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Characterization of Btbd9 Knockout Mice: a Potential Model of Restless Legs Syndrome

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CHARACTERIZATION OF BTBD9 KNOCKOUT MICE – A POTENTIAL MODEL OF RESTLESS LEGS SYNDROME

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ABSTRACT

Restless legs syndrome (RLS) is a neurological disorder that is manifested at rest by periodic movements in sleep and unpleasant sensations deep inside the legs that are relieved partially with movement. RLS has been associated with the central dopaminergic system and iron metabolism. Additionally, family and twin studies strongly support a genetic contribution to the pathogenesis of RLS. Two independent genomic studies published recently suggest that the BTBD9 gene plays a role in RLS.

We have created a line of Btbd9 mutant mice that mimic the mutation reported in RLS patients by an insertion of a gene trap vector into the Btbd9 gene. The Btbd9 knockout mice were born in a non-Mendelian ratio suggesting the knockout is partially lethal. Additionally, we have demonstrated hyperactivity, sensory deficits, altered iron metabolism and sleep structure in the Btbd9 knockout mice. This mouse model is the first genotypic mammalian model of restless legs syndrome, thus making it a relevant model for further study of RLS.

Keywords: Restless Legs Syndrome, RLS, Btbd9, Knock-out mouse, Iron metabolism

DEDICATION

To my parents, Avito and Neola, for their continuous guidance and support and to my brothers, Kevin and James, for their inspiration and motivation to strive for excellence.

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LIST OF IMPORTANT ABBREVIATIONS

ADHD – Attention Deficit Hyperactivity Disorder

BTBD9 – Human broad-complex, tram track and bric à brac domain 9 gene

Btbd9 – Mouse broad-complex, tram track and bric à brac domain 9 gene

CSF – Cerebral spinal fluid

EEG – Electroencephalography

EMG – Electromyography

GWAS – Genome Wide Association Study

Het – Heterozygous Btbd9 knockout

KO – Knockout Btbd9 knockout

L-DOPA – L-3,4-dihydroxyphenylalanine

PCR – Polymerase chain reaction

PLMD – Periodic Limb Movement Disorder

PLMS – Periodic leg movements in sleep

PSG - Polysomnography

REM – Rapid eye movement sleep

RLS – Restless Legs Syndrome

SNP – Single nucleotide polymorphism

SWS – Slow-wave sleep

WT – Wildtype

CHAPTER 1

INTRODUCTION

Background of Restless Legs Syndrome (RLS) and Treatments Options

Restless legs syndrome (RLS) is a common neurological disorder that has both a movement and a sleep component. It is characterized by unpleasant sensations in the legs, generally at night or rest, accompanied by an uncontrollable urge to move the legs for relief (Walters, 1995). RLS affects approximately 10% of the general population (5 to 9% of men, 9 to 14% of women). Periodic limb movements disorder (PLMD) has a high co-morbidity with RLS, appearing in 80% of RLS patients (Montplaisir et al., 1997). Other risk factors for RLS include advanced age, pregnancy, and diseases such as Attention Deficit Hyperactivity Disorder (ADHD), iron-deficiency anemia, and end-stage renal disease (Patrick, 2007; Stefansson et al., 2007). Minimal criteria for diagnosis of RLS are complaints of unpleasant sensations in the legs usually at night, and that these sensations are relieved by movement (Walters, 1995). The symptoms of RLS often lead to sleep disturbances, and can affect the patient's daytime function and quality of life (Abetz et al., 2004).

Currently there is not a cure for RLS; however, there are several treatments that relieve the symptoms of RLS. These primarily are dopaminergic agonists, but also include opioids and anticonvulsants. Dopamine agonist such as ropinirole, which is the only drug approved by the Food and Drug Administration (FDA) to treat RLS, and pramipexole modulate dopamine receptor levels in the central nervous system. L-3,4-

dihydroxyphenylalanine (L-DOPA) is the precursor for dopamine and has been examined as a potential therapeutic option but has been shown to cause rebound (the reoccurrence of symptoms 2 to 6 hours later), augmentation (occurrence of RLS symptoms earlier in the day), and dyskinesia (Comella, 2002). Opioids such as codeine, propozyphene, and oxycodone have been shown to be effective in suppressing RLS symptoms. However, their risk of abuse and danger of addiction limit their use in clinical settings (Karatas, 2007). Furthermore, anticonvulsants such as carbamazepine and gabapentin, can also help relieve symptoms of RLS and sleep structure (Karatas, 2007). Recently, intravenous and oral iron treatments have been the focus of recent therapeutic options for patients with some success in alleviating symptoms (O'Keeffe, 2005; Bhandal and Russell, 2006; Earley et al., 2009; Wang et al., 2009).

Iron, Dopaminergic, and Sensory Deficits in RLS Patients

A common symptom of RLS patients is an iron transport and/or storage deficit. Free iron levels and proteins involved in the transport and storage of iron have, therefore, been analyzed in RLS patients. Additionally, iron metabolism and homeostasis is tightly regulated in the body. Two major regulatory proteins of iron in the body are ferritin, which is an iron sequestering and storage protein, and transferrin, which is an iron transport protein. In the cerebrospinal fluid (CSF), ferritin has been demonstrated to be lower and transferrin higher in RLS patients (Earley et al., 2000; Earley et al., 2005; Mizuno et al., 2005). In addition, magnetic resonance imaging and transcranial ultrasound have been used to show that iron storage in the striatum and red nucleus are reduced in RLS patients (Allen et al., 2001; Schmidauer et al., 2005).

Analyses of the dopaminergic system in RLS patients are not as extensive as that of the iron system. One analysis of CSF revealed no difference in the dopaminergic metabolite homovanillic acid (HVA) between RLS and control patients. They, however, found a possible decrease for 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin, and tetrahydrobiopterin (BH_4), a cofactor for the enzyme to make dopamine and serotonin (Earley et al., 2001). Other studies have shown a slight decrease in fluro-dopa uptake (Turjanski et al., 1999; Ruottinen et al., 2000) and D2 receptor binding (Staedt et al., 1995; Turjanski et al., 1999; Michaud et al., 2002). Fluorodopa is a radiolabeled fluorine in L-DOPA that is used to assess the dopaminergic system using positron emission tomography (PET) scan. These studies collectively suggest that in RLS patients there is a hypofunctioning dopaminergic system.

In previous studies, it has been demonstrated that the iron and dopaminergic systems interact, and that these two deficits in RLS are potentially related. First, iron is a cofactor for tyrosine hydroxylase, the rate limiting enzyme that converts tyrosine to dopamine via L-DOPA. Second, iron-deprived rats show a 40-60% reduction in D2 dopamine receptors. This effect can be reversed by administering an iron supplement to the rats (Ben-Shachar et al., 1985).

As previously mentioned, RLS is a disorder with a movement and sensory component. These sensory system deficits give rise to the primary complaint of RLS patients which is that of unpleasant sensations in their legs at rest or night. Sensory studies of RLS patients have discovered a temperature perception alteration in RLS patients. One study found that RLS patients exhibit an increase in cold and warm perception (Iannaccone et al., 1995). Another study, however, found only a cold

perception difference (Happe and Zeitlhofer, 2003), while a third study found no difference but rather an abnormal temperature limens, which is the difference between cold and heat pain thresholds (Schattschneider et al., 2004). Additionally, iron-deficient mice exhibit increase sensitivity to pain and induced nociception by formalin, a chemical irritant (Dowling et al., 2009).

Genetics of RLS

Genetics have also been shown to contribute to RLS. In approximately 60% of RLS cases there is also a family history of RLS. 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. Two genome wide association studies (GWAS) were conducted to determine potential genes associated with RLS have been reported. A GWAS is a high-throughput genotyping strategy to assay over 100,000 single nucleotide polymorphisms (SNPs) and their association with a disease or condition (Pearson and Manolio, 2008). Generally, groups conducting a GWAS will probe an exploratory population. This exploratory population will reveal significantly associated genes and those that are possibly associated but not significant. To minimize bias, genes associated with the disease in the exploratory study will be examined in another independent population to test for replication of results (Pearson and Manolio, 2008).

In the first GWAS, the exploratory stage revealed a single SNP within MEIS1 to be significantly associated with RLS ($p<0.0002$). Additionally, there were 13 SNPs that were potentially associated with RLS patients but not significant. These 14 SNPs were analyzed in two replication populations and concluded that four genes, MEIS1 ($p<10^{-11}$),

BTBD9 ($p<^{-5}$), MAP2K5 and LBXCOR1 ($p<10^{-4}$) were potentially associated with RLS (function of genes below). Nominal significance for the gene PTPRD in its relationship to RLS was found. Later, Schormair and colleagues determined that PTPRD was in fact linked to RLS (p=0.018) (Schormair et al., 2008). The second GWAS study found a significant association in the BTBD9 gene, specifically within intron 6 ($p=2x10^{-9}$). This association was replicated in two independent populations ($p=4x10^{-3}$ and $p=4x10^{-3}$). Additionally, Stefansson and colleagues determined that serum ferritin levels were decreased by 13% per allele of the at-risk variant (p=0.002) (Stefansson et al., 2007).

These genes associated with RLS have a variety of functions that could potentially make them good candidate genes for studies in animal models of RLS. Ladybird homeobox corepressor 1 (LBXCOR1) is downstream of the mitogen-activated protein kinase kinase 5 (MAP2K5) and is a transcriptional corepressor of LBX1, which is critical in the development of sensory pathways in the dorsal horn of the spinal cord that relay pain and touch (Gross et al., 2002). Additionally, the mitogen-activating protein kinase family (MAP) is important in transducing extracellular signals to cytoplasmic nuclear effectors. Additionally, the MAP kinase cascade is crucial at early stages of muscle cell differentiation (Dinev et al., 2001). Myeloid ecotropic viral integration site 1 homolog (MEIS1) is essential for proximo-distal limb formation in *Drosophila melenagoster* and is part of a Hox transcriptional regulatory network that specifies spinal motor neuron pool identity and connectivity (Rajaram et al., 2004; Dasen et al., 2005). Mutations of protein tyrosine phosphatase, receptor type D (PTPRD) in mice are associated with deficient axon guidance and termination of motor neurons during embryonic development (Uetani et al., 2006). Broad complex, tram track, bric à brac

domain 9 (BTBD9) does not have any known functions, but is hypothesized to play a role in trace metal regulation.

Function of BTBD9

As previously mentioned, BTBD9 has been implicated in both of the GWAS as a potential gene of interest for RLS (Stefansson et al., 2007; Winkelmann et al., 2007). BTBD9 has also been implicated with PLMS (Stefansson et al., 2007; Winkelmann et al., 2007). The BTB or POZ domain is involved in a variety of cellular functions including transcriptional regulation, cytoskeleton organization, ion channels, and protein ubiquitination (Stogios et al., 2005). BTBD9 is expressed ubiquitously inside and outside of the central nervous system and expressed during development and adulthood. The Allen Mouse Brain Atlas shows that the Btbd9 protein is expressed heavily in the cerebellum, striatum, cortex, hippocampus, and olfactory bulb (Lein et al., 2007). Two proteins, CSN6 and USP21, were discovered recently to potentially interact with BTBD9 (Sowa et al., 2009). COP9 signalosome complex subunit 6 (CSN6) is a member of the COP9 signalosome and when it is cleaved from the signalsome, apoptosis occurs (da Silva Correia et al., 2007). Additionally the COP9 signalosome is important in the regulation of several important intracellular pathways, including the ubiquitin/proteasome system(da Silva Correia et al., 2007). Ubiquitin specific peptidase (USP21) is a deubiquitinating enzyme that has been shown *in vitro* to be important in the down-regulation of TNFα-induced NF-κB activation through deubiquitinating RIP1 (Xu et al., 2010).

Animal Models of RLS

The implication of how the genes identified in the GWAS are associated with RLS has not yet been elucidated. A few animal models of RLS have been developed based on current understandings of the disorder to help elucidate the pathophysiology of RLS. These models only provide some phenotypic characteristics that resemble RLS or PLMD. For example, by lesioning either the A11 diencephalic nucleus or the spinal cord at T9 a phenotypic model of RLS has been developed. The A11 nucleus consists of approximately 200 dopaminergic neurons symmetrically located in the posterior hypothalamus which receives afferents from the suprachiasmatic nucleus (SCN). Using 6-hydroxydopamine the A11 nucleus was partially lesioned in rats, reducing the dopaminergic neurons by approximately half. The rats exhibited an increase in standing episodes, sleep latency, and a decrease in total sleep time compared to sham operated rats (Ondo et al., 2000). Esteves and colleagues analyzed the effects of transverse spinal cord lesion by performing a stereotaxic lesion at the T9 level and conducted a polysomnography (PSG). A polysomnography is a sleep study that examines the electrical activity of several different parts such as the brain (electroencephalography or EEG), muscle (electromyography or EMG), and heart (electrocardiography, EKG). They observed a decrease in sleep efficiency and the appearance of pseudo-periodic gastrocnemious activation during sleep (Esteves et al., 2004).

Clemens and colleagues attempted to induce RLS-like behavior by injecting haloperidol, a non-selective dopamine receptor antagonist, intraperitoneally in rats. They conducted a PSG using an EEG and an EMG of a neck muscle. Additionally, they were able to monitor hindlimb movements using a magnetic inductive device located

subcutaneously in the hindlimb. However, haloperidol did not affect either the sleep pattern or the amount of periodic movements in the rats. However, in older rats that did not receive haloperidol there was sleep-related motor phenomenon. The significance of this study is that they have high prevalence of spontaneous, even not RLS-related PLMS (Clemens et al., 2005).

Lastly, a knockout mouse of the D3 dopamine receptor was generated and analyzed. The D3 receptor is present in the dorsal horn of the cervical and lumbar regions suggesting that the D3 receptor may play a role in sensation or nociception. Additionally the D3 receptor is present in the pars centralis which suggest that it may play a role in sensory-motor integration. The D3 receptor knockout exhibited several abnormalities, namely hyperactivity and hypertension (Clemens and Hochman, 2004). In another study of the D3 receptor knockout, the mouse exhibited an inverted circadian cycling in tyrosine hydroxylase and nitric oxide synthase (Clemens et al., 2005).

Currently, all the models of RLS are phenotypic models, in that they replicate RLS-like symptoms and behavior. There are no genetic mouse models of restless legs syndrome. In this present study we attempt to create and examine the first genetic mammalian model of RLS by creating a Btbd9 knockout mouse, a gene that has been associated with RLS.

As there are no standard tests for RLS in a mouse, *a priori* we designated the essential criteria of RLS as hyperactivity and hyperalgesia. Additionally, other criteria that can be present but not necessary include iron homeostasis imbalances, the existence of periodic limb movements in sleep, and alterations in sleep structure.

CHAPTER 2

CREATION OF THE BTBD9 KNOCKOUT MOUSE

Two genome-wide association studies (GWAS) have implicated BTBD9 as being potentially associated with restless legs syndrome (RLS) and periodic limb movements in sleep (PLMS) (Stefansson et al., 2007; Winkelmann et al., 2007). No previous mammalian model of a disrupted Btbd9 gene has been generated and characterized. We therefore thought it would be a good candidate gene for a mouse model of RLS and/or PLMS.

One common method of disrupting a normally functioning gene in mice is to insert a gene trap into the gene. A gene trap is a combination of two or more genes together. BayGenomics created a gene trap of the Btbd9 gene (gene trap clone number RRE078) in the sixth intron, the same intron that the GWAS showed a high correlation of mutations to RLS. In the case of the mutant Btbd9 gene there are two genes that are part of the gene trap. One is the bacterial gene *lacZ* which produces the enzyme β-galactosidase. *lacZ* is called a reporter gene because when transcribed and translated into β-galactosidase in the presence of X-gal (an organic compound containing β-galactoside linked to an indole) it produces a blue color from the breakdown of X-gal. The other gene in the gene trap is also from bacteria called *neomycin* which imparts resistance to the neomycin. As such the *neomycin* gene is considered a selective gene. At the end of this fusion gene is a stop sequence which will terminate transcription. When this fusion gene is inserted within a normally functioning gene, it will cause transcription to

terminate prematurely due to this stop sequence. This mRNA generated is translated into a protein, but generally the protein does not have the same function as the original unmodified gene (Figure 1).

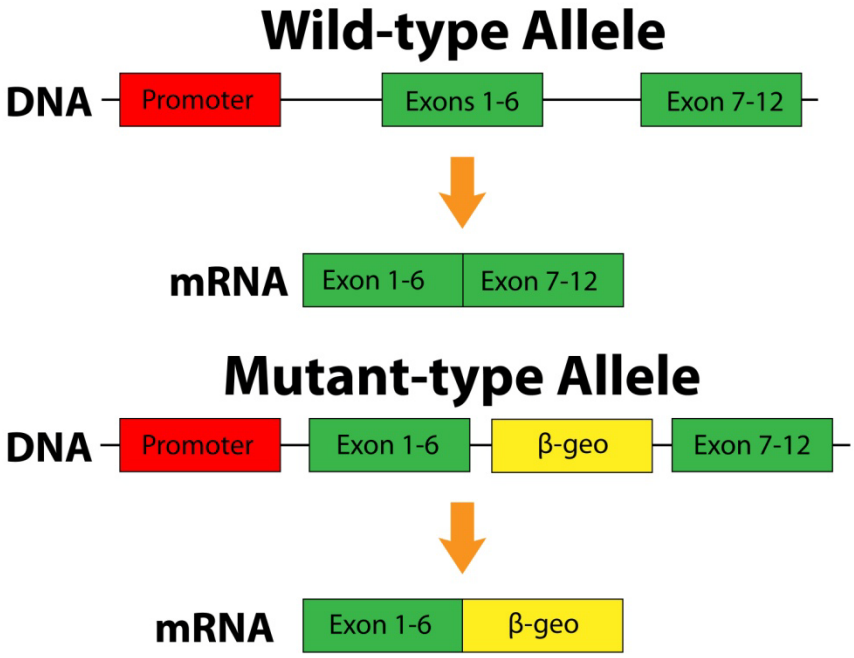

Figure 1. Schematic diagram of a normal gene translation of DNA to mRNA (Wild-type Allele) and an alternative splicing caused by the insertion of the β-geo gene (Mutant-type Allele).

We ordered the embryonic stem (ES) cell containing the gene trap in the Btbd9 gene and the insertion within the sixth intron was confirmed by 5'-RACE RT-PCR sequencing (data not shown). 5'-RACE RT-PCR results showed that the insertion is in indeed in intron six. The ES cells were then injected into C57BL/6 blastocysts and placed in pseudo-pregnant females, from which we obtained chimeras. These chimeras were then bred with C57BL/6 mice to transmit the mutation into its offspring, which was confirmed by PCR of the β-geo gene (data not shown). These heterozygous mice were

then interbred to generate knockout mice. Initially, knockout mice were identified by using MIT100 primer set that can distinguish 129 and C57BL/6 by PCR reactions. This primer set is specific for microsatellite markers located within 1 Mbp of the Btbd9 gene. However, this method of genotyping the mice has the disadvantage of about 1 to 5% error rate due to occasional recombination between the Btbd9 gene and the MIT100 marker. To accurately genotyping the mice, we then focused on locating the insertion site of the gene trap clone.

In order to determine the approximate location of the insertion site within intron six, we designed 19 pairs of primers spaced about 10 kilo base pairs (kb) apart within the intron covering the entire 179,223 base pairs (bp) of intron six. Long-arm (LA) PCR was then performed with various combinations of primers using an LA PCR kit (TaKaRa). The LA PCR was conducted using templates from control wild-type DNA and DNA from a knockout mouse. The insertion site was determined to be within a 12.5 kb range between 90 kb and 102.5 kb into the intron. In order to further narrow down the region primers were created at 500 bp intervals before 102.5 kb mark. Once again, LA PCR was conduction on various combinations of primers. This revealed that the insert was located between 102,000 to 102,501 bp into the intron 6 (Figure 2).

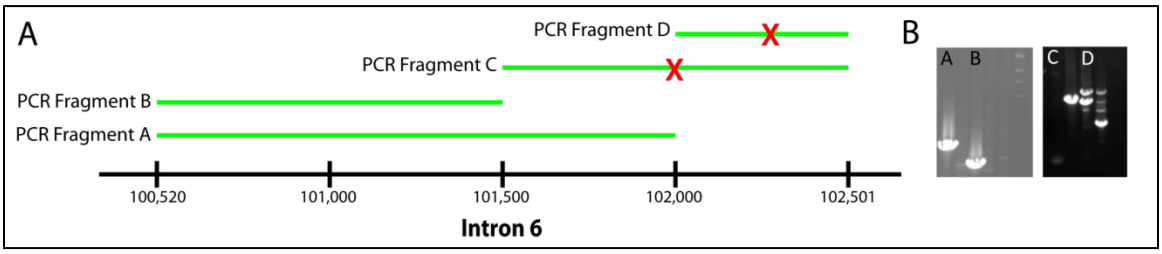

Figure 2. Location of Insertion. A. Schematic diagram of primer positions (vertical black bars) and various PCR fragments attempted to be generated (green horizontal bars). Red X's indicate PCR fragments that were not able to be generated. B. PCR fragments generated from A. Bands in lane D are non-specific PCR fragments determined by incorrect molecular weights. The appropriate bands were not generated in lanes C and D.

CHAPTER 3

MATERIALS AND METHODS

All experiments were carried in compliance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee under an approved protocol (Appendix A).

Mice. Heterozygous Btbd9 knockout mice were interbred to generate littermates of the three genotypes: wild-type (WT), heterozygous Btbd9 knockout mice (Het), and homozygous Btbd9 knockout mice (KO). Mice were housed under a 12-h-light and 12-h-dark cycle. Behavior tests were performed within the last 9 h of the light period after acclimation to a sound-attenuated testing room for 1 h. Mice were allowed to rest for one week in between the tests. Behavior experiments were performed in the following order: open field, tail flick, wheel running, PSG. All of the behavior and biochemical tests were performed by the investigator blind to the genotypes.

Genotyping. For genotyping purposes, PCR was first conducted on all mice to determine whether the β-geo gene was present in the mouse's genome (βGeoF: 5' GGTCCCAGGTCCCGAAAACCAAAGAAGA 3' and βGeoR: 5' ACAGTATCGGCCTCAGGAAGATCGC 3'). This would produce a band of approximately 360 bp. β-geo negative animals are WT and β-geo positive animals are mutant animals. To determine whether the β-geo animals are Het or KO, a set of primers were designed spanning the site of the insertion (Btbd9WTF: 5'

CTGAGATGATTAACAAGAGCTGAGGGCT 3' and Btbd9WTR: 5' TCAGCCACGT CTTCTAAATG TAATGGTT 3'). In WT animals this would produce a band approximately 500 bp long. However, in the KO between the forward and reverse primer is the location of the β-geo gene, which is approximately 8,500 bp long. This band will not be produced under regular PCR conditions.

Open field. Mice were tested for spontaneous activity level on the open field apparatus (AccuScan Instruments) using 16 WT (8 males and 8 females), 7 Hets (2 males and 5 females), and 6 KO (3 males and 3 females). Movements of individual mice were recorded by infrared light beam sensors in a 41×41 x 31 cm acrylic cage for 30 min at 5 min interval using DigiPro software. The test was conducted on the same day during 9 h of the light period.

For statistical analysis a two-way ANOVA (mixed model) was used to analyze total distance, movement time, center distance, and circling. Genotype and sex were treated as class variables. Means and standard errors were calculated least square method. Significance was assigned by $p < 0.05$.

Tail flick. Mice were tested for perception of pain using the Tail Flick Algesia Meter (San Diego Instruments). The same animals as above were used. The test was conducted within the last 6 hrs of the light period. An individual mouse was placed in an acrylic restrainer with the distal end of its tail protruding under a heat lamp. The heat lamp will then be turned on, at which time will heat up rapidly to a set temperature and a digital timer will record time. A sensor built into the heat lamp will detect movement of the tail

and shut the lamp off and stop the timer. If a mouse fails to react within 15s of the light being turned on, the lamp will be turned off manually by the investigator to prevent tissue damage.

For statistical analysis a two-way ANOVA (mixed model) was used to analyze total latency. Genotype and sex were treated as class variables. Means and standard errors were calculated by least square means. Significance was assigned by $p < 0.05$.

Wheel running. Mice were tested for voluntary activity using the wheel running chamber (Lafayette Instrument). The chamber records wheel revolutions, distance, and speed via a computer. The cage had minimum bedding to prevent blockage of the wheel. Food and water were accessible *ad libitum*. The same mice as above were used. The mice were recorded continuously for 6 days at 5 min intervals. Recordings from first three days were excluded from analysis due to acclimation and novelty of the wheel. Only data from the last 3 full days were analyzed.

For statistical analysis, a two-way ANOVA (mixed model) was used to analyze total distance and activity between 9 am to 3 pm (middle 6 hrs of light period). Genotype and sex were treated as class variables. Pairwise comparisons were derived from the least-square means and standard errors. Significance was assigned at $p < 0.05$.

Polysomnography. A total of 3 WT and 3 KO mice were implanted and analyzed. The wireless transmitter from DSI can have two channels of data transmitted. We used one channel for EEG and one for EMG. For EMG recording, only right leg of each mouse was used to implant the wire leads, in order to minimize variations. The mice was

anesthetized and shaved in order to perform surgery. A small vertical cut (about 1 cm) was made on one side of the skin near the hind limb and the tibialis cranialis muscle was localized. The leads were then inserted into the muscle and sutured in place along with glue. The body of the transmitter and any excess wire were inserted under the back of the skin and closed by suture. The other two leads from the other channel were placed on the top of the skull to obtain EEG data. A midline incision was made on the skin, 10 mm on the head and 5-10 mm down the neck. The superficial thin layer of the skull was then pretreated with 10% H_2O_2 and two burr holes, 0.7 mm in diameter, were drilled with a dental drill. Both electrodes were shortly bent at the tip and placed directly on the dura matter (epidural lead placement) and fixed in place with dental cement. After the cement had dried, the scalp was closed using sutures and the mouse was allowed to recover for 48 hours. The EEG signal along with the relative location signal was used to discern the animal's sleep status (active wake, wake, slow-wave sleep, and paradoxical sleep) while the EMG was used to detect movements of the leg. Features were extracted using Neuroscore software (Data Science International).

For statistical analysis, a Student t-test was used to analyze percentages of various sleep stages and Microsoft Excel were used to generate means and standard errors. Significance was assigned at $p < 0.05$.

Iron assay. Blood were collected by retro-orbital blood collection using a glass pipette on four WT and four KO female animals. The blood was fractionated by centrifugation at 1,500×g for ten minutes. The serum was removed and centrifuged again at 1,500×g

for ten minutes for further purification. The iron concentration was quantified using the QuantiChrom™ Iron Assay Kit (DIFE-250).

For statistical analysis, a two-way ANOVA (mixed model) was used to analyze iron concentration. Genotype was treated as a class variable. Pairwise comparisons were derived from the least-square means and standard errors. Significance was assigned at $p < 0.05$.

CHAPTER 4

RESULTS

The litters from the heterozygote interbreeding should be distributed in a Mendelian ratio of 1 wildtype (WT) to 2 heterozygous Btbd9 mutants (Het) to 1 homozygous Btbd9 mutant (KO). The ratio of Het and KO mice, however, was significantly reduced (Table 1). This data suggest that the mutant Btbd9 gene induces partial lethality in mice.

Table 1. Chi-square test to test for Mendelian ratio of inheritance.

Genotype	Expected	Observed	Chi-square value	p-val
WT	20.5	35	-	-
Het	41	34	9.391	0.0022*
KO	20.5	13	10.083	0.0015*
Total	82	82	14.195	.0008**

WT: Wild type, Het: Heterozygous Btbd9 knockout, KO: Homozygous Btbd9 KO. * $p < 0.01$, ** $p < 0.001$

In order to determine whether the mice exhibited hyperactivity, anxiety, or increased stereotypical behaviors 29 littermates were observed at approximately 6 months of age in an open-field test. The Het exhibited no significant difference when compared to WT in total distance traveled, movement time, time spent in the center, or circling behavior. The KO, however, did show a significant increase in these four criteria when compared to WT. Comparison of the KO to the WT showed the KO had a significant increase of approximately 37% in total distance traveled ($F=4.59$, $p<0.05$, Figure 3A).

Additionally, there was an approximately 33% increase in movement time in the KO when compared to the WT ($F=4.31$, $p=0.05$, Figure 3B). The open field apparatus box can be divided into two zones – the marginal, or the perimeter, and the center. Normal mice prefer to spend the majority of time in the marginal area of the box, however, KO mice showed a 94% increase in time spent in the center ($F=6.25$, $p<0.05$, Figure 3C). Lastly, all mice exhibited some circling behavior but the KO showed a 77% increase in anticlockwise circling ($F=7.26$, $p<0.05$, Figure 3D). There was no difference between any of the groups in clockwise circling (data not shown). Taken together, the KO mice showed hyperactivity and an increase in circling behavior.

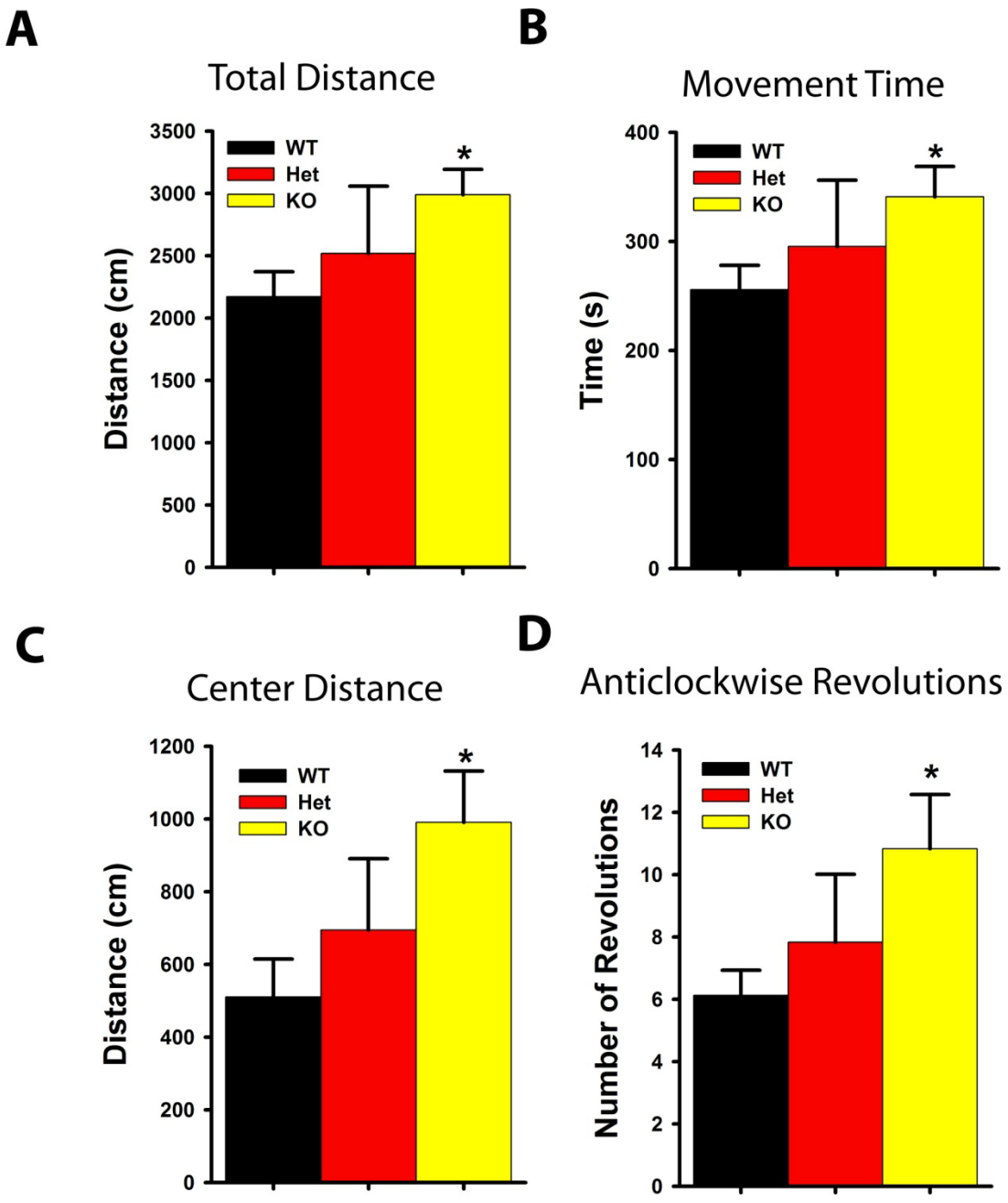

Figure 3. Open field test. (A) Total distance of the three genotypes. (B) Total movement time by the three genotypes. (C) Amount of time spent in the center of the open field apparatus by the three genotypes. (D) Anticlockwise revolutions by the three genotypes. WT: wild type, Het: heterozygous Btbd9 knockout, KO: homozygous Btbd9 knockout. Bars represent mean values and error bars are SEM. * $p < 0.05$

Next, to determine if the Het or KO mice exhibited an abnormal response to a warm stimulus, we subjected the same mice as before approximately a week later to a tail flick test. Comparison of the Het to the WT showed a significant 27% reduction in the latency to respond to the warm temperature stimulus ($t=-2.27$, $p<0.05$, Figure 4). Additionally, the KO when compared to the WT showed a significant reduction in latency of approximately 54% ($t=-4.15$, $p<0.001$, Figure 4). Taken together, the two mutant groups exhibit hyperalgesia.

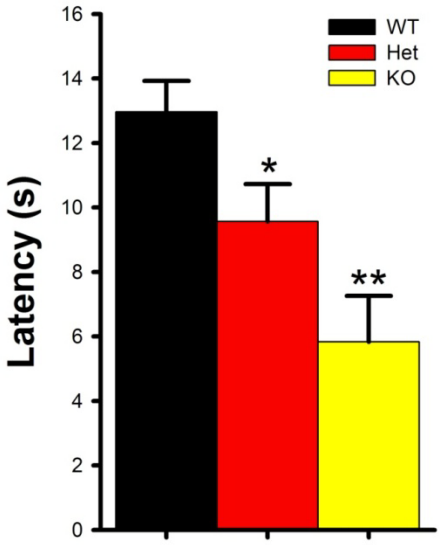

Figure 4. Latency in seconds to respond to temperature stimuli during a tail flick experiment. WT: wild type, Het: heterozygous Btbd9 knockout, KO: homozygous Btbd9 knockout. Bars represent mean values and error bars are SEM. * $p < 0.05$; ** $p < 0.001$.

To determine whether the Btbd9 mutant mice exhibit any alterations in voluntary hyperactivity and diurnal or circadian abnormalities, a wheel running experiment was performed the same set of the mice over 6 days, with data from the last 3 days being combined. Comparison of WT animals to both Het and KO showed no abnormalities in voluntary hyperactivity (data not shown).

To determine whether the KO mice exhibit alterations in time spent in different stages of sleep, we measured PSG of both WT and KO mice. A PSG covering two days was recorded using an EMG of the tibialis cranialis muscle and a dura EEG. It has been shown that RLS symptoms generally occur during non-REM sleep. The male KO exhibit a decrease (9.1% decrease, $t=6.58$, $p<0.05$, Figure 5A) in slow-wave sleep (SWS) but no change in REM sleep ($t=--0.26$, $p>0.5$) when compared to WT. Additionally, male KO show an increase (82% increase, $t=5.39$, $p<0.05$) in time awake compared to WT.

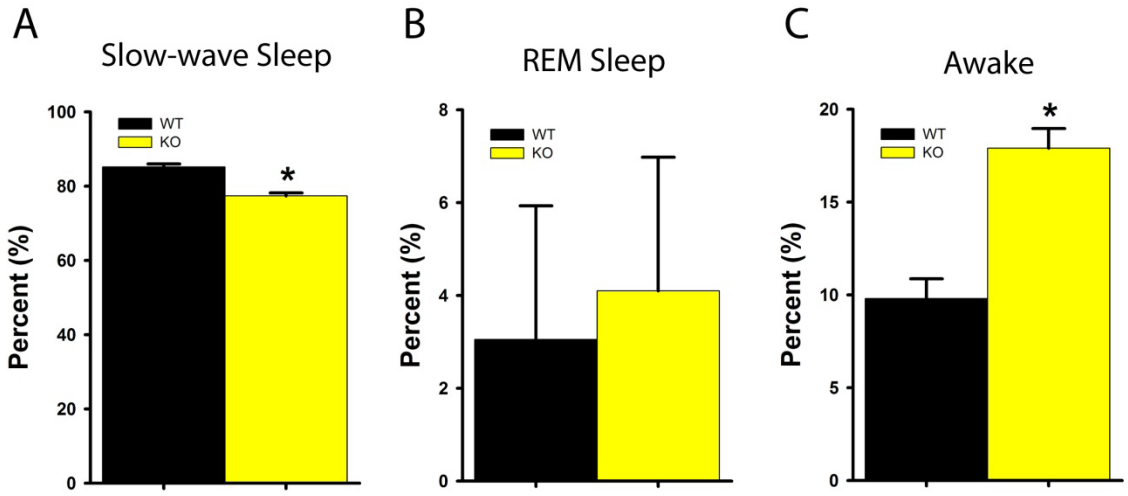

Figure 5. Percent of time animals spent in various stages of sleep between 9 am and 3 pm. WT: wild type, KO: homozygous Btbd9 knockout. Bars represent mean values and error bars are SEM. * $p < 0.05$.

To determine whether there is an imbalance in iron metabolism caused by the Btbd9 mutation, we measured serum iron levels of approximately 4 month old female littermates (n=4 each). The KO mice had on average 100 µg/dL increase of iron in the serum (36%) when compared the WT ($t=-9.09$, $p < 0.001$, Figure 6).

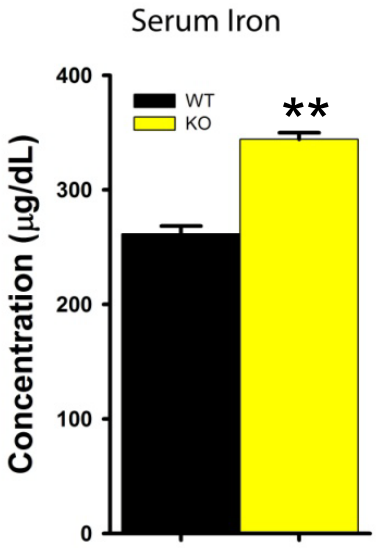

Figure 6. Total serum iron concentration as determined by a colorimetric assay. WT: wild type, KO: homozygous Btbd9 knockout. Bars represent mean values and error bars are SEM. ** $p < 0.001$.

CHAPTER 5

DISCUSSION

In this report, we have created a knockout mouse of the Btbd9 gene by inserting a β-geo gene trap vector into the sixth intron of the Btbd9 gene. The Btbd9 knockout (KO) was shown to be born in a non-Mendelian ratio, suggesting that the mutant mice are partially lethal. The KO mice showed several deficiencies. First, the mice showed hyperactivity in several different parameters but no change in voluntary activity levels. The KO mice showed increase sensitivity to a warm stimulus, indicative of a sensory system deficiency. Additionally, the mice showed disruptions in the normal sleep pattern.

Alterations in the dopaminergic system have been suggested in RLS patients (Staedt et al., 1995; Turjanski et al., 1999; Ruottinen et al., 2000; Earley et al., 2001). We have shown in our Btbd9 KO an increase in hyperactivity and circling, which has been linked in numerous studies to potential dopaminergic system alterations (Accili et al., 1996; Xu et al., 1997; Zhuang et al., 2001; Viggiano et al., 2003). We hypothesize that there will be alterations in the dopaminergic system that underlie the deficits we present here. Experiments such as high performance liquid chromatography (HPLC) of striatum, dopamine receptor binding levels, and quantification of dopamine receptors can be done to assess the dopaminergic system.

The primary complaint of RLS patients is that of unpleasant sensations in their legs at rest or night. Studies on sensory system have shown that there are abnormalities in the perception to warm and cold stimuli in RLS patient (Iannaccone et al., 1995;

Happe and Zeitlhofer, 2003; Schattschneider et al., 2004). In our Btbd9 KO we have found an increase in sensitivity to a warm stimulus, suggesting a sensory deficit in the KO similar to human RLS patients.

The unpleasant sensations in the legs and periodic limb movements in sleep in RLS patients lead to sleep disruptions and a decrease in quality of life (Abetz et al., 2004). In our Btbd9 KO we have found that there is a decrease in slow-wave sleep (non-REM sleep and an increase in time awake). The cause of the decrease in sleep time in the KO has not yet been determined but is the aim of future experiments, including analyzing electromyography (EMG) recordings during the polysomnography (PSG) for the presence of periodic limb movements in sleep.

Previous phenotypic animal models of restless legs syndrome (RLS) have shown similar findings to what we have found in our Btbd9 KO of hyperactivity and sleep disruptions. Lesioning of the A11 dopaminergic neurons in rat brains leads to hyperactivity and increase sleep (Ondo et al., 2000). The A11 neurons are believed to play a role in sensorimotor gating due to their primary projections onto the spinal cord. Additionally, a spinal cord lesion at the T9 level in rats showed alterations in sleep efficiency (Esteves et al., 2004). Whether A11 neurons are altered in Btbd9 knockout mice remains to be investigated.

It has also been demonstrated that RLS patients have alterations in iron metabolism, specifically decreases in ferritin and increases in transferrin. These taken together would point to an iron-deficiency. Animal models of iron-deficient mice show increased pain sensitivity (Dowling et al., 2009) and sleep disruptions (Dean et al., 2006) similar to what we have found. However, in our animal model we have found that there

is an increase in iron levels in the mice. Several hypotheses can be generated as to the cause of this. It is possible that the KO red blood cells are more prone to cell lysis, which would release hemoglobin molecules. Hemoglobin uses iron as a cofactor to bind to oxygen. If cell lysis occurs, this would potentially contaminate the purified serum and show a higher level of iron concentration in the KO. This could be investigated by electrophoretic protein analysis of serum proteins. Additionally, another possible explanation is that in the KO mice there is a compensatory mechanism occurring. It is possible that the KO mice are deficient in other locations of the body, such as the brain, and more iron is absorbed into the blood stream from the digestive system. This would also lead to more iron in the serum.

Some researchers have postulated that the GLO1 gene, which is directly downstream of the BTBD9 gene, is involved in RLS and iron metabolism and not the BTBD9 gene (Jones et al., 2008). The glyoxalase 1 (GLO1) gene encodes an enzyme that converts hemimercaptal to S-lactoylgutathione. However, Jellen and colleagues, using quantitative trait loci (QTL), was able to correlate alterations in iron concentration in the brain and the Btbd9 gene in the mouse (Jellen et al., 2009). This taken with our results of alterations in the Btbd9 KO, suggest that the Btbd9 gene is in fact involved in iron homeostasis. Experiments are needed to further characterize the iron system in the KO mice and define what the alteration is. These experiments include, but are not limited to, Western blots of transferrin, ferritin, and various other iron transport proteins in the mouse brain, serum, and liver. These experiments will show if there are any alterations in protein expression levels for iron transport proteins and indirectly the levels

of iron in those various regions. Additionally, as younger mice are potentially less prone to cell lysis, serum analysis at a younger age can be conducted.

The implications of these findings of this project are far reaching. First, as the function of the Btbd9 protein is currently unknown, this mouse model will help understand the function of the Btbd9 protein *in vivo* and the effects of altering protein levels. This mouse model and these findings will also further the field of RLS research by providing the first genetic mammalian model of the disease. This will allow further dissection of the pathophysiology of RLS and designing and testing of novel therapeutics.

In summary, we have created and generated the first Btbd9 KO mouse in an attempt to understand the normal function of the gene and its relation to RLS. No prior research has been conducted on the normal function of the Btbd9 protein and here we report that the Btbd9 gene plays a role in iron regulation. Alterations in the Btbd9 protein result in iron overload in the serum. Knocking-out the Btbd9 gene result in similar results to established phenotypic models of RLS, primarily hyperactivity and sleep disturbances. In addition, we report that the Btbd9 KO have sensory deficits which can be related to RLS. These data, taken together, support that the Btbd9 KO is a good animal model of RLS and could serve as the first genetic mammalian model of the disease.

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APPENDIX A

IACUC APPROVAL NOTICE

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL WITH STIPULATIONS

DATE: September 1, 2009

TO: Li, Yuqing
CIRC-545
996-6299

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Pathophysiology and Anial Model f Restless Legs Syndrome (RLS): Btbd9 Null Mice
Sponsor: NIH
Animal Project Number: 090908921

On September 1, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approved above referenced application for use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	C	40
Mice	A	2100

Approval is granted with the following stipulation(s):

The IACUC request that an ARP veterinarian observe the electrode implanted mice. Please contact Dr. Erik Dohm (4-5558) to arrange the observation. Once the IACUC is notified that the observation has taken place the stipulation can be lifted and animal procurement and initiation of the studies may commence.

Animal procurement and use of potentially hazardous agents in live animals in this project may not occur until the PI (or designee) has discussed safety precautions as indicated on the Animal Use Safety Information Sheet corresponding to this project (authorized by Occupational Health and Safety on 05/03/07) with the ARP Health and Safety Specialist, Earle Durboraw (934-3538). This stipulation will be removed when Mr. Durboraw informs the IACUC Office that a satisfactory discussion has occurred.

This protocol must be submitted to the IACUC for renewal no later than September 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.
Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 090908921 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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