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THE DYT1 CARRIER STATE INCREASES ENERGY DEMAND IN THE OLIVOCEREBELLAR NETWORK

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Abstract—DYT1 dystonia is caused by a GAG deletion in *TOR1A*, the gene which encodes torsinA. Gene expression studies in rodents and functional imaging studies in humans suggest that DYT1 dystonia may be a network disorder of neurodevelopmental origin. To generate high resolution metabolic maps of DYT1 dystonia and pinpoint dysregulated network elements, we performed 2-deoxyglucose autoradiography and cytochrome oxidase (CO) histochemistry in transgenic mice expressing human mutant (hMT1) torsinA and wild-type littermates. In comparison with controls, hMT1 mice showed increased glucose utilization (GU) in the inferior olive (IO) medial nucleus (IOM), IO dorsal accessory nucleus and substantia nigra compacta, and decreased GU in the medial globus pallidus (MGP) and lateral globus pallidus. The hMT1 mice showed increased CO activity in the IOM and Purkinje cell layer of cerebellar cortex, and decreased CO activity in the caudal caudate-putamen, substantia nigra reticulata and MGP. These findings suggest that (1) the DYT1 carrier state increases energy demand in the olivocerebellar network and (2) the IO may be a pivotal node for abnormal basal ganglia-cerebellar interactions in dystonia. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: dystonia, globus pallidus, glucose utilization, cytochrome oxidase histochemistry, inferior olive, Purkinje cells.

Dystonia is characterized by sustained and involuntary muscle contractions resulting in twisting and repetitive movements or abnormal postures (Fahn, 1988). Early-onset primary dystonia typically begins in the distal portions of a limb and often spreads to involve other regions of the body. A common genetic cause of early-onset primary dystonia is a heterozygous GAG deletion in the *TOR1A* gene (i.e., DYT1 dystonia) which results in the loss of a single glutamic acid residue near the C-terminus of the encoded protein torsinA (Ozelius et al., 1997). DYT1 dystonia is transmitted in an autosomal dominant fashion with reduced penetrance. Symptoms usually become manifest in childhood. Onset before the age of 4 or after the age of 28 is uncommon (Bressman et al., 1998, 2000; Xiao et al., 2009).

Using [18F]-fluorodeoxyglucose and positron emission tomography (PET) in humans, both manifesting and non-manifesting carriers of the DYT1 mutation have shown similar patterns of hypermetabolism when compared to neurologically-normal controls (Eidelberg, 1998). In movement-free conditions, both manifesting and non-manifesting DYT1 carriers showed increased metabolic activity in the lentiform nuclei, cerebellum and supplementary motor areas. In movement-related conditions, only manifesting DYT1 carriers showed increased metabolic activity in the midbrain, cerebellum and thalamus. More recently, magnetic resonance diffusion tensor imaging has demonstrated reduced integrity of cerebellothalamocortical fiber tracts in both manifesting and non-manifesting carriers of the DYT1 ΔGAG mutation (Argyelan et al., 2009). The nigrostriatal pathway has also been implicated in the network pathophysiology of DYT1 dystonia. PET studies have shown that non-manifesting carriers exhibit reduced dopamine D2 receptor binding (Asanuma et al., 2005). In addition, post-mortem neurochemical studies have noted a significant increase in dopamine turnover (Augood et al., 2002). Taken together, these findings suggest that DYT1 dystonia is a circuit disorder of the nervous system.

Mice that overexpress mutant human torsinA (hMT1) show abnormalities in nigrostriatal neurochemistry, similar to what has been found in humans with DYT1 dystonia. Zhao et al. (2008) detected increased striatal 3′,4′-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels in hMT1 mice consistent with increased dopamine turnover. In addition, hMT1 mice display attenuated amphetamine-induced dopamine release, altered activity of the dopamine transporter, and abnormal dopaminergic D2 receptor responses in striatal cholinergic interneurons (Pisani et al., 2006; Balcioglu et al., 2007; Sciamanna et al., 2009; Hewett et al., 2010). Thus, the physiological and neurochemical abnormalities found in humans with DYT1 dystonia can be exposed with greater clarity in animal models of this disorder.

Metabolic mapping in animal models has provided critical insights into the network pathophysiology of a variety

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Abbreviations: AInt, anterior interposed nucleus; CHem, cerebellar cortex hemisphere; CO, cytochrome oxidase; CPuC, caudate-putamen caudal; CPuRD, caudate-putamen rostral-dorsal; CPuRV, caudate-putamen rostral-ventral; CVer, cerebellar cortex vermis; GU, glucose utilization; hMT1, human mutant torsinA; hWT, human wild-type; IO, inferior olive; IOD, inferior olive dorsal accessory nucleus; IOM, inferior olive medial nucleus; Lat, lateral cerebellar nucleus; LGP, lateral globus pallidus; Med, medial cerebellar nucleus; MGP, medial globus pallidus; O.D., optical density; PB, phosphate buffer; PCA, principal component analysis; PCL, Purkinje cell layer; PET, positron emission tomography; Pn, pontine nuclei; RMC, red nucleus magnocellular; ROI, region of interest; SNC, substantia nigra compacta; SNR, substantia nigra reticulata; STh, subthalamic nucleus; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VP, ventral pallidum; WT, wild-type; 2-DG, 2-deoxy-D-glucose.

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of movement and neurodegenerative disorders (Kimura et al., 1980; Brown and Lorden, 1989; Mitchell et al., 1992; Vila et al., 1996; Nobrega et al., 1998; Richter et al., 1998). Functional neuroimaging in humans suffers from limited spatial resolution and may be confounded by the effects of genetic background, age and gender. To reduce these effects and generate data of higher spatial resolution, 2-deoxy-D-glucose (2-DG) autoradiography and cytochrome oxidase (CO) histochemistry were performed in transgenic mice expressing either human wild-type (hWT) or mutant (hMT1) torsinA and wild-type (WT) littermates. The combined use of CO histochemistry and 2-DG autoradiography provides complementary information regarding metabolic activity in specific cell groups and local circuits (Jacquin et al., 1993).

EXPERIMENTAL PROCEDURES

Animals

Adult male mice, between 3 and 4 months of age, that over express human mutant torsinA (hMT1; $n=9$), human wild type torsinA (hWT; $n=8$) and their non-transgenic littermates (WT; $n=9$) were used for quantitative analyses of brain metabolism using 2-DG autoradiography and CO histochemistry. CO histochemistry reflects oxidative energy metabolism of neural tissues, especially neurons and is thought to provide information reflecting long-term neuronal metabolic demand over days or weeks (Di Rocco et al., 1989; Wong-Riley, 1989; Gonzalez-Lima and Garrosa, 1991; Hevner et al., 1995). In contrast, the glucose analogue, 2-DG, is taken up by glucose transporters in neurons and glia but cannot undergo glycolysis. Thus, 2-DG reveals the instantaneous glucose demand of neural and extra-neural tissues and 2-DG autoradiography is thought to reflect short-term metabolic demands (Wree and Schleicher, 1988; Wree, 1990; Duncan and Stumpf, 1991; McCasland and Graczyk, 2001). Mice were maintained in a temperature-controlled environment with free access to food and water. Light was controlled on a 12 h light/dark cycle. All procedures were approved by the University of Tennessee Health Science Center Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tissue preparation

The protocol for combined 2-DG autoradiography and CO histochemistry was adapted from McCasland and Graczyk (2001). Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. After a single dose of 2-DG (PerkinElmer, Waltham, MA, USA; 0.165 μCi/g) given by i.p. injection, mice were placed in individual cages for 45 min, overdosed with pentobarbital (Butler Schein, Dublin, OH, USA) and rapidly perfused ($<$2 min) with heparinized saline followed by a mixture of 2.5% paraformaldehyde/1.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA)/4% sucrose in 0.1 M phosphate buffer (PB). Of note, perfusion-fixation with paraformaldehyde and/or glutaraldehyde for 2-DG autoradiography has been employed by numerous investigators as a means of preserving tissue integrity for CO histochemistry and immunocytochemistry (Silverman and Tootell, 1987; McCasland and Woolsey, 1988; Redies and Gjedde, 1989). Brains were rapidly removed, blocked, frozen in pre-chilled isopentane at -40 °C and stored at -80 °C. Twenty μm coronal tissue sections were prepared using a Leica CM3050 S cryostat (Leica Microsystems Inc., Bannockburn, IL, USA) and collected in five rostral-caudal coronal series onto SuperFrost®-plus glass slides (Fisher Scientific, Pittsburgh, PA, USA). One series of sections was air-dried overnight and stained with Cresyl Violet. The remaining four series of sections were stored at -80 °C in vacuum-sealed slide containers until further processing for 2-DG autoradiography imaging and CO histochemistry.

Regions of interest (ROIs)

The nomenclature and abbreviations used for glucose utilization (GU) and CO ROIs (Tables 1 and 2) were adapted from Franklin and Paxinos (1997). In general, CO histochemistry (tissue sections, Fig. 1) generated data of higher spatial resolution than 2-DG autoradiography (film, Fig. 2). For example, CO activity was well delineated among the layers of cerebellar cortex (molecular, Purkinje and granule cell) and regions of hippocampus. Accordingly, a larger number of ROIs were interrogated for CO activity than GU. Mean values for optical density (O.D.) were derived from four to six sections covering the rostal-caudal extent of each ROI. To generate O.D. values for the layers of cerebellar cortex, measurements were made from the vermis ($n=6$) and hemispheres ($n=6$).

Image acquisition and analysis of GU

Brain sections were exposed to Kodak Biomax MR Film together with ^{14}C Microscale™ autoradiography standards (RPA504, Amersham Biosciences, Piscataway, NJ, USA) for 7 days. Due to technical issues, data from four mice (one hMT1, two hWT, and one WT) was not suitable for subsequent analyses of GU. Autoradiographic images were acquired in transmission mode (ScanMaker 9800 XL, Microtek, Carson, CA, USA) and imported into ImageJ (Java version of NIH Image, http://rsb.info.nih.gov/ij/) for neuroanatomical ROI quantification of radioactivity. The investigator performing ROI analyses was blinded to genotype. O.D. was calibrated to the set of autoradiography standards to generate measures of radioactivity (nCi/g tissue) for each ROI. Due to potential confounding variables such as peritoneal absorption and global differences in GU, ROI GU data from each mouse was converted to Z-scores.

CO histochemistry

CO histochemistry was performed using a protocol modified from that of Gonzalez-Lima and Jones (1994). The reaction solution was freshly made in 0.1 M PB (pH. 7.4) and contained 0.06% diaminobenzidine, 0.02% cytochrome c and 4.5% sucrose. Brain sections and standards of brain homogenate were incubated in the reaction solution for 1.5 h in the dark at 37 °C in a shaking water bath. The reaction was stopped by washing the slides with 0.1 M PB/4% sucrose for 5 min$\times$3. After dehydration in ascending concentrations of ethanol (30%, 50%, 75%, 95%, and 100%), the slides were cleared with xylene and coversliped with Permount (Fisher Scientific, Pittsburgh, PA, USA).

Fresh whole brains from five wild-type C57BL/6 mice (littermates) were completely homogenized on ice with a manual Dounce-glass homogenizer. The homogenates were transferred to 2 ml microcentrifuge tubes. After centrifugation at 1000 rpm (4 °C) for 2 min, the tubes were fast frozen in isopentane (-40 °C) and stored at -80 °C. Brain homogenates were sectioned on a cryostat at a series of thicknesses covering the entire range of CO activity (5 μm, 10 μm, 20 μm, 30 μm, 40 μm, 50 μm and 60 μm), collected onto SuperFrost®-Plus glass slides and stored at -80 °C in a vacuum-sealed slide container for use as CO activity standards.

The CO activity of the brain homogenates was determined with a spectrophotometric method as modified from Hess and Pope (1953) by Gonzalez-Lima and Jones (1994). Briefly, the sample solution was made by mixing the brain homogenate in 0.75% deoxycholate at a ratio of 0.01 g/5 ml. Then, 0.1 ml of the sample solution was added to 0.9 ml of a 30 μM reduced cyto-