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# Subtle microstructural changes of the striatum in a DYT1 knock-in mouse model of dystonia

Chang-Hyun Song [a,1], Douglas Bernhard [a], Caroline Bolarinwa [c], Ellen J. Hess [a,b], Yoland Smith [c], H.A. Jinnah [a,d,*]

[a] Department of Neurology, Emory University, Atlanta, GA 30322, USA
[b] Department of Pharmacology, Emory University, Atlanta, GA 30322, USA
[c] Yerkes National Primate Research Center and Department of Neurology, Emory University, Atlanta, GA 30329, USA
[d] Department of Human Genetics and Pediatrics, Emory University, Atlanta, GA 30322, USA

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ABSTRACT

The dystonias are comprised of a group of disorders that share common neurological abnormalities of involuntary twisting or repetitive movements and postures. The most common inherited primary dystonia is DYT1 dystonia, which is due to loss of a GAG codon in the *TOR1A* gene that encodes torsinA. Autopsy studies of brains from patients with DYT1 dystonia have revealed few abnormalities, although recent neuroimaging studies have implied the existence of microstructural defects that might not be detectable with traditional histopathological methods. The current studies took advantage of a knock-in mouse model for DYT1 dystonia to search for subtle anatomical abnormalities in the striatum, a region often implicated in studies of dystonia. Multiple abnormalities were identified using a combination of quantitative stereological measures of immunohistochemical stains for specific neuronal populations, morphometric studies of Golgi-stained neurons, and immuno-electron microscopy of synaptic connectivity. In keeping with other studies, there was no obvious loss of striatal neurons in the DYT1 mutant mice. However, interneurons immunoreactive for choline acetyltransferase or parvalbumin were larger in the mutants than in control mice. In contrast, interneurons immunoreactive for neuronal nitric oxide synthase were smaller in the mutants than in controls. Golgi histochemical studies of medium spiny projection neurons in the mutant mice revealed slightly fewer and thinner dendrites, and a corresponding loss of dendritic spines. Electron microscopic studies showed a reduction in the ratio of axo-spinous to axo-dendritic synaptic inputs from glutamatergic and dopaminergic sources in mutant mice compared with controls. These results suggest specific anatomical substrates for altered signaling in the striatum and potential correlates of the abnormalities implied by human imaging studies of DYT1 dystonia.



## Introduction

The dystonias are a group of disorders that share the common abnormality of involuntary twisting or repetitive movements (Fahn, 1988; Tarsy and Simon, 2006). There are many different clinical manifestations and many different causes. The different types of dystonia traditionally are classified according to three independent axes that involve the region of the body affected, age at onset, and etiology. The etiological axis relies on a fundamental distinction between "primary" and "secondary" dystonias. The primary dystonias are defined as disorders where dystonia is the chief manifestation, while the secondary dystonias more often include other neurological features.

Although many investigators have suggested that the brain is structurally normal in primary dystonia (Breakefield et al., 2008; de Carvalho Aguiar and Ozelius, 2002; Nemeth, 2002; Schwarz and Bressman, 2009; Tanabe et al., 2009), neuroimaging studies repeatedly have implied the existence of microstructural defects in the basal ganglia, cerebellum, thalamus, or cerebral cortex (for reviews see: Neychev et al., 2011; Zoons et al., 2011). However, imaging studies provide only indirect measures of microstructure, and some of the methods used may be detecting functional rather than structural changes. Unfortunately, postmortem studies of brains from patients with dystonia are sparse (Standaert, 2011). The main reason for the paucity of histopathological data is that the dystonias are rare, making it difficult to collect the necessary specimens. A second reason is that the methods needed to reveal potential microstructural defects require special tissue preparation and processing methods that cannot be applied readily in postmortem

*Abbreviations:* ΔE, deletion of a single GAG codon in Tor1A gene; ChAT, choline acetyltransferase; PV, parvalbumin; nNOS, neuronal nitric oxide synthase; TH, tyrosine hydroxylase; AP, region from bregma in the anterior–posterior direction; EM, electron microscopic; vGluT, vesicular glutamate transporters; ANOVA, analysis of variance; DL, dorso-lateral; DM, dorso-medial; VL, ventro-lateral; VM, ventro-medial; RC, rostro-caudal; SP, axo-spinous; Den, axo-dendritic.

* Corresponding author at: 6300 Woodruff Memorial Research Building, Department of Neurology, Emory University, Atlanta, GA 30322, USA. Fax: +1 404 712 8576.
*E-mail address:* hjinnah@emory.edu (H.A. Jinnah).

**Available online on ScienceDirect (www.sciencedirect.com).**

[1] Current address: Department of Anatomy and Histology, College of Oriental Medicine, Daegu Haany University, Gyeongsan, 712-715, Republic of Korea.

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studies of human brains. The third reason for the limited information is that the types of neuropathological changes suspected for primary dystonias may not involve overt degeneration or emergence of unique pathological hallmarks, but rather quantitative changes in soma or neurite morphology, complexity or connectivity. Revealing these types of changes requires precise quantitative morphometric and ultrastructural comparisons with controls. These three limitations of human autopsy studies have hampered efforts to delineate potential microstructural defects in the human DYT1 brain.

In this situation, animal models can provide a valuable resource to reveal abnormalities that subsequently may be confirmed in more targeted human studies (Jinnah et al., 2005). Several genetically engineered mouse models have been created for DYT1 dystonia, the most common inherited primary dystonia that is caused by loss of a GAG codon (ΔE) in the *TOR1A* gene that encodes torsinA. Although overt signs of dystonia are lacking in the mouse models, several studies have shown poor motor skills, abnormalities in the physiology and neurochemistry of basal ganglia pathways, and subtle changes in the structure of the membranes surrounding nuclei of neurons of some brain regions (for review see Song et al., 2012). Nissl stains have revealed no obvious anatomical brain abnormalities, but histological methods capable of identifying microstructural defects in the basal ganglia have not been conducted.

In the current studies, the fine structure of neurons in the striatum and their connectivity were examined in a knock-in mouse model of DYT1 dystonia using a combination of quantitative stereological measures of immunohistochemical stains for specific neuronal populations, morphometric studies of Golgi-stained striatal neurons, and immunoelectron microscopy of synaptic connectivity.

## Materials & methods

### *Animals*

Animals used in these studies were heterozygous DYT1(ΔE) mutant knock-in mice (Goodchild et al., 2005) maintained congenically with C57BL/6J mice from the Jackson Laboratories (Bar Harbor ME). Animals were housed on a 12 h light/dark cycle, with ad libitum access to food and water. Animals were genotyped by DNA obtained from tail clips using a primer pair for the 34 base pair *loxP* site in the DYT1 mutant (forward primer, AGTCTGTGGCTGGCTCTCCC; reverse primer, CCTCA GGCTGCTCACAACCAC). The studies were approved by the Institutional Animal Care and Use Committee at Emory University.

### *Immunohistochemistry*

Because anatomical abnormalities may vary according to sex or age, a multivariate statistical strategy was used that enabled simultaneous exploration of these variables in mutant and normal mice. Specifically, immunohistochemical stains were conducted in parallel on a cohort of 24 animals that included 12 mutants and 12 littermate controls. Half of the mutants were male and half female. Half were 3 months of age and half were 6 months of age. Mice were anesthetized with 2,2,2-tribromoethanol and perfused through the heart with a rinse solution of 50 mL of 137 mM NaCl, 22.2 mM dextrose, 23.4 mM sucrose, 2 mM $CaCl_2$ and 1.6 mM sodium cacodylate at pH 7.2. They then were perfused with 4% paraformaldehyde containing 117 mM sucrose and 67 mM sodium cacodylate (pH 7.2) and immersion post-fixed for 16 h.

To ensure uniformity of tissue sections, all 24 brains were embedded in a block of gelatin and cut simultaneously in the coronal plane at 40 μm from the frontal pole through the caudal brainstem and cerebellum (Neuroscience Associates, Knoxville TN). To ensure uniformity of staining, each stain was conducted in parallel with an entire series of sections spaced at 240 μm intervals. One series of sections was thionin stained to demonstrate Nissl substance, and the remaining 5 series were used for immunohistochemical stains of specific neuronal populations. Immunostains targeted choline acetyltransferase (ChAT; Millipore, Billerica MA), calbindin (Swant, Bellinzona, Switzerland), parvalbumin (PV; Swant, Bellinzona, Switzerland), neuronal nitric oxide synthase (nNOS; Immunostar, Hudson WI), or tyrosine hydroxylase (TH; Pelfreez, Rogers AR).

### *Stereological quantifications*

For stereological analyses, sections were examined under an Olympus BX51 light microscope (Melville NY), with a motorized stage (MAC5000, Ludl Electronic Products, Hawthorne NY) controlled by a computer with StereoInvestigator software (MicroBrightField, Williston VT). In the rodent basal ganglia, the caudate nucleus and putamen are combined as the caudoputamen, here referred to as the dorsal striatum (excluding the accumbens and olfactory tubercle). To determine the volume of the dorsal striatum using the Cavalieri method (Oorschot, 1996), the entire region was outlined at 4× objective in every 6th Nissl-stained section extending approximately from +1.7 to −2.2 mm from bregma in the anterior–posterior (AP) direction according to the mouse brain atlas (Paxinos and Franklin, 2004).

The total numbers of neurons were counted at 100× in Nissl-stained material or at 60× in immunostained material using the optical fractionator with a 12 μm depth and 1 μm top guard zone, as previously described (Egami et al., 2007). Because the numbers of target neurons for counting varied according to the stain applied, stereological counting frames and grid sizes were adjusted to achieve a Gunderson coefficient of error below 0.10 for each stain. For Nissl-stained neurons, the counting frame was 20×20 μm in a sampling grid of 600×600 μm. For ChAT and PV+ cells, the frame was 100×100 μm in a grid of 250×250 μm. For nNOS+ cells, the frame was 120×120 μm in a grid of 330×330 μm. Cell volumes were determined using the optical rotator with a 3 μm focal plane separation and a 4 grid line separation of 4 μm for ChAT+ cells, or with a 2 μm focal plane separation and a 3 grid line separation of 4 μm for PV+ and nNOS+ cells.

The statistical approach for stereological measures involved Analysis of Variance (ANOVA) with genotype, sex, and age as potential explanatory variables. Since identifying abnormalities related to the mutant *Tor1A* gene was the primary focus, results were combined across ages and sex for final graphic summary of the data. For all analyses, statistical significance was defined as $p<0.05$. Because this study was exploratory and effect sizes sometimes were small, any result with $0.05<p<0.10$ was noted as being a trend of borderline statistical significance, to acknowledge places where Type II errors could occur.

### *Spatial distribution of immunostained neurons*

To explore potential differences in the spatial distributions in the numbers or sizes of immunostained neurons, stained neurons also were examined according to rostro-caudal, dorso-ventral, and medio-lateral orientations. Because stereological methods are not suitable for delineating 3-dimensional distributions, differences in the rostro-caudal orientation were explored by subdividing results from total stereological counts into 4 rostro-caudal subgroups to provide sufficient power for statistical analyses. For these analyses, stereological counts were grouped from the first 3 sections for AP +1.5 to +0.9 mm, second 3 sections for AP +0.7 to +0.1 mm, third 3 sections for AP −0.1 to −0.7 mm and remaining 6 sections for AP −0.9 to −2.0 mm. For dorso-ventral and medio-lateral gradients, total cell counts were taken at the level of the anterior commissure (AP +0.14 mm) and subdivided into four quadrants as previously described (Luk and Sadikot, 2001). For this analysis, all available neurons were re-counted, since stereological sampling yielded insufficient data for statistical analyses. Data regarding neuronal distributions were examined by ANOVA with genotype and subregion as explanatory variables.