

Cerebellothalamocortical pathway abnormalities in torsinA DYT1 knock-in mice

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The factors that determine symptom penetrance in inherited disease are poorly understood. Increasingly, magnetic resonance diffusion tensor imaging (DTI) and PET are used to separate alterations in brain structure and function that are linked to disease symptomatology from those linked to gene carrier status. One example is DYT1 dystonia, a dominantly inherited movement disorder characterized by sustained muscle contractions, postures, and/or involuntary movements. This form of dystonia is caused by a 3-bp deletion (i.e., ΔE) in the *TOR1A* gene that encodes torsinA. Carriers of the DYT1 dystonia mutation, even if clinically nonpenetrant, exhibit abnormalities in cerebellothalamocortical (CbTC) motor pathways. However, observations in human gene carriers may be confounded by variability in genetic background and age. To address this problem, we implemented a unique multimodal imaging strategy in a congenic line of DYT1 mutant mice that contain the ΔE mutation in the endogenous mouse torsinA allele (i.e., DYT1 knock-in). Heterozygous knock-in mice and littermate controls underwent microPET followed by ex vivo high-field DTI and tractographic analysis. Mutant mice, which do not display abnormal movements, exhibited significant CbTC tract changes as well as abnormalities in brainstem regions linking cerebellar and basal ganglia motor circuits highly similar to those identified in human nonmanifesting gene carriers. Moreover, metabolic activity in the sensorimotor cortex of these animals was closely correlated with individual measures of CbTC pathway integrity. These findings further link a selective brain circuit abnormality to gene carrier status and demonstrate that DYT1 mutant torsinA has similar effects in mice and humans.

connectivity | regional metabolism | brain networks

In recent years, advanced imaging technologies such as PET and magnetic resonance diffusion tensor imaging (DTI) have provided unique information regarding the impact of specific genetic mutations on brain structure and function. However, to control for variability in genetic background and additional confounders such as age and sex, many more mutation carriers (and control subjects) are required than can conveniently be scanned, even in a multicenter design. Human imaging studies may also suffer from relatively low spatial resolution and sensitivity. These considerations motivated the current study in which high field magnetic resonance DTI was performed ex vivo in an experimental genetic model of a brain disorder.

Primary dystonia is a childhood-onset neurological illness characterized by disabling abnormal involuntary movements without consistent brain lesions on routine structural brain imaging or at postmortem analysis (1). This disorder is associated with several genotypes (2). DYT1 dystonia, the most common inherited form of the disease, is caused by a dominantly inherited 3-bp in-frame deletion in the *TOR1A* gene that removes a single glutamic acid (ΔE) from the torsinA protein (3). This low-prevalence mutation (approximately 1 in 30,000) (4) is also incompletely penetrant, causing dystonic symptoms in only 30% to 40% of gene carriers (5).

Functional imaging studies comparing DYT1 mutation carriers with and without clinical manifestations to control subjects have

contributed to the current understanding of disease mechanisms (6, 7). DYT1 gene carriers exhibit abnormal increases in regional metabolic activity in the cerebellum, putamen/globus pallidus, and supplementary motor cortex, irrespective of clinical penetrance (8, 9). Indeed, these metabolic changes are present during sleep when no involuntary movements are present, suggesting an association with genotype rather than phenotype (8, 10, 11). Magnetic resonance DTI shows white matter abnormalities in dystonia gene carriers (12, 13), and tractographic analysis of these abnormalities has associated these changes with reduced integrity of cerebellothalamocortical (CbTC) motor pathways (6), which modulate the excitability and synaptic plasticity of the sensorimotor cortex (14). Irrespective of clinical penetrance, DYT1 carriers exhibited reduced connectivity in the proximal cerebellothalamic segment of this pathway. By contrast, nonmanifesting carriers were distinguished by additional tract deficits involving the distal thalamocortical segment of this projection system. The presence of tandem tract changes in these mutation carriers raises the possibility that the additional abnormality prevents clinical manifestations by interrupting the transmission of aberrant cerebellar output signals to motor cortex (6, 15).

In this study we use a unique multimodal animal imaging approach to control for the potential confounds described earlier in relation to human mutation carriers. Congenic C57Bl6/J DYT1 knock-in mice heterozygous for the ΔE mutation in the *TOR1A* gene (*Tor1a* ^{ΔE /+}) (16) and littermate controls (*Tor1a*^{+/+}) were scanned ex vivo with high field MR DTI followed by tractographic analysis. Because they are fully congenic, *Tor1a* ^{ΔE /+} and *Tor1a*^{+/+} mice are 99.9% genetically identical, enabling us to isolate and study the CNS effects of the ΔE mutation. Significant microstructural abnormalities were identified in the CbTC pathways of the mutant animals. The relationship of these tract changes to regional brain function was assessed by correlation with measurements of cerebral metabolism acquired in vivo with [¹⁸F]fluorodeoxyglucose (FDG) microPET. These studies allowed for the direct assessment of the effect of the DYT1 mutation on the structure and function of CbTC motor pathways.

Results

Several discrete regions with abnormal white matter microstructure (Fig. 1A) were identified in the *Tor1a* ^{ΔE /+} mice (Table 1). Significant reductions in fractional anisotropy (FA), a DTI index of axonal integrity and coherence, were present in the mutant animals relative to controls ($P < 0.0001$, Student *t* tests). These abnormalities were localized to the right superior cerebellar tract (cluster 1), the white matter subjacent to the right primary sensorimotor cortex (cluster 2), and the left caudate/

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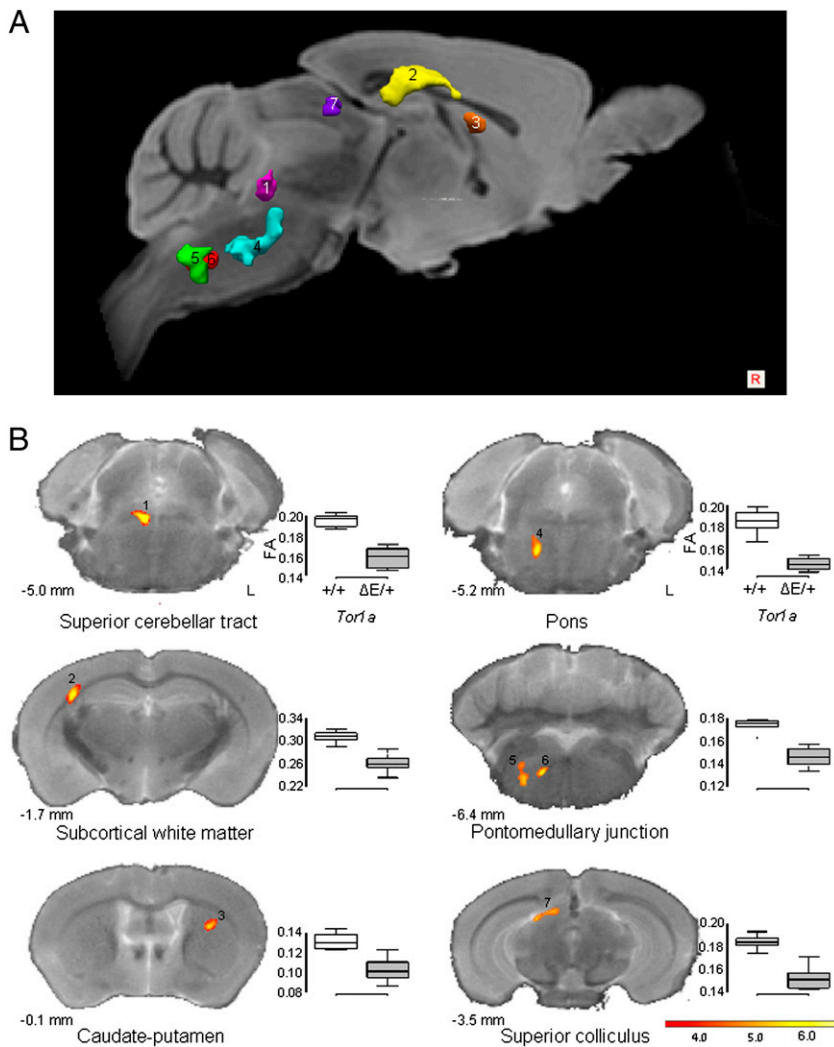


Fig. 1. Regions with abnormal microstructure in *Tor1a*^{ΔE/+} mice. (A) Three-dimensional display of regions with abnormal white matter microstructure in the mutant animals (Table 1). Significant reductions ($P < 0.0001$, Student *t* test) in FA were present in the following brain areas: superior cerebellar tract (marked by "1"), subcortical white matter in the primary sensory and motor regions ("2"), caudate/putamen ("3"), pons in the vicinity of the reticular nuclei ("4"), lateral pontomedullary junction ("5"), medial pontomedullary junction ("6"), and white matter adjacent to the superior colliculus ("7"). (B) Two-dimensional displays of these clusters overlaid on a standard mouse MRI template (37). The color scale represents *t* values threshold at 3.9 corresponding to $P < 0.001$ (uncorrected). Individual cluster values for the mutant and for the control groups are represented by box-and-whisker plots. All group differences were highly significant ($P < 0.0001$).

putamen (cluster 3). Of note, the FA reductions observed in these regions were also significant ($P < 0.05$) in homologous areas on the opposite side of the brain. Each of these clusters was then used as a seed region for fiber tracking. In the control animals, group tractography revealed extensive anatomical connections between these regions and the ventral thalamus (Fig. 2A). In each of these pathways, fewer tracts were visualized in the mutant group (Fig. 2B). This observation was substantiated by the quantitative fiber counts that were conducted in the individual animals. These measurements in the mutant animals revealed reduced tract numbers relative to littermate controls ($P < 0.001$, Student *t* tests) in cerebellothalamic (−49%), thalamocortical (−55%), and thalamostriatal (−86%) projection pathways.

Several additional regions (Fig. 1B) were identified in the mutant animals that were associated with unilateral abnormalities in pathway microstructure (Table 1). Two areas of reduced FA ($P < 0.0001$) were present in the right pons, in the vicinity of the reticular nuclei (clusters 4 and 5). Tractographic analysis in the control animals disclosed that both these areas were connected to the cerebellum through the inferior peduncle (Fig. 2C). The mutant group exhibited fewer tracts in these pathways (Fig. 2D), with significant fiber count reductions in the *Tor1a*^{ΔE/+} animals in the rostral (−59%, $P = 0.003$) and caudal (−86%, $P < 0.001$) pontocerebellar pathways. Additional FA reductions in the mutant animals ($P < 0.0001$) were noted medially in the right

caudal pons (cluster 6) and in the white matter adjacent to the right superior colliculus (cluster 7), without consistent tractographic correlates. No connections were discerned between basal ganglia structures and any of the significant clusters identified in the voxel-wise group comparison.

We next determined whether the pathway abnormalities identified in the *Tor1a*^{ΔE/+} mice were associated with discrete alterations in regional brain function analogous to those observed in human *DYT1* mutation carriers. Specifically, we examined the hypothesis that the changes in CbTC pathway microstructure seen in mutation carriers correlate with metabolic activity at downstream nodes of this network. FDG microPET disclosed a single brain region with abnormal glucose utilization in the *Tor1a*^{ΔE/+} mice (Table 1). This change (Fig. 3A) was localized to the superior cerebellar vermis (lobule VI) (17), with increased local metabolic activity in mutant relative to control animals ($P < 0.001$, Student *t* test).

We then examined the relationship between individual differences in CbTC tract integrity and resting metabolic activity in the mutant group. Given that all the *Tor1a*^{ΔE/+} mice exhibited proximal (cluster 1) as well as distal (cluster 2) tract abnormalities along this pathway, FA values from both these white matter regions were entered as covariates in a voxel-wise search for regions in which brain metabolism correlated with pathway microstructure. A significant structure–function correlation ($P < 0.0001$, multiple linear regression; Table 1) was identified in a

Table 1. Voxel-based comparison of pathway microstructure and regional metabolic activity in mutant versus control animals

| Region | Coordinates* | | | Cluster Size [†] | Z _{max} | P Value |
|--|--------------|------|------|---------------------------|------------------|----------------------|
| | x | y | z | | | |
| Regions with significant group differences in FA | | | | | | |
| Mutant < Control | | | | | | |
| 1. Superior cerebellar tract | 0.4 | -5.0 | -3.6 | 190 | 3.94 | 0.002 [‡] |
| 2. Subcortical white matter (sensorimotor) | 2.9 | -1.7 | -1.5 | 682 | 4.20 | <0.001 [‡] |
| 3. Caudate/putamen | -2.3 | -0.1 | -2.3 | 102 | 3.93 | 0.019 [‡] |
| 4. Pons | 0.4 | -5.5 | -5.1 | 661 | 4.28 | <0.001 |
| 5. Lateral pontomedullary junction | 1.7 | -6.7 | -5.2 | 192 | 4.54 | 0.002 |
| 6. Medial pontomedullary junction | 0.9 | -6.4 | -5.3 | 110 | 4.59 | 0.015 |
| 7. Superior colliculus | 1.0 | -3.5 | -1.5 | 121 | 3.91 | 0.011 |
| Region with a significant group difference in metabolic activity | | | | | | |
| Mutant > Control | | | | | | |
| Cerebellar vermis (lobule VI) [§] | -0.2 | -7.3 | -2.7 | 136 | 3.26 | <0.001 |
| Correlations between FA and glucose metabolism | | | | | | |
| Sensorimotor cortex | 3.8 | -2.2 | -2.0 | 418 | 4.10 | 0.002 |

*Coordinates according to the common space of SPMouse (37).

[†]Cluster size in voxels (1 voxel = 3.4×10^{-4} mm³).

[‡]Cluster significant at $P < 0.05$ (Student *t* test), corrected for cluster extent, with additional changes in the contralateral ("mirror") hemisphere.

[§]According to atlas of Franklin and Paxinos (17).

^{||}Uncorrected at voxel level.

discrete area within the sensorimotor cortex (Fig. 3B, yellow/red). In this gray matter region, metabolic activity exhibited a negative correlation ($P < 0.0005$) with FA values in the superior cerebellar tract (Fig. 3C, *Left*). Metabolism in this region also correlated ($P < 0.002$) with the tract abnormality (Fig. 3C, *Right*) that was observed in the underlying subcortical white matter (Fig. 3A, blue). This correlation was positive, however, and of smaller magnitude than that with the proximal pathway lesion. One interpretation of these findings is that the thalamocortical tract abnormality serves to modulate the increases in sensorimotor cortical activity that, in dystonia gene carriers, have been attributed to reduced cerebellothalamic facilitation of inhibitory cortical interneurons (6, 15, 18, 19).

These observations may also be consistent with the related hypothesis that the distal CbTC tract abnormality mitigates clinical penetrance in nonmanifesting gene carriers by interrupting aberrant signaling at the thalamocortical level (e.g., ref. 15). To explore this possibility, we determined whether the pattern of microstructural change in the mutant animals was consistent with the probabilistic model of gene penetrance that we proposed based on connectivity measurements in human gene carriers (6). Human carriers of autosomal dominant dystonia mutations, whether affected or not, exhibited abnormally reduced FA values proximally in cerebellothalamic projections ($P < 0.001$, post-hoc tests). By contrast, the integrity of the distal thalamocortical segment was significantly lower in the nonmanifesting mutation carriers relative to their affected counterparts ($P < 0.005$) as well as healthy control subjects ($P < 0.0001$). Given that heterozygous *Tor1a*^{ΔE/+} knock-in mice do not display abnormal movements (16), these animals would be expected to exhibit a substantial distal tract abnormality akin to that observed in the human nonmanifesting carriers. As noted earlier, highly significant FA reductions ($P < 0.0001$) involving both the proximal and distal segments of the CbTC pathway (clusters 1 and 2) were consistently present in the mutant animals. Moreover, the relationship of FA values in these two regions (Fig. 4B) was similar to that observed in nonmanifesting human subjects. That is, the reduction in tract integrity was greater in the distal segment relative to the proximal segment of the pathway ($P < 0.002$, paired Student *t* tests). Indeed, this relationship was evi-

dent at the individual animal level (see lines connecting the individual data points in Fig. 4B). That is, the *Tor1a*^{ΔE/+} mice, without exception, exhibited lower FA values distally, as was also the case in the human nonmanifesting carriers.

Discussion

We found that *Tor1a*^{ΔE/+} mice exhibited microstructural abnormalities in cerebellothalamic and thalamocortical pathways, and that these abnormalities correlated with local metabolic activity measured downstream in the sensorimotor cortex. These abnormalities resembled those previously identified in nonpenetrant human mutation carriers who, like the *Tor1a*^{ΔE/+} mice, do not exhibit abnormal involuntary movements (6). *Tor1a*^{ΔE/+} mice also exhibit increased metabolic activity in the dorsal cerebellar vermis, a finding consistently identified in manifesting and nonmanifesting human DYT1 carriers (7, 9) and in experimental rodent models of dystonia (20–22). The similarity of these findings indicate that DYT1 mutant torsinA can produce similar CNS changes in mice and humans, and suggest that *Tor1a*^{ΔE/+} mice are a model of human nonmanifesting DYT1 mutation carriers.

The *Tor1a*^{ΔE/+} knock-in mice also exhibited significant FA reduction in the caudate/putamen, along with a corresponding deficit in the number of visualized thalamostriatal tracts. Alterations in this tract may affect the regulation of neurotransmitter release in the striatum of these animals (23) and may also relate to the alterations in striatal synaptic plasticity that have recently been observed in a transgenic mouse model with overexpression of the human mutant protein (24). Interestingly, the thalamostriatal tract abnormality failed to reach significance in our earlier study (6) of pathway integrity in human dystonia mutation carriers. The striking deficits in this pathway that were observed in the mouse are attributable to the genetic homogeneity of the sample combined with high-field ex vivo imaging. Together, these features permit the detection of regional changes that may not be easily discerned in the corresponding human studies.

In this vein, the current approach allowed for the detection of tract reductions in projections from the pontine reticular nuclei to the cerebellum in the mutant animals. These small brainstem structures receive afferents from the subthalamic nucleus (25) and have been proposed as conduits of basal ganglia output

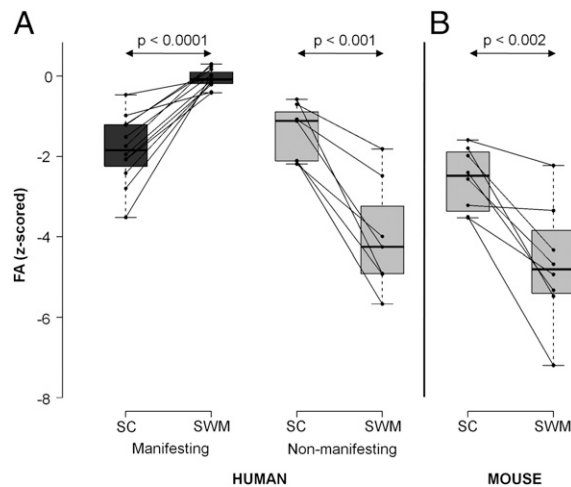


Fig. 4. Regional differences in pathway microstructure: mechanism of penetrance. (A) FA measured in the superior cerebellar (SC) pathway and in the subcortical white matter (SWM) subjacent to primary sensorimotor cortex from the previously described cohort of 12 clinically manifesting (MAN) and eight nonmanifesting (NM) dystonia mutation carriers (6). For each region, individual subject FA values were z-transformed with respect to the corresponding cluster values from eight age-matched normal control subjects. Each of the two groups of gene carriers exhibited significant FA reductions in CER relative to controls ($P < 0.001$). By contrast, FA values in SMC were normal in MAN but lower than control values in NM gene carriers ($P < 0.0001$, Student *t* test). (B) Plot of z-transformed FA values measured in the homologous regions of the *Tor1a*^{ΔE/+} mouse. As in human non-manifesting gene carriers, the mutant animals exhibited significant FA reductions relative to controls ($P < 0.0001$, Student *t* test) in the distal segment of the CbTC pathway in the subcortical white matter.

afferent synaptic activity. That FA values measured in cerebellar outflow pathways were found to correlate negatively with sensorimotor metabolic activity is consistent with the hypothesized role of the ascending CbTC system in facilitating intracortical inhibition via thalamocortical projections to inhibitory interneurons in the sensorimotor cortex (18, 19). In this regard, more severe involvement of the proximal (i.e., cerebellothalamic) pathway segment in mutant animals was associated with relatively greater loss of local cortical inhibition and corresponding increases in regional neural activity (15). In this vein, the additional presence of microstructural abnormalities in the distal (i.e., thalamocortical) pathway segment may serve to modulate the size of the mutation-related functional changes that occur at the cortical level.

Despite significant microstructural alterations in cerebellar pathways, the mutant mice did not exhibit abnormal motor behavior (16). In this regard, these animals resembled human nonmanifesting DYT1 carriers, who also do not develop signs of dystonia during their lifespan (5). In fact, the current data suggest that every heterozygous knock-in animal exhibited a tandem tract deficit at the distal end of the pathway, which served to reduce the likelihood of clinical penetrance. As in the human nonmanifesting carriers, this “second lesion” was of sufficient magnitude to interrupt the abnormal signaling generated proximally by the deficit in cerebellothalamic connectivity. The reason for the consistent presence of a clinically mitigating second lesion in the *Tor1a*^{ΔE/+} knock-in mice, as opposed to the partial penetrance observed in the human, is not known. Nonetheless, we have observed substantially tighter correlations between connectivity values for the two CbTC segments in the normal mouse relative to the healthy human subjects ($r = 0.9$ and $r = 0.5$, respectively; Pearson correlation coefficients). Indeed, simulations based on the probabilistic connectivity model that we

have proposed suggest that “hard wiring” of the circuit during development, as appears to be the case in the rodent, is associated with very low penetrance rates (figure 5C in ref. 6).

Whether the distal pathway abnormality is relatively less pronounced in mutant animals displaying a motor phenotype, similar to clinically manifesting dystonia gene carriers (6), is not currently known. Further studies comparing the current tractographic findings with those from experimental models with observable manifestations will be needed to address this issue. Nonetheless, the presence of consistent microstructural changes in the mutant animals, even if not overtly penetrant, can be regarded as a valuable endophenotype of the disease process. The multimodal animal imaging approach described in the present study can also be used to elucidate the biochemical mechanism of the DYT1 mutation during neurodevelopment. Thus, the findings in the *Tor1a*^{ΔE/+} knock-in mice can be compared directly with heterozygous mice carrying the analogous KO mutation (*Tor1a*^{+/-}). The presence of similar pathway changes in both groups of animals would suggest that the mutant torsinA protein is associated with a loss of function. By contrast, if significant changes in these pathways prove to be present only in the knock-in animals, the gain of an abnormal function becomes a more likely pathogenetic mechanism. In this vein, performing serial microPET and DTI studies in living mutant and control animals, as well as ex vivo tractographic assessments in animals of varying age, will provide valuable information regarding the developmental time course of these system-specific changes (32). Needless to say, it will be of great interest to determine whether similar structure–function abnormalities are discerned by this approach in other experimental models of brain disease.

Materials and Methods

Data Acquisition. Eight C57Bl/6J congenic heterozygous ΔE *Tor1a* knock-in mice (*Tor1a*^{ΔE/+}) and six WT littermate control (*Tor1a*^{+/+}) animals were studied at 18 wk of age. These mice were confirmed as fully congenic at Charles River Labs through the use of a panel of genotyping probes. *Tor1a*^{ΔE/+} and *Tor1a*^{+/+} mice were housed together from birth to assure that all animals experienced as identical an environment as possible. The animals were anesthetized by using ketamine (150 mg/kg) and xylazine (15 mg/kg) and scanned for 15 min on a Siemens Focus 220 microPET instrument, beginning 60 min after the injection of approximately 310 μCi FDG. The resulting images were reconstructed using an iterative algorithm (33, 34) to a matrix size of 128 × 128 × 95 (voxel size, 0.600 mm × 0.600 mm × 0.796 mm).

Following FDG microPET, the animals underwent transcardiac perfusion with 4% paraformaldehyde (35); the brains were harvested and stored in 4% paraformaldehyde for at least 2 wk. Magnetic resonance DTI was performed ex vivo on whole brain samples using the 9.4-T animal magnetic resonance scanner at Johns Hopkins Medical Center (36). Diffusion weighted images of these specimens were acquired by using a rapid acquisition with relaxation enhancement sequence in six directions with a maximum b-value of 2,138 s/mm². Two additional images with a b-value of 210 s/mm² were also acquired. Each acquisition included six echoes with two repetitions to improve the signal-to-noise ratio. The DTI data acquisition matrix was 136 × 84 × 64, which was zero-filled before Fourier transformation to 272 × 168 × 128. The field of view was 17 mm × 10.5 mm × 8 mm. The nominal resolution of the DTI images was 62.5 μm × 62.5 μm × 62.5 μm, with a total ex vivo imaging time of approximately 20 h. Institutional animal care and use committees at Johns Hopkins University School of Medicine and Weill Medical College of Cornell University approved all animal procedures.

Data Analysis. After DTI data acquisition, maps of FA were calculated, as well as the entire diffusion tensor for tractography. A nine-parameter affine registration was applied to the individual animal FA maps by using FSL software (<http://www.fmrib.ox.ac.uk/fsl/>). When they had been aligned, the FA images were smoothed by using a kernel of 312.5 μm (full width at half maximum) and the two groups were compared voxel-wise over the entire brain volume by using SPM10 software (<http://www.wbic.cam.ac.uk/~sjs80/spmmouse.html>), a new toolbox for statistical parametric mapping in the mouse (37). Group differences were considered significant at a voxel-level threshold of $P = 0.001$ with a correction for multiple comparisons at

$P < 0.05$. We also reported regional differences at a hypothesis testing threshold of $P < 0.001$ (voxel level uncorrected) with a cluster cutoff of 100 voxels. The individual data from the resulting clusters were measured post hoc. Values for the mutant and control groups were displayed graphically by using box-and-whisker plots to evaluate overlapping data and potential outlier effects. Each significant cluster within a given hemisphere was transposed to the opposite side and measured for the two groups. FA values for each significant cluster, on each hemisphere separately, were compared across groups by using Student t tests and were considered significant at $P < 0.05$.

Following the identification of discrete regions with significant FA abnormalities in the mutant animals, white matter pathways passing through these areas were reconstructed separately for the two groups. Tractography was performed on the images from each group using an early registration method by which the DTI scans were registered to a common template before tensor calculation (38). We used TrackVis software (<http://www.trackvis.org/>) to map white matter pathways coursing between volumes of interest delineated in the mutant and control animals; tracking parameters were identical for the two groups. The resulting tracts were verified using AKILIDTI software (39), which uses a different tractography algorithm (40). In this study, the significant clusters identified by voxel-based comparison of the FA maps for the two groups were used as seed volumes for tractography. Mean tracts were displayed for each group and compared qualitatively based on visualized tract size.

For quantitative comparison of the two groups, tractographic analysis was also performed in the native space of each animal. The significant clusters identified in standard space were reverse-transformed, and fiber tracking

was then performed on each sample individually. To account for fixation-dependent FA differences between specimens, tracking parameters were adjusted by using the FA histogram peaks as reference for the individual samples. Fiber counts were conducted for each reconstructed tract in every animal. Mean values for the mutant and control groups were compared by using Student t tests; group differences were considered significant for P values lower than 0.05.

The microPET data were analyzed by using a similar approach to that described for the DTI group comparisons. Individual images were registered to a standard template (41) by using an iterative algorithm. Group differences in regional metabolic activity were assessed on a voxel basis with SPMMouse; the threshold for significance was the same as for the voxel-based comparison of the FA maps. We also tested the hypothesis that, as in human DYT1 carriers (6), functional activity at the major nodes of the CbTC network is correlated with individual differences in the microstructural integrity of this pathway. This possibility was examined by conducting a voxel-wise multiple regression analysis in SPMMouse, and the results were considered significant at a voxel-level threshold of $P = 0.001$ with a correction for multiple comparisons at $P < 0.05$. The contribution of each FA regressor to metabolic activity in the significant brain regions was evaluated post-hoc by using leverage plot analysis (42) in JMP software (SAS Institute). These correlations were considered significant at $P < 0.05$.

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