardinal feature of RLS is a desire to move. Previous phenotypic mouse models of RLS have shown altered activity levels, including hyperactivity and periodic limb movement-like phenomena (32,37,38). Therefore, to assess the total activity levels of the *Btbd9* mutant mice, we measured activity using an open field activity chamber. We found that the homozygous *Btbd9* mutant mice had an increased total distance traveled compared with wild-type (WT) mice (Fig. 1A, $P < 0.05$). Furthermore, we found that the homozygous *Btbd9* mutant mice had an increase in counterclockwise (CCW) circling (Fig. 1B, $P = 0.05$), while no statistical difference in clockwise (CW) circling compared with WT mice was observed (Fig. 1B, $P > 0.05$). Finally, we saw no significant differences in stereotypical behavior or anxiety in the mice (Supplementary Material, Table S1). The results suggest that the homozygous *Btbd9* mutant mice are hyperactive. Furthermore, alterations in circling behavior in mice have been linked in other studies to imbalances in the dopaminergic system (43,44).

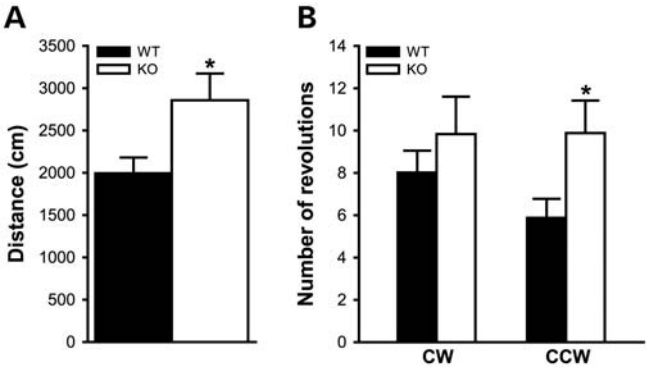

Figure 1. Open field test to measure total activity. (**A**) Homozygous *Btbd9* mutant mice showed an increased total activity compared with WT mice. (**B**) Homozygous *Btbd9* mutant mice showed no alteration in CW circling, but did show an increase in CCW circling. Bars represent means with standard errors of the mean. $^{*}P \leq 0.05$.

Next, we assessed wheel running activity, which measures voluntary activity in a home cage (Fig. 2). In normal 12 h light, 12 h dark (LD) conditions, homozygous *Btbd9* mutant mice exhibited a trend of increase in total counts, which are the counts during both day and night (Table 1, $P = 0.08$), and a trend of increase in counts during the lights-on phase, when the mice would normally be resting or sleeping (Table 1, $P = 0.07$). Next, the mice were placed in constant darkness (DD), during which they typically respond with increased activity. We found that the homozygous *Btbd9* mutant mice exhibited an increase in activity compared with WT mice (Table 1, $P < 0.05$). Additionally, we found that there are no significant alterations in circadian parameters, including period, alpha or amplitude in normal LD or DD ($P > 0.05$, Table 1). These data, taken together, suggest that there is an increase in voluntary activity that corroborates the previous finding of total activity being increased in the homozygous *Btbd9* mutant mice (Fig. 1A).

Thermal sensory alterations in *Btbd9* mutant mice

Commonly associated with the urge to move in RLS patients are uncomfortable sensations in the legs. Therefore, we tested the *Btbd9* mutant mice for abnormalities in the sensory system using the tail-flick test. Heterozygous *Btbd9*

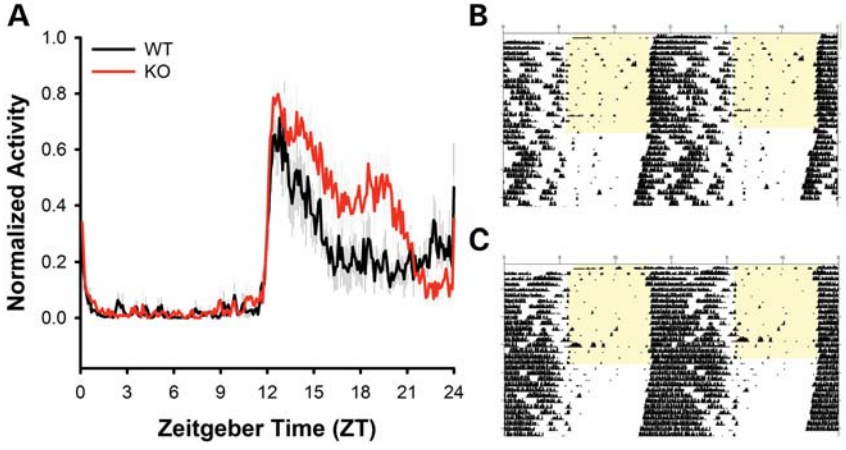

Figure 2. Wheel running analysis to measure voluntary activity. (**A**) Homozygous *Btbd9* mutant mice showed a trend of increased activity during the normal LD cycle. The light was on between ZT 0 and 12. (**B**) Actograms of WT and (**C**) homozygous *Btbd9* mutant mice. Yellow shaded region represents when the lights were on.

Table 1. Wheel running activity parameters during normal LD cycle and DD

Parameter	WT	KO	*P*-value
Normal LD cycle			
Alpha	12.3 ± 1.9	13.6 ± 0.6	0.519
Light counts	55.0 ± 16.2	112.8 ± 23.7	0.067
Dark counts	7.518 ± 1.829	13.440 ± 2.510	0.081
Total counts	7.573 ± 1.845	13.552 ± 2.532	0.081
DD			
Period	23.73 ± 0.05	23.80 ± 0.04	0.289
Amplitude	1.543.7 ± 256.8	1.812.3 ± 292.9	0.504
Alpha	11.5 ± 0.6	11.4 ± 0.6	0.853
Average counts	4.9 ± 2.2	11.1 ± 1.4	0.032*

Alpha (activity period length) and chi-square periodogram-determined period is presented in hours ± standard error of the mean (SEM). Light counts, dark counts, total counts and average counts are presented as number of counts ± SEM. WT, wild-type mice; KO, homozygous *Btbd9* mutant mice.
*$P < 0.05$.

mutant mice had a 27% decrease in time to respond to the warm stimulus (Fig. 3A, $P < 0.05$). Furthermore, the homozygous *Btbd9* mutant mice had a 53.4% decrease in time to respond to the warm stimulus (Fig. 3A, $P < 0.01$). We further dissected this sensory alteration in the heterozygous *Btbd9* mutant mice, as they may represent more of the RLS population, and showed an intermediary deficit. We observed that the heterozygous *Btbd9* mutant mice had no significant sensory alteration compared with WT mice during the middle of the active phase (Fig. 3B—midnight, $P > 0.05$). However, there was a significant sensory alteration in the heterozygous *Btbd9* mutant mice during the middle of the rest phase in comparison with WT mice (Fig. 3B—midday, $P < 0.01$), suggesting a circadian component to the sensory deficit. Next, we injected intraperitoneally WT and heterozygous *Btbd9* mutant mice with a 0.1 mg/kg injection of ropinirole, a common D2 receptor-like dopaminergic agonist given to

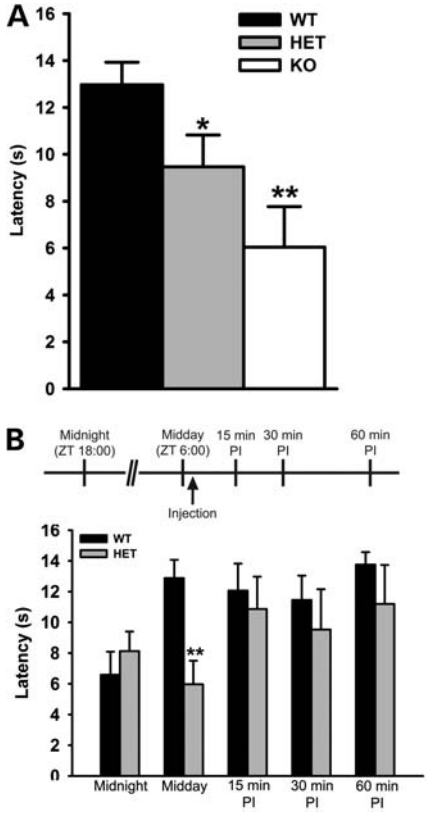

Figure 3. Tail-flick test to determine sensory perception to warm stimuli. (**A**) Heterozygous *Btbd9* mutant mice and homozygous *Btbd9* mutant mice showed a dramatic decrease in latency to respond to a warm stimulus. (**B**) Heterozygous *Btbd9* mutant mice showed no sensory alteration during the middle of the active phase (midnight), but showed a sensory alteration during the middle of the rest phase (midday). However, after a 0.1 mg/kg injection of ropinirole, a DA receptor agonist, there was no statistical difference in latency to respond 15, 30 or 60 min PI. Top schematic describes the experimental design and setup. Bars represent means with standard errors of the mean. *$P < 0.05$, **$P < 0.01$.

Table 2. Polysomnographic sleep parameters during the rest phase

Parameter	WT	KO	*P*-value
Awake	5.50 ± 1.10	15.26 ± 2.36	0.020*
SWS	93.05 ± 1.58	79.04 ± 1.23	0.002**
REM	1.44 ± 1.05	5.70 ± 3.03	0.255
Sleep onset	0.27 ± 0.27	1.43 ± 2.36	0.42
REM onset	141.00 ± 115.17	9.60 ± 4.86	0.32
WASO	5.15 ± 1.43	15.08 ± 2.50	0.03*
Arousal index	11.59 ± 1.24	24.93 ± 2.69	0.01*
TW:TS	5.85 ± 1.22	18.19 ± 3.35	0.03*

Awake, SWS, REM, sleep onset and REM onset were normalized to total time and are presented in percentage of time ± SEM. Wake after sleep onset (WASO) is the time awake after the initial sleep bout and is normalized to total time and presented in percentage of time ± SEM. Arousal index is the number of wake bouts per hour ± SEM. Total awake to total asleep (TW:TS) is the ratio of total awake time to total asleep time ± SEM.
WT, wild-type mice; KO, homozygous *Btbd9* mutant mice.
*$P < 0.05$.
**$P < 0.01$.

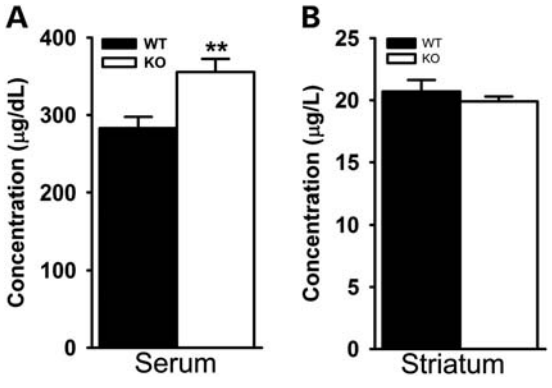

Figure 4. Iron concentrations in the serum and striatum. (**A**) Homozygous *Btbd9* mutant mice showed a significant increase in iron levels in blood serum compared with WT mice. (**B**) However, there was no significant alteration in iron levels in the striatum between homozygous *Btbd9* mutant mice and WT mice. Bars represent means with standard errors of the mean. **$P < 0.01$.

RLS patients (6,45–51). This treatment was able to rescue the sensory alterations at 15, 30 and 60 min post-injection (PI) (Fig. 3B, $P > 0.05$). These taken together suggest that the *Btbd9* mutant mice have a circadian-dependent sensory deficit. More importantly, this sensory deficit is responsive to dopaminergic treatment.

Sleep structure alterations in *Btbd9* mutant mice

Due to the uncomfortable sensations in the legs and the uncontrollable urge to move, patients with RLS often will have fragmented sleep (52–54). To investigate if similar sleep disruptions occur in the homozygous *Btbd9* mutant mice, we implanted homozygous *Btbd9* mutant mice and WT mice with a wireless telemetry system capable of electroencephalographic (EEG) and electromyographic (EMG) recordings of the right tibialis cranialis, which is equivalent to the tibialis anterior muscle in humans. Similarly, we observed that during the rest phase, the homozygous *Btbd9* mutant mice had decreased slow-wave sleep (SWS) (Table 2, $P < 0.01$), no statistical difference in rapid-eye movement (REM) sleep (Table 2, $P > 0.05$) and an increased awake time (Table 2, $P < 0.05$) compared with WT mice. Furthermore, there was an increase in arousals in the homozygous *Btbd9* mutant mice compared with WT mice (Table 2, $P < 0.05$), but no significant alteration in latencies to either sleep or REM sleep (Table 2, $P > 0.05$). These results, taken together, suggest that there is an imbalance in the normal sleep architecture of the *Btbd9* mutant mice similar to RLS patients.

Altered iron metabolism in *Btbd9* mutant mice

Analysis of the iron system has been an emphasis of RLS research. Therefore, to test whether the *Btbd9* mutant mice have an alteration in iron homeostasis, we measured serum iron using a colorimetric assay. We found that the homozygous *Btbd9* mutant mice have an increase in iron levels in the serum (Fig. 4A, $P < 0.01$). We then performed atomic absorption (AA) spectroscopy of homozygous *Btbd9* mutant mice striatum, a critical region of the basal ganglia in the brain. We found that there was no statistical difference in striatal brain iron levels between homozygous *Btbd9* mutant mice and WT mice (Fig. 4B, $P > 0.05$). These results taken together suggest that there is an imbalance in the iron homeostasis, at least in the periphery of the *Btbd9* mutant mice.

Altered serotonergic metabolism in *Btbd9* mutant mice

Another biochemical aspect of RLS that has been a focus of study in RLS is the monoamine neurotransmitter systems. We analyzed the striatum of homozygous *Btbd9* mutant mice using high-performance liquid chromatography (HPLC) for alterations in DA; serotonin [5-hydroxytryptamine (5-HT)] and their metabolites 3,4-dihyroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA). We observed no statistical difference between homozygous *Btbd9* mutant mice and WT mice in DA, serotonin, DOPAC and HVA (Table 3, $P > 0.05$). However, we did find an increase in 5-HIAA, a metabolite of serotonin, in the homozygous *Btbd9* mutant mice compared with WT mice (Table 3, $P < 0.05$). This suggests that while the gross levels of DA and serotonin are not altered, there may be alterations in the metabolism of the monoamine neurotransmitters in the striatum.

DISCUSSION

In this study, we examined a line of mutant mice lacking the complete Btbd9 protein for behavioral and biochemical deficits that could potentially be related to RLS, as recent genomic studies have suggested that polymorphisms in *BTBD9* impart an increased risk of having RLS. We found that *Btbd9* mutant mice had motor restlessness including increased total activity, voluntary activity, and wake time and arousals during the rest phase. Furthermore, the *Btbd9* mutant mice had alterations in thermal sensory perception that was likely limited to the rest phase. Additionally, this sensory alteration could be rescued using ropinirole, a common dopaminergic agonist used to treat RLS patients. Finally, we examined the

Table 3. Levels of DA, serotonin and their metabolites in the striatum

Neurochemical	WT	KO	*P*-value
Epinephrine	3.62 ± 1.468	3.21 ± 0.53	0.61
DA	44.05 ± 5.85	41.28 ± 4.53	0.25
Serotonin (5-HT)	1.30 ± 0.04	1.40 ± 0.07	0.50
DOPAC	15.60 ± 0.98	17.12 ± 1.40	0.74
5-HIAA	0.26 ± 0.04	0.47 ± 0.07	0.04*
HVA	20.20 ± 2.21	22.29 ± 1.57	0.80
DOPAC/DA	0.37 ± 0.08	0.43 ± 0.04	0.68
HVA/DA	0.43 ± 0.03	0.56 ± 0.05	0.11
5-HIAA/5-HT	0.20 ± 0.03	0.34 ± 0.06	0.07

The values of neurochemicals represent means ± SEM in pmol/g of tissue. The turnover of metabolites is shown as ratios of neurochemicals.
WT, wild-type mice; KO, homozygous *Btbd9* mutant mice.
*$P < 0.05$.

iron levels and monoamine neurotransmitter systems in the *Btbd9* mutant mice to examine potential molecular bases for these deficits and found an elevated level of iron in blood serum, an increase in the serotonin metabolite 5-HIAA and a preferential alteration in circling behavior.

Previous phenotypic models of RLS have shown motor restlessness or alterations in sleep efficiency. These include lesioning of the A11 dopaminergic nucleus in rats, which showed increased standing episodes and increased arousal from sleep compared with sham-operated rats (32,36). In another study, a transverse spinal cord lesion at the T9 level in rats showed decreased sleep efficiency and the appearance of pseudo-periodic gastrocnemious activation during sleep (37). Lastly, a mouse lacking the D3 DA receptor was found to have hyperactivity (38). Similarly, our *Btbd9* mutant mice have an increased voluntary activity, total activity and increased wake time and arousal during the rest phase. We speculate that this hyperactivity parallels either the symptoms of RLS, in that the animals are more active due to uncontrollable urges to move, or the symptoms of attention deficit hyperactivity disorder (ADHD) in step with the finding that there is comorbidity between RLS and ADHD (55–61). As the hyperactivity has been observed during the rest phase in both our *Btbd9* mutant mice and other models, this would lend support to hypothesis that the hyperactivity arises from an RLS-like phenotype. However, further studies will need to be conducted to examine whether the *Btbd9* mutant mice have ADHD-like phenotypes as well.

Sensory symptoms have been reported in a number of studies on RLS patients (62–66), including thermal sensory alterations (67,68). Furthermore, recent studies in the D3 DA receptor knockout mice have shown increased sensitivity to acute and persistent pain (31). Comparably, our homozygous *Btbd9* mutant mice had a thermal sensory alteration. Our finding also parallels a study conducted on RLS patients showing that immobility increases sensory deficits (69), as our tail-flick experiment was conducted on restrained mice. Furthermore, we showed that heterozygous *Btbd9* mutant mice have sensory alterations, though to a lesser extent than the homozygous *Btbd9* mutant mice, suggesting a relationship between Btbd9 expression and thermal sensory perception. Additionally, we showed that heterozygous *Btbd9* mutant mice have no significant sensory alteration during the middle of the active phase, but do have significant sensory alterations during the middle of the rest phase. This is similar to RLS patients, as symptoms predominately occur during rest or while sleeping. However, one report suggested that pain in response to mechanical stimulation in RLS patients is altered during both the day and night (66). However, it is possible that mechanical and thermal stimulation are processed differently. Furthermore, the majority of the patients in this study lacked a familial history of RLS, thereby potentially causing heterogeneity in the study population. Finally, we show that the sensory alteration in heterozygous *Btbd9* mutant mice can be rescued by a single injection of ropinirole, a common dopaminergic treatment for RLS patients (6). Taken together, the *Btbd9* mutant mice have a sensory alteration that is likely limited to the rest phase, which can be rescued using ropinirole, similar to RLS patients. Additional experiments will need to be conducted to determine the mechanisms that underlie this dopaminergic rescue and the effects of other RLS treatments.

To elucidate a possible biochemical mechanism underlying the behavioral deficits, we also examined the iron and monoamine neurotransmitter systems. Iron homeostasis has been a major area of research in RLS. Primarily, iron anemia or related conditions have been associated with RLS (70). Interestingly, we found in our *Btbd9* mutant mice an increase in iron levels in the serum. Furthermore, we found that there was no change in iron levels in the striatum in the *Btbd9* mutant mice. Further studies will need to be conducted to examine whether proteins involved in iron regulation are altered or whether iron is present in correct cell types in the *Btbd9* mutant mice. It is worth noting as well that while iron anemia may be a common occurrence in RLS patients, not all patients with iron anemia have RLS (71). Furthermore, RLS has been reported in patients with familial hemochromatosis, which causes excess iron in the body (72,73).

Finally, we also examined the monoamine neurotransmitter system using HPLC on striatal brain tissue. In RLS patients, varying results have been found on the dopaminergic and serotonergic systems. Two studies have shown that there are no alterations in the levels of DA, serotonin or their metabolites in cerebrospinal fluid (CSF) of RLS patients (74,75), whereas a third showed a decrease in 5-HIAA, a metabolite of serotonin, and tetrahydrobiopterin (BH4), an essential co-factor for the biosynthesis of the monoamine neurotransmitters, in CSF of RLS patients (76). We found that in the striatum of our *Btbd9* mutant mice there was no difference in DA, serotonin or DA metabolites, but an increase in the serotonin metabolite 5-HIAA. We also found that the *Btbd9* mutant mice have a preferential increase in circling behavior, which has been shown in other mouse models to be related to dopaminergic imbalance, in particular that arising from the striatum (43,44). Lastly, the *Btbd9* mutant mice showed sensory alterations that can be rescued by ropinirole, which targets the dopaminergic system. Taken together, these results suggest an altered dopaminergic system in addition to a serotonergic system in the *Btbd9* mutant mice. Further studies will need to be conducted on the serotonergic and dopaminergic systems to better understand the nature of these alterations.

In conclusion, we propose that the *Btbd9* mutant mice model several aspects similar to an RLS-like phenotype, including motor restlessness, sensory alterations, imbalances in iron homeostasis and alterations in the monoamine neurotransmitter systems, and therefore would be the first genotypic model of RLS. Furthermore, speculation has arisen about whether *BTBD9* is involved in RLS or whether its neighboring gene, *GLO1*, is in fact the true susceptibility gene (77). Our data suggest that BTBD9 is involved in the pathophysiology of RLS, and further research will need to be conducted to examine how these behavioral deficits arise and the role of BTBD9 in the pathophysiology of RLS. Finally, as the *Btbd9* mutant mice can be treated with dopaminergic treatments to rescue a sensory alteration, this supports the utility of this line of mice as a model of RLS and an animal model to discover and screen potential novel therapeutics.

MATERIALS AND METHODS

All experiments were carried out in compliance with the USPHS Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Use and Care Committee at the University of Alabama at Birmingham.

Mice

We previously generated a line of *Btbd9* mutant mice using a commercially available embryonic stem cell clone that contained a β-geo gene trap vector within the sixth intron of the *Btbd9* gene (RRE078, BayGenomics) (42). The sixth intron of the *Btbd9* gene corresponds to the fifth intron of the human *BTBD9* gene where the SNPs in the GWAS of RLS patients are located. In brief, this gene trap prematurely terminates *Btbd9* gene transcription, resulting in a truncated Btbd9 fused with β-galactosidase and neomycin. The animals were genotyped by PCR using a two-step process. First, a pair of primers was used to specifically detect the gene trap (v1531—5′-GGTCCCAGGTCCCGAAAACCAAAGAAGA-3′ and v1842R—5′-ACAGTATCGGCCTCAGGAAGATCGC-3′) along with a pair of primers serving as an internal control (10200F—5′-ACTCTGAGATGATTAACAAGAGCTCAGG GCTGA-3′ and 102200BR—5′-AGCCCTCAGCTCTTGTTA ATCATCTA-3′). Second, a pair of primers was used to detect the WT allele, if one was present (102000B—5′-AGATG ATTAACAAGAGCTGAGGGCT-3′ and 6ERA—5′-TCAG CCACGTCTTCTAAATGTAATGGTT-3). Mice were housed under a LD cycle, except when noted, with *ad libitum* access to food and water. Heterozygous *Btbd9* mutant mice were interbred to produce experimental mice. In all experiments, adult heterozygous *Btbd9* mutant mice and/or homozygous *Btbd9* mutant mice were used along with WT littermate mice as controls, and performed by investigators blind to the genotype of the mice. Advanced age in rats (>16 months) has been noted to cause sleep-related motor phenomena such as periodic limb movements (78). All of our studies were conducted in non-aged, adult mice typical of behavior and molecular experiments. The open field, first tail-flick and serum iron analysis were done in mice approximately between the ages of 4 and 6.7 months. The second tail-flick was performed in mice approximately between the ages of 2 and 2.3 months. The wheel running was performed in mice approximately between the ages of 2.5 and 4.3 months. The polysomnography was done in mice approximately between the ages of 7.5 and 8.3 months. The AA and HPLC were done in mice between the ages of 9.3 and 10 months.

Open field

An open field apparatus was used to measure total activity and circling behaviors, as previously described (79–81), in 5 homozygous *Btbd9* mutant mice and 16 WT mice. In brief, the open field apparatus (Lafayette Instruments) is equipped with infrared sensors that detect breaks in the beams. Software (DigiScan Systems, AccuScan Instruments) is then used to decode these beam breaks into varying behavioral patterns. This experiment was performed with 30 min of observation time during the rest phase.

Wheel running

Seven male homozygous *Btbd9* mutant mice and seven male WT mice were maintained on a LD cycle for 17 days and then followed by 17 days of DD. Wheel running activity was recorded as the number of wheel revolutions occurring during 5 min bins and analyzed using ClockLab software (Actimetrics). For the last 10 days in LD, the proportions of activity during lights on and lights off, as well as the total amount of activity per day and alpha length (time between onset and offset of primary activity bout) were determined. The activity profile feature of ClockLab was used to determine the proportion of activity over the course of the LD cycle averaged over a 7-day period. The averaged data for each animal was normalized to the maximum activity for that animal. Means from each group across 24 h is shown in Figure 2. For statistical comparison, data were binned into 3 h bins and analyzed with a two-way repeated-measures analysis of variance (ANOVA). In DD, activity was measured for the entire 17 days and ClockLab was used to determine average counts per minute, alpha length. In addition, the chi-squared periodogram analysis in ClockLab was used to determine period and rhythmic power as a measure of the amplitude and coherence of behavioral rhythms (82,83).

Tail-flick

Six heterozygous *Btbd9* mutant mice, 5 homozygous *Btbd9* mutant mice and 16 WT mice were tested for perception of warm stimuli using the Tail Flick Analgesia Meter (San Diego Instruments). The experiment was performed as previously described (84). In brief, the mouse was placed in an acrylic restrainer with the distal end of its tail protruding under a heat lamp, which was then manually turned on alongside a timer. Both the heat lamp and the timer stopped automatically when the mouse produced a strong reaction to the heat by moving its tail away from the light. The latency to respond was limited to 15 s to prevent injury to the mouse. In a separate cohort of mice, the tail-flick experiment was conducted during the middle of the active phase (approximately Zeitgeber time (ZT) 18, where ZT 12 refers to lights off);

during the middle of the rest phase (approximately ZT 6); and 15, 30 and 60 min following a 0.1 mg/kg of body weight (0.1 ml/1 ml of saline) intraperitoneal injection of ropinirole, a common dopaminergic treatment of RLS, and similar dosage has been used in mice with efficacy (33).

Polysomnography

To measure sleep architecture in the *Btbd9* mice, three male homozygous *Btbd9* mutant mice and three male WT mice were implanted with a wireless telemetry system (DSI Instruments). The mice were anesthetized and a small vertical cut (~1 cm) was made on one side of the skin near the hind limb and the tibialis cranialis muscle was localized. A pair of leads were then inserted into the muscle and sutured and glued in place to obtain EMG data. Another pair of leads was placed on the dura of the brain to obtain EEG data, as suggested by the manufacturer, and dental cemented in place. The body of the transmitter and any excess wire were inserted under the back of the skin and sutured close. The mice were then allowed 48 h to recover from surgery (Supplementary Material, Video 1). The EEG and EMG signals were processed and sleep patterns were analyzed by NeuroScore computer software (DSI Instruments).

Colorimetric assay for serum iron

Blood was collected by retro-orbital blood collection using a glass pipette on four homozygous *Btbd9* mutant mice and three WT mice. The blood was allowed to clot and then separated by centrifugation at 1500*g* for 10 min. The serum was removed and centrifuged again at 1500*g* for 10 min for further purification. The iron concentration was quantified using a colorimetric assay (QuantiChrom Iron Assay Kit, BioAssay Systems Inc.), according to the manufacturer's instructions.

AA spectroscopy for iron in striatal tissue

Striatum from seven homozygous *Btbd9* mutant mice and seven WT mice were dissected out and homogenized 1:10 in PBS (pH 7.4) containing protease inhibitors (Roche). Brain region aliquots were wet digested by published and standard procedures and analyzed for iron concentration by AA spectrometry (Perkin Elmer AAnalyst 600, Perkin Elmer) (85). Standards were prepared by diluting a Perkin Elmer iron standard (PE#N9300126) in 0.2% ultra-pure nitric acid and blanks prepared with digesting and diluting reagents to control for possible contamination. All standard curves exceeded $r > 0.99$.

High-performance liquid chromatography

Homogenate from the same striatal samples used for iron measurements was aliquoted for HPLC analysis. The homogenate (50 ml), 0.24 M perchloric acid (50 ml) and internal standard 3,4-dihydroxybenzylamine (DHBA) were passed through a 0.2 mm micro-Sephadex column (Spin-X Costar, Corning Inc.) to remove endogenous substrates. Samples were then loaded onto a refrigerated ESA model 542 autosampler and 10 ml of sample was injected onto an ESA MD-150 narrow-bore HPLC column (150 × 2 mm; ESA Inc.). The mobile phase consisted of 75 mM sodium phosphate, 1.7 mM 1-octanesulfonic acid, 25 μM ethylenediaminetetracetic acid, 7.0 μM triethylamine and 10% v/v acetonitrile in a volume of 2 l (pH 3.0). Once separated, compounds were measured with a coulometric detector (ESA model 5300: guard cell potential, +400 mV; working cell potentials, −174 mV and 350 mV). The neurotransmitter metabolite peak areas were integrated using EZ Chrom Elite Software (Scientific Software, Inc.) and quantified against known standards. The standard curves exceeded $r = 0.99$, and the relative standard deviation of DHBA between samples was less than 3%. DA, serotonin or 5-HT, DOPAC, HVA and 5-HIAA were measured. Samples were normalized to weight of tissue and reported in picomole of neurochemical per gram of tissue.

Statistics

All data were analyzed by Student's *t*-test, except those of open field. Open field data were analyzed using mixed model ANOVA taking into consideration genotype, age, sex and weight. SAS statistical package was used for ANOVA. Significance was assigned at $P \leq 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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