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Tremor and Other Hyperkinetic Movements

Articles

Reduced Number of Pigmented Neurons in the Substantia Nigra of Dystonia Patients? Findings from Extensive Neuropathologic, Immunohistochemistry, and Quantitative Analyses

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Abstract

Background: Dystonias (Dys) represent the third most common movement disorder after essential tremor (ET) and Parkinson's disease (PD). While some pathogenetic mechanisms and genetic causes of Dys have been identified, little is known about their neuropathologic features. Previous neuropathologic studies have reported generically defined neuronal loss in various cerebral regions of Dys brains, mostly in the basal ganglia (BG), and specifically in the substantia nigra (SN). Enlarged pigmented neurons in the SN of Dys patients with and without specific genetic mutations (e.g., GAG deletions in DYT1 dystonia) have also been described. Whether or not Dys brains are associated with decreased numbers or other morphometric changes of specific neuronal types is unknown and has never been addressed with quantitative methodologies.

Methods: Quantitative immunohistochemistry protocols were used to estimate neuronal counts and volumes of nigral pigmented neurons in 13 SN of Dys patients and 13 SN of age-matched control subjects (C).

Results: We observed a significant reduction (~20%) of pigmented neurons in the SN of Dys compared to C ($p<0.01$). Neither significant volumetric changes nor evident neurodegenerative signs were observed in the remaining pool of nigral pigmented neurons in Dys brains. These novel quantitative findings were confirmed after exclusion of possible co-occurring SN pathologies including Lewy pathology, tau-neurofibrillary tangles, β-amyloid deposits, ubiquitin (ubiq), and phosphorylated-TAR DNA-binding protein 43 (pTDP43)-positive inclusions.

Discussion: A reduced number of nigral pigmented neurons in the absence of evident neurodegenerative signs in Dys brains could indicate previously unconsidered pathogenetic mechanisms of Dys such as neurodevelopmental defects in the SN.

Keywords: Substantia nigra, pigmented neurons, neuronal reduction, neuronal loss, neurodevelopmental disorder, neurodegenerative disorder

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Introduction

Dystonias (Dys), which manifest either as an isolated abnormal motor phenomenon or in combination with other motor and nonmotor signs of more complex neuropsychiatric syndromes, represent a relatively frequent movement disorder.[1,2] Dys, in fact, represent the third most frequent movement disorder after essential tremor (ET) and Parkinson's disease (PD).[3]

A number of efforts have led to various classification systems of Dys,[4–8] attempts to characterize their biological bases,[9–13] and multiple proposals of clinical guidelines for their treatment.[14–16] However, although important progress has been achieved in understanding some of the neurological bases of Dys, especially in terms of molecular mechanisms[17] and genetic causes,[18] the distinct characteristic neuropathologic features of Dys remain poorly defined[19] with some very rare exceptions.[20,21]

There have been few clinicopathologic correlation studies of postmortem brain tissues from Dys patients, with most analyses described in sporadic case-reports.[22–27] These neuropathologic studies have proven to be mostly inconclusive due to the clinical heterogeneity of Dys cases examined, the different classification systems used, and more importantly, difficulty in obtaining brains from clinically well-characterized Dys patients.[28] However, a few neuropathologic studies have begun to shed light on possible neuropathologic and morphometric aspects of specific types of Dys, such as DYT1-dystonia (DYT1),[29] cervical dystonia (CD)[30], and dopa-responsive dystonia (DRD).[31] Rostasy and colleagues assessed brains from five patients with DYT1 (GAG-deletions) and three Dys patients without GAG-deletions and reported larger and more compacted pigmented neurons in the substantia nigra (SN) of both types of Dys compared to control subjects (C).[29] In contrast, Göttle et al. described smaller SN neurons in Lesch-Nyhan disease.[32] In general, these lines of evidence support the hypothesis that the SN is indeed involved in Dys pathogenesis.[33–35]

The primary aim of this study was to investigate morphometric aspects of neuromelanin-containing (pigmented) neurons in the SN of Dys patients by performing rigorous neuropathologic quantitative analyses. We aimed to estimate nigral pigmented neuronal counts and cell body, nuclear, and nucleolar volumes using established quantification methods and the largest number of publicly available Dys autopsy brains to date.

We observed a significant decrease in the number of pigmented neurons in the SN of Dys brains versus C. This reduction was present in the SN of patients with both adulthood- and childhood-onset Dys. Interestingly, fewer pigmented neurons in the SN were not associated with significant changes in cellular, nuclear, or nucleolar volumes of the remaining pool of nigral pigmented neurons, or with other major neuropathologic and neurodegenerative signs. These findings might indicate that a reduction in SN pigmented neurons in Dys patients could be due to a neurodevelopmental defect rather than a neurodegenerative process.

A nigral pigmented neuronal reduction in the absence of cellular (cell body) and subcellular (nuclear/nucleolar) volumetric changes in the SN of Dys brains was confirmed by excluding other possible confounding or co-occurring nigral pathologies with immunohistochemistry protocols. In this study, the Dys and C groups did not differ in terms of mean age at death, further minimizing the influence of the important confounding factor of age.[36]

Methods

A total of 13 SN from patients (9 females and 4 males) with various Dys subtypes were available for this study. The mean age at death was 68.8 ± 15.5 years (range: 44–95). These individuals had a history of childhood- or adulthood-onset Dys with focal, segmental, or generalized distribution. Dys cases did not show signs of any other major neurological, psychiatric, or medical disorders except for one patient with diagnosis of possible dementia with Lewy bodies (possDLB), and another with cardiovascular disease. Cases of DYT1, the most frequent genetic form of Dys, were excluded from this investigation to avoid performing measurements on neurons from the brains of patients with Dys attributable to specific genetic causes. With this exclusion, we aimed to obtain general but specific quantitative findings on non-DYT1 or idiopathic Dys only.

The 13 SN from Dys patients were compared to 13 SN from age-matched control subjects (C, 9 females and 4 males). The mean age at death was 64.3 ± 13.8 years (range: 44–88 years). C did not show clinical or pathologic evidence of any major neurological, psychiatric, or medical disorders except for heart disease in some cases.

In addition to the standard microscopic neuropathological assessments, all SNs were immunohistochemically assessed for the presence of: α-synuclein-positive Lewy bodies (LB) and Lewy neurites (LN), hyperphosphorylated-tau neurofibrillary tangles (tau-NFT), hyperphosphorylated-tau threads (tau-th), ubiquitin (ubiq), intraneuronal cytoplasmic inclusions of phosphorylated-TAR-DNA binding protein-43 (pTDP-43), and extracellular deposits of insoluble 1–42 β-amyloid (diffuse-Aβ and Aβ-neuritic plaques). Subjects with neuropathologic findings indicative of any other neurodegenerative disorder or severe cerebrovascular disease were excluded from the study.

All Dys and C brains were collected after obtaining written consent from the next-of-kin or legal representative and after approval from each institution where the case was examined. Tissues were obtained from the University of Maryland Brain and Tissue Bank (UMBTB). The use of these tissues was approved by UMBTB and the local Institutional Research Board. Each hemibrain was fixed in 10% buffered formalin for at least 2 weeks and then grossly and microscopically examined in coronal sections for a general neuropathologic assessment at UMBTB.

Neuropathology and quantitative methods

Each hemi-SN block was randomly cut at different anatomical levels along the rostro-caudal anatomical axis of the structure. This anatomically random cutting minimized possible anatomical selection biases. We were unable to obtain the entire SN for each subject, but we were confident that at least three quarters of the entire structure was actually sampled for each case. This conclusion was based on our samples measurements (see below) in comparison to previous anatomical studies that assessed the entire length of SN.[37,38]

Tissue cutting and procedures for morphometric-quantitative analyses

The total length of each block of formalin-fixed SN received ranged from 8–10 mm. Tissues were processed at the Neuropathology Research labs at the Biomedical Research Institute of New Jersey (BRInj). Tissue blocks were processed with an automated tissue-processor (Tissue-Tek V.I.P. 1000 Vacuum Infiltration Processor, Ames Division, Miles Laboratories, Inc., Elkhart, IN, USA) using standard protocols. Tissue blocks were then embedded in paraffin, oriented with the rostral area facing the bottom of the paraffin mold, and serially cut along the rostro-caudal direction using a semi-automatic microtome (Leica RM2255, Leica Biosystems, Nussloch, Germany). Each block was cut for its entire length in series of 40 μm-thick consecutive sections, alternating to a series of 16 consecutive 10 μm-thick

sections. These serial, consecutive, and alternating sectioning procedures guaranteed the constancy of the established sampling rate for this investigation (one every five 40 μm-thick sections) and simultaneously allowed the use of 10 μm-thick consecutive sections anatomically adjacent to the 40 μm-thick sections for further immunohistochemistry assessments and volumetric measurements.[39]

The mean length of the sampled SN did not differ between Dys and C brains (mean lengths of $9{,}392.31 \pm 1{,}518.99$ μm and $10{,}015.38 \pm 1{,}573.2$ μm, respectively). The total number of consecutive sections (including 40 μm- and 10 μm-thick sections) obtained for Dys and C were 792.9 ± 129.3 and 845.4 ± 132.3, respectively. No statistical differences in terms of total length of sampled tissue and number of sections obtained were present between the Dys and C groups. Means of 48.6 ± 7.5 and 51.6 ± 7.5 40 μm-thick consecutive sections were respectively obtained for Dys and C.

Each set of 40 μm-thick sections was stained with a 1.0% cresyl violet (CV) solution for neuronal counting.[40] Each set of 40 μm-thick sections was separately and randomly recoded by an investigator blinded to the clinical and pathologic diagnoses (M.G.E.). Quantitative measurements using well-established stereological probes (the Optical Fractionator and Nucleator, see below) were performed by a second investigator (D.I.). Slides codes were opened, and statistical analyses were performed only after all measurements were completed.

A single 10 μm-thick tissue section was randomly chosen, stained with hematoxylin and eosin (H&E), and microscopically inspected at low ($2.5\times$) and high ($20\times$) magnifications to assess for possible micro-ischemic vascular pathologies, microhemorrhages, tissue rarefaction, and other possible histologic abnormalities not visible on gross examination.

Procedures for SN neuropathologic quantitative measurements

Each section was stereoscopically inspected ($2\times$) across the entire sectional area to localize SN anatomical borders. The pars compacta and pars reticulata were identified and marked as based on previous detailed anatomical descriptions.[41]

For neuronal counting, the following histologic criteria were applied:

1) Use of 40 μm-thick sections;
2) Inclusion of any neuron, of any size, containing neuromelanin pigment;
3) Exclusion of nonneuronal cells containing neuromelanin pigment, such as macrophages.

For neuronal volumetric measurements, the following histologic criteria were applied:

1) Use of 10 μm-thick sections;
2) Individuation of a well-defined nonneuromelanin-covered nucleolus for each randomly sampled pigmented neuron;
3) Establishing the nucleolus as the only point of reference for all volumetric measurements in each randomly sampled pigmented neuron.

Measurements were performed using the Stereo-Investigator system, Version 10.0 (MBF Bioscience, Williston, VT, USA), equipped with a digital camera (AxioCam MRm, and MRc, Zeiss, Oberkochen, Germany) and multiple objective head (2.5–$100\times$ oil-immersion). The sampling grid area was 500×500 μm, the counting frame was 40×40 μm, and the disector height was 25 μm with guard zones of ± 2 μm. The total number of SN pigmented neurons was estimated using the Optical Fractionator probe.[42,43]

Series of 10 μm-thick consecutive sections adjacent to each previously analyzed 40 μm-thick section were stained with CV to measure the cellular, nuclear, and nucleolar volumes of pigmented neurons. Volumetric measurements were performed using the Nucleator probe.[38] Pigmented neuron volumes were measured by placing six rays that automatically centered on the nucleolus and randomly intersecting the cell membrane (Figure 1A). Pigmented neurons were measured if their nucleolus was inside the counting frame intersecting or touching the green inclusion line; they were excluded if their nucleolus intersected or touched the red exclusion line (Figure 1A, upper right quadrant). This second-run of volumetric measurements on separate sets of 10 μm-thick consecutive sections was chosen to increase the number of available pigmented neurons showing a well-defined nucleolus, that is, the established point of spatial reference for volumetric measurements in this study. In fact, a consistent number of pigmented neurons that could be clearly counted on 40 μm-thick sections could not be volumetrically measured due to the presence of neuromelanin covering, partially or totally, the nucleolus (Figure 1B).

Immunohistochemistry

Each Dys and C case underwent an extensive immunohistochemical assessment. For each case, we examined 16 10-μm thick sections adjacent to the median 40 μm-thick set of sections. The 10 μm-thick tissue sections were deparaffinized, hydrated, and treated to block endogenous peroxidase activity (3% hydrogen peroxide in water). They were then rinsed in buffer (Tris with 0.05% Triton-X), microwaved with antigen retrieval solution (sodium citrate, pH 6.0) for 10 minutes, and cooled. Protein block (1.5% horse serum) was applied for 30 minutes. The following antibodies were used: mouse anti-β-amyloid, 17–24 (dilution 1:500, 4G8; SIG-39220, Covance, Princeton, NJ, USA) with overnight incubation at 4°C (sections were pre-treated with 90% formic acid, 5 minutes); mouse anti-PHF-tau (dilution 1:500, MN1020; Thermo Fisher Scientific, Waltham, MA, USA); mouse anti-α-synuclein (dilution 1:500, Ab27766; Abcam, Cambridge, UK) rabbit anti-ubiq (dilution 1:250, ab7780, Abcam); rabbit anti phospho-TDP-43 (dilution 1:2,000, TIP-PTD-P02; Cosmo Bio Co. LTD, Carlsbad, CA, USA). With the exception of the anti-β-amyloid antibody, sections were incubated overnight at 4°C, rinsed with buffer, incubated with biotinylated horse anti-mouse or anti-rabbit secondary antibodies (30 minutes), Vector Kit reagents (Vector Labs, Inc., Burlingame, CA, USA), then with a 3, 3'-diaminobenzidine substrate system (D3939; Sigma, St. Louis, MO, USA) for 5 minutes.

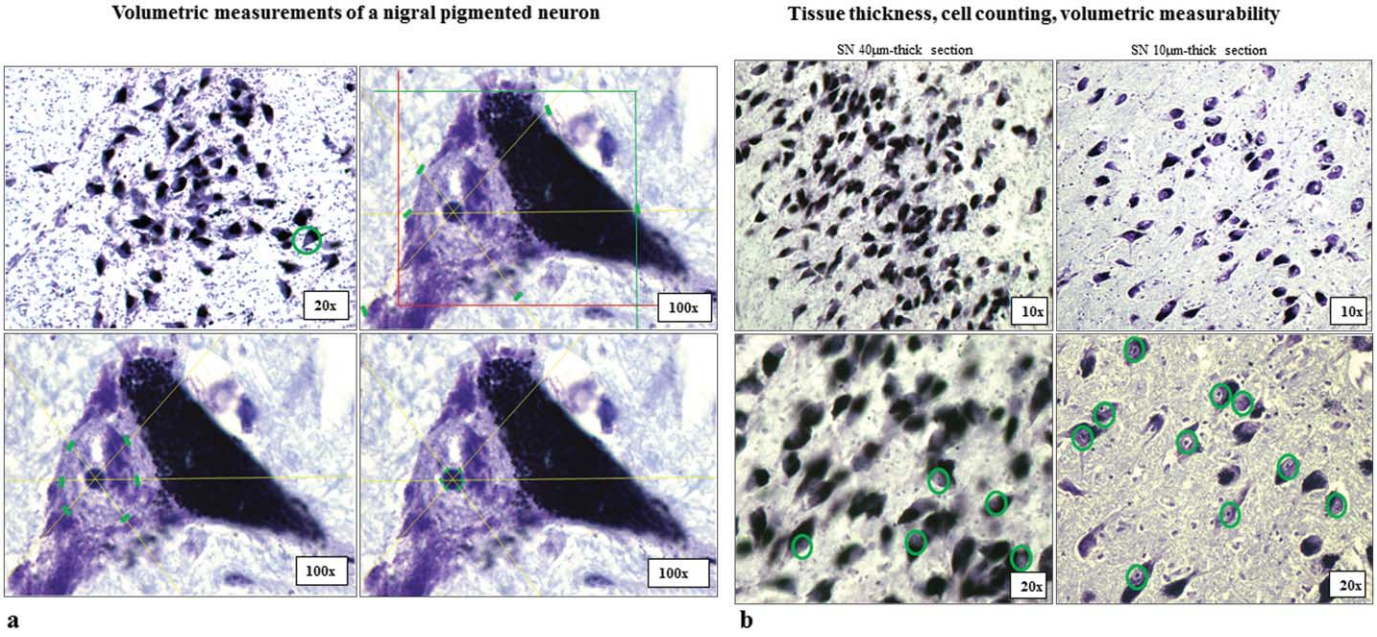

Figure 1A. Quantification of SN Pigmented Neurons. The quadrants in the figure show how a randomly sampled pigmented neuron (first upper-right quadrant, 20 × objective), and 6 randomly projected rays on that pigmented neuron intersect its cell body, nucleus, and nucleolus (respectively, upper-right, lower-left, and lower-right quadrant). A 100 × oil immersion objective was used for all volumetric measurements and for each pigmented neuron. The green markers in each quadrant indicate the point of measurement from which the areas of the cell body, nucleus, and nucleolus were calculated. The colored square upper-right quadrant represents the "counting frame" used for the Optical Fractionator probe. The Stereo-investigator software automatically calculates the volumes of each single neuron computing various parameters such as the thickness of the tissue, the disector height, and guard zones. Figure 1B. Tissue thickness, cell counting, volumetric measurability of nigral pigmented neurons. SNc from a C brain (case#16), cut at different levels of thicknesses (40 µm and 10 µm), stained with CV, and inspected at two different magnifications (10 × and 20 × objectives). The green circles in the inferior parts of the figure indicate the number of pigmented neurons measurable when pigmented neurons were sampled using 40 µm-thick and 10 µm-thick tissue sections, respectively, with a 20 × objective. Notably, the number of measurable pigmented neurons in SN cut at 10 µm of thickness (right of figure) is markedly higher due to the increased number of clear visible CV-stained nucleoli. The nucleolus was the established point of spatial reference on this study. All measurements were performed using a 100 × oil-immersion, NA 1.30, neofluor ∞/0.17 objective. Abbreviations: C, Control; CV, Cresyl Violet; SN, Substantia Nigra; SNc, Pars Compacta of Substantia Nigra.

Slides were counterstained with hematoxylin, dehydrated in xylene, and coverslipped.

Statistical analyses

Statistical analyses for quantification and volumes were performed to compare Dys and C, adulthood-onset Dys and C, childhood-onset Dys and C, childhood-onset Dys and adulthood-onset Dys, and generalized and segmental/focal Dys. All comparisons were computed before and after excluding co-occurring pathology (LB, LN, tau-NFT, tau-th, ubiq, and pTDP43 inclusions, β-amyloid deposits). The childhood-onset Dys group included subjects with early adulthood-onset diagnoses and the only subject with posttraumatic Dys (Dys#10, onset at age 12).

Neuronal counting results revealed a Gaussian distribution, and a *t*-test was initially performed to compare Dys and C and Dys and C without copathologies. Furthermore, we performed analyses of variance (ANOVAs) with Tukey's posthoc tests across all comparisons. The Tukey's test compares every mean with every other mean and takes into account multiple comparisons and adjusted *p*-values for each comparison. Statistical significance was established at $p<0.01$ (adjusted for all five types of comparisons).

The preliminary analyses for the obtained neuronal volumetric measurements did not show a Gaussian curve, so nonparametric Mann-Whitney tests were performed across all comparisons for those measurements. Data refer to the values estimated for the bilateral SN. All computations were performed using GraphPad Prism software, version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The demographic, medical, and genetic data for both the Dys and C groups are summarized in Table 1. Table 1 shows diagnoses of Dys as received from UMBTB records, while Table 2 classifies each Dys patient based on a review of supplemental medical records according to the newest Dys classification system.[4] This most recent classification system was not available for any of the subjects in this cohort at the time of their clinical diagnosis. Table 2 also summarizes all available data in terms of age at onset, body distribution, temporal pattern, variability, associated features, occurrence of other neurological and systemic manifestations, and etiologies (i.e., following the Axis I and II of the newer classification).[4] In terms of temporal patterns, 3 out of 13 Dys cases were classified as adulthood onset, 3 as early adulthood onset, 6 as childhood onset, and 1 case of childhood-onset

Table 1. Demographic, Diagnostic, Genetic, and Medical Data.

Subject#/ UMBTB Code	Age at Death	Sex	Diagnosis (As Received)	Genetics	Other Relevant Medical Data
1 (Dys)/1458	62	M	Dystonia	Neg for GAG del in DYT1	None
2 (Dys)/1484	81	F	Dystonia (started as blepharospasm)	Not tested for DYT1 mutations	None
3 (Dys)/1850	50	F	Dystonia (started at 42 years old: adulthood onset, focal and segmental)	Not tested for DYT1 mutations	Mother with PD, multiple drugs
4 (Dys)/4897	49	F	Dystonia (generalized)	Neg for GAG del in DYT1	None
5 (Dys)/4880	44	F	Dystonia (generalized dystonia with involvement of trunk, limbs, neck, and flexion contractures in the right leg; severe episodes of jerking [head back and forth] with generalized body contortions; possible seizures generalized)	Neg for GAG del in DYT1	Brain aneurysm, intracerebral hemorrhage, multiple drugs
6 (Dys)/5145	84	F	Dystonia (cervical dystonia for >4 decades, head begun to turn 43 years before death with pain 20 years later, mouth involvement 22 years later)	Not tested for DYT1 mutations	Father with tremor (unspecified)
7 (Dys)/5414	95	F	Dystonia (tremor since the 6th grade, myoclonic jerks and terminal intentional tremor, marked paraspinal muscles spasms with lordosis, spastic dysphonia)	Not tested for DYT1 mutations	None
8 (Dys)/5421	81	F	Dystonia	Not tested for DYT1 mutations	None
9 (Dys)/1457	72	M	Dystonia	Neg for GAG del in DYT1	None
10 (Dys)/1353	78	M	Dystonia (severe bike-automobile accident at 12 years old caused a fractured skull, tremor and posttraumatic dystonia followed)	Neg for GAG del in DYT1	None

Table 1. Continued

Subject#/ UMBTB Code	Age at Death	Sex	Diagnosis (As Received)	Genetics	Other Relevant Medical Data
11 (Dys)/4635	76	M	Dystonia (generalized in combination with cervical myelopathy, initial cervical dystonia at 27 years old)	Neg for GAG del in DYT1	History of familial dystonia symptoms (father and sister), possDLB
12 (Dys)/1481	64	F	Dystonia	Neg for GAG del in DYT1	Diabetes, congestive heart failure, angina, angioplasty, daughter with dystonia
13 (Dys)/1554	59	F	Dystonia	Neg for GAG del in DYT1	Small cell carcinoma, pneumonia, history of dystonia preceding lung carcinoma
14 (C)/4263	61	M	Control	Not tested	Ischemic cardiomyopathy, heart transplant, left ventricular dysfunction, depression, chronic renal failure, diabetes mellitus
15 (C)/4921	73	F	Control	Not tested	Gallbladder dysfunction, high blood pressure
16 (C)/5357	51	F	Control	Not tested	Drug overdose, suicidal attempt
17 (C)/4788	48	F	Control	Not tested	Multiple injuries resulting from a motorcycle accident
18 (C)/4228	44	F	Control	Not tested	None
19 (C)/5219	76	F	Control	Not tested	None
20 (C)/M3642M	88	F	Control	Not tested	Congestive heart disease
21 (C)/5274	64	F	Control	Not tested	Hypertension, heart disease, skin cancer
22 (C)/1818	76	M	Control	Not tested	None
23 (C)/5171	79	M	Control	Not tested	COPD
24 (C)/M3903M	71	M	Control	Not tested	Coronary artery disease, coronary angioplasty (twice), COPD
25 (C)/1503	53	F	Control	Not tested	Knee surgery after falling
26 (C)/1379	53	F	Control	Not tested	High blood pressure, asthma

Abbreviations: C, Control; Cauc, Caucasian; COPD, Chronic Obstructive Pulmonary Disease; del, Deletion; Dys, Dystonia; DYT1, Dystonia Gene; F, Female; GAG, Three-nucleotide Deletion; M, Male; Neg, Negative; PD, Parkinson's Disease; possDLB, Possible Dementia with Lewy Bodies; UMBTB, University of Maryland Brain and Tissue Bank.

Table 2. Dys Patients Classified Following the Newer Classification System of Dystonia. The table shows the specific diagnosis of Dys for each patient in study based on the Axis I (clinical features) and Axis II (etiologies) of the newer Dys classification system.[4]

Subject#	Diagnosis (As Received)	Dystonia Diagnosis based on the Newer Classification	Dystonia Diagnosis based on the Newer Classification
		Axis I	Axis II
		(Clinical Features)	(Etiology)
1 (Dys)	Dystonia	Age at onset: not reported (probably childhood-onset as for medical history)	Nervous system pathology: no evidence of degeneration or structural lesions
		Body distribution: not reported (probably generalized as for medical history)	Inherited: possible
		Temporal pattern: not reported	Acquired: no
		Variability: not reported	Idiopathic: yes
		Associated features: unknown/not clinically significant	
		Occurrence of other neurological/ systemic manifestations: unknown/not clinically significant	
2 (Dys)	Dystonia (started as blepharospasm in 1983, 22 years of dystonia history until death)	Age at onset: late adulthood	Nervous system pathology: no evidence of degeneration or structural lesions
		Body distribution: initially focal, then segmental (oromandibular dystonia with jaw closure and lip pursing, facial grimacing)	Inherited: no (aunt, mother's sister, with resting tremor)
		Temporal pattern: static	Acquired: no
		Variability: diurnal	Idiopathic: yes
		Associated features: headache (with temporal and orbital pain)	
		Occurrence of other neurological/ systemic manifestations: tardive dyskinesia secondary to adverse effects to metoclopramide, moderate bradykinesia, rigidity, and rest tremor; possible side effects of various drugs used for the treatment of the blepharospasm	

Table 2. Continued

Subject#	Diagnosis (As Received)	Dystonia Diagnosis based on the Newer Classification	Dystonia Diagnosis based on the Newer Classification
		Axis I	Axis II
		(Clinical Features)	(Etiology)
3 (Dys)	Dystonia (started at 42 years old: adulthood onset, focal and segmental)	Age at onset: late adulthood	Nervous system pathology: evidence of degeneration (SN)
		Body distribution: focal, segmental (face, neck, shoulders), dysphagia	Inherited: no (mother with PD)
		Temporal pattern: static	Acquired: no
		Variability: diurnal	Idiopathic: yes
		Associated features: unknown/not clinically significant	
		Occurrence of other neurological/ systemic manifestations: unknown/not clinically significant	
4 (Dys)	Dystonia (generalized)	Age at onset: childhood	Nervous system pathology: no evidence of degeneration or structural lesion
		Body distribution: generalized (with leg involvement)	Inherited: no
		Temporal pattern: progressive	Acquired: no
		Variability: diurnal	Idiopathic: yes
		Associated features: headache (not clinically significant)	
		Occurrence of other neurological/ systemic manifestations: unknown/not clinically significant	
5 (Dys)	Dystonia (generalized)	Age at onset: childhood	Nervous system pathology: cerebellar cortical degeneration
		Body distribution: multifocal neck, trunk, limbs), segmental (leg involvement)	Inherited: no
		Temporal pattern: progressive	Acquired: no
		Variability: paroxysmal	Idiopathic: yes
		Associated features: pain, headache	
		Occurrence of other neurological/ systemic manifestations: frontal lobe intracerebral hemorrhage (after many years of generalized dystonia)	

Table 2. Continued

Subject#	Diagnosis (As Received)	Dystonia Diagnosis based on the Newer Classification	Dystonia Diagnosis based on the Newer Classification
		Axis I	Axis II
		(Clinical Features)	(Etiology)
6 (Dys)	Dystonia (cervical dystonia for >4 decades)	Age at onset: early adulthood	Nervous system pathology: no evidence of degeneration or structural lesion
		Body distribution: segmental (started as cervical dystonia, then mouth and lingual involvement)	Inherited: unknown (father with head tremor)
		Temporal pattern: progressive (mount involvement, paraspinal muscles spasm, spastic dysphonia)	Acquired: no
		Variability: diurnal	Idiopathic: yes
		Associated features: facial pain	
		Occurrence of other neurological/ systemic manifestations: resting hand tremor (later, after many years cervical dystonia appearance)	
7 (Dys)	Dystonia	Age at onset: childhood	Nervous system pathology: no evidence of degeneration or structural lesion
		Body distribution: segmental (truncal, paraspinal spams with lordosis)	Inherited: no
		Temporal pattern: diurnal	Acquired: no
		Variability: diurnal	Idiopathic: yes
		Associated features: spastic dysphonia, tremor started in 6th grade	
		Occurrence of other neurological/ systemic manifestations: unknown	

Table 2. Continued

Subject#	Diagnosis (As Received)	Dystonia Diagnosis based on the Newer Classification	Dystonia Diagnosis based on the Newer Classification
		Axis I	Axis II
		(Clinical Features)	(Etiology)
8 (Dys)	Dystonia	Age at onset: late adulthood	Nervous system pathology: evidence of degeneration (SN), LB pathology
		Body distribution: segmental (started as blepharospasm then spasmodic dysphonia; lip pursing, grimacing, and jaw opening)	Inherited: no
		Temporal pattern: static	Acquired: no
		Variability: diurnal (getting worse with action); not action-specific but with associated	Idiopathic: yes
		features of late-onset tardive dyskinesia (oromandibular, pharyngeal, and facial dystonia) due to possible side effects of neuroleptic drugs (perphenazine/ amitriptyline)	
		Occurrence of other neurological/ systemic manifestations: unknown/not clinically significant	
9 (Dys)	Dystonia	Age at onset: childhood (probably)	Nervous system pathology: cerebellar atrophy (moderate)
		Body distribution: generalized	Inherited: no
		Temporal pattern: progressive (probably)	Acquired: no
		Variability: diurnal	Idiopathic: yes
		Associated features: unknown/not clinically significant	
		Occurrence of other neurological/ systemic manifestations: unknown/not clinically significant	

Table 2. Continued

Subject#	Diagnosis (As Received)	Dystonia Diagnosis based on the Newer Classification	Dystonia Diagnosis based on the Newer Classification
		Axis I	Axis II
		(Clinical Features)	(Etiology)
10 (Dys)	Dystonia (posttraumatic)	Age at onset: childhood	Nervous system pathology: cerebellar atrophy, LBs in the SN and LC
		Body distribution: focal (right hand)	Inherited: no
		Temporal pattern: static	Acquired: yes, (posttraumatic)
		Variability: persistent	Idiopathic: no
		Associated features: tremor (right hand)	
		Occurrence of other neurological/ systemic manifestations: unknown/not clinically significant	
11 (Dys)	Dystonia (generalized in combination with cervical myelopathy; initial cervical dystonia at 27 years old)	Age at onset: early adulthood	Nervous system pathology: diffuse LB pathology (brainstem, cortex), cerebellar heterotaxia (white matter)
		Body distribution: generalized	Inherited: possible (reported familiarity for movement disorders)
		Temporal pattern: progressive (started as cervical dystonia/torticollis then progressed with arms and legs)	Acquired: no
		Variability: diurnal	Idiopathic: yes
		Associated features: marked diffuse tremor	
		Occurrence of other neurological/ systemic manifestations: possible dementia	
12 (Dys)	Dystonia	Age at onset: early adulthood	Nervous system pathology: no evidence of degeneration or structural lesion
		Body distribution: generalized	Inherited: possible (daughter with dystonia)
		Temporal pattern: progressive	Acquired: no
		Variability: diurnal	Idiopathic: yes
		Associated features: unknown/not clinically significant	
		Occurrence of other neurological/ systemic manifestations: mastectomy for breast cancer	

Table 2. Continued

Subject#	Diagnosis (As Received)	Dystonia Diagnosis based on the Newer Classification	Dystonia Diagnosis based on the Newer Classification
		Axis I	Axis II
		(Clinical Features)	(Etiology)
13 (Dys)	Dystonia	Age at onset: childhood (probably)	Nervous system pathology: no evidence of degeneration or structural lesion (metastatic cells in cerebrum, cerebellum, brainstem)
		Body distribution: generalized	Inherited: no
		Temporal pattern: progressive	Acquired: no
		Variability: diurnal	Idiopathic: yes
		Associated features: unknown/not clinically significant	
		Occurrence of other neurological/ systemic manifestations: lung cancer	

Abbreviations: C, Control; Dys, Dystonia; LB, Lewy Body; LC, Locus Coeruleus; PD, Parkinson's Disease; SN, Substantia Nigra.

posttraumatic Dys. For body distribution, 6 out of 13 Dys cases were classified as generalized, 6 as segmental/focal, and 1 segmental/focal posttraumatic Dys.

All main neuropathologic data, postmortem delay (PMD, time between death and autopsy) values, and causes of death are shown in Table 3. The mean PMD was 11.7 ± 6.4 hours, with no significant differences between the Dys (13 ± 6.7 hours) and C (10.4 ± 6.0 hours) groups. The majority of Dys and C did not show signs of major recent or remote ischemic, hemorrhagic lesions, metastatic infiltration, or arteriovenous or congenital malformations. The exception was case#5 (Dys who showed microhemorrhages, probably due to the prolonged periagonal status and history of remote hemorrhage from an aneurysmatic rupture. Intima thickening was observed in most of the arteries and arterioles in case #3, #12, #13, and #23, which was compatible with arteriosclerotic disease (see Table 4).

Immunohistochemistry findings

Overall, 5/13 Dys and 5/13 C were positive for tau lesions. In the majority of cases, tau lesions were sparse or rare (tau-NFT or tau-th), and only two Dys cases (cases #7 and #8) had moderate levels of tau lesions, mostly tau-th.

No C cases had α-synuclein-positive lesions (LBs or LNs), whereas three Dys cases (#8, #10, and #11) did. These three cases were classified as possible Lewy Body disease (possLBD) of the brainstem.[40] None of the SN samples were positive for 1–42 β-amyloid (diffuse amyloid or neuritic plaques), ubiq, or pTDP43-positive cytoplasmic intraneuronal inclusions. Ubiq-positive intranuclear inclusions (Marinesco bodies [MB])[44] were observed in some cases, and more often in Dys than C. As a reminder, MB are eosinophilic intranuclear neuronal inclusions observed in human brains that have an uncertain origin and pathologic meaning. This intriguing finding has been reported previously;[30] however, larger studies are necessary to confirm the higher frequency of MB in Dys in general or in a specific Dys subtype. Table 4 summarizes the immunohistochemistry findings in both groups.

Quantitative findings: neuronal counting

A total number of $496{,}601 \pm 130{,}010$ and $648{,}926 \pm 162{,}475$ pigmented neurons were estimated in the SNs of the Dys and C groups, respectively. The estimated nigral neuronal population in C did not differ proportionally from previous stereologic studies that assessed the entire SN length in control subjects.[45,46]

Histograms depicting the estimated nigral pigmented neuronal counts and volumes are shown in Figure 2. The estimated nigral pigmented neuronal population was calculated using neuronal numbers weighted for each section thickness per each subject. A significant reduction of pigmented neurons in SN of Dys versus C was found ($p=0.01$). A separate computation after exclusion of all positive LB/LN and/or tau-NFT/th Dys (#6, #7, #8, #10, and #11) and C (#15, #19, #20, #21, and #24) cases was also performed. This second narrower computation confirmed a significant reduction of pigmented neurons in the SN of Dys (n=8) versus C (n=7) ($p=0.01$). No significant differences in terms of neuronal counts were observed between childhood-onset Dys and adulthood-onset Dys cases regardless of whether cases with co-occurring nigral pathologies were

Table 3. Causes of Death, Autopsy Data, and Main Neuropathologic Findings.

Subjects#	BW (Grams)	PMD (Hours)	Cause of Death	Main Neuropathologic Findings
1 (Dys)	1,455	12	Natural	No significant neuropathologic findings
2 (Dys)	N/A	16	Natural	Remote infarct in right parieto-occipital region; moderate cerebral artery atherosclerosis
3 (Dys)	N/A	3	Brain ischemic injury	Acute hypoxic-ischemic encephalopathy, idiopathic SN degeneration
4 (Dys)	N/A	16	Complications of the disorder (multiple falls)	No significant neuropathologic findings
5 (Dys)	N/A	20	Complications of the disorder	Encephalomalacia involving frontal lobes with subarachnoid hematoma due to ruptured berry aneurysm and surgical clip placement, cerebellar cortical degeneration
6 (Dys)	N/A	20	Complications of the disorder	Generalized mild atrophy, arteriosclerotic cerebrovascular disease
7 (Dys)	N/A	16	Complications of the disorder	Neocortical gyral atrophy, arteriosclerosis with leukomalacia
8 (Dys)	N/A	8	Complications of the disorder	Diffuse brain atrophy, arteriosclerotic cerebrovascular disease, SN degeneration with LBs in the SN and limbic cortex, AD-type lesions (amyloid neuritic plaques and neurofibrillary tangles) in the hippocampus
9 (Dys)	N/A	12	Natural	Microscopic remote infarct in the left caudate, moderate cerebellar atrophy
10 (Dys)	N/A	5	Septic shock, metabolic acidosis, respiratory failure	Mild cerebral hemispheric atrophy, rare LBs in the SN and LC, mild cerebellar atrophy
11 (Dys)	N/A	9	Complications of the disorder	Amyloid neuritic plaques in the cortex; diffuse LBs in the SN, limbic structures, and cortical regions; incidental cerebellar white matter heterotopia
12 (Dys)	N/A	26	Natural	No significant neuropathologic findings
13 (Dys)	N/A	6	Lung cancer	Metastatic poorly differentiated carcinoma in the cerebrum, cerebellum, and brainstem
14 (C)	1,309	6	Cardiac arrest	Small cystic infarcts in the left parieto-occipital and frontal lobes
15 (C)	N/A	13	Peritonitis	No significant neuropathologic findings
16 (C)	N/A	8	Atherosclerosis-Cardiovascular disease	No significant neuropathologic findings
17 (C)	N/A	17	Multiple traumatic injuries	No significant neuropathologic findings
18 (C)	1,260	16	Coronary artery atherosclerosis and thrombosis	Acute hypoxic-ischemic encephalopathy in hippocampus
19 (C)	N/A	3	Complications of cancer	No significant neuropathologic findings

Table 3. Continued

Subjects#	BW (Grams)	PMD (Hours)	Cause of Death	Main Neuropathologic Findings
20 (C)	N/A	8	Congestive heart failure	No significant neuropathologic findings
21 (C)	N/A	20	Acute cerebrovascular disease	No significant neuropathologic findings
22 (C)	N/A	3	Acute cerebrovascular disease	Mild to moderate atheromatosis of the circle of Willis, arteriosclerosis of the middle- and small-sized intraparenchymal arteries, microinfarct in the right Sommer's sector (cornu ammonis 1 of the hippocampus)
23 (C)	N/A	5	COPD, peripheral vascular disease	No significant neuropathologic findings
24 (C)	N/A	16	Cardiac arrest	Brain edema
25 (C)	N/A	5	Pulmonary thromboembolism	No significant neuropathologic findings
26 (C)	N/A	15	Respiratory distress	No significant neuropathologic findings

Abbreviations: AD, Alzheimer's Disease; BW, Brain Weight; C, Control; COPD, Chronic Obstructive Pulmonary Disease; Dys, Dystonia; LB, Lewy Body; LC, Locus Coeruleus; N/A, Not Applicable; PMD, Postmortem Delay; SN, Substantia Nigra.

excluded. Table 5 summarizes all comparisons for neuronal counting between Dys and C with their numerical values and statistical significance. See also Figure 4.

The mean for each type of volumetric measurement (cell body, nucleus, and nucleolus) performed on nigral pigmented neurons of Dys and C are shown in Table 5. A mean of 179.7 ± 56.3 nigral pigmented neurons were volumetrically measured (range: 97–297) across all cases. No difference was measured between the mean numbers of pigmented neurons volumetrically measured in Dys (157.5 ± 40.0) and C (201.9 ± 56.3). Importantly, no significant differences in mean cellular, nuclear, or nucleolar volumes were found across all types of comparisons between the Dys and C groups (Table 5). Table 6 shows the coefficient error (CE) values for each performed measurement (counting and volumes) for each subject in the study.

Discussion

To our knowledge, this is the first investigation to apply rigorous neuropathologic quantitative methods to estimate the numbers and volumes of nigral pigmented neurons in a consistent series of Dys and an equivalent number of age-matched control brains that were systematically assessed by specific immunohistochemistry protocols for the individuation and exclusion of possible co-occurring pathologies affecting the SN. Importantly, this is the largest quantitative analyses of Dys brain tissue to date. Our results show that both adulthood- and childhood-onset Dys patients exhibited significant reductions of pigmented SN neurons compared to the C groups. Furthermore, these quantitative analyses showed no significant differences between Dys and C in terms of nigral pigmented neuronal volumes. These findings would seem to exclude phenomena of atrophy or hypertrophy for this type of pigmented neuron in non-DYT1 idiopathic Dys brains. Previous studies describing semiquantitative or pathologic findings on Dys brains mainly focused on genetic Dys, specifically DYT1 cases.[20,29] Therefore, most of the findings from those studies do not seem to be directly applicable to the Dys cohort examined here.

The previous report of larger pigmented neurons in the SN of subjects with Dys (with and without GAG deletions)[29] was not confirmed in our non-DYT1 idiopathic Dys cohort. However, our findings do not exclude the possibility that certain genetic forms of Dys, such as DYT1 characterized by TorsinA protein dysfunction, could induce specific cellular adaptation phenomena during brain development. Indeed, it could be hypothesized that congenital reductions or enlargements of specific neuronal types are present in some specific sensory-motor cortical or subcortical areas[47] as adaptive or compensatory neuronal mechanisms against the subjacent initial pathology. Phenomena of neuroplasticity[48] in Dys patients illustrated by some recent functional neuroimaging studies[49] might support a neurodevelopmental hypothesis of idiopathic Dys.

Limitations and strengths

Although the quantitative methods used in this study were very similar to those used in unbiased stereology investigations, and we tried to minimize confounding factors, it is important to consider our results in the context of some important limitations:

Table 4. H&E and Immunohistochemistry Assessment of other SN Pathologies. The table shows the types and frequencies of co-occurring brain pathologies in the SN of Dys and C subjects. Co-occurring brain pathologies were assessed by specific immunohistochemistry protocols for the following antigens: 1–42 β-amyloid, hyperphosphorylated-tau, α-synuclein, ubiq, and phosphorylated-TDP43. The lesions considered were 1–42 β-amyloid diffuse and neuritic plaques, hyperphosphorylated-tau positive NFTs, tau threads, and ubiq- and phosphorylated-TDP43-positive intraneuronal cytoplasmic inclusions.

Case#	1–42 β-amyloid	Hyperphosphorylated-tau	α-synuclein	Ubiq-cytopl. incl./MB	Phosphorylated-TDP43	H&E
1 (Dys)	Neg	Neg	Neg	Neg/Neg	Neg	Normal
2 (Dys)	Neg	Neg	Neg	Neg/Pos	Neg	Normal
3 (Dys)	Neg	Neg	Neg	Neg/Pos	Neg	Intimal thickening
4 (Dys)	Neg	Neg	Neg	Neg/Neg	Neg	Normal
5 (Dys)	Neg	Neg	Neg	Neg/Neg	Neg	Microhemorrhages
6 (Dys)	Neg	NFT and Th (sparse), tau-glia	Neg	Neg/Pos	Neg	Normal
7 (Dys)	Neg	NFT and Th (moderate)	Neg	Neg/Pos	Neg	Normal
8 (Dys)	Neg	Th (moderate)	Pos (sparse)	Neg/Neg	Neg	Normal
9 (Dys)	Neg	Neg	Neg	Neg/Neg	Neg	Normal
10 (Dys)	Neg	NFT (rare)	Pos (rare)	Neg/Pos	Neg	Normal
11 (Dys)	Neg	Th (sparse), pre-NFT (rare)	Pos (rare)	Neg/Neg	Neg	Normal
12 (Dys)	Neg	Neg	Neg	Neg/Neg	Neg	Intimal thickening
13 (Dys)	Neg	Neg	Neg	Neg/Pos	Neg	Intimal thickening
14 (C)	Neg	Neg	Neg	Neg/Neg	Neg	Normal
15 (C)	Neg	Th (sparse)	Neg	Neg/Neg	Neg	Normal
16 (C)	Neg	Neg	Neg	Neg/Neg	Neg	Normal
17 (C)	Neg	Neg	Neg	Neg/Neg	Neg	Normal
18 (C)	Neg	Neg	Neg	Neg/Neg	Neg	Normal
19 (C)	Neg	Th (rare)	Neg	Neg/Neg	Neg	Normal
20 (C)	Neg	NFT (rare)	Neg	Neg/Neg	Neg	Hyperemia
21 (C)	Neg	Th (rare)	Neg	Neg/Neg	Neg	Normal
22 (C)	Neg	Neg	Neg	Neg/Pos	Neg	Normal
23 (C)	Neg	Neg	Neg	Neg/Neg	Neg	Intimal thickening
24 (C)	Neg	Th (sparse)	Neg	Neg/Neg	Neg	Normal
25 (C)	Neg	Neg	Neg	Neg/Neg	Neg	Normal
26 (C)	Neg	Neg	Neg	Neg/Neg	Neg	Normal

Abbreviations: C, Control; Dys, Dystonia; H&E, Hematoxylin and Eosin; Neg, Negative; NFT, Neurofibrillary Tangle; Pos, Positive; SN, Substantia Nigra; TDP43, TAR DNA-binding Protein 43; Th, Thread

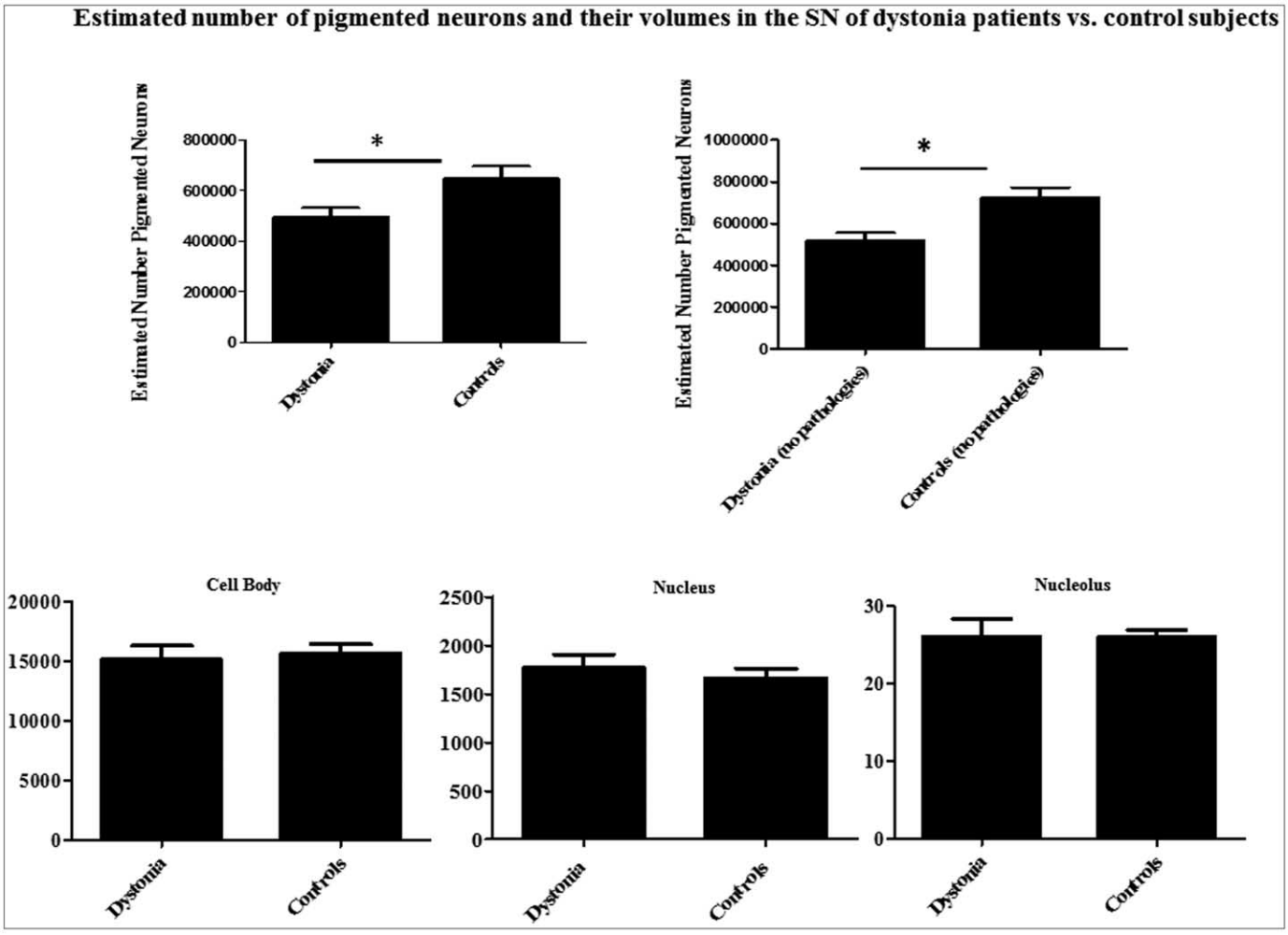

Figure 2. Histograms of the Estimated Mean Number of SN Pigmented Neurons in 13 Dys Patients and 13 C Subjects. The histograms on the left side include all Dys and C brains, while those on the right show Dys and C data after exclusion of all brains with immunohistochemical evidence of co-occurring pathologies. The lower part of the figure shows histograms of cell body, nuclear, and nucleolar mean volumes of pigmented neurons in the SN. The y-axis indicates mean volumetric values expressed in μm^3. *$p<0.01$. Abbreviations: C, Control; Dys, Dystonia; SN, Substantia Nigra.

1) The obtained quantitative estimations were not based on the entire length of the SN. Although we used well-established and reliable stereology tools (such as the Optical Fractionator and Nucleator probe) and precise histological, neuropathologic, and clinical approaches (i.e., serial sectioning, anatomical randomization, exclusion of co-occurring pathologies, same mean age at death, etc.), this investigation cannot be defined as an unbiased stereology study mainly due to the impossibility of obtaining the entire structure of interest (SN). Our preliminary novel findings need to be confirmed by unbiased stereological investigations that include the entire anatomical extension of SN.
2) We assessed a low number of cases for each specific type of Dys. It is highly probable that different types of Dys have different causes and unique pathologic features. For example, it is highly probable that adulthood- and childhood-onset forms of Dys represent two different pathogenetic pathways. However, it cannot be totally excluded that they share some common clinical and pathologic features. Then, we cannot be certain that future larger neuropathologic quantitative stereologic studies focusing on a single type of Dys could confirm our findings only on some specific type or group of Dys.

This investigation, however, also has several strengths:

1) To the best of our knowledge, this is the first clinicopathologic correlative study performed on a relatively large number of autopsy-Dys brains (n=13) and an equivalent amount of autopsy-confirmed age-matched control brains applying rigorous neuropathologic quantitative methods to describe possible specific characteristics (e.g., a reduced number of pigmented neurons in the SN).

Table 5. Estimated Number of Pigmented Neuron Population and their Cellular and Subcellular Volumes in the SN of Dys and C Subjects. The table shows the estimated number and cell body, nuclear, and nucleolar volumes of pigmented neurons (mean ± SD) in the SN of Dys and C across all considered subtypes. The significance of *p*-values is indicated for each type of comparison. The significance was established when $p<0.01$.

Comparisons	Estimated Number of Pigmented Neurons (±SD)	Cell Body	Nuclear	Nucleolar
		Volume (μm^3)	Volume (μm^3)	Volume (μm^3)
Dys (n=13) vs. C (n=13)	496,601.6 ± 130,009.4 vs. 648,925.7 ± 169,700.2 (p=0.0186)	15,136.1 ± 4,122.2 vs. 15,632.1 ± 2,718.2 (ns)	1,768.1 ± 505.4 vs. 1,662.2 ± 347.0 (ns)	26.0 ± 8.2 vs. 25.9 ± 3.1 (ns)
*Dys (n=8) vs. *C (n=8)	518,991.3 ± 139,210.6 vs. 725,432.2 ± 16,531.8 (p=0.0045)	14,340.1 ± 5,097.2 vs. 15,936.1 ± 2,992.1 (ns)	1,627.1 ± 560.1 vs. 1,767.2 ± 399.2 (ns)	25.8 ± 8.9 vs. 26.0 ± 3.8 (ns)
Adulthood-onset Dys (n=3) vs. C (n=13)	408,748.1 ± 85,747.9 vs. 648,925.7 ± 169,700.2 (p=0.0366)	13,669.1 ± 3,557.2 vs. 15,936.1 ± 2,992.1 (ns)	1,661.1 ± 491.9 vs. 1,662.2 ± 347.0 (ns)	24.0 ± 6.8 vs. 25.9 ± 3.1 (ns)
*Adulthood-onset Dys (n=2) vs. *C (n=8)	408,748.1 ± 85,747.9 vs. 725,432.2 ± 16,531.8 (p=0.0366)	11,709.5 ± 1,510.5 vs. 15,222.7 ± 766.4 (ns)	1,491.1 ± 556.6 vs. 1,796.1 ± 372.0 (ns)	24.1 ± 9.7 vs. 26.0 ± 5.2 (ns)
Childhood-onset Dys (n=10) vs. C (n=13)	503,648.9 ± 124,734.1 vs. 648,925.7 ± 169,700.2 (p=0.0436)	15,764.1 ± 4,569.2 vs. 15,632.1 ± 2,718.2 (ns)	1,764 ± 533.1 vs. 1,662 ± 347.0 (ns)	27.1 ± 9.1 vs. 25.9 ± 3.1(ns)
*Childhood-onset Dys (n=6) vs. *C (n=8)	541,369.8 ± 112,651.7 vs. 725,432.2 ± 16,531.8 (p=0.0192)	15,216.7 ± 1,373.2 vs. 15,222.7 ± 766.4 (ns)	1,671.7 ± 400.9 vs. 1,796.1 ± 372.0 (ns)	25.6 ± 5.2 vs. 26.0 ± 5.2 (ns)
Childhood-onset Dys (n=10) vs. Adulthood-onset Dys (n=3)	504,647.0 ± 371,697.8 vs. 463,247.9 ± 97,180.9 (ns)	15,764.1 ± 4,569.2 vs. 13,669.1 ± 3,557.2 (ns)	1,764 ± 533.1 vs. 1,662.2 ± 347.0 (ns)	27.1 ± 9.1 vs. 25.9 ± 3.1 (ns)
*Childhood-onset Dys (n=6) vs. *Adulthood-onset Dys (n=2)	535,589.0 ± 371,697.8 vs. 512,103.1 ± 67,582.0 (ns)	15,216.7 ± 1,373.2 vs. 11,709.5 ± 1,510.5 (ns)	1,671.7 ± 400.9 vs. 1,796.1 ± 372.0 (ns)	25.6 ± 5.2 vs. 26.0 ± 5.2 (ns)
Generalized Dys (n=6) vs. segmental/focal Dys (n=7)	514,023.1 ± 98,491.31 vs. 481,668.9 ± 163,184.3 (ns)	14,379.1 ± 1,004.9 vs. 15,784.8 ± 3,587.4 (ns)	1,767.6 ± 409.5 vs. 15,784.8 ± 3,587.4 (ns)	28.9 ± 3.5 vs. 23.4 ± 4.2 (ns)
*Generalized Dys (n=5) vs. *segmental/focal Dys (n=3)	513,204.1 ± 139,210.5 vs. 528,636.7 ± 139,513.19 (ns)	13,984.5 ± 1,373.2 vs. 14,932.3 ± 5,683.2 (ns)	1,655.5 ± 400.9 vs. 1,982.4 ± 312.7 (ns)	27.2 ± 1.0 vs. 32.1 ± 5.5 (ns)

Abbreviations: C, Control; Dys, Dystonia; ns, not significant; SD, Standard Deviations; SN, Substantia Nigra.

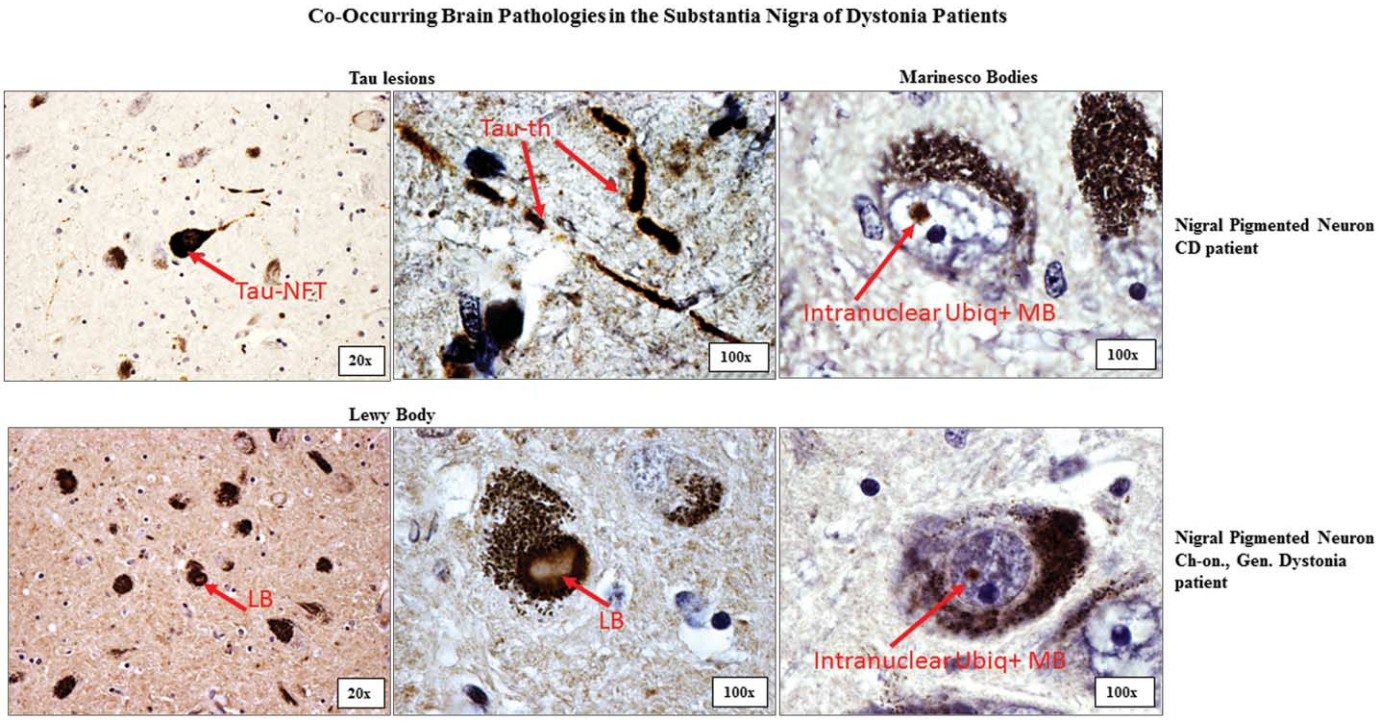

Figure 3. Co-occurring Pathologies in the SN of Dys Patients. Abbreviations: CD, Cervical Dystonia (subject Dys#6); Ch-on, Childhood-onset; Gen., Generalized (Subject Dys#13); LB, α-synuclein-positive Lewy Body; MB, Marinesco Body (intranuclear eosinophilic body); Tau-NFT, Tau-positive Neurofibrillary Tangle; Tau-th, Tau-positive Thread; Ubiq+, Ubiquitin Positivity.

2) Extensive histopathologic analyses were performed on each single Dys and C case, including possible co-occurring pathologies affecting the SN. In our opinion, this type of immunohistochemistry assessment is essential to decrease the possibility that a lower number of nigral pigmented neurons could be due to the simultaneous presence of nigral degenerative pathologies. These could indeed represent important confounders that are not directly or necessarily related to the pathogenesis of idiopathic forms of Dys. However, our findings do not exclude the possibility that nigral or extranigral neurodegenerative phenomena (e.g., during aging), could contribute to the progression or initial disease manifestations.
3) There was a remarkable effort in reclassifying most of the non-DYT1 Dys autopsy cases available at UMBTB following the most recent classification system changes.[4] The reclassification was based on the careful re-examination of all available medical records. This effort was an important attempt to describe new neuropathologic findings using precious brain tissues previously donated and already available to the scientific community in consideration of newer clinical views and modern neuropathologic quantitative and immunohistochemistry techniques.

Our neuropathologic morphometric-quantitative findings, which are similar to the results of other recent pathologic studies,[31,32] seem to confirm the direct involvement of the SN in the pathomechanisms of Dys. Importantly, the involvement of the SN in Dys with regard to a reduced number of pigmented neurons (mainly dopaminergic cells) is not necessarily or directly related to dopaminergic dysfunction *per se* (i.e., dopaminergic function loss), or to a specific clinical phenomenology of Dys. In fact, in a hypothetical and more global context of a neurodevelopmental disorder, it is possible that other nondopaminergic circuits and neurotrasmettitorial abnormalities (i.e., gamma-aminobutyric acid, acetylcholine)[50,51] could play important or even major roles. Moreover, results from two clinical and functional neuroimaging series[52,53] support a more global view on Dys phenomenology where the motor and nonmotor abnormalities observed in Dys patients are associated with a broad spectrum of abnormalities in multiple cerebral regions, some of which are nondopaminergic. Furthermore, at a higher speculative level, a neurodevelopmental hypothesis for idiopathic Dys (or some specific form of it) that would not primarily involve dopaminergic dysfunction (i.e., dopaminergic function loss) could help to explain the constant and frustrating inefficacy of dopaminergic therapies in most Dys patients, with the remarkable exception of DRD.

Larger studies are needed to confirm our novel findings on idiopathic Dys. However, these preliminary results seem to suggest the presence of a specific neuropathologic substrate in idiopathic Dys in the form of a reduced number of nigral pigmented neurons that is not due to a neurodegenerative process.

Some very important questions remain:

Table 6. Estimated Number of Nigral Pigmented Neuronal Populations and Volumetric Measurements with Corresponding Values of Gunderson's Coefficient Errors in the Dys and C Groups. The table shows single values obtained in each Dys and C subject in terms of estimated number of pigmented nigral neurons counted. Each mean neuronal counting estimation has a corresponding mean cell body, nuclear, and nucleolar volume for all examined subjects. CE is an indicator of precision for the performed estimations and is generally acceptable if <0.10.

Subject#	Mean Pigmented Neuronal Count	CE	Mean Cellular Volume	CE	Mean Nuclear Volume	CE	Mean Nucleolar Volume	CE
1 (Dys)	530,990.7	0.05	18,457.9	0.006	2,332.3	0.006	38.1	0.007
2 (Dys)	409,690.0	0.07	12,777.6	0.003	1,884.7	0.003	31.0	0.004
3 (Dys)	494,021.3	0.06	10,641.4	0.005	1,097.5	0.006	17.2	0.022
4 (Dys)	604,631.9	0.06	12,589.7	0.004	1,730.0	0.005	27.3	0.005
5 (Dys)	682,198.8	0.05	21,377.8	0.005	1,752.9	0.005	24.4	0.006
6 (Dys)	491,138.2	0.05	17,297.2	0.007	1,510.4	0.007	24.8	0.006
7 (Dys)	275,364.9	0.08	16,926.6	0.009	1,840.5	0.009	21.6	0.011
8 (Dys)	322,533.2	0.07	17,586.4	0.007	2,002.1	0.007	23.7	0.062
9 (Dys)	405,516.6	0.06	5,273.8	0.006	581.8	0.009	8.0	0.008
10 (Dys)	696,735.9	0.05	13,886.4	0.004	2,286.8	0.005	21.3	0.049
11 (Dys)	518,118.1	0.06	16,352.0	0.006	2,328.5	0.007	37.4	0.007
12 (Dys)	414,003.9	0.06	17,771.6	0.006	1,533.1	0.006	30.6	0.079
13 (Dys)	610,877.4	0.05	15,829.5	0.005	2,100.2	0.005	32.0	0.005
14 (C)	787,705.8	0.06	13,858.9	0.004	1,115.2	0.005	22.0	0.016
15 (C)	648,925.7	0.06	11,253.3	0.007	1,390.6	0.008	23.6	0.045
16 (C)	351,255.9	0.05	15,631.9	0.003	1,663.0	0.003	25.8	0.004
17 (C)	856,559.1	0.06	11,864.0	0.003	1,600.5	0.003	24.0	0.004
18 (C)	621,172.8	0.07	15,532.5	0.007	2,084.9	0.008	29.0	0.053
19 (C)	370,591.1	0.06	20,711.7	0.010	1,585.1	0.009	21.1	0.010
20 (C)	571,960.5	0.06	18,168.9	0.003	1,704.5	0.004	27.3	0.048
21 (C)	696,553.0	0.06	15,519.3	0.003	1,365.8	0.003	26.8	0.034
22 (C)	488,401.4	0.05	14,754.4	0.004	1,327.2	0.005	25.1	0.057
23 (C)	800,241.8	0.05	18,947.8	0.004	1,660.0	0.004	28.4	0.004
24 (C)	718,722.8	0.04	15,375.8	0.004	2,007.7	0.003	29.7	0.004
25 (C)	750,282.5	0.05	16,342.6	0.004	1,788.9	0.005	23.0	0.059
26 (C)	773,662.1	0.05	15,258.7	0.003	2,315.1	0.003	30.5	0.004

Abbreviations: C, Control; CE, Coefficient of Error (of Gunderson); Dys, Dystonia.

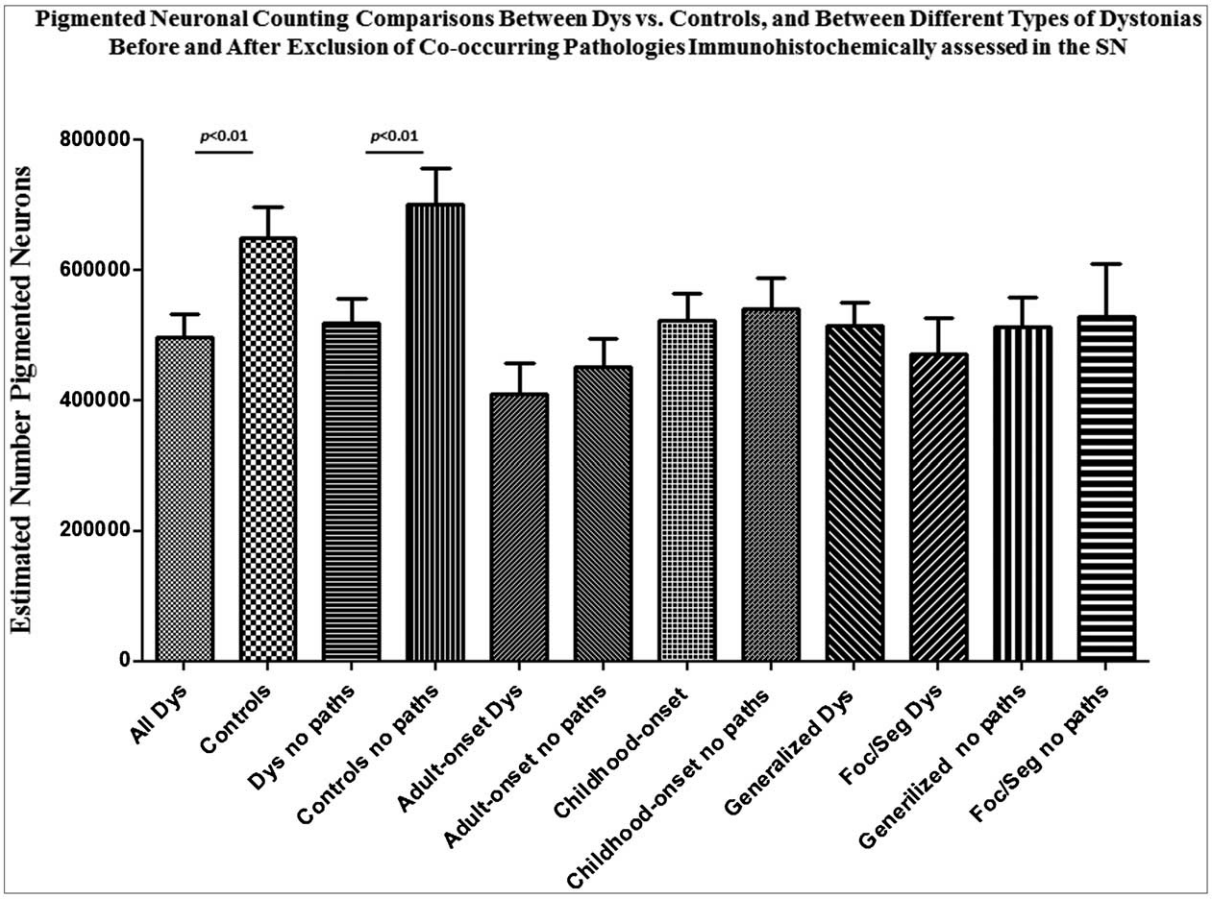

Figure 4. Histograms of Estimated Mean Numbers of Pigmented Neurons. We quantified neurons in the substantia nigra (SN) of dystonia (Dys) vs. age-matched control subjects (C) and compared counts in types of dystonia (adulthood-onset, childhood-onset, generalized, focal/segmental [foc/seg] dystonias). For each measurement there are two histograms showing the obtained estimated number of neurons before and after exclusion of all nigral co-occurring pathologies (β-amyloid, tau, Lewy bodies, and ubiquitin). All co-occurring nigral pathologies were assessed using immunohistochemistry protocols.

1) In Dys brains, is the nigral pigmented neuronal reduction a loss of neurons (neurodegenerative hypothesis), or does it represent, as we hypothesize, a possible neurodevelopmental defect (neurodevelopmental hypothesis)?[54] In support of the latter hypothesis is the observation that volumetric measurements of nigral pigmented neurons in Dys cases were not significantly different from those in C brains as would be expected if atrophic-neurodegenerative processes were actually involved. These morphometric findings are especially relevant considering that the reduction of nigral pigmented neurons in Dys versus C remained significant even after immunohistochemical exclusion of some copathologies frequently associated with SN neuronal degeneration.

In addition, the absence of a difference in the number of nigral pigmented neurons between the only available posttraumatic dystonia case (#10) and C brains seems to support a neurodevelopmental hypothesis for idiopathic Dys. In the future, larger studies of posttraumatic and nonposttraumatic/idiopathic Dys could provide pathologic findings that support or exclude a neurodevelopmental defect of the SN in idiopathic Dys.

2) Does the nigral pigmented neuronal reduction primarily or exclusively affect dopaminergic neurons? Does it specifically impact the pars compacta or pars reticulata of the SN? It is important to recall that the SN (and basal ganglia [BG] in general) contains a considerable level of somatotopic neuronal organization and neurotrasmettitorial complexity. It is not possible to exclude then that fewer nigral pigmented neurons could mainly affect specific subregions of the SN, thus influencing specific cellular and pathogenetic mechanisms and causing various clinical phenotypes and motor and nonmotor manifestations of Dys.
3) Does the nigral neuronal reduction (or loss) represent a cause or an effect of other pathologic (or neurodevelopmental) phenomena?

Our preliminary quantitative findings, if confirmed, would offer novel insight into specific neuronal alterations in idiopathic Dys. However, these new findings need to take into account very complex pathophysiologic mechanisms and possible pathologic differences across the various forms of Dys.[55–58] Nonetheless, partial overlapping or common mechanisms among groups or subgroups of Dys with similar clinical phenotypes remain possible.[59]

Future major efforts should include unbiased stereological analyses in larger groups of different genetic and nongenetic forms of Dys to confirm our observation of fewer pigmented neurons in the SN, and if so, clarify whether this decrease is "congenital" or neurodegenerative in nature. Another important issue is whether this cellular abnormality is unique to the SN or specific forms of Dys.

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