eNeuro

Research Article: New Research | Disorders of the Nervous System

The role of BTBD9 in striatum and restless legs syndrome

https://doi.org/10.1523/ENEURO.0277-19.2019

Cite as: eNeuro 2019; 10.1523/ENEURO.0277-19.2019

Received: 15 July 2019
Revised: 22 July 2019
Accepted: 2 August 2019

Title: The role of BTBD9 in striatum and restless legs syndrome

Abbreviated title: BTBD9, striatum and restless legs syndrome

Authors and affiliations

Shangru Lyu [1*], Hong Xing [1*], Mark P. DeAndrade [1], Yuning Liu [1], Pablo D. Perez [2], Fumiaki Yokoi [1], Marcelo Febo [2], Arthur S. Walters [3], Yuqing Li [1].

1. Norman Fixel Institute for Neurological Diseases, Department of Neurology, College of Medicine, University of Florida Gainesville, Florida, USA
2. Department of Psychiatry, College of Medicine, University of Florida Gainesville, Florida, USA
3. Division of Sleep Medicine Vanderbilt University Medical Center, Nashville, Tennessee, USA

Corresponding author:

Yuqing Li, Ph.D., Department of Neurology, College of Medicine, University of Florida, PO Box 100236, Gainesville, Florida 32610-0236; Phone 352-273-6546; Fax: 352-273-5989; Email: yuqing.li@neurology.ufl.edu

Pages: 38

Figures: 6; Table: 1.

Word count: Abstract 249/250; Introduction 628/750; Discussion 1626/3000.

[*] The first two authors contributed equally

Conflict of interest: The authors declare no competing financial interests.

Acknowledgments

Authors acknowledge the support from the National High Magnetic Field Laboratory's Advanced Magnetic Resonance Imaging & Spectroscopy (AMRIS) Facility (National Science Foundation Cooperative Agreement No. DMR-1157490 and the State of Florida). We thank Dr. David Lovinger and his lab for helping with striatal brain slice recording. This study was sponsored by grants from the National Institute of Health (R01NS082244, R21NS065273), and the Restless Legs Syndrome Foundation. The views expressed in the submitted article are our own and not an official position of the National Institute of Health or Restless Legs Syndrome Foundation.

Abstract

Restless legs syndrome (RLS) is a sensory-motor neurological disorder characterized by uncomfortable sensations in the extremities, generally at night, which is often relieved by movements. Genome-wide association studies have identified mutations in *BTBD9* conferring a higher risk of RLS. Knockout of the *BTBD9* homolog in mice (*Btbd9*) and fly results in motor restlessness and sleep disruption. Clinical studies have found RLS patients have structural and functional abnormalities in the striatum; however, whether and how striatal pathology contributes to the pathogenesis of RLS is not known. Here, we used magnetic resonance imaging to map regions of altered synaptic activity in basal ganglia of *Btbd9* knockout mice. We further dissected striatal circuits using patch-clamp electrophysiological recordings in brain slices. Two different mouse models were generated to test the effect of specific knockout of *Btbd9* in either

striatal medium spiny neurons (MSNs) or cholinergic interneurons (ChIs) using the electrophysiological recording, motor and sensory behavioral tests. We found that *Btbd9* knockout mice showed enhanced neural activity in the striatum, increased postsynaptic currents in the MSNs, and decreased excitability of the striatal ChIs. Knocking out *Btbd9* specifically in the striatal MSNs, but not the ChIs, led to rest-phase specific motor restlessness, sleep disturbance, and increased thermal sensation in mice, which are consistent with results obtained from the *Btbd9* complete knockout mice. Our data establish the role of *Btbd9* in regulating the activity of striatal neurons. Increased activity of the striatal MSNs, possibly through modulation by the striatal ChIs, contributes to the pathogenesis of RLS.

Significance Statement

Restless Legs Syndrome (RLS) is a common movement disorder affecting up to 10% of the population and its pathophysiology is largely unknown. Brain imaging studies have shown striatal involvement. However, whether and how striatal pathology contributes to the pathogenesis of RLS is not known. Polymorphisms in the *BTBD9* gene are associated with RLS. *Btbd9* complete knockout mice have RLS-like phenotypes. With a combination of methods including functional magnetic resonance imaging, brain slice electrophysiology, cell type-specific knockout, and behavioral tests, we demonstrate the importance of the striatum, especially the MSNs, in the pathogenesis of RLS. Our results also suggest a novel mechanism that can explain the effectiveness of dopaminergic drugs for the treatment of RLS patients.

Introduction

Restless leg syndrome (RLS) is a sensorimotor neurological disease affecting up to 10% of the general population (Garcia Borreguero et al., 2017). Characteristic symptoms of RLS include an urge for patients to move their legs often accompanied by, or felt to be caused by, uncomfortable sensations in the legs (Lanza and Ferri, 2019). The symptoms of RLS generally occur or worsen at rest or inactivity in the evening, which can be at least partially relieved by movements (Trenkwalder et al., 2018). Previous studies have emphasized the major role of iron in the disease (Connor et al., 2017). In addition, one of the primary medications for the disease is D_2/D_3 dopamine (DA) agonists (Garcia-Borreguero and Cano-Pumarega, 2017), whereas refractory RLS can be treated with opioids (Silber et al., 2018).

To date, no neurodegeneration has been found in RLS patients. However, emerging studies suggest that changes in the striatum may underlie the pathogenesis of RLS (Earley et al., 2017; Lanza et al., 2017; Rizzo et al., 2017). The striatum, which is comprised of the caudate and putamen, serves as the first recipient for most of the excitatory input from the cortex and thalamus to the basal ganglia (Haber, 2016). About 95% of striatal neurons are GABAergic medium spiny neurons (MSNs), which are traditionally subdivided into two subtypes by dopamine receptor expression (Purves et al., 2001b). Generally, D_1 dopamine receptor (D_1R)-expressing MSNs function in the direct pathway and are thought to facilitate wanted movements and pronociceptive effects (Soares-Cunha et al., 2016). In contrast, MSNs in the indirect pathway mainly express D_2 dopamine receptors (D_2Rs). These MSNs are likely to be involved in the suppression of unwanted movement and the generation of antinociceptive effects (Soares-Cunha et al., 2016). Additionally, 1-2% of striatal neurons are cholinergic interneurons (ChIs) (Zucca et al., 2018). MSNs regulate the activity of ChIs through GABA and endogenous opioids

(Lim et al., 2014), while ChIs influence the activity of MSNs through M1, M4 and possibly nicotinic acetylcholine (ACh) receptors on MSNs (Bordia et al., 2016). Previous clinical studies have shown decreased D_2R expression but increased phosphorylated TH in the putamen (Connor et al., 2009). Brain imaging studies show decreased striatal dopamine transporter (DAT) (Earley et al., 2011) and D_2R binding potential (Michaud et al., 2002; Earley et al., 2013). Despite these observed alterations of the striatal dopaminergic system associated with RLS, the exact functions of the striatum, especially MSNs, in the disease development are largely unknown.

Genome-wide association studies (GWAS) have implicated up to 19 risk loci, including *BTBD9*, as genetic risk factors of RLS (Schormair et al., 2017; Jimenez-Jimenez et al., 2018). *BTBD9* codes for a protein belonging to the BTB (POZ) protein family, which modulates transcription, cytoskeletal arrangement, ion conductance and protein ubiquitination (Stogios and Prive, 2004; Stogios et al., 2005). An alteration in hippocampal synaptic plasticity and neurotransmission has been found in *Btbd9* knockout (KO) mice (DeAndrade et al., 2012b). Loss of the BTBD9 homolog in *Drosophila melanogaster* results in increased motor activity, decreased DA levels, and disrupted sleep patterns (Freeman et al., 2012). Similarly, the systematic *Btbd9* KO mice showed motor restlessness, thermal hypersensitivity, and a disruption in sleep structure (DeAndrade et al., 2012a). Therefore, the systematic *Btbd9* KO can be considered as a valuable disease mouse model to study the pathophysiology of RLS (DeAndrade et al., 2012a; Allen et al., 2017).

To elucidate the impact of striatal neurons on the generation of RLS-like phenotypes, we performed *in vivo* manganese-enhanced magnetic resonance imaging with the systematic *Btbd9*

KO mice and mapped the functional neural activity in the basal ganglia circuits. We next conducted electrophysiological recordings to observe both intrinsic and spontaneous firing activity of MSNs and ChIs in the striatum. Moreover, we selectively deleted *Btbd9* in either striatal MSNs or ChIs and conducted behavioral studies.

Material and methods

Mice

The generation of the systematic *Btbd9* KO mice

The homozygous *Btbd9* KO male mice used in MRI imaging were generated as described previously (DeAndrade et al., 2012a). The systematic *Btbd9* KO mice used in the electrophysiological recording were generated from a line of *Btbd9 loxP* mice imported from the European Mouse Mutant Archive (EMMA; ID: 05554). In this line, the fourth exon of the *Btbd9* gene was flanked by *loxP* sites (floxed). We first removed neomycin selection cassette by crossing with FLP mice (Jackson Laboratory stock 126 no. 003946) to obtain *Btbd9 loxP* mice, which was then crossed with a general cre deletor to obtain *Btbd9* KO allele. Heterozygous *Btbd9* KO mice were interbred to produce experimental homozygous *Btbd9* KO mice and the wild type (WT) littermate controls.

The generation of specific *Btbd9* knockout mice

The MSN-specific *Btbd9* knockout mice (*Btbd9* sKO) were generated by breeding *Btbd9 loxP* mice with *Rgs9-cre* mice, in which the *cre* gene was inserted at the 3' end of the *Rgs9* gene (Dang et al., 2006). Double heterozygous mice (*Rgs9-cre+/-Btbd9 loxP+/-*) were used for breeding with hetero- (*Btbd9 loxP+/-*) or homozygous Btbd9 loxP mice (*Btbd9 loxP-/-*) to

generate the conditional KO animals (*Rgs9-cre+/-Btbd9 loxP-/-*) and control groups, including WT littermates, animals only expressing *Rgs9-cre* (*Rgs9-cre+/-*) and animals only having loxP sites in one (*Btbd9 loxP+/-*) or both of the DNA strands (*Btbd9 loxP-/-*). PCR was used for genotyping the *Rgs9-cre* (forward: TGC TCA AAA ATT GTG TAC CTT TAG C; reverse: CAA CAC CCC ATT CGC TTT TTC CA) and the *loxP* sites (forward: ACA TCA CCC ATT ACT TAG AAC CTC; reverse: CAC AGC TAT TTC CTG TCA TTC TGG ACA).

The ChI-specific *Btbd9* knockout mice (*Btbd9* ChKO) were generated by breeding *Btbd9 loxP* mice with *Chat-cre* mice (The Jackson Laboratory; Stock No. 006410), in which the neo cassette had been removed by crossing with FLP mice. Breeding was conducted as outlined for *Btbd9* sKO above. PCR was used for genotyping the *Chat-cre* (forward: ATC TCC GGT ATT GAA ACT CCA GCG C; reverse: CAC TCA TGG AAA ATA GCG ATC). To confirm the specific deletion of *Btbd9* in the striatum, we dissected out brain regions following the protocol (Spijker, 2011) and PCR was conducted with primers specific for recombined locus (forward: AAG GCG CAT AAC GAT ACC ACG AT; reverse: TGG TGA TTC AAA TCT CCT TCC AAC ACA) (Fig. 4C). Experimental mice were housed in standard mouse cages at 21°C under normal 12-hour light, 12-hour dark (12 LD) cycle condition. The protocol for the study received prior approval by the Institutional Animal Care and Use Committee, and all studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed as described before (Yokoi et al., 2011) to determine whether exon 4 was deleted in mice after *cre*-mediated recombination. In brief, 3

160 *Btbd9* sKO and 3 control adult male mice were sacrificed, and several brain regions (striatum,
161 cerebral cortex and cerebellum) were harvested and flash frozen in liquid nitrogen. RNA was
162 extracted using an RNAeasy Mini kit (Qiagen) according to the manufacturer's instructions.
163 Next, cDNA was made using SuperScript III reverse transcriptase (Invitrogen). PCR primers
164 specific to *Btbd9* exons 4 and 5 (forward: GAC TCT TGT CTC CGG ATG CT; reverse: TCA
165 CAA CCT GAG CCC CAT AC); β-actin (forward: CAC CCG CGA GCA CAG CTT CTT TG;
166 reverse: AAT ACA GCC CGG GGA GCA TCG TC). The expression of *Btbd9* mRNA was
167 measured and normalized by Bio-Rad CFX manager 3.1.
168
169 **Manganese-enhanced magnetic resonance imaging (MEMRI)**
170 **$MnCl_2$ (manganese chloride) pretreatment**
171 MEMRI was performed as described previously (Perez et al., 2018; Zubcevic et al., 2018).
172 Before the treatment, the animals used in the experiment were handled every two weeks and
173 acclimatized to the investigator. Manganese (II) chloride tetrahydrate (Sigma-Aldrich Chemical
174 Co., St. Louis, MO, USA) was dissolved in distilled deionized water and sterilely filtered before
175 administered intraperitoneally at a dose of 70 mg/kg/ml. After injections, mice were returned to
176 their home cage and imaged after 20-24 hrs as previously reported (Perez et al., 2013).
177 **MRI**
178 Images were collected by a 4.7 Tesla Magnex Scientific scanner under the control of Agilent
179 Technologies VnmrJ 3.1 console software. A 38-mm quadrature transmit/receive radiofrequency
180 coil tuned to 200MHz was used (Insight NeuroImaging Systems, LLC, Leominster, MA). Mice
181 were anesthetized with 2.0% (0.1 L/min) delivered in 100% oxygen for 30-60 s. Then the level
182 of isoflurane was maintained between 1.0-1.25% throughout the entire setup and imaging session,

during which the respiratory rates were monitored continuously and sustained between 20-30 beats per minute by adjusting isoflurane levels between the range. Placed prone on custom-size plastic bed with a respiratory pad placed underneath the abdomen, body temperatures of the mice were maintained using a warm air recirculation system (SA Instruments, Inc., New York). The head and incisors of mice were secured on the front end of the plastic bed to minimize motion. The front half of the bed was aligned and clamped inside the quad RF coil and placed inside the isocenter of the scanner. Images were acquired at 4.7 Tesla using a T1-weighted spin echo pulse sequence with the following parameters: repetition time = 300 ms, echo time = 12 ms, the field of view = 19.2 × 19.2, slice thickness = 0.8 mm, 12 slices. Total scan time per mouse was 30 min.

Electrophysiological recording

Slice preparation

Experiments were conducted as described previously (Pappas et al., 2015; Augustin et al., 2018). Recordings of MSNs were conducted with 3 *Btbd9* KO and 5 WT male littermates at an average age of 4 months. Recordings of ChIs were performed with 4 *Btbd9* KO and 5 WT male littermates with an average age of 4 months, or with 3 *Btbd9* ChKO and 3 control males with an average age of 5 months. Investigators who conducted the experiments were blind to the genotypes. Animals were sacrificed, and the brains were rapidly removed. 300 μm-thick coronal brain slices containing the dorsal striatum were cut in ice-cold, oxygenated cutting saline (in mM): 180 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 D-glucose, 1 $CaCl_2$, 10 $MgCl_2$, and 10 glucose with a Vibratome (Leica VT 1000s). Slices were recovered in a holding chamber for 30 minutes at 35°C with artificial cerebrospinal fluid (ACSF). Final concentrations of ACSF (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $MgCl_2$, 2 $CaCl_2$, and 10 glucose, pH =

206 7.3 with KOH, osmolality = 290–300 mOsm. The slices were then incubated at room
207 temperature.

208 **Cell identification**

209 The slices were placed in a recording chamber and continuously perfused with ACSF that was
210 bubbled via 5% CO_2 and 95% O_2 at a rate of 1.5 ml/min while being visualized with an upright
211 microscope (Zeiss, Germany) using a 40× water-immersion objective with infrared optics. MSNs
212 were identified by the somatic size and basic membrane properties including input resistance,
213 membrane capacitance, and time constant. ChIs were recognized based on morphology and size,
214 as they are irregularly polygonal with large cell soma (>20 μm).

215 **Cell-attached and whole-cell recordings**

216 For MSN, all experiments were recorded at 32°C by a dual automatic temperature controller
217 (TC-344B). Cell-attached recording patch pipette (6–10 MΩ) contained following solutions (in
218 mM): 125 K-gluconate, 8 NaCl, 10 HEPES, 2 MgATP, 0.3 NaGTP, 0.2 EGTA (pH 7.25–7.3,
219 osmolality, 290-300 mOsm) and was used for voltage and current clamp recordings. Access
220 resistances were <30MΩ. Spontaneous postsynaptic currents were recorded in ACSF. To
221 minimize the contribution of $GABA_A$ receptors, we held cells at −70 mV with an application of
222 50 μM picrotoxin solution, which can abolish the activation of $GABA_A$ receptors. Next, at
223 holding potential -65 mV, injection of depolarizing 50 pA current pulse of 300 ms duration
224 evoked spike firing when the membrane potential reaches the firing threshold under current
225 clamp configuration in a brain slice. This process was repeated at 10 increasingly depolarized
226 potentials with incremental current steps (50pA).
227 For ChIs, electrodes for cell-attached recordings were filled with a K-gluconate-based solution
228 containing the following concentrations (in mM): 112.5 K-gluconate, 4 NaCl, 17.5 KCl, 0.5

$CaCl_2$, 5 MgATP, 1 NaGTP, 5 EGTA, 10 HEPES; with pH of 7.2 (270–280 mOsm) and resistance of 5–10 MΩ. Positive pressure was applied to the patch electrode as it approached the ChIs. Suction was applied to the electrode to create a seal (> 5 GΩ) between the recording pipette and cell membrane. Action potential current was recorded in a voltage-clamp mode that maintained an average of 0 pA holding current. After breaking through the cell membrane, cellular properties (capacitance, input resistance, and time constant) were recorded at a membrane potential of −70 mV. Electrode access resistance was maintained throughout at <30 MΩ. Resting membrane potential was recorded in current clamp mode. Action potential for current step recording was triggered using depolarizing currents steps of 300 ms.

Data acquirement and detection were the same as previously described (DeAndrade et al., 2012b). Recordings were made from targeted cells in the striatum using infrared differential interference contrast microscopy and an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Data were acquired using pClamp 10 software. Signals were filtered at 5 kHz, digitized at 10 kHz with a DigiData 1440 (Molecular Devices, Union City, CA). Events were detected using the Mini Analysis Program (Synaptosoft) with parameters optimized for each cell and then visually confirmed prior to analysis. The peak amplitude, 10–90% rise time and the decay time constant were measured based on the average of all events aligned by the rising phase.

Immunofluorescence staining

Rgs9-cre mice were bred with GFP mice imported from the Jackson Lab (Stock No. 007906) to obtain *Rgs9-cre* and GFP double heterozygous mice to map *Rgs9-cre* positive neurons. As described previously (Dang et al., 2006), the mice were anesthetized and perfused with ice-cold 0.1 M phosphate-buffered saline (pH7.4; PBS) followed by 4% paraformaldehyde in 0.1 M

phosphate-buffer (pH 7.4; PB). The brains were soaked in 4% paraformaldehyde-PB at 4°C overnight and then incubated in 30% sucrose in 0.1 M PBS at 4°C until the brains sank to the bottom. The brains were frozen with dry-ice powder and cut coronally into 40 μm sections with a Histoslide 2000 sliding microtome (Reichert-Jung). Sections were sequentially rinsed 5 min each in 0.5% Triton-X-100, 0.02 M PBS; 0.1% Triton-X-100, 0.02 M PBS; 10mM glycine in 0.1 M PBS for 3 times; 0.5 ml 2% gelatin in 0.1 M PBS; 10mM glycine in 0.1 M PBS; and 0.1% BSA in 0.1 M PBS. Then tissues were incubated with the primary antibody, 1:50 goat anti-choline acetyltransferase (AB144P; Millipore, Billerica, MA), dissolved in 100 ul 1% BSA, 0.1 M PBS at 4°C overnight. The next day, tissues were washed for 6 times with 0.1% BSA, 0.1 M PBS, followed by incubation with secondary antibody, 1:200 Cy3-conjugated AffiniPure donkey anti-goat IgG (705-265-003; Jackson Immuno Research Laboratories, West Grove, PA). After washing, the sections were mounted on glass slides with VECTASHIELD ® Antifade Mounting Medium (H-1400) and cover-slipped.

Behavioral studies

30 min open field

Eight *Btbd9* sKO male mice and 7 male littermates with an average age of 15 months, or 7 *Btbd9* ChKO (3 males, 4 female) and 9 controls (5 males, 4 female) with an average age of 7 months, were used in the 30 min open field analysis as previously described (DeAndrade et al., 2012a). Briefly, each mouse was placed in the center of a VersaMax Legacy open field apparatus connected to a computerized Digiscan System (Accuscan Instruments, Inc. OH) and continuously monitored for 30 min. The apparatus contains infrared sensors along the walls that

detect any breaks in the beams. Bright illumination (approximately 1 k lux at the center by a 60 W white bulb) was focused on the center of each field.

Wheel running

Eight *Btbd9* sKO mice (7 males, 1 female) and 13 control mice (11 males, 2 females) with an average age of 4 months, or 6 *Btbd9* ChKO male mice and 7 male littermates with an average age of 2 months, were maintained on a 12 LD cycle for 7 days. Wheel-running activity (DeAndrade et al., 2012a) was recorded as the number of wheel revolutions occurring during 5 min bins and analyzed using Lafayette Instrument Activity Wheel Monitor software. The activity from the last four days was included in the data analysis, grouped by light phase and dark phase.

Continuous open field

Seven male *Btbd9* sKO mice and 5 male littermates with an average age of 5 months or 3 *Btbd9* ChKO male mice and 4 male littermates with an average age of 2 months were used in the long-term open field analysis modified from 30 min open field test (Meneely et al., 2018). Each mouse was placed in the center of a VersaMax Legacy open field apparatus with enough corncob bedding, food, and water. Breaks in the beams were decoded by VERSDATA version 2.70-127E (AccuScan Instruments INC.) into behavioral patterns. Batch 1 data for *Btbd9* ChKO mice were collected every 1 hour. Other data were recorded every 15 min throughout the experiment. Data from the last four days were separated into light and dark phases, and the total distances during each phase were combined and coded as day 4-7, and night 4-7, respectively. The analysis was conducted based on all four periods in each phase. Separately, the total distances for each 15 min of the last four days were recoded for sleep analysis. If the total distance traveled in 15 min was 0, the mouse was considered as sleeping, and the data were coded as 0; otherwise, the mouse was considered as awake, and the data were coded as 1.

297 **Tail flick test**

298 Nine male *Btbd9* sKO mice and 9 male littermates with an average age of 8 months were tested
299 for the perception of warm stimuli. Each mouse was placed in an acrylic restrainer with the distal
300 end of its tail protruding on a metal surface maintained at 55°C. The timer was turned on once
301 the tail touched the surface and immediately stopped when the mouse flicked its tail away from
302 the heat. The latency to respond was limited to 90 s to prevent injury to the mouse.

303

304 **Experimental design and statistical analysis**

305 Images were processed and analyzed as previously reported (Perez et al., 2013). Mn^{2+}
306 accumulation in active neurons produces signal intensity increases in T1 images. However, as
307 this is a non-quantitative approach to measure activity and because there is scan-to-scan intensity
308 variation independent of Mn^{2+}, we normalized images based on their individual variance. Using
309 this normalization approach, where surpassing a normalized threshold value of 1 indicates
310 increased activity associated with Mn^{2+} administration, we have observed significant differences
311 between Mn^{2+} administered and non-treated rodents. Image processing was carried out using
312 ITK-SNAP (http://www.itksnap.org), and image math scripts were available on FSL (fslmaths
313 http://www.fmrib.ox.ac.uk/fsl/). Scans were aligned with a segmented atlas of the adult mouse
314 brain using an automated affine linear registration tool from FSL (Jenkinson et al., 2012). Each
315 scan was converted to a z value map through a voxel-wise normalization procedure. The mean
316 signal intensity across the entire extracted brain volume ($\bar{x}$) was subtracted from each voxel (xi)
317 and then divided by the variance (σ). A pre-set threshold of $z \geq 1$ was selected based on prior
318 observation of individual datasets and a close inspection of their intensity distribution histograms.
319 All voxels with z score values below this threshold were set to zero. Thus, the voxels exceeding

the threshold value of $z \geq 1$ were considered in our statistical analysis as having higher signal intensities (quantified as the number of voxels above a z value of 1). Mean number of voxels for each region of interest (ROI) was compared using an unpaired two-tailed t-test (homoscedastic variances, $\alpha \leq 0.05$).

Electrophysiological data were analyzed by logistic regression (not normally distributed) or mixed model ANOVA (SAS statistic package, normally distributed) with cell identification number nested within animal identification number. Open field data were analyzed by mixed model ANOVA and adjusted for multiple comparisons using the Benjamini-Hochberg-Yekutieli false discovery rate [FDR ($p < 0.05$)]. Data obtained from wheel running study was analyzed by logistic regression with a negative binomial distribution. Total distances of continuous open field were analyzed by logistic regression with a gamma distribution while sleep analysis was conducted with binomial logistic regression modeling the probability of waking. Tail flick data were processed by logistic regression with a gamma distribution. GEE model in the logistic regression normalized WT or control groups in terms of current steps, wheel running, continuous open field, and tail flick to 0 without the error bar. Age and gender were used as covariates in all analysis.

To generate the hourly wheel-running activity presented in Figure 5B, we summed the interval counts during each hour for each animal. Wheel-running activity during the last 96 hours was analyzed. Hence each animal had 4 data points for each hour. The average interval counts within each hour were calculated for each genotype. The *p* values, calculated by the unpaired Student's test, were marked above hours in the figure. To generate the hourly probability of waking in Figure 5I, we determined the sleep status by the total distance traveled during 15 min in the long-

342 term open field test as mentioned above. Therefore, there were 4 data points for each animal
343 during each hour, which were coded from 1 to 4 as "sample". Last 4 days' open field activity
344 was analyzed. Hence each animal had 4 days of data, which were coded from 1 to 4 as "period".
345 The probability of waking was calculated from a genotype and hour two-way interaction with
346 repeated measurement of the period, hour, and sample using SAS logistic regression with a
347 binomial distribution. For each genotype, SAS normalized probability of waking during each
348 hour to the probability of the last hour either during the day or night period. The probability of
349 waking at 6 PM of the control mice was set as 1, and the probabilities of the waking of other
350 hours of the control mice were calculated relative to that of 6 PM. To calculate the relative
351 difference between ChKO mice and controls during each hour, we sorted the data by the hour
352 and analyzed the probability of waking for each genotype with repeated measurement of period
353 and sample using SAS logistic regression with a binomial distribution. The *p* values were
354 marked above hours in the figure. The probability of waking of the ChKO mice was derived
355 from the relative difference between the ChKO mice and the control mice.
356

357 ## Results

358

359 ### Increased striatal neural activity in fMRI study with the systematic *Btbd9* KO mice

360

361 To study the role of the striatum in RLS pathogenesis, we first used MEMRI imaging to
362 determine the striatal neural activity in the systematic *Btbd9* KO mice. MEMRI has been
363 extensively used to track Ca^{2+}-dependent synaptic activity (Lu et al., 2007; Hsu et al., 2008; Chiu
364 et al., 2015; Dudek et al., 2015; Perrine et al., 2015). As a calcium analog, Mn^{2+} enters active

synapses through voltage-gated calcium channels (Fukuda and Kawa, 1977; Narita et al., 1990) and is sequestered and transynaptically transported antero- and retrogradely across active neural circuits (Sloot and Gramsbergen, 1994; Pautler et al., 1998; Takeda et al., 1998a; Takeda et al., 1998b; Saleem et al., 2002; Murayama et al., 2006). The presence of the paramagnetic Mn^{2+} ion in the brain increases longitudinal relaxation rates and enhances signal intensity in T1 weighted scans, and is utilized for functional mapping of synaptic activity (Duong et al., 2000). Here, after Mn^{2+} was injected into the mice, the images of ROI were acquired (Figure 1A). The result showed increased neuronal activity in the cerebral cortex of the systematic *Btbd9* KO mice, indicating an increased cortical input to the striatum. This will be addressed in detail in a separate manuscript. In the striatum, which is the focus of the current study, there was a significant increase in the caudate/putamen (Figure 1B, $p = 0.006$, unpaired two-tailed t-test), indicating increased neural activity specifically in the striatum. Increased entry of Mn^{2+} likely through voltage-gated Ca^{2+} channels also suggest an increase in Ca^{2+}-dependent neural activity.

More excitable MSNs but decreased ChI activity in the systematic *Btbd9* KO mice

As the first recipient for the excitatory input from almost all of the cortex, the striatum is mostly composed of MSNs (> 95%) (Cox and Witten, 2019). To determine the source of increased neural activity in striatum revealed by fMRI study, we did whole cell patch-clamp recording in brain slices. The result indicated that the resting membrane potential of the systematic *Btbd9* KO cells was higher than the WTs (Figure 2D, $p = 0.03$, logistic regression with a gamma distribution). No change was found in membrane capacitance (Figure 2A, $p = 0.93$, logistic regression with a gamma distribution), input resistance (Figure 2B, $p = 0.58$, logistic regression

388 with a gamma distribution) and decay time constant (Figure 2C, $p = 0.58$, logistic regression
389 with a gamma distribution). We then tested whether the intrinsic excitability of striatal MSNs
390 was affected by the loss of *Btbd9*. Depolarizing current steps were injected to the MSNs of both
391 *Btbd9* KOs and WTs (Figure 2E). There was no significant difference in the frequency-current
392 relationship (Figure 2F, G, $p = 0.51$, logistic regression with a negative binomial distribution),
393 amplitude, rise, and decay time (data not shown). Our results indicated that there was no change
394 in the intrinsic excitability of the KO MSNs.
395
396 MSNs are strongly driven by glutamatergic inputs (Purves et al., 2001a). To test whether loss of
397 BTBD9 affects excitatory synaptic transmission in MSNs, we recorded sEPSCs (Figure 2H).
398 There were no alterations in the rise time (Figure 2K, $p = 0.60$, logistic regression with a gamma
399 distribution), the decay time (Figure 2L, $p = 0.33$, logistic regression with a gamma distribution),
400 and the frequency (Figure 2I, $p = 0.11$, logistic regression with a gamma distribution) of
401 spontaneous postsynaptic currents. However, the systematic *Btbd9* KO mice showed a
402 significantly larger amplitude of sEPSC (Figure 2J, $p = 0.004$, logistic regression with a gamma
403 distribution). The increased amplitude may be due to the increased presynaptic quantal size,
404 increased postsynaptic functional AMPA receptor, or both. The result suggests that there are
405 significantly enhanced excitatory inputs to the KO MSNs. In combination with the increased
406 resting membrane potential found in the KO MSNs, the result indicates that BTBD9 deficiency
407 may cause striatal MSNs to be more excitable.
408
409 Abnormality in ACh neurotransmission plays an important role in movement disorders like
410 Parkinson's disease and dystonia (Bohnen and Albin, 2011; Dang et al., 2012; Lim et al., 2014;

411 Eskow Jaunarajs et al., 2015). Furthermore, another RLS susceptibility gene, *MEIS1*, has been
412 linked to the development of striatal ChIs (Spieler et al., 2014). Here, to better understand the
413 striatal physiology in *Btdb9* KO mice and RLS, we recorded both spontaneous activity (Figure
414 3C) and intrinsic excitability of ChIs (Figure 3A). We found that ChIs of the systematic *Btbd9*
415 KO mice had decreased intrinsic excitability (Figure 3B, $p = 0.049$, logistic regression with a
416 negative binomial distribution) and spontaneous firing activity (Figure 3D, $p = 0.04$, ANOVA).
417 It is known that MSNs inhibit the activity of ChIs through GABA and opioid receptors (Lim et
418 al., 2014). Therefore, the alterations found in ChIs can either be a cell-autonomous effect of
419 BTBD9 deficiency or the response to increased activity of MSNs. This was further explored in
420 *Btbd9* ChKO mice.
421
422 **Generation and molecular characterization of *Btbd9* sKO mice**
423
424 To further study the critical role of the striatum in RLS, we generated two conditional knockout
425 mouse models in which the *Btbd9* gene was selectively knocked out either in the MSNs or ChIs
426 (Figure 4A). Specifically, we interbred *Rgs9-cre* mice with *Btbd9 loxP* mice to obtain *Btbd9*
427 sKO mice. The transcription of *Btbd9* gene was quantified by qRT-PCR. As expected, there was
428 a significant reduction of *Btbd9* mRNA in the striatum compared to control littermates (Figure
429 4B, $p = 0.047$, paired two-tailed t-test), but not in the cerebral cortex or cerebellum (Figure 4B,
430 cerebral cortex, $p = 0.51$; cerebellum, $p = 0.28$; both paired two-tailed t-test). To future confirm
431 tissue specificity of the knockout, we dissected out different brain regions from the *Btbd9* sKO
432 mice and their controls. DNAs were extracted from these different brain regions, and PCR
433 reactions were conducted. Only the DNA extracted from the striatum of *Btbd9* sKO mice showed

434 the recombined band (Fig. 4C), indicating that the knockout was restricted to the striatum. It is
435 worth noting that the remaining *Btbd9* expression in the striatum can be accounted for by the
436 small subset of interneurons (< 4%) that do not express *cre*, and non-neuronal cells such as glial
437 cells. To determine whether ChIs express *Rgs9-cre*, we crossed *Rgs9-cre* mice with GFP
438 indicator mice and generated *Rgs9-cre*-GFP mice. There were no overlaps between 100
439 randomly selected, ChAT-positive neurons with any of the GFP-positive neurons (Figure 4D).
440 The results suggest that *Rgs9-cre* does not induce gene recombination in ChIs and *Btbd9* gene is
441 mostly knocked out in the MSNs of *Btbd9* sKO mice.
442
443 **RLS-like phenotypes in *Btbd9* sKO but not in *Btbd9* ChKO mice**
444
445 A principal feature of RLS is a desire to move (Ferre et al., 2019). Previous mouse or fruit fly
446 models of RLS have shown increased activity levels (DeAndrade et al., 2012a; Freeman et al.,
447 2012). Therefore, we utilized an open field activity chamber to assess the total activity levels of
448 the *Btbd9* sKO and *Btbd9* ChKO mice. In the short-term 30 min open field test, we observed that
449 although there was no alteration found with *Btbd9* ChKO mice (Figure 5F, total distance, p =
450 0.69; CW, p = 0.29; CCW, p = 0.70; All ANOVA), *Btbd9* sKO mice exhibited significantly
451 increased total distance traveled (Figure 5A, total distance, adjusted p = 0.01, ANOVA) and
452 vertical activity (Table 1, adjusted p = 0.049, ANOVA) compared with control mice. Vertical
453 activity here represents rearing behavior (Tatem et al., 2014). Furthermore, *Btbd9* sKO mice had
454 a significant increase in clockwise (CW) circling, while there was no statistical difference in
455 counterclockwise (CCW) circling compared with control mice (Figure 5A, CW, adjusted p =
456 0.02, ANOVA; CCW, adjusted p = 0.095, ANOVA). Finally, there were no significant

differences in stereotypical behavior or anxiety in the mice (Table 1). The increased activity level suggests that *Btbd9* sKO mice are hyperactive. Furthermore, alterations in circling behaviors indicate imbalances in the striatal dopaminergic system (Fornaguera and Schwarting, 2002). In the long-term open field test, *Btbd9* sKO mice showed no change (Figure 5C, left panel, $p = 0.35$, logistic regression with a gamma distribution) in the total distance traveled in the light phase, when mice are usually sleeping or resting. However, the sleep analysis indicates that the probability of waking of *Btbd9* sKO mice significantly increased in the light phase (Figure 5D, left panel, $p = 0.03$, logistic regression with a binomial distribution), but did not change in the dark phase, when mice are usually active (Figure 5D, right panel, $p = 0.63$, logistic regression with a binomial distribution). The symptoms of RLS patients usually occur or become worse in the evening or at night (Garcia Borreguero et al., 2017). With opposite day-night rhythms to human, *Btbd9* sKO mice had the motor restlessness with a similar circadian predominance as patients. In contrast, although *Btbd9* ChKO mice did not show any difference in total distance traveled compared with the controls (Figure 5H, left panel, $p = 0.28$; right panel, $p = 0.16$; both logistic regression with a gamma distribution), they had a decreased probability of waking during the light phase (Figure 5I, left panel, $p < 0.0001$, logistic regression with a binomial distribution), especially at 1:00PM, 3:00PM and 5:00PM (Figure 5I, right panel), and increased probability of waking during the dark phase (Figure 5I, middle panel, $p = 0.0029$, logistic regression with a binomial distribution), suggesting that the animals sleep better than their controls. It should also be noticed that the increased probability of waking mainly appeared during the 2nd half of the rest phase, which is consistence with clinical observations.

Next, we conducted a wheel running study to measure the voluntary activity of these mice under the normal 12 LD condition. *Btbd9* sKO mice showed a significantly elevated level of activity compared with controls during the light and rest phase (Figure 5B, light phase, $p = 0.0004$, logistic regression with a negative binomial distribution), but a similar level of activity as control mice during the dark and active phase (Figure 5B, dark phase, $p = 0.32$, logistic regression with a negative binomial distribution). On the other hand, *Btbd9* ChKO mice showed a significantly decreased activity level during the light and rest phase (Figure 5G, left panel, $p < 0.0001$, logistic regression with a negative binomial distribution) but increased activity level during the dark and active phase (Figure 5G, right panel, $p = 0.0015$, logistic regression with a negative binomial distribution). These data are consistent with the long-term open field test and suggest that only *Btbd9* sKO mice have an increase in voluntary activity during their rest period. The increased voluntary activity mainly appeared during the 2nd half of the rest phase and the first half of the active phase. Taken together, both total activity and voluntary activity were increased in the *Btbd9* sKO mice in the rest phase, which resembles aspects of nocturnal RLS activity found in patients. The circadian component-involved behavior of *Btbd9* ChKO mice is completely contradictory to *Btbd9* sKO mice. Loss of BTBD9 protein only in MSNs, but not ChIs, can cause diurnal motor restlessness in mice.

Uncomfortable sensations in lower limbs are another common phenotype of RLS (Garcia Borreguero et al., 2017). Therefore, we tested the *Btbd9* sKO mice for abnormalities in the sensory system using the tail-flick test. The mutant mice had a higher level of sensitivity to the heat stimuli (Figure 5E, $p = 0.005$, logistic regression with a gamma distribution), indicating that

mice lacking BTBD9 specifically in MSNs developed alterations in thermal sensation as the systematic *Btbd9* KO.

Increased ChIs excitability in *Btbd9* ChKO mice

The behavioral data showed that BTBD9 deficiency in MSNs, but not in ChIs, can lead to RLS-like phenotypes. Therefore, it is likely that the KO of *Btbd9* in ChIs alone did not contribute directly to the behavioral and electrophysiological alterations observed in the systematic *Btbd9* KO mice. To explore this, we recorded the ChIs in *Btbd9* ChKO mice for both spontaneous activity (Figure 6C) and responses to the injected currents (Figure 6A). We did not find the decreased activity of ChIs in *Btbd9* ChKO mice, as observed in the systematic *Btbd9* KO (Figure 3). Instead, spontaneous activity of ChIs in the mutant mice was significantly increased (Figure 6D, left panel, $p = 0.02$, ANOVA), although there was no change found in intrinsic excitability (Figure 6B, $p = 0.70$, logistic regression with a negative binomial distribution). Hence, a lack of *Btbd9* in ChIs alone led to elevated ChI activity.

Discussion

In this study, we determined how the loss of *Btbd9* affects striatal physiology and focused on the role of MSNs in RLS pathogenesis. Using both brain imaging *in vivo* and electrophysiological recording *in vitro*, we found that the systematic *Btbd9* KO mice had enhanced neural activity in the striatum, more excitable MSNs, and decreased activity in the ChIs. In addition, specific loss of *Btbd9* in the MSNs was sufficient to cause RLS-like phenotypes. When *Btbd9* was

524 conditionally knocked out in the ChIs, the mice showed neither RLS-like behavioral phenotypes
525 or maintained decreased activity in the ChIs as observed with the systematic *Btbd9* KO mice.
526 The results suggest that activity changes in the ChIs in the systematic *Btbd9* KO mice are not
527 cell-autonomous but result from alteration of striatal circuits, including changes in the MSNs. It
528 should be noted that the current study is not aimed at generating new animal models for RLS.
529 Instead, our findings demonstrate that striatum, especially MSNs, is critically involved in the
530 development of RLS-like phenotypes in mice.
531
532 Our imaging study revealed increased neural activity in the systematic *Btbd9* KO striatum.
533 Close to 95% of cells in the striatum are MSNs. Therefore, the result suggests a possible overall
534 increase in the Ca^{2+}-dependent neural activity of MSNs. Furthermore, electrophysiological
535 results showed an increased excitatory synaptic transmission onto striatal MSNs while their
536 intrinsic neuronal properties were not altered. No change in the frequency, but the higher
537 amplitude of sEPSC suggest that the MSNs of the KO mice seem to have increased excitatory
538 synaptic inputs. Combined, our data suggest an enhancement of corticostriatal or thalamostriatal
539 synaptic activity. Similarly, in iron deprived rat, which is thought to be an RLS rodent model,
540 corticostriatal excitability is elevated (Yepes et al., 2017). Here, it is not known whether inputs
541 from corticostriatal, thalamostriatal, or both contributed to the increased striatal sEPSC
542 amplitude in the systematic *Btbd9* KO mice. Differential alterations of thalamostriatal and
543 corticostriatal synapses have been found in mouse models of Huntington's disease (Deng et al.,
544 2014; Kolodziejczyk and Raymond, 2016; Parievsky et al., 2017), an MPTP-treated monkey
545 model of parkinsonism (Raju et al., 2008) and a rat model with L-DOPA-induced dyskinesias

(Zhang et al., 2013). Future studies will be focused on dissecting the differential effects of the systematic *Btbd9* KO on corticostriatal and thalamostriatal synaptic transmission.

The electrophysiological recording revealed a decreased excitability in striatal ChIs in the systematic *Btbd9* KO mice. It has been found that *Gbx2*, a gene which is essential for the proper development of striatal ChIs, is downed-regulated in heterozygous *Meis1* knockout embryos (E12.5) (Spieler et al., 2014). *MEIS1* is another gene implicated in RLS (Schormair et al., 2017). With conditional knockout mice and electrophysiological recordings, we found that like *Meis1* mice, *Btbd9* mutation in the ChIs alone can cause functional abnormalities in striatal ChIs, yet it was insufficient to produce RLS-like behaviors. In the rest phase, *Btbd9* ChKO mice had decreased locomotor activity with increased excitability of striatal ChIs, while the systematic *Btbd9* KO mice showed increased locomotor activity (DeAndrade et al., 2012a) with decreased excitability in striatal ChIs. The results suggest a critical role of ChIs in movement control in the rest phase, and the decreased ChI activity found in the systematic *Btbd9* KO mice is not a cell-autonomous effect. It is known that ACh can regulate striatal circuit through its receptors on MSNs, GABAergic interneurons, glutamatergic and DA terminals (Lim et al., 2014). It is possible that excess release of ACh in *Btbd9* ChKO mice downregulated activities of MSNs directly through the nAChRs (Liu et al., 2007) and M4 receptors (Howe and Surmeier, 1995) or indirectly by increasing GABAergic inhibition on MSNs (English et al., 2011). As the sole output of the striatum, MSNs may lead to decreased locomotion through their decreased activities. However, when *Btbd9* was knocked out systematically, neuronal activities were changed in both MSNs and ChIs. Our results showed that MSNs became more excitable and may have increased activity. These changes in MSNs were likely to be dominant and overcame the

influence of ChIs. MSNs, via their inhibition on the ChIs, in turn, led to decreased activity in ChIs as showed by the electrophysiological recording. Although it is not clear if the activity level of MSNs in the *Btbd9* sKO mice is the same as what we found in the systematic *Btbd9* KO, *Btbd9* sKO mice did show an opposite output in behavioral tests as *Btbd9* ChKOs, which support the overwhelmingly inhibitory effect of MSNs to ChIs as mentioned above.

MSN-specific *Btbd9* KO was found to be sufficient to induce rest-phase specific hyperactive movement, sleep disturbance, and increased thermal sensation in mice. The findings support the idea that striatum is critical for the pathogenesis of RLS motor phenotypes. Postmortem studies comparing RLS patients and control group show a decreased D_2R expression in the putamen, but increased phosphorylated tyrosine hydroxylase (TH), a rate-limiting enzyme for DA synthesis, in both putamen and SN (Connor et al., 2009). Brain imaging studies indicate decreased membrane-bound dopamine transporter (DAT) level and D_2R binding potential in the striatum (Rizzo et al., 2017). Additionally, changes in iron hemostasis have been found in SN and putamen of RLS patients (Allen et al., 2001; Earley et al., 2014). Striata of iron-deprived RLS rodent models show a reduced density of DAT and DA receptors (Erikson et al., 2000; Erikson et al., 2001) and enhanced release at corticostriatal terminals (Yepes et al., 2017). Taken together, these studies suggest functional alterations in the striatum are correlated with RLS (Rizzo et al., 2017). In addition, a recent GWAS study demonstrated that striatal MSNs are associated with insomnia (Jansen et al., 2019), which can be caused by RLS.

However, dysfunctional striatal circuit caused by the loss of BTBD9 might not be the only mechanism in the systematic *Btbd9* KO mice. *Btbd9* is also expressed in the spinal cord,

592 although our genomic PCR failed to detect any deletion of the *Btbd9* gene in the spinal cord of
593 the *Btbd9* sKO mice. Alterations in the striatum can lead to alterations in the spinal cord. The
594 basal ganglia output modulates the spinal cord through feedback to the cortex. Additionally,
595 recent evidence has suggested that a microcircuit exists between the corticostriatal tract and the
596 corticospinal tract, starting in the striatum and ending in the spinal cord (Kiritani et al., 2012).
597 Spinal neural circuits have been proposed to be central in RLS development (Clemens et al.,
598 2006; Zwartbol et al., 2013; Koblinger et al., 2014; Kumru et al., 2015). It has been found that a
599 lesion in a dorsoposterior hypothalamic dopaminergic A11 cell group, which is the sole source of
600 spinal dopamine, leads to increased wakefulness across the rest phase (Ondo et al., 2000) and a
601 long-lasting reduction in sensory thresholds (Clemens et al., 2006) in rats. D_3 receptors are
602 mostly present in the dorsal spinal cord where it has been shown to modulate sensory pathways
603 (Meneely et al., 2018). Mice knocked out of D_3 receptors (D_3KO) show increased locomotion
604 (Accili et al., 1996; Clemens et al., 2006) and decreased paw withdrawal latencies to thermal
605 pain stimulation. Furthermore, D_3KO mice have a reduction in frequency-dependent modulation
606 of the longer-latency reflex (LLRs) in the spinal cord (Keeler et al., 2012). Pharmacological
607 experiments indicate that D_3KO mice exhibit a reversal of the modulatory actions of DA on
608 spinal reflexes from depression to facilitation (Clemens and Hochman, 2004), and are responsive
609 to D_1 and D_2 receptor agonists (Keeler et al., 2012). These findings emphasize the role of spinal
610 dopamine in the etiology of RLS.
611
612 Our results provide a novel mechanism for the efficacy of dopaminergic agonists as treatments
613 for RLS. The dopaminergic system plays a critical role in RLS pathogenesis. In addition to the
614 evidence mentioned above, RLS patients have upregulated levels of L-DOPA metabolites and

615 TH activity in cerebrospinal fluid (Earley et al., 2001; Allen et al., 2009). RNAi-mediated
616 knockdown of *BTBD9* homolog gene in a subset of dopaminergic neurons can reproduce sleep
617 phenotype in fruit flies (Freeman et al., 2012). Ropinirole rescues the sensory deficit found in the
618 systematic *Btbd9* KO mice (DeAndrade et al., 2012a). It is possible that increased activity of
619 MSNs leads to a decreased firing in the ChIs and a lower level of ACh. Nigrostriatal DA
620 terminals express both ionotropic nicotinic ACh (nACh) and $G_{q/11}$-coupled muscarinic ACh
621 (mACh) receptors (Cachope and Cheer, 2014). The activation of nACh receptors elicits DA
622 release, while the downstream pathway of mACh receptor inhibits DA release. Overall,
623 optogenetic stimulation of ChIs evokes DA release in slice preparation (Cachope et al., 2012;
624 Cachope and Cheer, 2014). Therefore, a decreased level of ACh may cause a deficiency in
625 dopamine release. It has also been found that activation of MSN through AMPA receptor leads
626 to the generation of a diffusible messenger, hydrogen peroxide (H_2O_2), which can inhibit DA
627 release via ATP-sensitive potassium channels (Avshalumov et al., 2008; Sulzer et al., 2017).
628 Either way, increased MSN activity is predicted to reduce striatal DA release in the systematic
629 *Btbd9* KO mouse model, which is consistent with the clinical finding that DA agonists can be
630 used to treat RLS.
631
632 In summary, our results suggest that alteration in the striatal circuit, especially increased activity
633 of the striatal MSNs, could potentially serve as a main pathogenetic mechanism of the motor and
634 sensory dysfunction in RLS. It also supports an indirect role of the striatal ChIs in the disease
635 development. Finally, these data present a plausible explanation for the therapeutic efficacy of
636 the dopamine receptor agonists in RLS that includes the inhibition of the MSN activity, the
637 regulation of ChI excitability, and the resetting of the striatal circuit. Further investigations are

needed to dissect the complex interactions among the striatal neurons and modulatory neurotransmitters, which will aid the development of highly selective anti-RLS drugs with fewer side effects.

References:

Accili D, Fishburn CS, Drago J, Steiner H, Lachowicz JE, Park BH, Gauda EB, Lee EJ, Cool MH, Sibley DR, Gerfen CR, Westphal H, Fuchs S (1996) A targeted mutation of the D3 dopamine receptor gene is associated with hyperactivity in mice. Proc Natl Acad Sci U S A 93:1945-1949.

Allen RP, Connor JR, Hyland K, Earley CJ (2009) Abnormally increased CSF 3-Ortho-methyldopa (3-OMD) in untreated restless legs syndrome (RLS) patients indicates more severe disease and possibly abnormally increased dopamine synthesis. Sleep Med 10:123-128.

Allen RP, Barker PB, Wehrl FW, Song HK, Earley CJ (2001) MRI measurement of brain iron in patients with restless legs syndrome. Neurology 56:263-265.

Allen RP, Donelson NC, Jones BC, Li Y, Manconi M, Rye DB, Sanyal S, Winkelmann J (2017) Animal models of RLS phenotypes. Sleep Med 31:23-28.

Augustin SM, Chancey JH, Lovinger DM (2018) Dual Dopaminergic Regulation of Corticostriatal Plasticity by Cholinergic Interneurons and Indirect Pathway Medium Spiny Neurons. Cell Rep 24:2883-2893.

Avshalumov MV, Patel JC, Rice ME (2008) AMPA receptor-dependent H2O2 generation in striatal medium spiny neurons but not dopamine axons: one source of a retrograde signal that can inhibit dopamine release. J Neurophysiol 100:1590-1601.

Bohnen NI, Albin RL (2011) The cholinergic system and Parkinson disease. Behav Brain Res 221:564-573.

Bordia T, Perez XA, Heiss J, Zhang D, Quik M (2016) Optogenetic activation of striatal cholinergic interneurons regulates L-dopa-induced dyskinesias. Neurobiol Dis 91:47-58.

Cachope R, Cheer JF (2014) Local control of striatal dopamine release. Front Behav Neurosci 8:188.

Cachope R, Mateo Y, Mathur BN, Irving J, Wang HL, Morales M, Lovinger DM, Cheer JF (2012) Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing. Cell Rep 2:33-41.

Chiu CH, Siow TY, Weng SJ, Hsu YH, Huang YS, Chang KW, Cheng CY, Ma KH (2015) Effect of MDMA-Induced Axotomy on the Dorsal Raphe Forebrain Tract in Rats: An In Vivo Manganese-Enhanced Magnetic Resonance Imaging Study. PLoS One 10:e0138431.

Clemens S, Hochman S (2004) Conversion of the modulatory actions of dopamine on spinal reflexes from depression to facilitation in D3 receptor knock-out mice. J Neurosci 24:11337-11345.

Clemens S, Rye D, Hochman S (2006) Restless legs syndrome: revisiting the dopamine hypothesis from the spinal cord perspective. Neurology 67:125-130.

678 Connor JR, Patton S, Oexle K, Allen R (2017) Iron and restless legs syndrome: Treatment,
679 genetics and pathophysiology. Sleep Med 31:61-70.
680 Connor JR, Wang XS, Allen RP, Beard JL, Wiesinger JA, Felt BT, Earley CJ (2009) Altered
681 dopaminergic profile in the putamen and substantia nigra in restless leg syndrome.
682 Brain 132:2403-2412.
683 Cox J, Witten IB (2019) Striatal circuits for reward learning and decision-making. Nat Rev
684 Neurosci.
685 Dang MT, Yokoi F, Yin HH, Lovinger DM, Wang Y, Li Y (2006) Disrupted motor learning and long-
686 term synaptic plasticity in mice lacking NMDAR1 in the striatum. Proc Natl Acad Sci U S A
687 103:15254-15259.
688 Dang MT, Yokoi F, Cheetham CC, Lu J, Vo V, Lovinger DM, Li Y (2012) An anticholinergic reverses
689 motor control and corticostriatal LTD deficits in Dyt1 DeltaGAG knock-in mice. Behav
690 Brain Res 226:465-472.
691 DeAndrade MP, Johnson RL, Jr., Unger EL, Zhang L, van Groen T, Gamble KL, Li Y (2012a) Motor
692 restlessness, sleep disturbances, thermal sensory alterations and elevated serum iron
693 levels in Btbd9 mutant mice. Hum Mol Genet 21:3984-3992.
694 DeAndrade MP, Zhang L, Doroodchi A, Yokoi F, Cheetham CC, Chen HX, Roper SN, Sweatt JD, Li
695 Y (2012b) Enhanced hippocampal long-term potentiation and fear memory in Btbd9
696 mutant mice. PLoS One 7:e35518.
697 Deng YP, Wong T, Wan JY, Reiner A (2014) Differential loss of thalamostriatal and corticostriatal
698 input to striatal projection neuron types prior to overt motor symptoms in the Q140
699 knock-in mouse model of Huntington's disease. Front Syst Neurosci 8:198.
700 Dudek M, Abo-Ramadan U, Hermann D, Brown M, Canals S, Sommer WH, Hyytia P (2015) Brain
701 activation induced by voluntary alcohol and saccharin drinking in rats assessed with
702 manganese-enhanced magnetic resonance imaging. Addict Biol 20:1012-1021.
703 Duong TQ, Silva AC, Lee SP, Kim SG (2000) Functional MRI of calcium-dependent synaptic
704 activity: cross correlation with CBF and BOLD measurements. Magn Reson Med 43:383-
705 392.
706 Earley CJ, Hyland K, Allen RP (2001) CSF dopamine, serotonin, and biopterin metabolites in
707 patients with restless legs syndrome. Mov Disord 16:144-149.
708 Earley CJ, Uhl GR, Clemens S, Ferré S (2017) Connectome and molecular pharmacological
709 differences in the dopaminergic system in restless legs syndrome (RLS): plastic changes
710 and neuroadaptations that may contribute to augmentation. Sleep Medicine 31:71-77.
711 Earley CJ, Connor J, Garcia-Borreguero D, Jenner P, Winkelman J, Zee PC, Allen R (2014) Altered
712 brain iron homeostasis and dopaminergic function in Restless Legs Syndrome (Willis-
713 Ekbom Disease). Sleep Med 15:1288-1301.
714 Earley CJ, Kuwabara H, Wong DF, Gamaldo C, Salas R, Brasic J, Ravert HT, Dannals RF, Allen RP
715 (2011) The dopamine transporter is decreased in the striatum of subjects with restless
716 legs syndrome. Sleep 34:341-347.
717 Earley CJ, Kuwabara H, Wong DF, Gamaldo C, Salas RE, Brasic JR, Ravert HT, Dannals RF, Allen
718 RP (2013) Increased synaptic dopamine in the putamen in restless legs syndrome. Sleep
719 36:51-57.

720 English DF, Ibanez-Sandoval O, Stark E, Tecuapetla F, Buzsaki G, Deisseroth K, Tepper JM, Koos T
721 (2011) GABAergic circuits mediate the reinforcement-related signals of striatal
722 cholinergic interneurons. Nat Neurosci 15:123-130.
723 Erikson KM, Jones BC, Beard JL (2000) Iron deficiency alters dopamine transporter functioning
724 in rat striatum. J Nutr 130:2831-2837.
725 Erikson KM, Jones BC, Hess EJ, Zhang Q, Beard JL (2001) Iron deficiency decreases dopamine D1
726 and D2 receptors in rat brain. Pharmacol Biochem Behav 69:409-418.
727 Eskow Jaunarajs KL, Bonsi P, Chesselet MF, Standaert DG, Pisani A (2015) Striatal cholinergic
728 dysfunction as a unifying theme in the pathophysiology of dystonia. Prog Neurobiol 127-
729 128:91-107.
730 Ferre S, Garcia-Borreguero D, Allen RP, Earley CJ (2019) New Insights into the Neurobiology of
731 Restless Legs Syndrome. Neuroscientist 25:113-125.
732 Fornaguera J, Schwarting RK (2002) Time course of deficits in open field behavior after
733 unilateral neostriatal 6-hydroxydopamine lesions. Neurotox Res 4:41-49.
734 Freeman A, Pranski E, Miller RD, Radmard S, Bernhard D, Jinnah HA, Betarbet R, Rye DB, Sanyal
735 S (2012) Sleep fragmentation and motor restlessness in a Drosophila model of Restless
736 Legs Syndrome. Curr Biol 22:1142-1148.
737 Fukuda J, Kawa K (1977) Permeation of manganese, cadmium, zinc, and beryllium through
738 calcium channels of an insect muscle membrane. Science 196:309-311.
739 Garcia Borreguero D, Winkelmann J, Allen RP (2017) Introduction: Towards a better
740 understanding of the science of RLS/WED. Sleep Med 31:1-2.
741 Garcia-Borreguero D, Cano-Pumarega I (2017) New concepts in the management of restless
742 legs syndrome. Bmj 356:j104.
743 Haber SN (2016) Corticostriatal circuitry. Dialogues Clin Neurosci 18:7-21.
744 Howe AR, Surmeier DJ (1995) Muscarinic receptors modulate N-, P-, and L-type Ca2+ currents in
745 rat striatal neurons through parallel pathways. J Neurosci 15:458-469.
746 Hsu YH, Chen CC, Zechariah A, Yen CC, Yang LC, Chang C (2008) Neuronal dysfunction of a long
747 projecting multisynaptic pathway in response to methamphetamine using manganese-
748 enhanced MRI. Psychopharmacology (Berl) 196:543-553.
749 Jansen PR et al. (2019) Genome-wide analysis of insomnia in 1,331,010 individuals identifies
750 new risk loci and functional pathways. Nat Genet 51:394-403.
751 Jenkinson M, Beckmann CF, Behrens TE, Woolrich MW, Smith SM (2012) FSL. Neuroimage
752 62:782-790.
753 Jimenez-Jimenez FJ, Alonso-Navarro H, Garcia-Martin E, Agundez JAG (2018) Genetics of
754 restless legs syndrome: An update. Sleep Med Rev 39:108-121.
755 Keeler BE, Baran CA, Brewer KL, Clemens S (2012) Increased excitability of spinal pain reflexes
756 and altered frequency-dependent modulation in the dopamine D3-receptor knockout
757 mouse. Exp Neurol 238:273-283.
758 Kiritani T, Wickersham IR, Seung HS, Shepherd GM (2012) Hierarchical connectivity and
759 connection-specific dynamics in the corticospinal-corticostriatal microcircuit in mouse
760 motor cortex. J Neurosci 32:4992-5001.
761 Koblinger K, Fuzesi T, Ejdrygiewicz J, Krajacic A, Bains JS, Whelan PJ (2014) Characterization of
762 A11 neurons projecting to the spinal cord of mice. PLoS One 9:e109636.

763 Kolodziejczyk K, Raymond LA (2016) Differential changes in thalamic and cortical excitatory
764 synapses onto striatal spiny projection neurons in a Huntington disease mouse model.
765 Neurobiol Dis 86:62-74.
766 Kumru H, Vidal J, Benito J, Barrio M, Portell E, Valles M, Flores C, Santamaria J (2015) Restless
767 leg syndrome in patients with spinal cord injury. Parkinsonism Relat Disord 21:1461-
768 1464.
769 Lanza G, Ferri R (2019) The neurophysiology of hyperarousal in restless legs syndrome: Hints for
770 a role of glutamate/GABA. Adv Pharmacol 84:101-119.
771 Lanza G, Bachmann CG, Ghorayeb I, Wang Y, Ferri R, Paulus W (2017) Central and peripheral
772 nervous system excitability in restless legs syndrome. Sleep Med 31:49-60.
773 Lim SA, Kang UJ, McGehee DS (2014) Striatal cholinergic interneuron regulation and circuit
774 effects. Front Synaptic Neurosci 6:22.
775 Liu Z, Otsu Y, Vasuta C, Nawa H, Murphy TH (2007) Action-potential-independent GABAergic
776 tone mediated by nicotinic stimulation of immature striatal miniature synaptic
777 transmission. J Neurophysiol 98:581-593.
778 Lu H, Xi ZX, Gitajn L, Rea W, Yang Y, Stein EA (2007) Cocaine-induced brain activation detected
779 by dynamic manganese-enhanced magnetic resonance imaging (MEMRI). Proc Natl Acad
780 Sci U S A 104:2489-2494.
781 Meneely S, Dinkins M-L, Kassai M, Lyu S, Liu Y, Lin C-T, Brewer K, Li Y, Clemens S (2018)
782 Differential dopamine D1 and D3 receptor modulation and expression in the spinal cord
783 of two mouse models of Restless Legs Syndrome. Frontiers in Behavioral Neuroscience
784 12.
785 Michaud M, Soucy JP, Chabli A, Lavigne G, Montplaisir J (2002) SPECT imaging of striatal pre-
786 and postsynaptic dopaminergic status in restless legs syndrome with periodic leg
787 movements in sleep. J Neurol 249:164-170.
788 Murayama Y, Weber B, Saleem KS, Augath M, Logothetis NK (2006) Tracing neural circuits in
789 vivo with Mn-enhanced MRI. Magn Reson Imaging 24:349-358.
790 Narita K, Kawasaki F, Kita H (1990) Mn and Mg influxes through Ca channels of motor nerve
791 terminals are prevented by verapamil in frogs. Brain Res 510:289-295.
792 Ondo WG, He Y, Rajasekaran S, Le WD (2000) Clinical correlates of 6-hydroxydopamine
793 injections into A11 dopaminergic neurons in rats: a possible model for restless legs
794 syndrome. Mov Disord 15:154-158.
795 Pappas SS, Darr K, Holley SM, Cepeda C, Mabrouk OS, Wong JM, LeWitt TM, Paudel R, Houlden
796 H, Kennedy RT, Levine MS, Dauer WT (2015) Forebrain deletion of the dystonia protein
797 torsinA causes dystonic-like movements and loss of striatal cholinergic neurons. Elife
798 4:e08352.
799 Parievsky A, Moore C, Kamdjou T, Cepeda C, Meshul CK, Levine MS (2017) Differential
800 electrophysiological and morphological alterations of thalamostriatal and corticostriatal
801 projections in the R6/2 mouse model of Huntington's disease. Neurobiol Dis 108:29-44.
802 Pautler RG, Silva AC, Koretsky AP (1998) In vivo neuronal tract tracing using manganese-
803 enhanced magnetic resonance imaging. Magn Reson Med 40:740-748.
804 Perez PD, Hall G, Zubcevic J, Febo M (2018) Cocaine differentially affects synaptic activity in
805 memory and midbrain areas of female and male rats: an in vivo MEMRI study. Brain
806 Imaging Behav 12:201-216.

807 Perez PD, Hall G, Kimura T, Ren Y, Bailey RM, Lewis J, Febo M, Sahara N (2013) In vivo functional
808 brain mapping in a conditional mouse model of human tauopathy (tauP301L) reveals
809 reduced neural activity in memory formation structures. Mol Neurodegener 8:9.
810 Perrine SA, Ghoddoussi F, Desai K, Kohler RJ, Eapen AT, Lisieski MJ, Angoa-Perez M, Kuhn DM,
811 Bosse KE, Conti AC, Bissig D, Berkowitz BA (2015) Cocaine-induced locomotor
812 sensitization in rats correlates with nucleus accumbens activity on manganese-enhanced
813 MRI. NMR Biomed 28:1480-1488.
814 Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia A-S, McNamara JO, Williams SM (2001a)
815 Projections to the Basal Ganglia.
816 Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia A-S, McNamara JO, Williams SM (2001b)
817 Circuits within the Basal Ganglia System.
818 Raju DV, Ahern TH, Shah DJ, Wright TM, Standaert DG, Hall RA, Smith Y (2008) Differential
819 synaptic plasticity of the corticostriatal and thalamostriatal systems in an MPTP-treated
820 monkey model of parkinsonism. Eur J Neurosci 27:1647-1658.
821 Rizzo G, Li X, Galantucci S, Filippi M, Cho YW (2017) Brain imaging and networks in restless legs
822 syndrome. Sleep Med 31:39-48.
823 Saleem KS, Pauls JM, Augath M, Trinath T, Prause BA, Hashikawa T, Logothetis NK (2002)
824 Magnetic resonance imaging of neuronal connections in the macaque monkey. Neuron
825 34:685-700.
826 Schormair B et al. (2017) Identification of novel risk loci for restless legs syndrome in genome-
827 wide association studies in individuals of European ancestry: a meta-analysis. Lancet
828 Neurol 16:898-907.
829 Silber MH, Becker PM, Buchfuhrer MJ, Earley CJ, Ondo WG, Walters AS, Winkelman JW (2018)
830 The Appropriate Use of Opioids in the Treatment of Refractory Restless Legs Syndrome.
831 Mayo Clin Proc 93:59-67.
832 Sloot WN, Gramsbergen JB (1994) Axonal transport of manganese and its relevance to selective
833 neurotoxicity in the rat basal ganglia. Brain Res 657:124-132.
834 Soares-Cunha C, Coimbra B, Sousa N, Rodrigues AJ (2016) Reappraising striatal D1- and D2-
835 neurons in reward and aversion. Neurosci Biobehav Rev 68:370-386.
836 Spieler D et al. (2014) Restless Legs Syndrome-associated intronic common variant in Meis1
837 alters enhancer function in the developing telencephalon. Genome Res 24:592-603.
838 Spijker S (2011) Dissection of Rodent Brain Regions. Neuroproteomics 57:13-26.
839 Stogios PJ, Prive GG (2004) The BACK domain in BTB-kelch proteins. Trends Biochem Sci 29:634-
840 637.
841 Stogios PJ, Downs GS, Jauhal JJ, Nandra SK, Prive GG (2005) Sequence and structural analysis of
842 BTB domain proteins. Genome Biol 6:R82.
843 Sulzer D, Cragg SJ, Rice ME (2017) Chapter 19 - Regulation of Extracellular Dopamine: Release
844 and Uptake. In: Handbook of Behavioral Neuroscience (Steiner H, Tseng KY, eds), pp
845 373-402: Elsevier.
846 Takeda A, Ishiwatari S, Okada S (1998a) In vivo stimulation-induced release of manganese in rat
847 amygdala. Brain Res 811:147-151.
848 Takeda A, Kodama Y, Ishiwatari S, Okada S (1998b) Manganese transport in the neural circuit of
849 rat CNS. Brain Res Bull 45:149-152.

Tatem KS, Quinn JL, Phadke A, Yu Q, Gordish-Dressman H, Nagaraju K (2014) Behavioral and locomotor measurements using an open field activity monitoring system for skeletal muscle diseases. J Vis Exp:51785.

Trenkwalder C, Allen R, Hogl B, Clemens S, Patton S, Schormair B, Winkelmann J (2018) Comorbidities, treatment, and pathophysiology in restless legs syndrome. Lancet Neurol 17:994-1005.

Yepes G, Guitart X, Rea W, Newman AH, Allen RP, Earley CJ, Quiroz C, Ferre S (2017) Targeting hypersensitive corticostriatal terminals in restless legs syndrome. Ann Neurol 82:951-960.

Yokoi F, Dang MT, Li J, Standaert DG, Li Y (2011) Motor deficits and decreased striatal dopamine receptor 2 binding activity in the striatum-specific Dyt1 conditional knockout mice. PLoS One 6:e24539.

Zhang Y, Meredith GE, Mendoza-Elias N, Rademacher DJ, Tseng KY, Steece-Collier K (2013) Aberrant Restoration of Spines and their Synapses in l-DOPA-Induced Dyskinesia: Involvement of Corticostriatal but Not Thalamostriatal Synapses.

Zubcevic J, Watkins J, Perez PD, Colon-Perez LM, Long MT, Febo M, Hayward L (2018) MEMRI reveals altered activity in brain regions associated with anxiety, locomotion, and cardiovascular reactivity on the elevated plus maze in the WKY vs SHR rats. Brain Imaging Behav 12:1318-1331.

Zucca S, Zucca A, Nakano T, Aoki S, Wickens J (2018) Pauses in cholinergic interneuron firing exert an inhibitory control on striatal output in vivo.

Zwartbol RT, Jellema K, Boiten J, Rijsman R (2013) Acute exacerbation of restless legs due to cervical spinal cord ischaemia. BMJ Case Rep 2013.

Figure Legends

Fig. 1. MEMRI of neural activity in striatal and forebrain regions of the systematic *Btbd9* KO mice and their WT littermates. (A) Coronal views of the averaged signal intensity from *Btbd9* KO and WT controls. (B) *Btbd9* KO had significant increased neural activity in caudate and putamen and in the cerebral cortex. No difference was observed in other brain regions under investigation. Bars represent means plus standard errors. Cpu: caudate/putamen; AcbC: nucleus accumbens core; AcbSh: nucleus accumbens shell; GP: globus pallidus; LS: lateral septal nucleus; MS: medial septal nucleus; BNST: bed nucleus of the stria terminalis. *, $p < 0.05$.

884 Fig. 2. Whole-cell patch-clamp recording of MSNs from the systematic *Btbd9* KO mice and their
885 WT littermates. (A, B, C) *Btbd9* KO MSNs (n=20) did not have changes in membrane
886 capacitance, input resistance and decay time constant compared with the WT MSNs (n=33). (D)
887 *Btbd9* KO MSNs (n=22) had increased resting membrane potential compared with the WTs
888 (n=34). (E) Representative responses to the injected currents at 250pA of KO (n=21) and WT
889 (n=34) MSNs. (F) The frequency-current relationship for WT and *Btbd9* KO MSNs. (G) The
890 response of KO MSNs to the injected currents was not significantly different from the WT
891 MSNs. (H) Representative sEPSC traces of KO (n=20) and WT (n=30) MSNs. (I) The frequency
892 of spontaneous firing was similar between the two groups. (G) *Btbd9* KO MSNs had a higher
893 amplitude of sEPSC than the WT. (K, L) Both the rise and decay time were not different
894 between *Btbd9* homozygous KO and WT MSNs. GEE model normalized the WT group in the
895 bar graph of B to 0 without the error bars. Data in A-D and I-L were presented as median with 95%
896 confidence intervals (CI). ***, $p < 0.005$, *, $p < 0.05$.
897
898 Fig. 3. Electrophysiological recording of ChIs from the systematic *Btbd9* KO mice and their WT
899 littermates. (A) Representative responses to the injected currents at 200pA of KO (n=20) and
900 WT (n=14) ChIs. (B) The frequency-current relationship for KO and WT ChIs. KO showed
901 decreased firing frequency compared with the WT. WT was normalized to 0 in log
902 transformation. (C) Representative traces of spontaneous firings of KO (n=19) and WT (n=17)
903 ChIs. (D) *Btbd9* KO mice had decreased firing frequency, but no change in firing regularity in
904 ChIs. GEE model normalized the WT group in the bar graph of B to 0 without the error bars.
905 Data in D were presented as median with 95% CI. CV: coefficient of variance. *, $p < 0.05$.
906

907 Fig. 4. Generation of conditional KO mice and validation of the loss of *Btbd9* in the striatum by
908 qRT-PCR. (A) Schematic diagram of the generation of conditional KO mice. Filled boxes
909 represent exons. Filled triangles indicate *loxP* sites (around the 4th exon of the *Btbd9* gene). Open
910 triangles indicate the *FRT* sites that were incorporated to remove the neo cassette. *Btbd9 loxP*
911 mice were crossed with *Rgs9-cre* or *ChAT-cre* mice to obtain double heterozygotes. The double
912 heterozygotes were crossed with *Btbd9 loxP* homozygotes to obtain *Btbd9* sKO or *Btbd9* ChKO
913 mice. In conditional KO mice, exons 4 is deleted in specific types of neurons where *cre* is
914 expressed. Recombination occurs in these cells, while other brain regions and the rest of the
915 body still retain the intact exons. (B) *Btbd9* sKO mice showed a decreased level of *Btbd9* mRNA
916 in the striatum, but not in the cerebral cortex and cerebellum. Bars represent means plus standard
917 errors. *, $p < 0.05$. (C) Tissue-specific deletion of *Btbd9* exon 4 in *Btbd9* sKO mice was
918 confirmed by PCR using DNA isolated from each brain region. The deletion was detected only
919 in the striatum of *Btbd9* sKO mouse as predicted (Δ). (D) A representative immunohistochemical
920 image of a coronal section of the striatum from an *Rgs9-cre*/GFP mouse. Scale bars represent 25
921 μm. Enlarged images captured with ×40 objective lens showed that the ChAT staining (red) did
922 not overlap with GFP staining (green). The results suggest that *Rgs9-cre* does not have cre-
923 mediated recombination in ChIs.
924
925 Fig. 5. Behavior studies of the conditional KO mice. (A) *Btbd9* sKO mice traveled more and
926 showed an increase in clockwise circling in the 30 min open field test. (B) *Btbd9* sKO mice had
927 increased activity in the wheel running test compared with the controls during the light phase,
928 but not in the dark phase. Detailed hourly differences are listed (see Method). Significant *p*
929 values are marked above the corresponding time points. (C) In the long-term open field test,

930 *Btbd9* sKO mice did not show a significant change in the total distance traveled during both the
931 light and the dark phases. (D) *Btbd9* sKO showed an increased probability of waking in the light
932 phase of the long-term open field test. (E) *Btbd9* sKO mice showed shorter latencies to respond
933 to a warm stimulus in the tail flick test. (F) *Btbd9* ChKO mice did not show activity alteration in
934 the 30 min open field test. (G) *Btbd9* ChKO mice ran less during the light phase but ran more
935 during the dark phase in the wheel running test. (H) In the long-term open field test, *Btbd9*
936 ChKO mice did not have a significant change in the total distance traveled during both the light
937 and the dark phases. (I) The probability of waking significantly decreased during the light phase
938 and significantly increased during the dark phase for the *Btbd9* ChKO mice. Detailed hourly
939 differences are listed (see Method). Significant *p* values are marked above the corresponding
940 time points. GEE model normalized the WT or control group in the bar graphs of B-E and G-I to
941 0 without error bars. Bars represent means plus standard errors. ***, $p < 0.005$, **, $p < 0.01$, *, p
942 < 0.05.
943
944 Fig. 6. Electrophysiological recording of ChIs from *Btbd9* ChKO mice and their control
945 littermates. (A) Representative responses to the injected currents at 200pA of ChKO (n=16 cells)
946 and control (n=18 cells) mice. (B) The frequency-current relationship for ChKO and control ChIs.
947 Firing frequency of mutant ChIs did not change compared with the controls. (C) Representative
948 traces of spontaneous firings of mutant (n=22) and control (n=22) ChIs. (D) ChKO mice had
949 increased ChIs firing frequency. (E) Firing regularity of ChIs was not different between mutants
950 and controls. GEE model normalized the control group in the bar graph of B to 0 without the
951 error bars. Data in D were presented as median with 95% CI. CV: coefficient of variance. *, $p <$
952 0.05.

953 Table 1. A higher level of vertical activity and no alteration in anxiety or stereotypical behaviors
954 in the open field.
955

Genotype	Control (n=7)	*Btbd9* sKO (n=8)	*p* value	Adjusted *p* value
Vertical Activity	410 ± 48	580 ± 45	0.026	0.049 *
Center time	328 ± 43	370 ± 41	0.50	0.6
Center distance/ Total distance	0.27 ± 0.01	0.29 ± 0.01	0.43	0.5
Stereotypy Count	2071 ± 120	2308 ± 112	0.18	0.3

956
957 Vertical activity is presented as the mean number of beam breaks ± standard errors (SEs). Center
958 time is presented in s. Stereotypy count is presented as the number of counts. *p* values have been
959 adjusted for multiple comparisons using the Benjamini-Hochberg-Yekutieli false discovery rate
960 [FDR ($p < 0.05$)].