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short report

A Preliminary Characterization of Btbd9 Knockout Mice

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Abstract

Family and twin studies strongly support a genetic contribution to the pathogenesis of Restless Legs Syndrome (RLS). Two independent 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 Mendelian ratio suggesting the knockout is not lethal. However, the knockout mice showed a retarded growth pattern and were approximately 20% smaller. Our preliminary experiments show that the knockout mice had periodic wakefulness in sleep and had increased pain sensitivity.

Introduction

Restless Leg Syndrome (RLS) is a 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. Genome-wide association studies have implicated several genes in RLS including BTBD9. In the mouse, Btbd9 is expressed almost ubiquitously in the brain and the rest of the body and expressed both during development as well as in adults. The function of Btbd9 protein is not known, however the family of proteins it belongs to has function in transcriptional regulation, ion channels, and protein ubiquitination. Additionally, the protein family has important dopaminergic and glutamatergic functions in the brain.

The human BTBD9 protein is 544 amino acids long while the mouse Btbd9 protein is 612 amino acids long. The extra 68 amino acids in the mouse protein are located at the N-terminal of the protein, produced by an additional exon with a methionine start codon. Excluding the 68 amino acids of the N-terminal addition, the remaining 544 amino acid sequences share 95.4% amino acid residue identity and 96.5% similarity. The high homology between mouse and human BTBD9 proteins makes it feasible to model human RLS in mice by manipulating Btbd9 gene in embryonic stem (ES) cells.

The goal of the present study is to understand the function of Btbd9 protein by using Btbd9 knockout mice. Here we analyzed the sensory and circadian function of the mutant mice. Our research should provide insight into the pathogenesis of RLS and eventually new therapeutic treatments for RLS.

Methods and Materials

Generation of Btbd9 knockout mice. Considering the large proportion of mutations in humans is contained within the 6th intron of the BTBD9 gene, we looked for mutations in the mouse homolog gene, Btbd9. We found a gene trap clone, BayGenomics RRE078, which contained a promoterless β-geo gene inserted inside the 6th intron. The β-geo protein is a fusion protein of β-galactosidase and neomycin selection gene. The fusion protein has the activity of both β-galactosidase and neomycin. The β-geo gene contains a stop codon at the 3' end thus causing an alternative splicing when inserted into a gene (Figure 1).

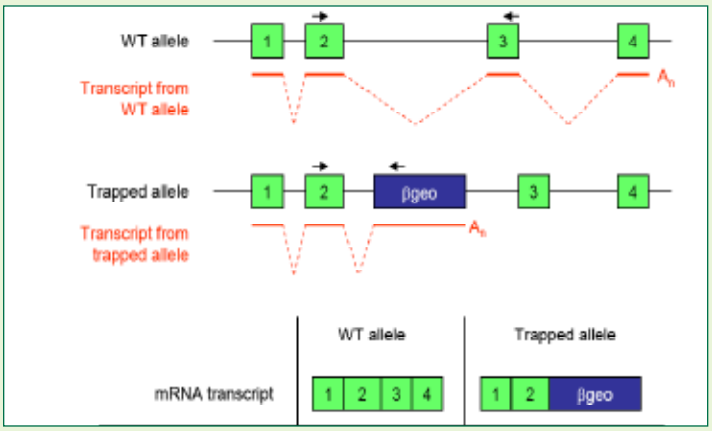


Figure 1. Diagram to show insertional mutations in mouse ES cells and the production of truncated fusion proteins (from www.mmrrc.org). Red lines: predicted splicing patterns in WT and trapped allele.

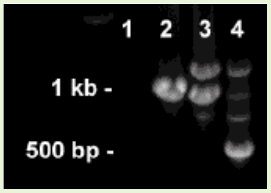


Figure 2. LA PCR genotyping of Btbd9 KO mice. Template DNA used: 1 and 3 from a KO mouse, 2 and 4 from wild type ES cells. Primers used for lanes 1 and 2 generate a predicted band of 1 kb (lane 2) and primers used for lanes 3 and 4 should produce a band of 500 bp. Top three bands in lanes 3 and 4 are non-specific PCR reaction products. Both reactions failed in lanes 1 and 3.

We obtained the ES cell clone containing this insertion and confirmed the insertion of site by 5'-RACE RT-PCR sequencing (data not shown) and the results showed that the insertion is in intron 6. The ES cells were then injected into C57BL6 blastocytes, from which we obtained 4 chimeras. One of the chimeras transmitted the mutation to germline which was confirmed by PCR genotyping of the tails (data not shown). These heterozygous mice born from that chimera were then bred to start building up a colony.
In order to determine the approximate location of the insertion site within intron 6, 19 pairs of primers spaced

about 10 kb apart within the intron covering the entire 179.223 kb intron 6 and exons 6 and 7 were designed. Each pair of primers then underwent long-arm (LA) PCR using an LA PCR kit (TaKaRa). The LA PCR was conducted using templates from control wild type DNA and DNA from a knockout mouse. After a series of LA PCR reactions we narrowed down the insertion site to approximately 500 bp (Figure 2).

Wheel running. Animals were housed in a plastic cage equipped with a steel running wheel (Lafayette Instruments). The cages contained little bedding with one nestlet as to avoid blockage of the wheel. Food and water were accessible *ad libitum*. Wheel revolutions were measured by a small sensor. Signals were registered on a computer using a data acquisition system. The mice were monitored continuously for 7 days with a 12 hr light/12 hr dark cycle. The data were imported to ClockLab for analysis of circadian activity.

Tail flick. The distal half of the animal's tail was placed on a platform under a heat lamp. The lamp was rapidly heated up to a temperature of approximately 49-53°C and the time taken to vigorous reflex withdrawal of the tail was measured. The cut-off time for this test in the absence of a withdrawal was 15s to prevent tissue damage.

Statistical analysis. Statistical analyses were performed using SAS Analyst (version 9) for wheel running distance and tail flick latency. Data was analyzed using ANOVA taking into consideration genotype, sex, age, and weight in the models. Significance was assigned at $P < 0.05$.

Results

The Btbd9 knockout mice were born in a Mendelian fashion and appeared to be healthy, suggesting that the Btbd9 knockout mice are not lethal. It was found that the body weight of the male homozygous knockout mice was approximately 78% of their control heterozygous male littermates (Figure 3; $p = 0.078$). The body weight difference was maintained at least up to 5 months of age. There was no significant body weight difference between wild type and Btbd9 heterozygous knockout mice.

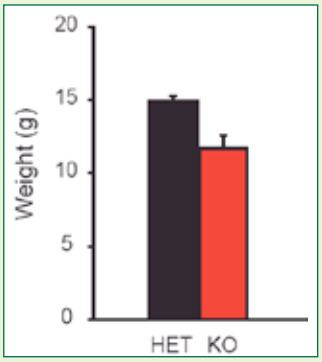


Figure 3. Body weight of Btbd9 mutant mice. HET: Btbd9 heterozygous mice; KO: Btbd9 knockout mice.

To determine if there was a sensory alteration in the heterozygous Btbd9 mice we used the tail flick experiment. The heterozygous mutant mice exhibited a significantly reduced latency to exhibit a withdrawal reflex compared to their wild type litter mates, by approximately 53 percent (Figure 4; $p = 0.0183$).

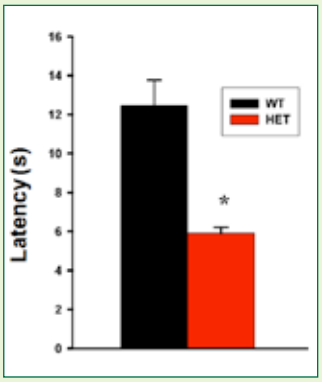


Figure 4. Tail flick experiment for pain sensation. WT: Wild type mice; HET: Heterozygous mutant mice.

For wheel running we wanted to analyze the data for hyperactivity and periodic wakefulness during the day as mice are nocturnal. Data from the wheel running experiment showed that the heterozygous Btbd9 mutants had a significant increase in wheel running activity during the day compared to that of the wild type mice ($p=0.0295$). The heterozygous mice showed an activity score of 130 while the wild type mice had a score close to 0. Additionally, the typical heterozygous mice appeared to have disrupted day activity (Figure 5). The typical heterozygous mutant mouse had brief, regular active periods during the day from 6:00 AM to 6:00 PM.

Discussion

We created a line of Btbd9 knockout mice by inserting a gene trap vector inside intron 6 of the Btbd9 gene. Using long range PCR we have identified an approximate location of the insert and confirmed the insert of the vector in the Btbd9 knockout mice. The knockout was not lethal and the mice have survived more than 6 months, albeit with a slower growth.

Preliminary behavioral and physiological experiments in the heterozygous mice have shown hyperactivity and periodic wakefulness during the day. Additionally, the heterozygous mice showed increased pain sensitivity suggesting a sensory abnormality in the mice. This coincides with the sensory and circadian abnormalities that are found in human patients with RLS.

Further experiments will be conducted to test for periodic leg movements in sleep (PLMS), decreases in dopamine and its metabolites, and decreases in iron and its transporter proteins in the Btbd9 knockout mice.

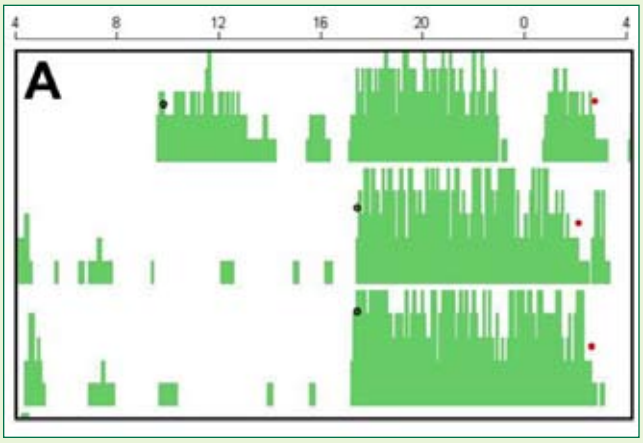
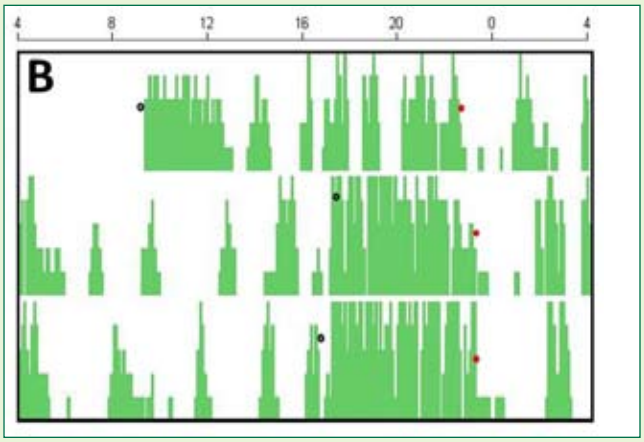


Figure 5. (A-figure above) A typical activity plot across three

days for a wild type mouse. (B-figure below) A typical activity plot across three days for a heterozygous mutant mouse, which includes periodic periods of wakefulness. Red dots signify the approximate time the mouse goes to sleep and the black dots predict the approximate time the mouse wakes up predicted by ClockLab. Heights of the bars are amount of activity.



Acknowledgments

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