Parkinsonism and Related Disorders 58 (2019) 63–69

Contents lists available at ScienceDirect

Parkinsonism and Related Disorders

journal homepage: www.elsevier.com/locate/parkreldis

# Hypertrophy of nigral neurons in Torsin1A deletion (DYT1) carriers manifesting dystonia

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ARTICLE INFO

*Keywords:*
Generalized dystonia
Substantia Nigra
Neuronal Hypertrophy
torsinA
DYT1
Dopamine

ABSTRACT

*Objective:* To individuate morphometric changes and prevalent types of intraneuronal inclusions in nigral neurons of DYT1 dystonia autopsy-brains.
*Methods:* Using precise methods of quantification, such as unbiased stereology, we measured cellular and subcellular volumes of neuromelanin-containing (pigmented) neurons in the substantia nigra (SN) of DYT1 carriers with and without manifestation of generalized dystonia (manif-DYT1 and non-manif-DYT1, respectively), non-DYT1 carriers manifesting generalized dystonia (manif-non-DYT1) patients, and age-matched control subjects (controls). A total of four DYT1 carriers (two manif-DYT1 and two non-manif-DYT1), six manif-non-DYT1 carriers, and six controls autopsy-brains were available for these neuropathological-morphometric analyses. The search of brain lesions was performed for: tau neurofibrillary tangles and neurites, extracellular $\beta$-amyloid deposits, Lewy bodies and neurites, TorsinA, Laminin A + C, Ubiquitin, p62, pTDP43 intraneuronal inclusions; and Negri, Bunina, Hirano, Marinesco, Nissl, and Buscaino bodies.
*Results:* An increased mean cell body, nuclear, and nucleolar volume of nigral neurons in manif-DYT1 vs. non-manif-DYT1 ($p < 0.0001$), manif-non-DYT1 ($p < 0.0001$), and controls ($p < 0.00001$) was found. Increased nuclear and nucleolar volumes in manif-non-DYT1 vs. controls were also found. None of the considered possible intraneuronal lesions were more frequent or prevalent in nigral neurons of manif-DYT1 vs. all the other groups.
*Conclusions:* Unbiased stereology-based measurements of nigral neurons enlargement in manif-DYT1 in the absence of intraneuronal inclusions or neurodegenerative processes, is novel. These findings suggest distinct pathogenetic mechanisms between manif-DYT1 vs. non-manif-DYT1 and manif-non-DYT1 dystonia, especially in terms of possible nigral dopaminergic abnormalities. These data could open new pathophysiologic views on specific dopamino-associated pathomechanisms related to the clinical manifestation of generalized dystonia.

## 1. Introduction

Among the inherited forms of dystonia, the autosomal dominant childhood-onset DYT1 dystonia (DYT1) is by far, the most frequent [1,2]. DYT1 is linked to a three base pair GAG deletion (ΔGAG) inside the exon 5 of TORSIN1A gene (TOR1A) localized on chromosome 9q.3411, which codifies for TorsinA protein, a member of the AAA + superfamily of ATPases [3,4]. While genetic discoveries helped to formulate useful classification systems [5], specific neuropathologic features of dystonia are generally lacking [6], with some rare exceptions [7,8]. In particular, investigations analyzing postmortem brains from ascertained ΔGAG_TOR1A carriers (DYT1) are indeed extremely rare [9]. Among those rare neuropathologic investigations on DYT1 brains, there is one published by Rostasy K. and colleagues in 2003 [10]. These authors described an enlargement of pigmented neurons in the substantia nigra (SN) of dystonia subjects in comparison to control subjects (controls). However, those data were not obtained using unbiased morphometric methods such as unbiased stereology methods, and have never been confirmed by other investigators.

We aimed to: a) perform rigorous quantitative measurements of cell body, nuclear, and nucleolar volumes of nigral pigmented neurons from brains of ascertained-DYT1 carriers subjects using unbiased stereology methods; b) compare the morphometric measurements obtained from manif-DYT1 carriers to the ones obtained from non-manif-DYT1 carriers, manif-non-DYT1 subjects, and age-matched controls to determine if significant nigral neurons volumetric differences existed across all

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https://doi.org/10.1016/j.parkreldis.2018.08.020
Received 13 March 2018; Received in revised form 14 August 2018; Accepted 28 August 2018

those groups; and c) perform an extensive and systematic immunohistochemistry assessment of nigral neurons of manif-DYT1, non-manif-DYT, and manif-non-DYT1 subjects to detect possible co-occurring brain pathologies (i.e. Lewy bodies, tau-neurofibrillary tangles [tau-NFTs] and others) and verify if a more frequent or prevalent type of intraneuronal lesion was present in nigral neurons of manif-DYT1 in comparison to non-manif-DYT1 carriers and manif-non-DYT1 patients.

## 2. Material and methods

We investigated three ascertained-DYT1 mutation carrier brains, all members of the same family. One person (a niece) was diagnosed with childhood-onset generalized dystonia with onset at age 12, while two other family members (two aunts) never manifested dystonia phenomenology, even after reaching the 9 t h decade of life (mean age at death for the two aunts was of 88 years). Other members of the same family (not tested for DYT1 or other possible dystonia-related genetic mutations or risk factors) were diagnosed with various types of movement disorders: cervical dystonia, writer's cramp, torticollis, as well as "*dystonia musculorum deformans*" (now referred as DYT1 dystonia). In addition to those three DYT1 carrier family cases, a further ascertained-DYT1 postmortem brain was available for quantitative neuropathologic measurements. Cumulatively, a total of four hemi-SN were available and sampled from confirmed-DYT1 mutation carriers' postmortem brains. Furthermore, six manif-non-DYT1 (generalized dystonia) hemi-SN were also available. All dystonia-brain samples were obtained from the University of Maryland Brain and Tissue Bank (UMBTB), member of the NIH NeuroBioBank consortium (https://neurobiobank.nih.gov). In addition, six hemi-SN from age-matched control subjects (controls) were available and obtained from the Brain and Body Donation Program (BBDP) at Banner Sun Health Research Institute, AZ (https://www.brainandbodydonationprogram.org).

All specimens were obtained after the next-of-kin or legal representative signed a written consent for their use in research, and after obtaining the required approval from each Institutional Review Board (IRB) where each case was examined.

## 3. DYT1 carriers, Non-DYT1 carriers and age-matched controls clinical features

Two cases (Table 1: case #1 and #4) out of all four DYT1 carrier individuals had clinical manifestations of generalized dystonia.

Case #1 had generalized dystonia with childhood-onset around 12 years of age. Her symptoms were initially diagnosed as a psychiatric condition (she was considered as having a "… neurotic condition and a way to get attention …" for a couple of decades). Only at the age of 34 was a formal diagnosis of dystonia made. Symptoms started (as confirmed by her sister) as a "tremor" of the body. Later the diagnosis was changed to torsional dystonia.

Case #2 and Case #3, which were ascertained to be DYT1 carriers, did not show any sign of dystonia or other movement disorders during their lifespan.

Case #4 had a diagnosis of generalized dystonia with probable childhood age at onset.

None of the four DYT1 carrier subjects were described to have signs of other movement disorders, or other neurologic, psychiatric, or major illnesses except for atherosclerotic disease in case #2, #3, and #4; and a subacute brain infarct in case #3. Case #3, was also diagnosed with clinical dementia, but only confirmed as possible Alzheimer's disease (possAD) at autopsy.

All DYT1 carrier subjects were genetically-tested and documented to be positive for ΔGAG codon in TOR1A on certified genetic reports.

Only four out of six hemi-SN of previously analyzed manif-non-DYT1 [11] were considered for this investigation. In fact, for the specific aims of this investigation two out of those six previously examined manif-non-DYT1 cases were excluded for the presence of LB pathology in the SN of one case and brainstem microhemorrhages in the other case. All manif-non-DYT1 patients were primary dystonia cases based on a previous large investigation, which excluded most causes of secondary dystonia [11].

None of the controls, based on the available clinical history and medical records, had clinical or pathological evidence of cerebrovascular diseases, neurodegenerative illnesses, or other neurological, psychiatric, or major medical disorders. None of the controls had a history of movement disorders or dystonia.

### 3.1. Neuropathology procedures

At autopsy, a half-brain from each DYT1 and non-DYT1 dystonia and control subjects, was fixed in 10% buffered formalin for at least two weeks, then grossly and microscopically examined on coronal sections as for a standard neuropathologic assessment at the corresponding brain bank (UMBTB or BBDP). All SNs samples were shipped and processed at the Neuropathology Research labs, Biomedical Research Center of New Jersey, BRInj. All tissue-blocks were processed by an automated tissue-processor (Tissue-Tek V.I.P. 1000 Vacuum Infiltration Processor, Ames Division, Miles Laboratories, Inc. IN, USA) as per standard protocols. The blocks were then embedded in paraffin, oriented with their rostral area facing to the bottom of the paraffin mold and serially cut along the rostro-caudal anatomical direction using a semi-automatic microtome (Leica RM2255, Leica Biosystems, Nussloch, Germany). Tissue cuts were performed by following an alternating 40- and 10-μm thick serial sectioning scheme [11]. The total length of each received formalin-fixed hemi-SN blocks ranged between 6 and 8 mm. Although we were unable to obtain the entire SN for each DYT1 carrier and non-DYT1 subject, we estimated that at least a 50% or more of the entire length of the SN was indeed received for each DYT1 carrier and non-DYT1 subject. Our anatomical metric estimations were based on previous anatomical studies that included the entire SN [12]. As for the controls, we were able to obtain the entire serially cut SN for each subject. In general, we were able to measure cellular and subcellular volumes of a consistent number of nigral pigmented neurons in manif-DYT1 and non-manif-DYT1 (mean number = 119), manif-non-DYT1 (mean number = 165) and controls (mean = 201).

### 3.2. Neurohistology procedures

A single 10 μm-thick tissue section was randomly chosen and stained with hematoxylin and eosin (H&E) and microscopically inspected at lower (2.5× objective) and higher (20× objective) magnification to detect possible ischemic vascular pathologies, microhemorrhages, tissue rarefactions, metastases, and other histologic microscopic abnormalities.

### 3.3. Morphometric methodologies and quantitative procedures

For morphometric purposes, separated series of 10 μm-thick sections across the entire available length of each SN from manif-DYT1 and non-manif-DYT1 carriers, manif-non-DYT1, and controls were consecutively chosen with a rate of one out twenty-four, with a starting section randomly chosen among the first five consecutive serially-cut sections. All sections were stained with 1.0% solution of cresyl violet (CV) and randomly re-coded by an investigator (MGE) blinded for both clinical and pathologic diagnoses. All volumetric measurements were performed by employing stereological tools such as Optical Fractionator and Nucleator probe [13,14]. The volumetric measures were performed by a second investigator (DI) blind for both clinical and pathologic diagnoses and for the re-coding system.

Each CV-stained section was stereoscopically inspected (x 2.0) for the entire sectional area to precisely localize SN anatomical borders. The SN anatomical borders included both *pars compacta* and *pars reticulata* of SN, and were identified and marked as based on previous

**Table 1**
The table summarizes main demographics, dystonia-related clinical manifestations, and ascertained type of genetic mutation data. Manif-DYT1 (DYT1 carrier subjects manifesting generalized dystonia); non-manif-DYT1 (DYT1 carrier subjects non-manifesting dystonia or other movement disorders); manif-non-DYT1 (not carrying DYT1 mutation subjects manifesting generalized dystonia); control (subjects without genetic mutations associated to dystonia and negative for neurological and psychiatric diseases). Δ = deletion; GAG = 3-base pair (guanine, adenine, guanine). Gen = generalized dystonia; f = female; m = male; C= Caucasian; N/A = not applicable.

| Subject# | Same Family | Age at death | Sex | Race | Manifesting dystonia | Ascertained type of genetic mutation | Presumptive age of onset for Dystonia Symptomatology (as for medical records or family interview) | Family History for Dystonia or other Movement Disorders |
|---|---|---|---|---|---|---|---|---|
| #1 (#2′and#3′niece) (manif-DYT1) | Yes | 67 | f | C | Yes (Gen.) | ΔGAG (pos) | Presumptive onset at 12–15; at 34 first diagnosis of dystonia; at 44 diagnosis of "torsion dystonia" | Yes. Two aunts confirmed ΔGAG_TOR1A-carriers |
| #2 (#3's sister) (non-manif-DYT1) | Yes | 89 | f | C | No | ΔGAG (pos) | Asymptomatic for dystonia | Yes. One sister (case#3) confirmed ΔGAG- TOR1A carrier; one niece (case#1) confirmed ΔGAG-TOR1A -carrier with generalized dystonia |
| #3 (#2's sister) (non-manif-DYT1) | Yes | 87 | f | C | No | ΔGAG (pos) | Asymptomatic for dystonia | Yes One sister (case#2) confirmed ΔGAG- TOR1A carrier; one niece (case#1) confirmed ΔGAG-TOR1A-carrier with generalized dystonia |
| #4 (manif-DYT1) | No | 88 | f | C | Yes (Gen) | ΔGAG (pos) | Clinical diagnosis of dystonia with uncertain age of onset | Unknown |
| #5 (manif-non-DYT1) | No | 49 | f | C | Yes (Gen) | ΔGAG (neg) | Childhood | Unknown |
| #6 (manif-non-DYT1) | No | 72 | m | C | Yes (Gen) | ΔGAG (neg) | Childhood | Unknown |
| #7 (manif-non-DYT1) | No | 64 | f | C | Yes (Gen) | ΔGAG (neg) | Early adulthood | Unknown |
| #8 (manif-non-DYT1) | No | 59 | f | C | Yes (Gen) | ΔGAG (neg) | Childhood | Unknown |
| #9 (control) | No | 81 | m | C | No | None | N/A | Unknown |
| #10 (control) | No | 80 | m | C | No | None | N/A | Unknown |
| #11 (control) | No | 86 | m | C | No | None | N/A | Unknown |
| #12 (control) | No | 89 | m | C | No | None | N/A | Unknown |
| #13 (control) | No | 91 | m | C | No | None | N/A | Unknown |
| #14 (control) | No | 87 | f | C | No | None | N/A | Unknown |

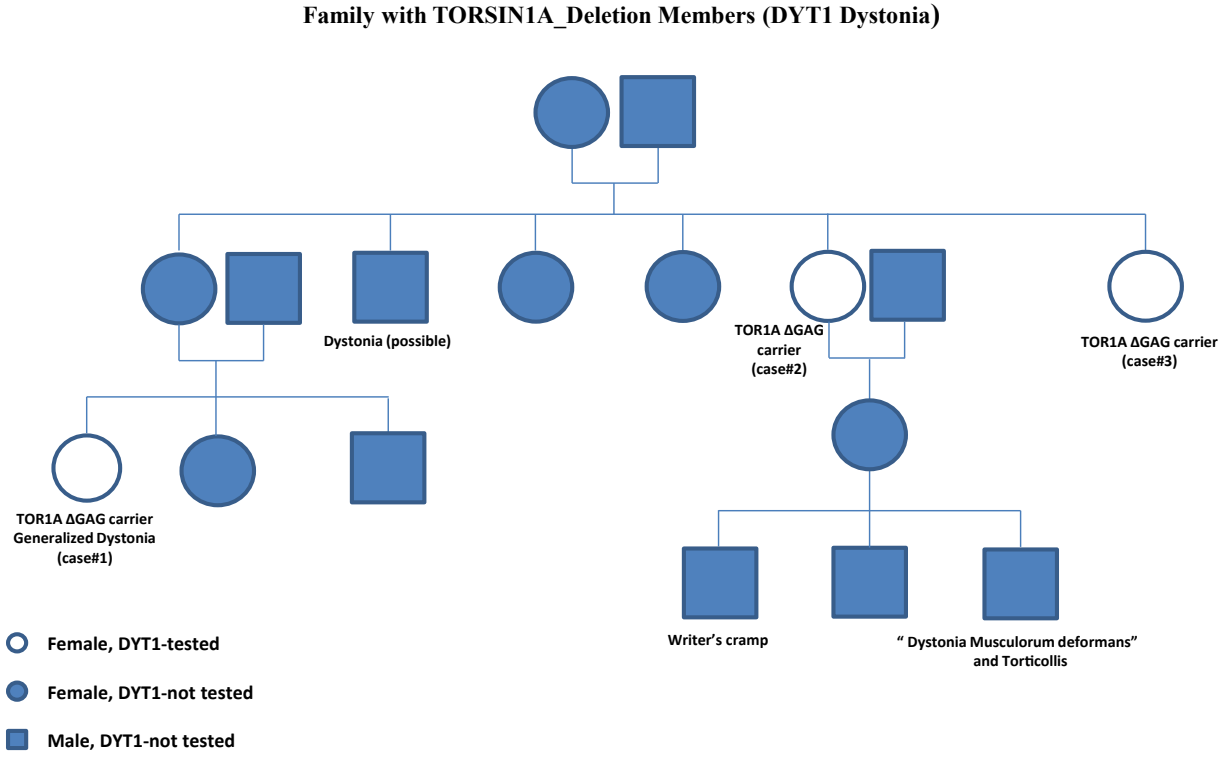


**Fig. 1.** The figure shows the pedigree of the family with three members ascertained for a three-base pair deletion in the TORSIN1A gene (TOR1A ΔGAG carriers). This mutation is associated with an early-onset generalized form of dystonia: DYT1 dystonia (DYT1). The three TOR1A ΔGAG carriers (case#1,#2,#3) received a complete postmortem brain evaluation, an extensive immunohistochemistry assessment for various co-occurring brain pathologies, and stereology-based morphometric measurement of their nigral neuromelanin-containing neurons (dopaminergic cells). Other members of the same family (three males), whom status of TOR1A ΔGAG carrier is unknown, were diagnosed with some type of dystonia -like movement disorders as well: (possible) dystonia, writer's cramp (WC), and "*dystonia musculorum deformans*" with torticollis. Two out of these three cases could potentially fit well the more updated definition of DYT1 dystonia.

**Table 2**
The table shows mean cell body, nuclear, and nucleolar volumes of pigmented neurons in the Substantia Nigra (SN) of manif-DYT1 (subjects manifesting generalized dystonia), non-manif-DYT1 (subjects non-manifesting dystonia or other movement disorders), manif-non-DYT1 (subjects manifesting generalized dystonia not associated with DYT1 mutation), and control subjects obtained by stereological measurements. Inclusions: intracytoplasmic or intranuclear inclusions. H&E = hematoxylin and eosin; Aβ = Aβ-Amyloid pathology (neuritic and diffuse plaques); Tau = PHF-positive tau pathology; LB = α-synuclein positive Lewy Body; Lam A-C = Laminin A-C intraneuronal inclusions; Ubiq = ubiquitin Inclusions; MB = Marinesco bodies; p62 = protein p62 inclusions. NFT = neurofibrillary tangles; tau-th = tau-threads. NSG = not significant changes; Neg = absence of lesions as detectable by immunohistochemistry. Numeric values express mean ± standard deviations.

| Subject# | Cell Body Volume ($\mu m^3$) | Nuclear Volume ($\mu m3$) | Nucleolar Volume ($\mu m3$) | H/E | Aβ | Tau | LB | TorsinA | Lam A + C | Ubiq | MB (H&E/Ubiq) | p62 | pTDP43 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| #1 (manif-DYT1) | 24,311 ± 16,017 | 2871 ± 2055 | 36 ± 25 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | 1 MB positive for Ubiq | Neg | Neg |
| #2 (non-manif-DYT1) | 12,092 ± 7327 | 1052 ± 909 | 17 ± 14 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #3 (non-manif-DYT1) | 7993 ± 2710 | 574 ± 318 | 5 ± 3 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #4 (manif-DYT1) | 21,460 ± 14,345 | 1573 ± 1099 | 28 ± 24 | NSG | Neg | Rare tau-NFT and tau-th | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #5 (manif-non-DYT1) | 12,589 ± 9903 | 1725 ± 1461 | 27 ± 26 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #6 (manif-non-DYT1) | 5273 ± 4650 | 1269 ± 797 | 8 ± 7 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #7 (manif-non-DYT1) | 17,771 ± 14,408 | 1533 ± 1254 | 30 ± 29 | Intimal thickening | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #8 (manif-non-DYT1) | 15,829 ± 14,-359 | 2100 ± 1805 | 32 ± 31 | Intimal thickening | Neg | Neg | Neg | Neg | Neg | Neg | Pos | Neg | Neg |
| #9 (control) | 16,123 ± 10,360 | 1344 ± 1010 | 21.26 ± 14 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #10 (control) | 13,763 ± 8982 | 1114 ± 776 | 20.70 ± 14 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #11 (control) | 20,532 ± 13,519 | 1594 ± 1171 | 24.08 ± 14 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #12 (control) | 16,851 ± 12,502 | 1392 ± 947 | 21.48 ± 14 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #13 (control) | 17,130 ± 13,337 | 1537 ± 1287 | 17.03 ± 12 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| #14 (control) | 16,323 ± 13,551 | 1332 ± 1123 | 20.96 ± 14 | NSG | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |

anatomical descriptions [15].

For neuronal volumetric measurements the following histologic criteria were applied:

1) 10 μm-thick sections;
2) a well-defined, not-neuromelanin-covered nucleolus for each randomly-sampled pigmented neuron;
3) nucleolus as only point of spatial reference for all volumetric measurements for the same randomly-sampled pigmented neuron.

Following identical protocols, series of 10 μm-thick sections adjacent and consecutive to each CV-stained section previously selected were collected to perform cell body volumetric measurements of all considered cases based on tyrosine hydroxylase (TH) immunohistochemistry. TH is the catalyzing enzyme for the formation of L-DOPA, the rate-limiting step in the biosynthesis of dopamine (DA) and it is normally expressed in nigral neurons [15]. For this investigation, it was decided to perform stereology-measurements for the cell body volume only since subcellular structures, such as nucleus and nucleolus, although sometimes recognizable, were often disturbed along their contour by partial covering of the TH positivity. The importance for cell body volumetric quantifications of TH positive nigral neurons (TH + nigral neurons) consisted in the opportunity to verify if possible volumetric differences found among manif-DYT1, non-manif-DYT1, manif-non-DYT1, and controls, could be also confirmed in terms of neurochemistry (most of the pigmented nigral neurons are indeed dopaminergic cells).

All stereological measurements were performed using Stereo-Investigator system, Version 10.0 (MBF Bioscience, Williston, VT), equipped with digital camera (AxioCam MRm, and MRc, Zeiss, Germany) and multiple objectives-head (2.5x-100× oil-immersion). Sampling grid area was 500 × 500 μm, counting frame of 40 × 40 μm, disector height of 25 μm with guard zones of ± 2 μm. Cell body, nuclear, and nucleolar volumes of each pigmented neuron were measured placing six rays automatically centered on the nucleolus, randomly intersecting the cell membrane. Pigmented neurons were measured if their nucleolus intersected or touched the inclusion (green) line and were excluded if their nucleolus intersected or touched the exclusion (red) line. All measurements were performed using a 100× oil-immersion, NA 1.30, neofluor ∞/0.17 objective [11].

All stereologically-measured volumes of the nigral pigmented neurons from manif-DYT1, non-manif-DYT1, manif-non-DYT1 and controls were compared as follows: manif-DYT1 vs. non-manif-DYT1 vs. manif-non-DYT1 vs. controls.

Section codes were broken and statistical analyses were performed only after all measurements and immunohistochemistry assessments were completed.

### 3.4. Intraneuronal lesions (H&E)

The search for possible generalized dystonia-associated intraneuronal (intracytoplasmic or intranuclear) lesions were mainly based on hematoxilin and eosin (H&E) stain (with possible immunohistochemistry co-assessment when available, i.e. ubiquitin), and focused on the following possible types of eosinophilic lesions: Negri bodies, Bunina bodies, Hirano bodies, and Marinesco bodies; or basophilic lesions: Nissl bodies and Buscaino bodies. In addition, possible elements of granulovacuolar degeneration were accounted for. Furthermore, possible abnormalities of the usual cellular distribution pattern (i.e. cellular spacing or disarrangement) of nigral pigmented neurons in manif-DYT1 vs. non-manif-DYT1, manif-non-DYT1, and controls were considered.

### 3.5. Immunohistochemistry assessment

In addition to standard gross and microscopic neuropathological

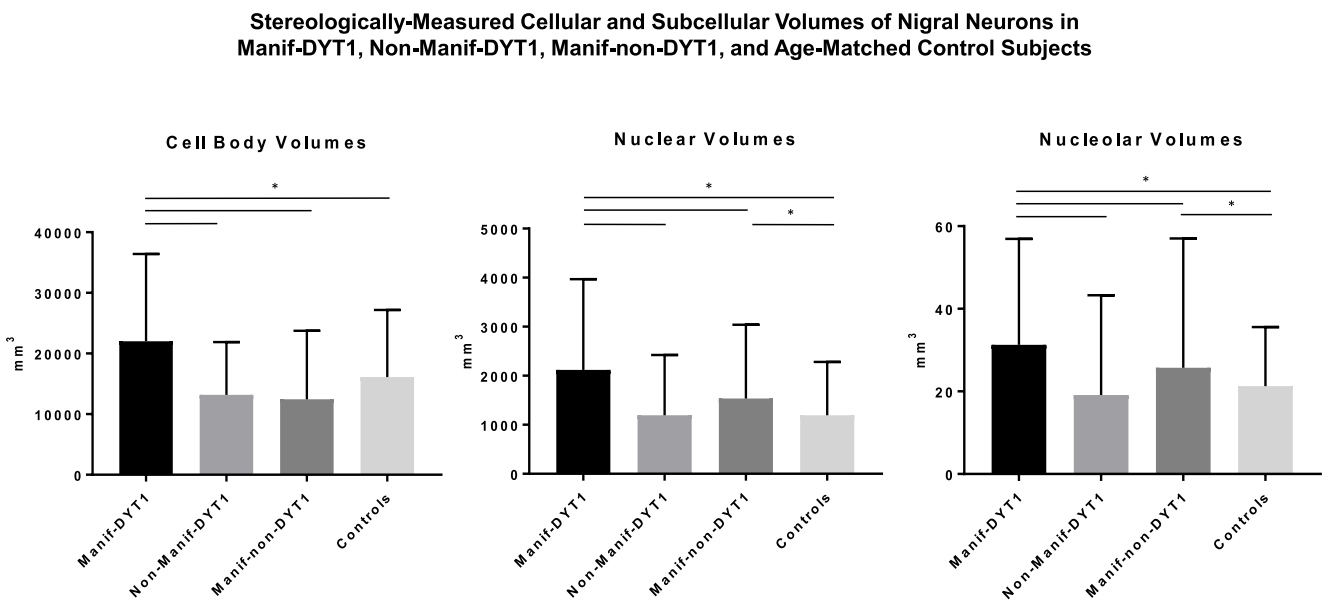


**Fig. 2.** The figure shows the histograms of the mean of cell body, nuclear, and nucleolar volume of nigral dopaminergic neurons of manif-DYT1, non-manif-DYT1, manif-non-DYT1, and controls subjects groups.
Manif-DYT1 = subjects ascertained for TOR1A-ΔGAG (deletion of the GAG inside the gene TORSIN1A) manifesting generalized dystonia (DYT1); Non-Manif-DYT1 = subjects ascertained for TOR1A-ΔGAG but not manifesting signs of dystonia; Manif-non-DYT1 = subjects without TOR1A-ΔGAG and manifesting generalized dystonia; Controls = age-matched subjects without any neurological or psychiatric disease. $\mu m^3$: cubic microns. * = $p < 0.01$.

assessments for all SNs (DYT1, non-DYT1, controls), each SN was assessed by an extensive series of specific immunohistochemistry protocols for the possible detection of the following: α-synuclein-positive Lewy bodies (LB) and Lewy neurites (LN), hyperphosphorylated-tau neurofibrillary tangles (tau-NFT) and tau-threads (tau-th), extracellular deposits of insoluble *β*-amyloid (A*β*-diffuse and A*β*-neuritic plaques), ubiquitin (Ubiq), phosphorylated-TAR-DNA binding protein-43 (pTDP-43), protein p62 (p62), TorsinA, and Laminin A + C intraneuronal cytoplasmic/intranuclear inclusions. For the evaluation of possible neuroinflammatory processes, immunohistochemistry was performed using an antibody against the antigen CD68 which is expressed in activated microglia. As per our standard procedures, each immunohistochemistry stain was performed always with a corresponding positive-control section.

Briefly, three sections (from the most rostral, medial, and caudal regions of each SN) were deparaffinized, hydrated, and treated with endogenous peroxidase blocking with 3% hydrogen peroxide in water, rinsed in buffer (TRIS with 0.05% Triton-X), microwaved with antigen retrieval (sodium citrate pH 6.0) for 10 min, and then cooled down. Protein block was applied for 30 min using 1.5% horse serum. The following specific antibodies were used: mouse anti-*β*-Amyloid, 17–24 (4G8) (Covance, SIG-39220; dilution 1:500); mouse anti-PHF-tau (Thermo Scientific, MN1020; dilution 1:500); mouse anti-α-synuclein (Abcam, Ab27766; dilution 1:500); rabbit anti-ubiquitin (Abcam, ab7780; dilution 1:250); rabbit anti phospho-TDP-43 (Cosmo Bio Co. LTD, TIP-PTD-P02, dilution 1:2000); mouse anti-p62 (Abcam, ab56416; dilution 1:1000), anti-Laminin A + C (Abcam, ab8984; dilution 1:100), anti-TorsinA (Santa Cruz, Sc-373915; dilution 1:10), and mouse anti-human TH (Millipore, MAB318; dilution 1:50). The sections were then incubated overnight at $4C^0$ (sections for 4G8 were pre-treated with 90% formic acid, 5 min), rinsed with buffer, and incubated with biotinylated horse anti-mouse, anti-rabbit secondary antibodies (30 min), Vector Kits (Vector Labs, Inc., CA), then with DAB substrate system (Sigma, D3939) for 5 min. Slides were counterstained with hematoxylin and dehydrated to xylene.

### 3.6. Statistics

Before conducting statistical the Kolmogorov–Smirnov tests confirmed that all volumetric data were distributed along a normal curve (Gaussian distribution). ANOVAs with both Bonferroni's and Tukey's test correction for multiple comparisons were performed to detect significant differences of the cell body, nuclear, and nucleolar mean volumes of nigral pigmented neurons across all groups. Adjustments for multiple comparisons (four groups) established a statistical significance if $p$ was $\leq 0.01$.

All single volumetric value for each measured cell body, nucleus, and nucleolus across each case was clustered as belonging to one of the four groups (manif-DYT1, non-manif-DYT1, manif-non-DYT1, controls group) to obtain one total-mean-volume value for each established group.

## 4. Results

All DYT1 carrier subjects were female with a mean age at death of 82.7 ± 10.5 year, manif-non-DYT1 subjects were three female and one male with a mean age at death of 61.0 ± 9.6 year, and controls were one female and five male with a mean age at death of 85.6 ± 4.3 year. While the mean age between DYT1 and manif-non-DYT1 subjects was different (with manif-non-DYT1 being younger), the mean age at death between all DYT1 carriers and controls was not statistically different. Demographic, clinical, genetic and family history data of manif-DYT1, non-manif-DYT1, manif-non-DYT1, and controls are summarized in Table 1. Fig. 1 shows the pedigree of the DYT1 family.

The gross neuropathologic examination did not reveal evidence of any major abnormality in case #1. In case #2, mild atrophic changes (age-related neuronal loss and gliosis in the CA1 region and subiculum bilaterally) were observed. In case #3 there was presence of an infarct in the territory of the right middle cerebral artery and Alzheimer's disease pathologic lesions (amyloid neuritic plaques in the entorhinal cortex and neocortical regions) not sufficient for a diagnosis of definite AD. In case #4, age-related generalized cortical atrophy with post-mortem petechial hemorrhages were found. Among the four manif-non-DYT1 cases examined, cerebrovascular and co-occurring brain

pathologies (such as tau or Lewy body pathology) were excluded; no other major neuropathologic features were observed in these brains.

Table 2 describes postmortem intervals (PMI), causes of death, relevant medical history, and general neuropathologic changes found at autopsy in DYT1 carriers, manif-non-DYT1 subjects, and controls.

Cellular (cell body) and subcellular (nucleus and nucleolus) volumes of nigral pigmented neurons and brain co-pathologies found in the SN of manif-DYT1 and non-manif-DYT1, manif-non-DYT1, and controls are illustrated in Table 2. Noticeably, nigral pathologies most frequently associated with age-related neurodegenerative diseases (i.e. Lewy bodies or tau-NFT) were absent, except for tau-NFTs in case #4, and isolated Marinesco bodies (eosinophilic and ubiq-positive intranuclear lesion) in case #1.

Statistical analyses (ANOVA corrected for multiple comparisons by Bonferroni's and Tukey's tests) showed an increased mean cell body nigral neuronal volume in manif-DYT1 carriers (case #1, #4) vs. non-manif-DYT1 carriers (case #2, #3) [$p < 0.0001$] vs. manif-non-DYT1 (case #5-#8) [$p < 0.0001$] subjects and vs. controls [$p < 0.0001$]. In addition, the mean nigral neuronal nuclear and nucleolar volumes of manif-DYT1 carriers were higher in comparison to non-manif-DYT1 ($p < 0.0001$) and controls ($p < 0.0001$). Moreover, nuclear and nucleolar volumes in nigral neurons of manif-non-DYT1 subjects were higher in comparison to controls. Histograms in Fig. 2 summarize all volumetric data and statistical significance.

Importantly, the stereology-based morphometric analyses performed on TH + nigral neurons across all groups showed comparable results to those obtained using CV-stained sections. TH + nigral neurons were significantly larger in manif-DYT1 vs. non-manif-DYT1 ($p < 0.0001$), manif-non-DYT1 ($p < 0.0001$), and controls ($p < 0.0001$). Th + nigral neurons in manif-non-DYT1 vs controls were also larger. These findings suggest that the observed volumetric changes in manif-DYT1 and manif-non-DYT1 are possibly associated to volumetric changes in dopaminergic nigral neurons.

## 5. Discussion

These new unbiased stereology-based quantitative-morphometric findings show that DYT1 carrier subjects who manifested generalized dystonia are associated with increased cell body, nuclear, and nucleolar volumes of nigral pigmented dopaminergic neurons in comparison to asymptomatic DYT1 carrier subjects, manif-non-DYT1 generalized dystonia patients and controls. Intriguingly, also subcellular (nuclear and nucleolar) nigral volumes in manif-non-DYT1 patients were increased in comparison to controls. These stereology data confirm and expand the previous findings described by Rostasy K. et al. [10]. However, while Rostasy K. et al. included subjects with different types of dystonia and with a variable age at death, we tried to minimize those possible confounding factors mainly focusing on one type of dystonia (DYT1) and having age-matched control subjects. The importance of considering the age for this type of morphometric analyses consists in the fact that normal aging, *per se,* can be associated with nigral neuronal volumetric changes [16].

These new morphometric findings seem to be coherent with previous stereology measurements of pigmented/tyrosine hydroxylase (TH)-stained neurons in SN of DYT1 knock-in vs. wild-type mice, which have shown a reduced number of nigral neurons in association with their enlargement [17]. Whereas in the current investigation, we could not estimate the total number of nigral pigmented neurons in DYT1 subjects, those previous animal findings [17] showing a reduced number of nigral neurons in DYT1 knock-in vs. wild-type mice in association with their enlargement seem to be consistent with other previously morphometric-quantitative findings obtained from human postmortem brains of subjects affected by different types of non-DYT1 dystonia, which showed indeed a significant reduction of nigral neurons in dystonia brains (non-DYT1) vs. age-matched control subjects [11]. If there is a reduction of the number of nigral neurons in human DYT1 carriers remain to be established.

In pathophysiologic terms, these morphometric findings could be explained by the fact that cellular alterations determined by protein folding, cell trafficking, disturbed cytoskeletal, and vesicle disorders in association to DYT1 mutations have been already observed. Moreover, these subcellular disorders were also found to interact with dopaminergic signaling, transcriptional factors and cellular stress activation responses [18]. In addition, patterns of possible adaptive microstructural cellular have been detected in both BG and cerebello-thalamo-cortical anatomical pathways of DYT1 mice [19]. Altogether, these animal and human morphometric findings seem to be coherent with the hypothesis that DYT1 mutation might be determine different types of neurodevelopmental abnormalities, which could include, among others, neuronal numerical, and volumetric changes of specific groups of neurons.

Importantly, we were unable to identify a higher frequency or a specifically-associated pattern for any of the intraneuronal inclusions considered in this study. These immunohistochemistry findings confirm the results obtained from other investigators [20]. Here though, as a novelty, we provided a quite extensive search for other possible intracytoplasmic and intranuclear lesions that were not previously considered (Table 2).

The main findings of our investigation are limited by the sample size of available SN from manif-DYT1, non-manif-DYT1, and manif-non-DYT1 subjects. Larger stereological studies including a higher number of DYT1 subjects, both manifesting and non-manifesting dystonia, and other types of non-DYT1 manifesting generalized dystonia should be performed to confirm these initial findings. However, a series of studies from humans and animals, have been already associated with enlargements of neuronal soma in the context of other neurodevelopmental diseases, such as autism [21], genetic forms of epilepsy [22], and prenatal neocortical lesions [23]. Altogether, these series of data seem to match with the hypothesis that DYT1 is indeed a neurodevelopmental disorder.

As we currently know, DYT1 carriers do not always manifest dystonia, and some subjects can remain completely asymptomatic until late in life or never show any dystonia symptomatology [24]. This possibility occurred indeed in the two oldest DYT1 positive family members examined in this study. The molecular mechanisms able to explain the incomplete penetrance of DYT1 mutation and unpredictability of the dystonia manifestations across different periods of life remain to be clarified [25]. Importantly, our new neuropathological-morphometric findings do not exclude the possibility that dopaminergic abnormalities could be variably combined with other neurochemical anomalies (i.e., cholinergic cells abnormalities), inside (i.e. putamen) or outside the BG (i.e. supplementary motor cortex), and consequently generate variable endo- (e.g. cognitive) and exo-phenotypic (dystonic) manifestations caused by DYT1 mutation [26]. In theory, multiple specific cellular-type abnormalities could be alternatively and variably present across different types or subgroups of dystonia, and neuroanatomical regions, as related to each specific gene mutation linked to dystonia. While a possible unifying “cholinergic dysfunction” hypothesis for DYT1 dystonia has been proposed [27], striatal dopaminergic abnormalities for some specific type of dystonia (i.e., spasmodic dystonia) have been implicated as well [28]. We retain that possible cholinergic abnormalities do not necessarily exclude dopaminergic or other neurotrasmettitorial abnormalities, especially in the context of a possible neurodevelopmental disorder, which can be potentially associated with various neuroplasticity/adaptive phenomena [29].

## Author contributions

Diego Iacono, study concept and design, acquisition of data.
Maria Geraci-Erck, acquisition of data.
Hui Peng, acquisition of data.
Marcie L. Rabin, critical revision of manuscript for intellectual

content.

Roger Kurlan, critical revision of manuscript for intellectual content.

## Authors disclosure

Diego Iacono – Report no disclosures.

Maria Geraci-Erck – Report no disclosures.

Hui Peng – Report no disclosures.

Marcie L. Rabin – Personal fees from Teva, Allergan, and Ipsen.

Roger Kurlan – Grants from NIH, Astra-Zeneca, Psyadon, Rhythm, Kyowa, and Teva.

## Funding

Study Funded by The Parkinson's & Movement Disorder Foundation (PDMD), Fountain Valley, CA, Grant 2012; Biomedical Research Institute of New Jersey, BRInj, NJ, “Dystonia Grant” 2013.

## Acknowledgements

We are deeply grateful to all dystonia patients, carrier persons, control subjects, caregivers and their families for their precious gifts and generosity. The human tissues were obtained from the NIH NeuroBioBank Brain at University of Maryland Baltimore, MD, and from the Brain and Body Donation Program, Banner Sun Health Research Institute (BBPD), AZ.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.parkreldis.2018.08.020.

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