Cerebellar Degeneration Correlates with Motor Symptoms in Huntington Disease

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Objective: Huntington disease (HD) is an autosomal dominant neurodegenerative disorder characterized by variable motor and behavioral symptoms attributed to major neuropathology of mainly the basal ganglia and cerebral cortex. The role of the cerebellum, a brain region involved in the coordination of movements, in HD neuropathology has been controversial. This study utilizes postmortem human brain tissue to investigate whether Purkinje cell degeneration in the neocerebellum is present in HD, and how this relates to disease symptom profiles.

Methods: Unbiased stereological counting methods were used to quantify the total number of Purkinje cells in 15 HD cases and 8 neurologically normal control cases. Based on their predominant symptoms, the HD cases were categorized into 2 groups: “motor” or “mood.”

Results: The results demonstrated a significant 43% loss of Purkinje cells in HD cases with predominantly motor symptoms, and no cell loss in cases showing a major mood phenotype. There was no significant correlation between Purkinje cell loss and striatal neuropathological grade, postmortem delay, CAG repeat in the IT15 gene, or age at death.

Interpretation: This study shows a compelling relationship between Purkinje cell loss in the HD neocerebellum and the HD motor symptom phenotype, which, together with our previous human brain studies on the same HD cases, provides novel perspectives interrelating and correlating the variable cerebellar, basal ganglia, and neocortical neuropathology with the variability of motor/mood symptom profiles in the human HD brain.

Huntington disease (HD) is an autosomal dominant neurodegenerative disorder characterized by progressive motor dysfunction, impaired cognition, and mood/psychiatric disturbance. HD is caused by a CAG trinucleotide repeat expansion in the IT15 (interesting transcript 15) gene on chromosome 4, encoding a mutant protein called huntingtin. Huntingtonin aggregates in the nuclei and cytoplasm of neurons are thought to be involved in the initiation of a pathogenic cascade, leading to neuronal death throughout the basal ganglia and cerebral cortex.

Despite the single-gene etiology, HD individuals show clear and considerable phenotypic variability. Recent studies suggest a strong association between clinical symptom variability in HD and the pattern of efferent neuronal loss in the basal ganglia and cerebral cortex. In the striatum, the major afferent nucleus of the basal ganglia, the variable degeneration of striatal medium spiny efferent neurons in the striosome and matrix compartments is found to be associated with mood and motor symptoms, respectively. Degeneration within other basal ganglia nuclei, such as the globus pallidus externus, also correlates with HD symptom heterogeneity. Furthermore, our detailed pathological studies on various functional cerebral cortical regions have demonstrated an association between variable cortical efferent pyramidal cell loss and clinical symptom profiles in HD.

Although it is well established that the cerebellum has a major role in the coordination of movements, and HD patients commonly show movement coordination deficits,
cerebellar involvement in HD neuropathology and symptomatology is controversial. Early pathological examinations of HD postmortem cases report relative preservation of the cerebellum. More recent neuropathological examinations report the HD cerebellum to be smaller than normally expected, but less atrophic compared to the cerebral cortex and basal ganglia. However, despite a loss of volume, neuronal density appears within normal limits. In contrast, a recent nonstereological quantitative study in postmortem HD cases reported considerable cerebellar atrophy, the Purkinje cells. Adding to the controversy, magnetic resonance imaging (MRI) studies in symptomatic patients with adult onset HD report contrasting findings for cerebellar volumes. Fennema-Notestine et al reported reduced cerebellar white and gray matter volumes, whereas Rosas et al reported no significant cerebellar atrophy in HD, suggesting cerebellar preservation with significant cortex and basal ganglia degeneration.

The cerebellum is known to play a crucial role in movement coordination, as well as posture and balance regulation, all of which have been shown to be impaired in HD. Furthermore, it is well established that the neocerebellar region of the cerebellum plays a major role in the coordination of goal-directed voluntary movements, which are especially affected in HD. It therefore follows that degeneration of the major cerebellar efferent neurons, the Purkinje cells, may also be a feature in HD cases with predominant motor symptom profiles. Therefore, the overall aim of the present study is to investigate the validity of this hypothesis by determining whether there are changes in the number of Purkinje cells in the HD neocerebellum, and whether this loss relates to symptom heterogeneity.

Subjects and Methods

Processing of Human Brain Tissue
The brain tissue was obtained with the full consent of all family members, and the research protocols were approved by the University of Auckland Human Participants Ethics Committee (2008/279 and 011654). Blocks from the right cerebellar hemisphere of 15 HD cases and 8 neurologically normal control brains, matched for age and postmortem delay, were used in this study. The HD cases included 10 males and 5 females, aged 35 to 68 years (mean = 59 years), with a postmortem delay of 4 to 25 hours (mean = 13.6 hours; Table 1). The control cases included 7 males and 1 female, aged 41 to 68 years (mean = 54 years), with a postmortem delay of 8 to 21 hours (mean = 13.5 hours; Table 2). The brains were perfused according to previously published protocols, and in each case the cerebellum was cut into 4 equal-size blocks from lateral to medial (CB0, CB1, CB2, and CB3) regions of the cerebellum, as shown in Figure 1. For this study, the CB1 block of the neocerebellum from the right cerebellar hemisphere was used (see Fig 1). The blocks were then postfixed by immersion in fixative for 24 to 48 hours and transferred into a cryoprotective 20% sucrose solution for 2 weeks. The blocks were subsequently immersed in a 30% sucrose solution for another 2 weeks before being frozen using powdered dry ice and stored at −80°C.

Clinical Assessments
As detailed previously, clinical data to assess HD symptom profiles were collected retrospectively from clinical records and from family members for each case at the Neurological Foundation of New Zealand Human Brain Bank. The clinical data were collected using a semistructured interview and a questionnaire, administered by researchers blind to the neuroanatomical analyses of the brains. Total clinical data for each HD case were reviewed independently by 2 neuropsychologists experienced with HD symptom assessments and were classified using the following definitions (Due to the retrospective nature of data collection, clinically validated assessment of cognitive symptoms was not possible.).

HD Motor. Individuals displayed a clear movement disorder with no significant presence of mood symptoms during the symptomatic course of the disease.

HD Mood. Individuals demonstrated predominant mood disturbances during the symptomatic course of the disease. However, some degree of motor symptoms were also present, but these were either very mild or only emerged during the very late stages of the disease.

HD Mixed Motor–Mood. Significant levels of both symptom types were present during a large part of the disease.

The classifications of the 2 psychologists were concordant for 90% of cases in the larger study. For any case where there was a difference, the case materials were reviewed by both psychologists until a consensus was reached. The 15 HD cases included in this study were selected because their clinical symptoms were dominated by either motor or mood symptoms as defined above.

Immunohistochemistry and Stereology
For each case, the entire CB1 block was cut into serial sagittal sections of 50 µm thickness. Free floating immunohistochemistry was carried out on the cerebellum sections using standard single peroxidase labeling techniques as detailed previously. Every 10th section in each case, beginning at a random start point (1 to 10), was immunolabeled with rabbit anti-calbindin-D28k (CB) (1:20,000; Swant, Marly, Switzerland) to specifically and reliably identify the cerebellar Purkinje cells. The immunostained sections were subsequently counterstained with cresyl violet. Double-blind unbiased stereological methods were then performed to obtain an unbiased estimate of the total number of Purkinje cells in each case. Digital microscopic images were acquired using a Nikon Eclipse Ni-E motorized microscope.
coupled to a high-resolution Nikon DS-Ri2 digital camera (Nikon, Tokyo, Japan). The final figures were processed and assembled using Photoshop v13 (Adobe Systems, San Jose, CA).

Design-based stereology is a quantification method to obtain a precise and unbiased estimate of the total number of cells in a well-defined region. In this study, the Optical Fractionator probe was performed using StereoInvestigator software (v10, MBF Bioscience, MicroBrightField, Williston, VT) to estimate the total number of Purkinje cells in the control and HD cases, based on the following formula: \( N = \frac{P \times Q - t/h \times 1/asf \times 1/ssf}{27} \). Each section was traced using a 1× ultra wide 0.04 numerical aperture (NA) air objective, and a grid of known dimensions (1,458 × 837 μm) was placed at

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F = female; HD = Huntington disease; M = male.

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F = female; M = male.
random on top of each tracing. A 40 × Fluor 0.75 NA air objective was used to count cells. Pilot studies, using both control and HD cases, were conducted to determine the stereological parameters, to ensure that the coefficient of error (CE) was within the acceptable limits (CE ≤ 0.1). These parameters were kept constant for all cases.

**Statistical Analysis**

Note that nonparametric tests were used due to the small sample size, unequal variances, and departures from normality. Average changes in the total number of Purkinje cells in the HD cases compared to control cases were assessed using a 2-tailed Mann–Whitney test. Multiple comparisons between subgroups (classified according to grade or symptoms) were carried out using Kruskal–Wallis 1-way analysis of variance on ranks, with Dunn’s method of subgroup comparisons versus control group. Lower and upper 95% confidence intervals (CIs) were included for between-group comparisons for the Mann–Whitney and Kruskal–Wallis tests where p values were reported. The associations between the total cell number and CAG repeat, postmortem delay, and age at death were examined for the control and HD cohorts using nonparametric Spearman rank order correlations. Spearman rank order correlations were classified according to the strength of the r value, with 0 ≤ r ≤ 0.350 considered weak, r = 0.351 to 0.670 considered moderate, r = 0.671 to 0.900 considered strong, and r > 0.900 considered very strong correlations. Ninety-five percent CIs were reported for Spearman rank order r values obtained from analysis of the HD cohort. All statistical analyses were performed using Prism v6 (GraphPad Software, La Jolla, CA), and p values <0.05 were considered statistically significant.

**Results**

**Overall Pattern of Cerebellar Purkinje Cell Loss in HD**

When comparing the pooled results from the 15 HD cases with 8 control cases, there was no significant loss of calbindin-positive Purkinje cells in the neocerebellum in HD (p = 0.21, 95% CI of difference = −676,463 to 299,678; Fig 2A).

Next, the relationship between Purkinje cell loss and variable HD symptom profiles was investigated. To achieve this, the HD cases were further subgrouped according to their predominant symptomatology (HD motor or mood), and the average total number of calbindin-positive Purkinje cells from each group was compared with the control group. Most interestingly, the HD motor cases showed a significant 43% loss of calbindin-positive Purkinje cells in the neocerebellum compared to the control group (p = 0.048; see Fig 2B). In contrast, in the HD mood subgroup, no significant changes were found in Purkinje cell numbers compared to controls (p > 0.99).

**Relationship between Purkinje Cell Numbers and Striatal Neuropathological Grade, CAG Repeat, Age at Death, and Postmortem Delay**

First, we confirmed that there was no significant association between the motor and mood HD symptomatology classification and their corresponding striatal neuropathological grades (Mann–Whitney analysis, p = 0.34, 95% CI of difference = −2 to 1). Hence, the striatal pathological grade did not confound the results for disease symptoms. Next, to investigate the relationship between Purkinje cell number and striatal pathology, the HD stereological counting results were subgrouped according to Vonsattel striatal neuropathological grades (grade 0–1, grade 2, and grade 3–4) and compared with the entire control group (see Fig 2C). In our group of 15 HD cases, there was no clear statistical association between the total number of...
Purkinje cells within the neocerebellum and striatal neuropathological grades ($p > 0.7$ for each comparison).

The relationship between the total number of Purkinje cells with respect to CAG repeat length, age at death, and postmortem delay was also investigated by calculating Spearman correlations (Fig 4). There were no significant correlations between the total number of Purkinje cells and any of these variables (all $p$ values $>0.40$, and all $r$ values were classified as “weak” $0 \leq r \leq 0.350$ and $-0.350 \leq r \leq 0$).

For all stereological analyses of the HD and control cases, the average CE for the total cell number was $<0.10$. This indicates that the observed variability is due to a true difference in the number of Purkinje cells between cases rather than a lack of precision in the stereological counting methods employed.

Discussion

This is the first detailed quantitative study, using rigorous design-based stereological approaches, to determine the pattern of Purkinje cell loss in the human neocerebellum in HD. This study of 15 HD and 8 control cases demonstrates, for the first time, a compelling relationship between Purkinje cell loss in the neocerebellum and motor symptoms in HD.

Cerebellum Is Not A Quiescent Region In HD

Due to its functional connectivity with major cortical and subcortical motor structures, the human cerebellum is known to play a critical role in movement initiation and termination, coordination, and fine tuning of limb, trunk, and eye movements, and modulation of posture and balance. Through its afferent and efferent connections, the neocerebellum of the lateral cerebellar hemisphere is known to play a major role in the overall initiation and coordination of goal-directed voluntary movements. Despite its major involvement in the coordination of voluntary movements, the cerebellum has been generally neglected in research studies on the neuropathology of HD. From the

620,252 Purkinje cells (95% confidence interval [CI] = 147,315–1,294,590) compared to the control cohort with 1,269,881 ± 897,436 cells (95% CI = 519,606–2,020,157). Note the HD cases with motor symptoms contained an average of 548,928 fewer Purkinje cells relative to control cases. Thus, a significant 43% loss of Purkinje cells was evident within the HD motor subgroup compared to controls ($p = 0.048$). In comparison, HD cases with mood symptoms contained 1,230,135 ± 664,325 Purkinje cells (95% CI = 674,745–1,785,524), which was not significantly different from the control cohort ($p > 0.99$). Data are expressed as mean ± standard deviation of the mean. Lower and upper 95% CIs were included for mean comparisons with reported $p$ values. Significance values are based on a 2-tailed $p$ value from a Mann–Whitney test in A and a Kruskal–Wallis test combined with Dunn multiple comparisons post-test in B and C. [Color figure can be viewed at www.annalsofneurology.org]
FIGURE 3: Representative photomicrographs showing the qualitative distribution of cerebellar calbindin-positive Purkinje cells from control case H108 (A, D, G, and J), Huntington disease (HD) case HC126 with predominant mood symptoms (B, E, H, and K), and HD case HC116 with predominant motor symptoms (C, F, I, and L). Arrows denote representative Purkinje neurons. Note the distinct loss of calbindin-positive Purkinje cells is only seen in the HD motor case (C), which is also accompanied by the complementary loss of dendritic branches. To ensure the apparent cell loss is not simply due to loss of calbindin expression, the sections illustrated in panels A–C were counterstained with cresyl violet (D–F). Careful examination at high power (J–L) shows all Purkinje cells were double stained for cresyl violet and calbindin. Panels G–L are high-magnification inserts of panels A–F. Scale bars correspond to 500 μm in A–F and 100 μm in G–L. Gra. = granule cell layer; Mol. = molecular layer.
limited literature available, there appears to be controversy involving the significance of cerebellar pathology in the pathogenesis and clinical presentation of HD. Earlier neuropathological studies in HD postmortem tissue have reported rare cases of cerebellar atrophy, gliosis, and “possible patchy loss of Purkinje cells.”11,36–38 More recent neuropathological studies by Vonsattel et al identified that the HD cerebellum is atrophic in cases of Vonsattel striatal grades 3 or 4. This study reported sporadic loss of Purkinje cells accompanied by the presence or absence of gliosis within the HD cerebellum.16 Collectively, these studies using conventional neuropathological examination suggest the involvement of the cerebellum in HD.

This aforementioned view has been challenged by a more recent quantitative study, where the cerebellum has been shown to be a site of primary degeneration in HD.14 This study of 8 HD cases and 8 control cases reported a 48% loss of calbindin-positive Purkinje cells in 2 representative sections at vermal (paleocerebellum) and hemispheric (neocerebellum) levels of the cerebellum. This morphometric study is concordant with recent MRI studies, where HD patients with mild to moderate symptom severity demonstrated significant atrophy of cerebellar gray and white matter.12,17 Taken together, these studies suggest that cerebellar degeneration is possibly an earlier and more significant event in the pathogenesis of HD than previously thought. However, more in-depth studies are needed to elucidate the relationships between cerebellar degeneration and key features of HD such as CAG repeat length, striatal neuropathological grade, and variable symptomatology. The present study examines the relationship between Purkinje cell loss in the neocerebellum with clinical symptom profiles of HD and the other key parameters of HD pathogenesis, CAG repeat length, and striatal neuropathological grade.

**No Overall Loss of Purkinje Cells in HD**

When comparing the pooled stereological cell count data for 15 HD cases and 8 controls (see Fig 2A), there was no overall significant loss of calbindin-positive Purkinje cells in the neocerebellum in HD. However, a significant loss number and CAG repeat length in both control ($r_s = -0.17$, $p = 0.69$) and HD ($r_s = -0.21$, $p = 0.45$, 95% confidence interval [CI] = −0.67 to 0.36) groups. (B) A nonsignificant weak negative and weak positive correlation was found between total Purkinje cell number and age at death in control ($r_s = -0.05$, $p = 0.93$) and HD ($r_s = 0.03$, $p = 0.93$, 95% CI = −0.51 to 0.54) groups, respectively. (C) A nonsignificant weak correlation was found between total Purkinje cell number and postmortem delay in both control ($r_s = 0.20$, $p = 0.63$) and HD ($r_s = 0.09$, $p = 0.75$, 95% CI = −0.46 to 0.59) groups. All $p$ values were > 0.05; 95% confidence intervals were reported for $r$ values in the HD cohort.
of Purkinje cells was found when the HD cases were further subgrouped according to the predominant HD symptomatology, as detailed below. Our findings differ from those of Rüü et al, who reported a significant overall loss of calbindin-positive Purkinje cells in the cerebellum in HD cases without any reference to symptom profiles. This variability in results is likely to be attributable to differences in study design and case selection. The methods used in the present study followed strict design-based stereological criteria, as outlined by West and Gundersen, to ensure the Purkinje cells counted represent the entire population of cells in the region of interest (neocerebellum). Designed-based stereological methods have been employed in our earlier studies investigating the pattern of cell loss in the basal ganglia and cerebral cortex in HD. In comparison, Rüü et al conducted quantitative studies using 4 representative sections selected from 2 separate subregions of the cerebellum with different functional and anatomical connectivity. Furthermore, the majority of the HD cases examined by Rüü et al displayed motor abnormalities, with only 1 case presenting personality changes. In contrast, the clinical symptom profiles were subgrouped in our study, with 8 cases classified as predominantly mood (with mood disturbance the dominant clinical feature across the disease course), and 7 cases classified as motor (significant motor symptoms with no significant presence of mood dysfunction). This subgrouping of clinical profiles could account for differences in our results compared to Rüü et al, as we did not find an overall loss of Purkinje cells when the cases of motor and mood symptomatology were pooled together and compared with the control cohort.

**Relationship between Purkinje Cell Loss and Motor Symptomatology in HD**

A great deal of variability was observed in Purkinje cell number in both the HD and control cohorts (see Fig 2A), which reinforces the natural variation observed in human populations. We hypothesized that variation within the HD cohort may be attributable to either differences in striatal neuropathological grade or the clinical symptoms of the HD cases. Previously, our cortical and basal ganglia studies have shown that variable patterns of efferent neuronal loss correlate with HD symptomatology, based on the functionality of the area of interest. Because the cerebral cortex is highly connected to the cerebellum via corticopontocerebellar circuits, which is known to influence voluntary movements, we first investigated the relationship between Purkinje cell number and variable symptomatology in HD.

This is the first study to show that there is a significant loss of Purkinje cells (43%) in the neocerebellum of HD cases with predominant motor symptom profiles (see Fig 2B), with no significant loss of Purkinje cells in HD cases with predominantly mood symptoms. Purkinje cells are inhibitory neurons, containing γ-aminobutyric acid as the main neurotransmitter. Purkinje cells are the major efferent neurons of the cerebellar cortex, which connect the cerebellum via the deep cerebellar nuclei and thalamus to the motor cortical areas. Other studies have also shown that the cerebellum is widely connected to both the motor nuclei of the basal ganglia and cortical motor areas, including the primary and premotor cortices. Due to this extensive connectivity, loss of Purkinje cells in the HD motor cases would cause disinhibition of the glutamatergic deep cerebellar nuclei, leading to overexcitation of thalamocortical glutamatergic neurons projecting to the premotor and primary motor cerebral cortex. Therefore, loss of Purkinje cells in the cerebellar cortex, in concert with basal ganglia changes, would result in increased excitation of the motor areas of the cerebral cortex in HD. This would, in turn, contribute to motor dysfunction characterized by hyperactive choreiform movements (hyperkinesia) in HD. This view is supported by our previous studies, where significant loss of cells in cortical motor areas or motor regions of the basal ganglia was correlated with motor symptomatology in HD.

**Purkinje Cell Loss Does Not Correlate with Striatal Neuropathological Grade**

As illustrated in Figure 2C, the extent of Purkinje cell loss in our group of 15 HD cases does not appear to correlate with striatal neuropathological grade. These results would support the notion that Purkinje cell loss and cerebellar atrophy do not necessarily develop simultaneously with striatal neuropathology. This is in agreement with recent neuropathological and MRI studies in early HD. Nevertheless, our recent neuropathological studies on the cerebral cortex and basal ganglia in the same HD cases as those used in this study also show that the pattern of pathology strongly correlates with disease symptom profiles. Collectively, these studies show that the cerebellum, basal ganglia, and cerebral cortex all play an interactive role in the pathogenesis of HD symptomatology. These findings emphasize that the widespread nature of the variable neuropathology of HD in the human brain correlates with symptom profiles. These general conclusions have major implications for correlating pathology and symptom profiles in the human brain in other neurodegenerative diseases such as Parkinson disease and Alzheimer disease.

In conclusion, our stereological study detailing the loss of Purkinje cells in HD motor dominant cases demonstrates that the neocerebellum plays a significant role in the
clinicopathological features of HD. This study also supports the notion that along with the cerebral cortex and the basal ganglia, the cerebellum needs to be considered as a contributor to the symptom manifestations of HD.

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Author Contributions

M.K.S.-B. and N.F.M. contributed to study design, data acquisition and analysis, and production of the manuscript and figures. T.S., L.J.T., M.D.R.A., and A.Y.S.T. contributed to data acquisition and analysis and production of the manuscript and figures. R.L.M.F., H.J.W., and M.D. contributed to study concept and design and production of the manuscript and figures.

Potential Conflicts of Interest

Nothing to report.

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