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Neuropathology of Cervical Dystonia

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Abstract

The aim of this study was to search for neuropathological changes in postmortem brain tissue of individuals with cervical dystonia (CD). Multiple regions of formalin-preserved brains were collected from patients with CD and controls and examined with an extensive battery of histopathological stains in a two-stage study design. In stage one, 4 CD brains underwent a broad screening neuropathological examination. In stage two, these 4 CD brains were combined with 2 additional CD brains, and the subjective findings were quantified and compared to 16 age-matched controls. The initial subjective neuropathological assessment revealed only two regions with relatively consistent changes. The substantia nigra had frequent ubiquitin-positive intranuclear inclusions known as Marinesco bodies. Additionally, the cerebellum showed patchy loss of Purkinje cells, areas of focal gliosis and torpedo bodies. Other brain regions showed minor or inconsistent changes. In the second stage of the analysis, quantitative studies failed to reveal significant differences in the numbers of Marinesco bodies in CD versus controls, but confirmed a significantly lower Purkinje cell density in CD. Molecular investigations revealed 4 of the CD cases and 2 controls to harbor sequence variants in non-coding regions of *THAPI*, and these cases had lower Purkinje cell densities regardless of whether they had CD. The findings suggest that subtle neuropathological changes such as lower Purkinje cell density may be found in primary CD when relevant brain regions are investigated with appropriate methods.

Keywords

spasmodic torticollis; autopsy study; Marinesco bodies; *THAPI*; DYT6

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Introduction

Dystonia is a disorder characterized by sustained or intermittent involuntary muscle contractions leading to twisting movements and abnormal postures (Tarsy and Simon, 2006). The most frequent manifestation is regional involvement of isolated muscle groups, with the neck as the most commonly affected area (Defazio, 2010). People with cervical dystonia (CD, or torticollis) suffer from over-activity of neck muscles with involuntary turning, tilting, or twisting of the head. A cause is not identifiable in most cases, where the disorder is known as primary or isolated CD. In contrast, a cause is identifiable in secondary or acquired CD and may include medications, trauma, space-occupying lesions, and developmental or degenerative conditions. Although CD is the most common primary dystonia, conclusive evidence implicating specific regions of the nervous system is lacking (Hedreen *et al.*, 1988; McGeer and McGeer, 1988; Standaert, 2011). A review of the literature disclosed only 15 reports, and no consistent neuropathological changes were noted (Table 1). The failure to detect overt changes has contributed to belief that there are no neuropathological defects in primary CD.

On the other hand, imaging techniques consistently reveal abnormalities in primary CD (Neychev *et al.*, 2011; Zoons *et al.*, 2011). Positron emission tomography has revealed abnormal activity of the cerebral cortex, basal ganglia, cerebellum, and thalamus. Voxel-based morphometry has shown abnormal white and gray matter volumes in the cerebral cortex, basal ganglia, cerebellum and thalamus. Diffusion tensor magnetic resonance imaging (MRI) also has suggested the existence of microstructural anomalies in white matter tracts within the prefrontal cortex, basal ganglia, thalamus and corpus callosum. Studies of secondary CD also have pointed to the cerebellum, brainstem and basal ganglia (LeDoux and Brady, 2003). These imaging studies raise the possibility that subtle anatomical defects may occur within specific brain regions.

Results from animal studies have further pointed to specific regions where anatomical abnormalities might be expected. Multiple studies in different species have implicated both the basal ganglia and cerebellum in different types of dystonia (Breakefield *et al.*, 2008; Neychev *et al.*, 2011). Animal models of potential relevance to cervical dystonia have more specifically implicated the interstitial nucleus of Cajal (INC), red nucleus, and nearby regions (Carpenter *et al.*, 1958; Malouin and Bedard, 1982; Fukushima, 1987; Walker *et al.*, 1990; Klier *et al.*, 2002; Neychev *et al.*, 2011). The human imaging studies, combined with results from animal studies, provide clues for locations where neuropathological changes might be expected in patients with primary CD. The purpose of the present study was to use recent imaging and animal studies as a guide to delineate potential neuropathological changes in primary CD.

Materials and Methods

Autopsy material

The National Institute of Child Health and Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland was searched for autopsy specimens of subjects with CD. Twenty cases of possible primary CD were reviewed with approval by the Institutional Review Board of the Johns Hopkins University. Among these, 14 had histories compatible with primary CD according to current criteria, but only 4 had sufficient tissue of good quality for study (CD1-4). Tissue from 2 additional cases (CD5 and CD6) became available after the initial screening study and was included in the second part of the study. Because these samples came from a public brain bank, systematic sampling of multiple regions for stereological studies was not feasible, and clinical information was

sometimes limited. Tissue from normal controls was obtained from the Johns Hopkins Brain Resource Center and the Emory Alzheimer's Disease Research Center.

Formalin-preserved tissue was methodically sampled with regions of interest based on recent human imaging investigations, animal studies, and findings from secondary CD. The regions sampled included the somatosensory cortex, caudate nucleus, putamen, globus pallidus, midbrain (including substantia nigra, red nucleus, INC), brainstem, cerebellar hemispheres and vermis, and deep cerebellar nuclei. Tissue was embedded in paraffin and sectioned at 10 μ m.

Genetic testing

Genotyping was performed on unstained sections from all CD cases and controls. Brain tissue was scraped from slides and transferred to microcentrifuge tubes. DNA was extracted with the Classic™ Genomic DNA Isolation Kit (Lamda Biotech, St. Louis, MO) and examined for sequence variants in exon 5 of *TOR1A* and all exons and introns of *THAP1* in 2 independent runs involving sections from different brain regions, with Sanger sequencing as described previously (Xiao *et al.*, 2009; Xiao *et al.*, 2010).

Histological procedures

The staining battery was designed to detect inclusion bodies, in view of studies of other forms of dystonia (McNaught *et al.*, 2004). Stains for degenerative processes were included in view of the association between CD and degenerative Parkinsonian disorders (Boesch *et al.*, 2002; Papapetropoulos and Singer, 2006). Stains for inflammatory processes were included because of prior associations of focal dystonia with autoimmune diseases (Moore *et al.*, 1986; Rajagopalan *et al.*, 1989; Deitiker *et al.*, 2011). Staining methods included hematoxylin/eosin (H&E), cresyl violet, Hirano silver, and immunohistochemistry. For immunohistochemistry, sections were stained for neuronal markers (parvalbumin, calbindin, calretinin), markers for intracellular inclusions (IC2 for polyglutamine sequences, TAR DNA binding protein 43, ubiquitin), neuroglial markers (glial fibrillary acidic protein, human leukocyte antigen-DR and ionized calcium binding adapter molecule 1), and cluster of differentiation 3, a T-cell marker. Sections first were heated at 37°C overnight, then heated to 60°C for 1 hr and deparaffinized. For antigen retrieval, sections were boiled in distilled water for 7 min. Nonspecific binding was blocked with 5% goat serum in phosphate-buffered saline, containing 0.4% Triton X-100 for 1 hr at room temperature. Slides were incubated with primary antibody overnight at room temperature and then with secondary antibody for 1 hr. Sections then were incubated with streptavidin-peroxidase complex and developed for 5 min with diaminobenzidine, and counterstained with cresyl violet.

Two-stage analysis

In the first stage of the analysis, an experienced neuropathologist (C.A. Pardo) examined the first 4 brains for overt changes. This subjective assessment revealed frequent ubiquitin-positive inclusions in melanized nigral neurons and a patchy but prominent loss of cerebellar Purkinje cells. In the second stage of the study, material from the original 4 brains plus 2 additional brains that became available after the initial study were analyzed. The two main subjective findings identified in stage one were quantified and compared to age-matched controls by a microscopist blinded to phenotype. Tissue from 12 controls was investigated for the analysis of nigral inclusions, and 13 controls were studied for the Purkinje cells analysis. Quantification of the number of nigral inclusions and Purkinje cells was performed using Stereo Investigator (MicroBrightField, Inc., Williston, VT, USA). Two ubiquitin-stained sections from different regions of the substantia nigra were randomly selected for

counting intranuclear inclusions for each case. Two H&E-stained sections from different regions of the cerebellum were randomly selected for Purkinje cell counts for each case.

Statistical analyses

To examine the association between CD and the number of nigral intranuclear inclusions, two statistical approaches were used. In one approach, the analysis was based on the proportion of cells with at least one inclusion. A logistic regression model was fitted to the proportion of cells with inclusions, and both age and group (CD or control) were analyzed as explanatory variables. To adjust for the correlation among neuronal cells within a given case, the Williams weighting option was used as implemented in SAS PROC LOGISTIC (SAS Institute Inc., Cary, NC, USA), which assumed an exchangeable correlation structure. The above approach had the limitation that it ignored information about the number of inclusions per cell. To use this cell-specific information, we considered an alternative approach based on the number of inclusions (ranging from 0 to 6) for each neuron. A log-linear regression model was fitted for the number of inclusions, and age and group were analyzed as explanatory variables. The generalized estimating equation method (Liang and Zeger, 1986) was used to adjust for intra-individual correlation among the cells.

Purkinje cell loss was investigated by examining linear Purkinje cell density measured as the number of cells/mm of Purkinje cell layer. Fitted linear regression models employing the generalized estimating equation approach were used to examine the association between CD and Purkinje cell density, with adjustments for age and the intra-individual correlation of Purkinje cell density measurements (Liang and Zeger, 1986). In this model, Purkinje cell density was the response variable and both age and group (CD or control) were explanatory variables. Residual plots were used to determine if there were departures from model assumptions, such as evidence of nonlinearity or non-constant variance.

A linear regression analysis using the generalized estimating equation approach also was conducted to investigate the association between the occurrence of *THAP1* sequence variants and Purkinje cell density, adjusted for age and group. In all regression analyses described, two persons with ages reported as “over 80” were assigned arbitrarily the age of 85.

Results

Samples

The CD cases consisted of 5 women and 1 man (Table 2). Mean age at onset was 45 ± 10.7 years, and mean age at death was 70.0 ± 10.4 years. Mean duration of CD was 25 ± 15.3 years. Post-mortem intervals ranged from 5 to 25 hours. None of the 6 CD cases examined had clinical histories of prolonged agonal states with hypoxia, nor histories of taking drugs known to cause neuropathological changes. In stage 2 of the study, findings from CD individuals were compared to 16 age-matched controls (average age at death of 69.5 ± 9.8 years, Table 2).

Although none of the CD cases had a family history of dystonia, all were examined for mutations associated with DYT1 and DYT6 dystonia. There were no c.904_906delGAG mutations or other variants in exon 5 of *TOR1A*. In contrast, 2 single nucleotide sequence variants in the non-coding region of *THAP1* (c.71+9C>A and c.268-31A>G) were identified in both cases and controls (Table 2). The intron 1 variant (c.71+9C>A) was found in 3 CD cases. The intron 2 variant (c.268-31A>G) was found in 2 CD cases and 1 control. The c.268-31A>G variant is novel, but the c.71+9C>A variant has been reported (Xiao *et al.*, 2010; LeDoux *et al.*, 2012).

Screening phase

Tissue quality for all CD cases chosen for the final examinations showed good preservation and no signs of prolonged hypoxia before death. The most salient findings in the midbrain included focal areas of gliosis, satellitosis (accumulation of glial cells around neurons), and frequent ubiquitin-positive intranuclear inclusions in the substantia nigra (Figure 1). These small and homogeneous round inclusions resembled Marinesco bodies, a common finding in melanin-pigmented neurons in the substantia nigra pars compacta and locus ceruleus of normal aged humans (Yuen and Baxter, 1963). Findings in the cerebellum included focal areas of Bergmann gliosis, patchy loss of Purkinje cells, and torpedo bodies in the granule cell layer (Figure 2). Torpedo bodies are axonal swellings of Purkinje cells that are thought to be a non-specific result of abnormal accumulation of cellular constituents (Mann *et al.*, 1980; Louis *et al.*, 2009). No consistent abnormalities were seen in regions implicated in recent imaging and animal studies including the cerebral cortex, basal ganglia, INC, red nucleus or pons. Findings for each case are summarized in Table 3.

Quantification phase

Ubiquitin-positive intranuclear inclusions in the substantia nigra were quantified in 6 CD individuals and 12 controls (Table 4). The number of melanized neurons available for counting ranged from 139 to 1393. The number of inclusions in individual melanized neurons ranged from 0 to 6. Among CD individuals, 8% of the cells counted had at least one inclusion, as compared to 11% of cells counted in the control group. The mean $\pm$ standard deviation for the total number of inclusions identified was 35.8 ± 25.2 for the CD group and 63.0 ± 81.3 for the controls. The logistic regression analysis results indicated that neither group (Wald test: $W=0.44$; 1 df; $p=0.51$) nor age (Wald test: $W=1.41$; 1 df; $p=0.24$) was a significant predictor of the proportion of cells with inclusions. The estimated intra-individual correlation was $r=0.06$ among the cells counted. Further analysis included an investigation of the association between number of inclusions per cell, group and age. Log-linear regression analysis based on the number of inclusions (ranging from 0 to 6) per cell failed to converge to a solution, owing to numerical difficulties. As an alternative, computationally simpler approach, a square root transformation was applied to the inclusion counts in order to stabilize their variances, and a linear regression model was fitted using the generalized estimating equation method. The results of this cell-specific regression analysis indicated that neither group (Wald test: $W=0.89$; 1 df; $p=0.37$) nor age (Wald test: $W=-0.74$; 1 df; $p=0.46$) was a significant predictor of the number of inclusions. The quantitative study therefore failed to confirm any abnormality of nigral inclusions in the CD brains.

Main subjective findings in the cerebellum included loss of Purkinje cells, areas of focal gliosis and torpedo bodies. Due to difficulties in quantifying gliosis and the low frequency of torpedo bodies, the only variable analyzed was Purkinje cells. The linear density of Purkinje cells was measured in two separate cerebellar sections from each of 6 CD individuals and 13 controls (Figure 3A). The CD group had an average linear density of 2.26 ± 0.48 cells/mm, whereas controls had an average linear density of 2.70 ± 0.58 cells/mm. Regression analysis indicated that CD was significantly associated with lower linear density of Purkinje cells (Wald test: $W=-2.06$; 1 df; $p<0.05$). The estimated intra-individual correlation was $r=0.73$ for the linear density of Purkinje cell measurements. Age was not a significant predictor of Purkinje cell linear density ($p=0.72$). Moreover, dropping age from the model did not affect the fitted regression coefficient for group, indicating that age was not a confounding variable. Residual plots did not reveal any apparent departures from model assumptions. These results confirm a lower linear density of Purkinje cells in CD brains in comparison with controls.

Independent from patient group (CD vs controls), samples with a *THAP1* sequence variant had a lower linear density of Purkinje cells (2.14 ± 0.54 cells/mm) compared to *THAP1* negative cases (2.75 ± 0.48 cells/mm) as revealed by linear regression analysis (Wald test: $W = -2.00$; 1 df; $p < 0.05$; Figure 3B). The controls with *THAP1* sequence variants (N7 and N12) fell at the lower limits of normal for linear density. The unexpected finding of *THAP1* sequence variants in the control group led to a more detailed investigation of the available medical records. N7 had no neurological problems, but N12 had a history of hand tremors. The neuropathological evaluation of N12 was not consistent with Parkinson's disease, but available records were insufficient to determine if the tremors were consistent with dystonic or essential tremor.

Discussion

This study provides neuropathological findings from the largest cohort of CD patients ever evaluated. Guided by recent findings from human neuroimaging studies and animal models, it provides a more thorough and quantitatively rigorous evaluation than previously available. In the more traditional subjective phase of these studies when 4 CD brains were available, two changes appeared salient. The CD brains appeared to have a relatively large number of Marinesco bodies in the substantia nigra. The CD brains also appeared to show reduced cerebellar Purkinje neurons, along with related findings of increased torpedo bodies and focal regions of gliosis. Extensive stains failed to reveal any consistent defects in the basal ganglia, cerebral cortex, red nucleus, or INC. The absence of consistent structural defects in these regions was disappointing, considering that these areas have been implicated in multiple prior studies relevant to CD (Carpenter *et al.*, 1958; Malouin and Bedard, 1982; Fukushima, 1987; Walker *et al.*, 1990; Neychev *et al.*, 2011). In the second quantitative phase of these studies, the reduced number of Purkinje neurons was confirmed, but the increase in nigral inclusions was not. Although the study did not intend to address neuropathology associated with *THAP1* sequence variants, an incidental finding was a significantly lower linear density of Purkinje cells among cases harboring such variants, regardless of whether they had CD or not. The results are valuable for three main reasons. First, they highlight some general weaknesses in the way that human neuropathological studies for dystonia have been conducted, and they lead to suggestions for potential solutions. Second, they question recent classifications of dystonia that propose to discriminate secondary from primary forms by the presence or absence of neuropathological correlates. Third, they highlight limitations inherent to all studies that involve attempts at clinico-pathological correlations, including dystonia.

Limitations of human neuropathology

While it may initially seem surprising that no prior study has identified the changes described in the current studies, an analysis of the prior reports reveals several explanations. One reason is that there are only 15 reported cases, with most studies including only 1-2 cases, making it difficult to appreciate subtle defects (Foerster, 1933; Grinker and Walker, 1933; Alpers and Drayer, 1937; Garcia-Albea *et al.*, 1981; Jankovic *et al.*, 1987). Moreover, all prior studies relied exclusively on subjective impressions of a neuropathologist, with no quantification of suspected abnormalities, eliminating the possibility of identifying subtle quantitative differences. Additionally, several of these reports included cases with clinical or neuropathological findings suggesting secondary CD (Table 1), and others combined different primary dystonias, presuming a common neuropathological substrate (Gibb *et al.*, 1988; Zweig *et al.*, 1988; Holton *et al.*, 2008). By mixing different disorders in the same study, any potentially consistent defect limited to primary CD becomes difficult to discern. With a single exception (Holton *et al.*, 2008), all prior reports were published more than two

decades ago; and they did not include special methods such as immunohistochemistry with the power to detect specific neuropathological processes.

Perhaps the most serious shortcoming of prior studies is that many focused only on specific regions of interest. As summarized in a recent review (Neychev *et al.*, 2011), the belief that all forms of dystonia arise from dysfunction of the basal ganglia has dominated scientific thought for decades, despite accumulating evidence that other brain regions are involved. Some of the prior autopsy studies highlighted abnormalities in the striatum and/or globus pallidus in tissue of CD individuals (Foerster, 1933; Alpers and Drayer, 1937; Holton *et al.*, 2008). However, brain regions other than the basal ganglia were often not analyzed in detail, so it is possible that additional defects in other areas were overlooked. Furthermore, several prior studies of CD did not address the cerebellum (Alpers and Drayer, 1937; Tarlov, 1970; Zweig *et al.*, 1986; Gibb *et al.*, 1988; Holton *et al.*, 2008). Among the few studies that reported a broader analysis in cases of probable primary CD, one described patchy loss of Purkinje cells, but quantitative comparisons to controls were lacking (Garcia-Albea *et al.*, 1981). As a result, the authors interpreted these findings as normal for age.

Wherever possible, the current study aimed to avoid the limitations of prior studies. To avoid problems associated with mixing different forms of dystonia together, we focused on cases with primary CD. Cases of secondary CD were excluded. Although dystonia outside of the neck region was allowed, CD was the main clinical problem. We also employed an extensive battery of histological stains sensitive for detecting inclusion bodies, degeneration, or inflammatory processes. We also took advantage of evidence from modern human neuroimaging studies and animal studies to guide selection of brain regions. The Marinesco bodies in the substantia nigra initially were intriguing, in view of evidence that they may be more frequent in dopamine-related disorders (Beach *et al.*, 2004), combined with evidence of dysfunction of dopamine pathways in dystonia (Perlmutter and Mink, 2004; Wichmann, 2008). The cerebellar defects also were intriguing, in view of recent studies suggesting abnormal cerebellar Purkinje neuron activity in dystonia (Pizoli *et al.*, 2002; Neychev *et al.*, 2008; Chen *et al.*, 2009). As a final step, we sought to confirm subjective findings with rigorous quantitative methods. We were unable to confirm an increase in Marinesco bodies, highlighting the weakness in relying on subjective impressions. However, we were able to confirm a reduction in the linear density of cerebellar Purkinje neurons. Purkinje neuron densities in CD overlapped with those of controls (Figure 2), explaining how such a defect might be overlooked in prior studies.

After the histopathology was completed, molecular studies were conducted to identify sequence variants associated with DYT1 and DYT6 dystonia. Although none had the GAG deletion of DYT1 dystonia, 4 cases and 2 controls unexpectedly were found to harbor sequence variants in *THAP1*. Because the spectrum of *THAP1* mutations responsible for causing DYT6 dystonia is heterogeneous and not yet fully defined, it is not clear if these variants are pathogenic (Xiao *et al.*, 2010; LeDoux *et al.*, 2012; Paudel *et al.*, 2012). The c. 71+9C>A variant was first reported by Xiao and colleagues (Xiao *et al.*, 2010) with an allele frequency of 7/2420 in mainly adult-onset primary dystonia and 1/1200 in matched controls. Although this variant could alter splicing efficiency and the ratio of the two known *THAP1* isoforms, follow-up studies (Groen *et al.* 2010, Lohmann *et al.* 2011, Kamm *et al.* 2011, Groen *et al.* 2011) have been underpowered to determine if this variant is statistically associated with risk for dystonia (LeDoux *et al.*, 2012). The c.268-31A>G variant is novel. In comparison to previous work using DNA derived from whole blood (Xiao *et al.*, 2010), the relatively high frequency of *THAP1* variants in the post-mortem samples examined in this study suggests the possibility that *THAP1* is prone to somatic mutations (Kennedy *et al.*, 2012). Nonetheless, *THAP1* sequence variants appear to be associated with significantly lower linear density of Purkinje cells regardless of the existence of CD, a finding consistent

with observations that Purkinje cells express the highest levels of this gene in the brain (LeDoux *et al.*, 2012). The existence of *THAPI* sequence variants in 2 controls led to questions regarding whether they should be included in the final analysis, especially since one clinically had overt tremor. However, eliminating these controls *improved* the strength of the association between lower Purkinje cell density and CD, because both of these controls fell at the lowest limits of the control group (Figure 3). These findings emphasize the importance of molecular diagnosis in both cases and controls.

Despite an improved study design, our study still had several shortcomings. We examined only 6 CD brains. Based on the results we obtained for Purkinje cell linear density, a power analysis indicates the need for 15 CD cases for a confirmatory follow-up study. Because CD is so rare, obtaining this many cases could take many years without a large-scale cooperative effort. The rarity of CD and the small number of brains evaluated also creates a risk of missing subtle changes elsewhere in the brain, but were not sufficiently obvious to be detected in the screening neuropathological portion of our study. Many histological findings were not quantified, so it remains possible that significant changes exist in brain regions other than the cerebellum.

Another limitation of our study was that it involved tissue available through a public brain bank, where precise selection of matched brain regions was not feasible. Tissue selection is important, because different body regions are represented somatotopically in many brain regions, including the cerebellum. Tissue from the medial cerebellum where proximal body regions such as the neck are represented could not be compared to tissue from the hemispheres, which are more relevant for the distal limbs. It also was not possible to compare the corresponding right and left sides of the brain, which may be important for a disorder such as CD, where the head and neck are consistently turned or tilted in one direction. A related limitation was the paucity of detailed clinical information, such as the direction of head movements, which could be important for interpreting asymmetrical findings in the brain, or presence of tremor, which is associated with neuropathological findings similar to those reported here (Louis *et al.*, 2006; Louis and Vonsattel, 2008; Shill *et al.*, 2008; Louis *et al.*, 2009; Louis *et al.*, 2011).

A final limitation was the inability to include truly stereological methods for counting inclusions and cells. Truly unbiased stereological studies of human autopsy material are rarely feasible because of specific requirements including sectioning of the entire region of interest, systematic random sampling through the tissue, and particularly the requirement for random planes of section (Kristiansen and Nyengaard, 2012). Finally, despite a comprehensive battery of stains, none was sensitive for the detection of changes in the fine structure of neurons, such as dendrites. Most of these limitations are a common problem in autopsy studies and they might be solved by the establishment of a centralized brain bank, where the collection and processing of clinical information together with post-mortem brains could be standardized. Any follow-up study also would have to account for *THAPI* sequence variants, and ideally incorporate new evidence regarding which of these variants may be pathogenic.

Relevance for classifying the dystonias

The results of these studies are directly relevant to efforts that classify the many different dystonias by etiology. Traditionally, primary and secondary dystonias are distinguished largely based on whether the clinical phenotype is pure or mixed with non-dystonic features. However, some investigators have suggested that the distinction also includes the occurrence of histopathological defects (Neychev *et al.*, 2011). For example, some authors have described primary dystonia as a “neurofunctional disorder” because of the absence of apparent neurodegeneration (Breakefield *et al.*, 2008). Others have similarly stated that

primary dystonia is characterized by “abnormal functioning of a structurally-normal appearing brain” (Tanabe *et al.*, 2009). Several additional authors have implied that primary dystonias are defined in part by the absence of neuropathological abnormalities (De Carvalho Aguiar and Ozelius, 2002; Nemeth, 2002; Schwarz and Bressman, 2009).

The current studies showing a lower linear density of Purkinje neurons in the cerebellum in CD demonstrate that neuropathological changes may be revealed in primary dystonia when appropriate methods are applied. In fact, prior studies of human DYT1 primary generalized dystonia have shown peri-nuclear inclusions in the brainstem, although frank cell loss was not apparent (McNaught *et al.*, 2004). Histological studies of animal models of DYT1 dystonia also have revealed subtle changes of the structure of the dendrites of cerebellar Purkinje neurons (Zhang L *et al.*, 2011) or size of midbrain dopamine neurons (Song *et al.*, 2012). Finally, modern human neuroimaging studies of CD have repeatedly provided evidence for microstructural defects (Neychev *et al.*, 2011; Zoons *et al.*, 2011). In view of these findings, it seems likely that additional neuropathological changes will be uncovered. If this is the case, then reliance on the presence or absence of neuropathological abnormalities to discriminate primary from secondary dystonia becomes increasingly problematic.

Inferring causation from clinico-pathological studies

Results from clinico-pathological studies inevitably lead to questions regarding a causal link between the clinical features and the histopathological defects. The results presented here are correlative and cannot establish a causal link between CD and a lower linear density of Purkinje neurons. In fact, Purkinje cell loss is a non-specific finding that is associated with a variety of conditions. Purkinje cell loss is typical of a large group of disorders known as the spinocerebellar ataxias (Yang *et al.*, 2000; Sarna and Hawkes, 2003). However, Purkinje cell loss sometimes is associated with other degenerative disorders such as Parkinson's disease (Takada *et al.*, 1993; Wenning *et al.*, 1996) or Alzheimer's disease (Sarna and Hawkes, 2003; Mavroudis *et al.*, 2010). Purkinje cell loss also occurs in non-degenerative disorders including essential tremor (Louis *et al.*, 2006; Louis and Vonsattel, 2008; Louis *et al.*, 2011), hypoxia/ischemia (Sarna and Hawkes, 2003; Kern and Jones, 2006), traumatic brain injury (Sarna and Hawkes, 2003; Park *et al.*, 2007), autism (Sarna and Hawkes, 2003; Kern and Jones, 2006), a variety of drugs and toxins (Sarna and Hawkes, 2003), and even normal aging (Sarna and Hawkes, 2003; Zhang C *et al.*, 2010). Cerebellar Purkinje neurons therefore seem unusually vulnerable to many biological processes.

It is important to note that our cases had a relatively narrow age range that statistically did not contribute to Purkinje cell density, none had clinical histories of using drugs known to affect Purkinje neurons, none had evidence of another neurodegenerative disease or trauma, and tissue quality was good with no evidence of prolonged hypoxia before death. Thus, it is unlikely that Purkinje cell loss resulted from these other conditions. However, the association of Purkinje neuron loss with other disorders emphasizes that the lower linear density of Purkinje neurons found in CD is not specific to this disorder, and probably not responsible for causing dystonic symptoms.

The relationships between CD and essential tremor are important to consider. Clinically, up to two thirds of patients with CD have a tremor that resembles essential tremor (Jankovic *et al.*, 1991; Pal *et al.*, 2000), and recent studies have suggested that CD and essential tremor may share similar genetic substrates (Hedera *et al.*, 2010). The neuropathology of essential tremor is similar to our findings, with subtle reductions in the linear density of Purkinje neurons, prominent torpedo bodies, and focal areas of Bergmann gliosis (Louis *et al.*, 2006; Louis and Vonsattel, 2008; Shill *et al.*, 2008; Louis *et al.*, 2009; Louis *et al.*, 2011). In view of the many relationships between CD and essential tremor, it is perhaps not surprising that

they may share some similar pathological features. In essential tremor, the loss of Purkinje neurons may not cause tremor, but instead may reflect a pathological neurophysiological process involving aberrant oscillatory activity in circuits that involve the cerebellum, thalamus, and motor cortex (Raethjen and Deuschl, 2012). The reduction in the linear density of Purkinje neurons in CD may similarly reflect abnormal cerebellar physiology, although the nature of the physiological changes probably differs from essential tremor. Animal studies have suggested that abnormal bursting patterns of cerebellar Purkinje neurons may underlie dystonia, and this abnormal bursting may predispose to torpedo body formation, gliosis, and loss of these neurons (LeDoux and Lorden, 2002; Chen *et al.*, 2009). Thus a reduction in Purkinje cell density is neither specific nor causal in CD, but may instead provide a clue to an abnormal physiology. This interpretation is consistent with recent genetic studies suggesting that CD may result from mutations affecting Purkinje neuron function (Xiao *et al.*, 2012).

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Abbreviations

ACC	nucleus accumbens
AD	Alzheimer's disease
BG	basal ganglia
BL	blepharospasm
BS	brainstem
CAUD	caudate
CD	cervical dystonia
COD	complication of the disorder
CNF	cuneiform nucleus
CN III	cranial nerve III
CN IV	cranial nerve IV
CRB	cerebellum
CTX	cerebral cortex
CVD	cerebrovascular disease
F	female
FD	facial dystonia
FHD	focal hand dystonia
GP	globus pallidus

HT	hand tremor
H&E	hematoxylin/eosin
HIP	hippocampus
IC2	anti-polyglutamine antibody
ID	identifier
INC	interstitial nucleus of Cajal
LC	locus ceruleus
M	male
MB	midbrain
MRI	magnetic resonance imaging
NA	not available
NFT	neurofibrillary tangles
NBM	nucleus basalis of Meynert
NR	not relevant
NT	neck tremor
PAG	periaqueductal gray
PMI	post-mortem interval
PPN	pedunculopontine nucleus
PUT	putamen
RF	reticular formation
RN	red nucleus
SC	spinal cord
SN	substantia nigra
STN	subthalamic nucleus
sv	sequence variant
TDP-43	TAR DNA binding protein 43
TG	tegmentum
THAL	thalamus
uINI	ubiquitinated intranuclear inclusions

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Highlights

- Subjects with cervical dystonia had a lower density of cerebellar Purkinje cells.
- Subjects with *THAPI* variants had a lower density of cerebellar Purkinje cells.
- No consistent abnormalities were identified in the basal ganglia, or other regions.

Figure 1.

Histopathology in the midbrain. Panels A-B show typical ubiquitin-positive inclusions in the substantia nigra (Marinesco bodies). Nigral neurons with one or multiple inclusions were observed (black arrows). Panel C shows an example of satellitosis (arrow) around a dopaminergic neuron in the substantia nigra. Panel D shows increased number and activation of astrocytes (gliosis, arrows) in a midbrain section of a cervical dystonia case. Panels A-B: ubiquitin immunostaining; Panel C: hematoxylin and eosin; Panel D: glial fibrillary acidic protein.

Figure 2.

Histopathology in the cerebellum. Panels A-B shows areas with patchy loss of Purkinje cells, as indicated by the black arrows. Torpedo bodies (white arrows) can be identified in Panels B-C. Panel D shows a typical Purkinje cell layer with morphologically normal cell bodies. Panels A-D: silver impregnation.

Figure 3.

Box and whisker plot comparing Purkinje cell linear density in 6 cervical dystonia (CD) cases and 13 controls. In this type of plot, the upper and lower limits of the box show the upper and lower quartiles of the data, with the horizontal line in between showing the median. The whiskers show the entire data range, and circles depict each data point. Two independent sections for each subject were analyzed, and all 12 points for the CD group and 26 for the controls are shown. Regression analysis indicated that CD was significantly associated with lower density of Purkinje cells at $p < 0.05$ (A). Regression analysis also revealed that cases with a *THAP1* sequence variant (*THAP1* sv+) had a significantly lower linear density of Purkinje cells compared to *THAP1* negative cases at $p < 0.05$ (*THAP1* sv-) (B). In A, open and closed circles represent the data for *THAP1* positive and negative cases within each group. In B, open and closed circles represent the data for CD and controls within each *THAP1* group.

Postmortem studies of cases with cervical dystonia

Table 1

Age at onset	Age at death	Sex	Body region affected	Brain regions investigated	Pathological findings	Probability of primary CD	Source
23	24	M	neck	BG, MB/BS, CRB	loss of neurons, gliosis, multiple small and bilateral lacunes in PUT, substantia innominata; perivascular lymphocytic infiltration suggestive of vasculitis	low	(Foerster, 1933)
21	25	F	neck	CTX, THAL, CAUD, PUT, GP, SN, CRB	cell loss in CTX; cell loss in the molecular and Purkinje cell layers in CRB; diffuse perivascular lymphocytic infiltration and meningeal thickening consistent with chronic encephalitis	low	(Grinker and Walker, 1933)
43	90	M	neck, tongue, forearm, hand, fingers	CTX, GP, CAUD, PUT, THAL, SN, RN	atrophy of CAUD and PUT; cell shrinkage in CAUD, PUT and GP bilaterally, with vacuolization, pyknotic nuclei, neuronophagia, gliosis, and presence of lipid pigment; cell loss in GP	low	(Alpers and Drayer, 1937)
59	65	F	neck	CTX, THAL, GP, CAUD, PUT, CRB (vermis, hemispheres and peduncles), MB, NBM, RN, SN, INC, pons, medulla, SC	normal	high	(Tarlov, 1970)
NA	NA	NA	neck	upper pons	normal	indeterminate	(Tarlov, 1970)
56	62	F	neck, upper and lower face	BG, SC, BS, CRB, cerebrum	normal	high	(Garcia-Albea et al., 1981)
NA	50	F	neck	NA	normal	indeterminate	(Zweig et al., 1986)
61	68	F	upper face, larynx, neck	CTX, HIP, CAUD, PUT, GP, hypothalamus, THAL, MB, pons, medulla, CRB	normal	high	(Jankovic et al., 1987)
47	50	F	neck	HIP, amygdala, GP, CAUD, PUT, basal forebrain, THAL, STN, BS, CRB	normal	high	(Zweig et al., 1988)
<33	68	M	upper and lower face, neck	HIP, amygdala, CAUD, PUT, ACC, GP, THAL, STN, CRB, SN, LC, NBM, raphe, PPN, medulla, CN III, CN IV	depigmentation in SN, LC; NFT in NBM; neuronal loss in SN, raphe, PPN, LC; astrogliosis in SN	low	(Zweig et al., 1988)
59	68	F	neck, lower face	CTX, CAUD, PUT, GP, NBM, THAL, STN, RN, SN, LC, raphe, inferior olive, SC (cervical and thoracic)	normal	high	(Gibb et al., 1988)
54	72	F	neck, face	PAG, PPN, RF, CNF, GP, CAUD, PUT, HIP, CTX (anterior frontal, parietal, temporal)	diffuse gliosis in GP; patchy gliosis in CAUD, PUT; signs of AD, Lewy bodies in BS	moderate	(Holton et al., 2008)
46	79	F	neck, lower face, hand	same as above	cystic infarct in CAUD; diffuse gliosis in GP; patchy gliosis in CAUD, PUT; small vessel disease in BG	low	(Holton et al., 2008)

Age at onset	Age at death	Sex	Body region affected	Brain regions investigated	Pathological findings	Probability of primary CD	Source
47	65	M	axial, larynx	same as above	diffuse gliosis in GP; patchy gliosis in CAUD, PUT; mild small vessel disease	moderate	(Holton <i>et al.</i> , 2008)
73	80	F	neck	same as above	diffuse gliosis in GP; patchy gliosis in CAUD, PUT	high	(Holton <i>et al.</i> , 2008)

This table includes only cases originally described or typically cited as examples of primary or idiopathic dystonia. Segmental and multifocal dystonia cases were included if dystonia of the neck also was a prominent feature of the disorder. Probability of primary cervical dystonia is classified as low, moderate and high according to current clinical and pathological criteria. Cases were classified with high probability if the clinical description was consistent current clinical criteria. Cases with one atypical feature were considered with moderate probability, whereas cases with two or more atypical features were classified with a low probability of primary CD. The limited information available for some cases did not allow proper classification and, thus, they were listed as indeterminate. ACC, nucleus accumbens; AD, Alzheimer's disease; BG, basal ganglia; BS, brainstem; CAUD, caudate; CD, cervical dystonia; CNF, cuneiform nucleus; CN III, cranial nerve III; CN IV, cranial nerve IV; CRB, cerebellum, CTX, cerebral cortex; GP, globus pallidus; HIP, hippocampus; ID, identifier; INC, interstitial nucleus of Cajal; MB, midbrain; NA, not available; NFT, neurofibrillary tangles; NBM, nucleus basalis of Meynert; PAG, periaqueductal gray; PPN, pedunculopontine nucleus; PUT, putamen; RF, reticular formation; RN, red nucleus; SC, spinal cord; STN, subthalamic nucleus; THAL, thalamus.

Table 2

Clinical information for cervical dystonia cases and controls

ID	Diagnosis	Sex	Age at onset	Age at death	PMI	Cause of death	Family history	THAP1 gene
CD1	CD, BL, FD	F	39	60	18	COD	negative	c.71+9C>A, heterozygous
CD2	CD	F	53	75	20	COD	NA	c.268-31A>G, heterozygous
CD3	CD, BL	M	52	58	5	temporal glioma	negative	c.71+9C>A, homozygous
								c.268-31A>G, homozygous
CD4	CD	F	40	65	25	CVD	negative	c.71+9C>A, heterozygous
CD5	CD, FHD	F	29	82	6	COD	negative	normal
CD6	CD, NT, HT	F	57	80	22	respiratory arrest	NT	normal
N1	normal	M	NR	55	NA	lung carcinoma	NA	normal
N2	normal	M	NR	59	NA	multiple sclerosis, stroke	NA	normal
N3	normal	M	NR	60	NA	cardiopulmonary arrest	NA	normal
N4	normal	F	NR	61	NA	cardiac failure	NA	normal
N5	normal	M	NR	62	NA	carcinoma of lymph nodes, liver, lung	NA	normal
N6	normal	F	NR	62	NA	leiomyosarcoma	NA	normal
N7	normal	M	NR	64	NA	lung carcinoma	NA	c.268-31A>G, heterozygous
N8	normal	M	NR	70	NA	nasopharyngeal carcinoma, leukemia	NA	normal
N9	normal	F	NR	74	NA	aortic aneurysm	NA	normal
N10	normal	F	NR	76	NA	lung carcinoma, aortic aneurysm	NA	normal
N11	normal	M	NR	76	NA	cardiac arrest	NA	normal
N12	normal	M	NR	77	NA	pneumonia	NA	c.71+9C>A, homozygous
N13	normal	M	NR	84	NA	bronchopneumonia, rheumatoid myocarditis	NA	normal
N14	normal	F	NR	80+	NA	NA	NA	normal
N15	normal	M	NR	80+	NA	NA	NA	normal
N16	normal	M	NR	69	NA	aortic aneurysm	NA	normal

Information was obtained from the University of Maryland Brain and Tissue Bank for Developmental Disorders, Johns Hopkins Brain Resource Center and the Emory Alzheimer's Disease Research Center. Age is expressed in years. BL, blepharospasm; CD, cervical dystonia; COD, "complication of disorder"; CVD, cardiovascular disease; F, female; FD, focal hand dystonia; FHD, focal hand dystonia; HT, hand tremor; ID, identifier; M, male; NA, not available; NR, not relevant; NT, neck tremor; PMI, post-mortem interval in hours. The glioma in CD3 was restricted to the temporal lobe on one side of the brain.

Table 3

Main findings of the subjective screening study

ID	BS	MB	INC	RN	BG	CRB	CTX
CD1	normal	gliosis, ballooned neurons, satellitosis, uINI in SN	normal	normal	arrested migration of neuroblasts	gliosis, slight microgliosis, cell loss in dentate nucleus	normal
CD2	normal	corpora amylacea, isolated axonal swellings, satellitosis, uINI in SN	normal	normal	hyalinosis and mineralization (GP)	frequent torpedo bodies, patchy loss of Purkinje cells	normal
CD3	uINI (LC)	slight microgliosis, slight satellitosis, patchy microgliosis in TG, uINI in SN	normal	normal	satellitosis	patchy microgliosis in penduncle, gliosis around Purkinje cells, slight satellitosis, Bergmann gliosis, torpedo bodies, patchy loss of Purkinje cells	normal
CD4	normal	isolated axonal swellings, clusters of oligodendrocytes, focal astrogliosis, satellitosis, uINI in SN	normal	normal	hemosiderin loaded macrophages	corpora amylacea, Bergmann gliosis in vermis, frequent torpedo bodies, patchy loss of Purkinje cells	normal

Tissue from cases CD5 and CD6 was not analyzed during the initial screening study. BG, basal ganglia (caudate, putamen, globus pallidus); BS, brainstem; CRB, cerebellum; CTX, cerebral cortex; GFAP, glial fibrillary acidic protein; GP, globus pallidus; ID, identifier; INC, interstitial nucleus of Cajal; LC, locus ceruleus; MB, midbrain; RN, red nucleus; SN, substantia nigra; TG, tegmentum; uINI, ubiquitinated intranuclear inclusions.

Table 4

Number of nigral inclusions in melanized neurons in the substantia nigra

ID	Total cell count	Total number of cells w/inclusions	Proportion of cells with inclusions (%)	Number of cells with 1 to 6 inclusions per cell					
				1	2	3	4	5	6
CD1	698	58	8.3	28	12	12	5	0	1
CD2	407	12	2.9	6	3	2	1	0	0
CD3	263	8	3.0	5	0	2	1	0	0
CD4	396	31	7.8	16	12	1	1	1	0
CD5	365	72	19.7	37	24	9	2	0	0
CD6	531	34	6.4	24	8	2	0	0	0
N2	262	43	16.4	29	8	4	2	0	0
N3	197	0	0.0	0	0	0	0	0	0
N5	139	3	2.2	2	0	1	0	0	0
N6	727	53	7.3	40	10	3	0	0	0
N7	141	8	5.7	2	4	2	0	0	0
N8	705	15	2.1	10	4	1	0	0	0
N9	1393	252	18.1	130	71	32	16	3	0
N10	200	47	23.5	28	17	2	0	0	0
N12	980	199	20.3	78	52	40	21	7	1
N14	353	7	2.0	6	0	0	1	0	0
N15	355	35	9.9	21	9	4	1	0	0
N16	504	94	18.7	53	21	12	7	0	1

Ubiquitin-positive intranuclear inclusions in the substantia nigra were quantified in 6 CD individuals and 12 controls. The number of inclusions in each melanized neurons ranged from 0 to 6. Logistic regression analysis indicated that neither group nor age was a significant predictor of the proportion of cells with inclusions, or the number of inclusions per cell. ID, identifier.