

# Purkinje cell axonal anatomy: quantifying morphometric changes in essential tremor versus control brains

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Growing clinical, neuro-imaging and post-mortem data have implicated the cerebellum as playing an important role in the pathogenesis of essential tremor. Aside from a modest reduction of Purkinje cells in some post-mortem studies, Purkinje cell axonal swellings (torpedoes) are present to a greater degree in essential tremor cases than controls. Yet a detailed study of more subtle morphometric changes in the Purkinje cell axonal compartment has not been undertaken. We performed a detailed morphological analysis of the Purkinje cell axonal compartment in 49 essential tremor and 39 control brains, using calbindin D<sub>28k</sub> immunohistochemistry on 100- $\mu$ m cerebellar cortical vibratome tissue sections. Changes in axonal shape [thickened axonal profiles ( $P = 0.006$ ), torpedoes ( $P = 0.038$ )] and changes in axonal connectivity [axonal recurrent collaterals ( $P < 0.001$ ), axonal branching ( $P < 0.001$ ), terminal axonal sprouting ( $P < 0.001$ )] were all present to an increased degree in essential tremor cases versus controls. The changes in shape and connectivity were significantly correlated [e.g. correlation between thickened axonal profiles and recurrent collaterals ( $r = 0.405$ ,  $P < 0.001$ )] and were correlated with tremor duration among essential tremor cases with age of onset  $> 40$  years. In essential tremor cases, thickened axonal profiles, axonal recurrent collaterals and branched axons were 3- to 5-fold more frequently seen on the axons of Purkinje cells with torpedoes versus Purkinje cells without torpedoes. We document a range of changes in the Purkinje cell axonal compartment in essential tremor. Several of these are likely to be compensatory changes in response to Purkinje cell injury, thus illustrating an important feature of Purkinje cells, which is that they are relatively resistant to damage and capable of mobilizing a broad range of axonal responses to injury. The extent to which this plasticity of the Purkinje cell axon is partially neuroprotective or ultimately ineffective at slowing further cellular changes and cell death deserves further study in essential tremor.

**Keywords:** essential tremor; Purkinje cell; neurodegenerative; axon; recurrent collateral

**Abbreviations:** LH&E = Luxol fast blue counterstained with haematoxylin and eosin

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## Introduction

Essential tremor is among the most prevalent neurological diseases (Benito-Leon *et al.*, 2003; Louis and Ferreira, 2010); prevalence rises in an exponential manner during the later decades of life (Das *et al.*, 2009; Louis *et al.*, 2009b). The precise pathogenesis is not understood, although recent post-mortem studies have identified several structural changes within the cerebellum of patients with essential tremor, with these changes centred on or around the Purkinje cell (Louis *et al.*, 2007; Erickson-Davis *et al.*, 2010; Louis, 2010; Kuo *et al.*, 2011; Yu *et al.*, 2012). Prominent among these changes is a substantial increase in the number of torpedoes (i.e. swellings of the proximal portion of the Purkinje cell axon) (Louis *et al.*, 2007, 2011b). Electron microscopic studies have revealed that these axonal swellings contain an excess and disorganization of neurofilaments (Louis *et al.*, 2009c). These accumulations, in general, can either be a cause or result of damaged axonal transport (Beaulieu *et al.*, 1999; Liem and Leung, 2003).

Interest in axonal neurobiology and pathology has recently increased in the setting of neurodegenerative disorders, including Parkinson's disease (Li *et al.*, 2009; Burke and O'Malley, 2013) and Alzheimer's disease (Stokin and Goldstein, 2006). The size difference between neuronal cell soma and their axons (ratio of lengths up to 1:100 000) (Petzold, 2005) puts intense metabolic demand on the neuron to maintain this elongated cellular architecture. Thus, the axon compartment rather than the soma compartment might be more vulnerable to and a better marker of early and subtle metabolic and degenerative changes (Burke and O'Malley, 2013). Recent association of LINGO1 variants with essential tremor is of particular interest, as LINGO1 has potent and negative regulatory influences on axonal extension (Stefansson *et al.*, 2009; Clark *et al.*, 2010; Deng *et al.*, 2012).

The changes in Purkinje cell axon morphology after injury, disease or genetic mutation have been well documented (Hamori and Lakos, 1980; Sotelo, 1990; Takahashi *et al.*, 1992; Rossi *et al.*, 1995b; Dusart *et al.*, 1999; Sarna and Hawkes, 2011), and involve the formation of torpedoes, followed by a hypertrophy of the axonal recurrent collateral system and terminal axonal sprouting. We set out to thoroughly study the axonal compartment of Purkinje cells in essential tremor, focusing on structural changes such as torpedoes and changes in axonal connectivity. We used 100- $\mu$ m thick tissue sections, which allow greater visualization of the course of Purkinje cell axons in the cerebellar granule cell layer, in order to identify these axonal morphological alterations. *A priori*, we quantified the presence of thickened axons, torpedoes, recurrent collaterals, branched axons, terminal axonal sprouting, arciform axons and the extent of the recurrent collateral plexus in the Purkinje cell layer in essential tremor cases versus controls. Our primary aim was to move beyond our observation of excessive torpedoes in essential tremor as quantified in paraffin sections (Louis *et al.*, 2007), and to conduct more detailed morphological studies of Purkinje cell axons in essential tremor versus control brains. Through this aim, we tested the hypotheses that (i) other axonal changes (aside from torpedoes) would be more common in essential tremor than control brains; and (ii) in

essential tremor, that these changes would be more common in Purkinje cells with torpedoes than in Purkinje cells without torpedoes. For these analyses, we capitalized on the resources of the Essential Tremor Centralized Brain Repository, which stores a large collection of essential tremor brains. We now report our results.

## Materials and methods

### Clinical evaluation

All essential tremor cases were collected prospectively through the Essential Tremor Centralized Brain Repository in the New York Brain Bank, Columbia University Medical Centre; this is a centralized repository of brains from cases with essential tremor living in the United States. Essential tremor cases learn about the Essential Tremor Centralized Brain Repository through several sources, including advertisements on the two main organizational websites (International Essential Tremor Foundation, Tremor Action Network) and through an Essential Tremor Centralized Brain Repository study website. Interested cases, once enrolled, signed a written informed consent form approved by Columbia University Medical Centre Internal Review Board.

Essential tremor diagnoses were carefully assigned using each of the following three sequential methods. First, the vast majority (>95%) of cases were diagnosed clinically with essential tremor by their treating physician; the remaining few were self-diagnosed cases, and included healthcare workers and individuals with strong family history of essential tremor. Second, cases were asked to complete a series of semi-structured clinical questionnaires (demographic data, general medical data including medications, tremor-specific data), which included data on age of onset (i.e. age at first symptoms and signs of essential tremor) and family history information (i.e. reportedly affected relatives). Each case then submitted four standardized hand-drawn Archimedes spirals (two right and two left hand, each drawn on a 8.5  $\times$  11 inch sheet of white paper). These drawings were supplemented with additional clinical information (from clinical records, treating physicians, family members). Essential tremor diagnoses were then confirmed by a senior neurologist specializing in movement disorders (E.D.L.) who used the following criteria: (i) moderate or greater amplitude arm tremor (rating of 2 or higher) in at least one of the submitted Archimedes spirals; (ii) no history of Parkinson's disease or dystonia; and (iii) no other aetiology for tremor (e.g. medications, hyperthyroidism). Third, essential tremor cases then underwent a standardized, videotaped neurological examination, including a detailed assessment of tremor (Louis *et al.*, 2005). The videotape protocol included assessments of postural tremor (two positions), kinetic tremor (five activities with each arm), and intention tremor of the arms, as well as neck, voice and jaw tremors. The videotaped examination also included the motor portion of the Unified Parkinson's Disease Rating Scale, including assessments of speech, facial expression, rest tremor (with arms in four positions: resting in the lap, relaxed at sides while standing, while walking, and while lying down), bradykinesia, posture, arising from a chair, and gait while walking and turning (Louis *et al.*, 2011a). Each videotape was reviewed (E.D.L.) and, based on the questionnaire and videotape data, the diagnosis of essential tremor was re-examined in each case using published diagnostic criteria (moderate or greater amplitude kinetic tremor (tremor rating  $\geq$  2) during three or more activities or a head tremor in the absence of Parkinson's disease or other known causes) (Louis *et al.*, 1997).

Data on lifetime exposure to medications known to cause cerebellar damage (e.g. lithium, diphenylhydantoin, chemotherapeutic agents) were collected. In essential tremor cases, the amount of beer, wine, and liquor were carefully quantified (i.e. the average number of daily drinks of each during adult lifetime). Heavy ethanol use was also defined previously as consumption of an average of four or more standard drinks (15 ml of absolute ethanol) per day for a male, or three or more per day for a female, at any point in their lives (Harasymiw and Bean, 2001; Louis *et al.*, 2007).

Every 6 months, cases completed a follow-up telephone questionnaire, which included a series of screening questions for Parkinson's disease and dystonia; they also submitted four new standardized Archimedes spirals (two right and two left) on 8.5 × 11 inch sheets of paper. A follow-up videotaped neurological examination was performed if any screening question was positive for Parkinson's disease or dystonia, or if the spiral showed signs of micrographia. Between late 2003 and the end of 2011, 86 essential tremor cases died and their brains were prospectively collected.

Available normal elderly control brains ( $n = 25$ ) were control subjects from the New York Brain Bank, derived from the Alzheimer's Disease Research Centre and the Washington Heights Inwood Columbia Ageing Project; they were free of clinical diagnoses of Alzheimer's disease, essential tremor or Parkinson's disease and without neuropathological diagnoses of neurodegenerative disease. We selected controls that were elderly ( $\geq 60$  years of age) to lessen the disparity between case and selected control ages. The New York Brain Bank operates under approval of the Institutional Review Board of Columbia University Medical Centre. Additional normal controls were obtained from the Harvard Brain Tissue Resource Centre (McLean Hospital, Belmont, MA) ( $n = 14$ ).

## Neuropathological assessment

All brains were well characterized at the New York Brain Bank, which operates under the approval of the Columbia University Medical Centre Institutional Review Board. Each brain underwent a comprehensive neuropathological assessment for determination of pathological findings (nybb.hs.columbia.edu). Standardized measurement of brain weight (g) and post-mortem interval (hours between death and placement of brain in a cold room or upon ice) were recorded. All brains underwent Braak and Braak Alzheimer's disease staging for neurofibrillary tangles (Braak *et al.*, 2006), Braak Parkinson's disease staging of Lewy bodies (Braak *et al.*, 2003), and Consortium to Establish a Registry for Alzheimer's disease (CERAD) ratings for neuritic plaques (Mirra, 1997). Similar assessments took place at the Harvard Brain Tissue Resource Centre.

Blocks were taken from standardized brain regions and embedded in paraffin; 7- $\mu$ m thick sections were stained with Luxol fast blue counterstained with haematoxylin and eosin (LH&E) (Louis *et al.*, 2007; Vonsattel *et al.*, 2008). Additional sections from selected blocks were stained with modified Bielschowsky silver stain, and others, with antibodies directed against  $\alpha$ -synuclein (1:40, Leica), amyloid- $\beta$  (1:400, Biocare Medical), hyperphosphorylated tau (AT8) (1:200, ThermoScientific), and glial fibrillary acidic protein (GFAP) (Ventana) proteins. Selected blocks were stained with antibodies directed against ubiquitinated proteins (1:300, Dako).

A standard 3 × 20 × 25 mm parasagittal, formalin-fixed, tissue block was also harvested from the neocerebellum (Louis *et al.*, 2007); the block included the cerebellar cortex, white matter and dentate nucleus. A senior neuropathologist (P.L.F.) who was blinded to all clinical information counted torpedoes throughout one entire LH&E 7- $\mu$ m thick section and, when available, a Bielschowsky stained 7- $\mu$ m

thick section. Purkinje cells were counted and averaged from 15 × 100 fields (LH&E) (Louis *et al.*, 2007). As described (Erickson-Davis *et al.*, 2010), a semiquantitative (0–3) rating of the appearance of the basket cell plexus surrounding Purkinje cell bodies throughout Bielschowsky preparations was carried out by the same neuropathologist (P.L.F.) and included the following ratings: 0 (few, or no discernible processes); 1 (sparse number of processes); 2 (moderate number of processes); and 3 (dense tangle of processes). In some instances, the rater used intermediate values (0.5, 1.5, and 2.5).

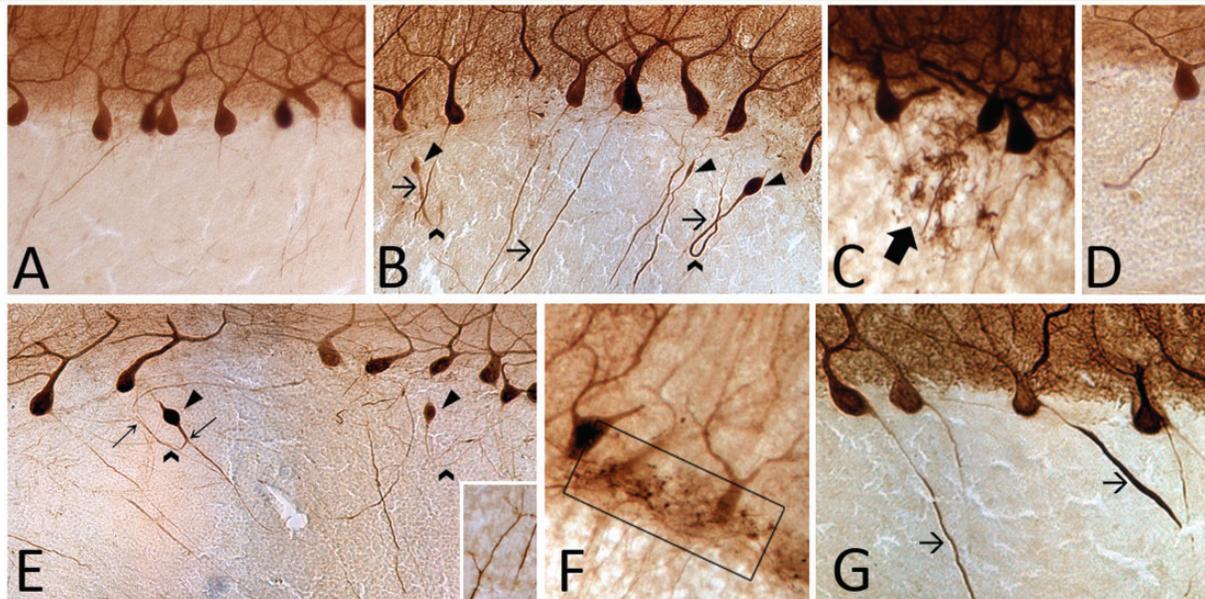
## Calbindin D<sub>28k</sub> immunohistochemistry

Calbindin immunohistochemistry was used for these analyses as it is specifically expressed by Purkinje cells in cerebellum, in contrast to the Bielschowsky silver stain, a less specific method, which stains many axons and other structures in the cerebellar cortex. Formalin-fixed cerebellar cortical tissue from standard 0.3 cm parasagittal slices was embedded in agarose and tissue sections prepared with a vibratome (Ted Pella, Inc). Free-floating 100- $\mu$ m thick neocerebellar sections were heated at 37°C for 10 min in 20  $\mu$ g/ml Proteinase K (Roche Applied Science) in 10 mM Tris, 0.1 mM EDTA, pH 8, followed by 1% hydrogen peroxide in PBS for 30 min and serum blocking solution [10% normal goat serum, 1% IgG-free bovine serum albumin (Jackson ImmunoResearch), 1% Triton<sup>TM</sup>X-100, in PBS] for 1 h. Rabbit polyclonal anti-calbindin D<sub>28k</sub> (1:1000, Swant, Marly) was applied overnight at 4°C in antibody diluent (1% IgG-free bovine serum albumin, 1% Triton<sup>TM</sup>X-100 in PBS). Secondary antibody [1:200, 2 h, biotin-SP goat-anti-rabbit (Fisher Scientific)], followed by streptavidin-horseradish peroxidase (1:200, 1 h, AbD Serotec, for biotinylated antibodies) was developed with 3,3' diaminobenzidine Chromogen Solution (Dako).

## Calbindin D<sub>28k</sub> immunohistochemical quantification method

For each brain, three vibratome sections of neocerebellum were stained with calbindin D<sub>28k</sub> and images were obtained using a Zeiss Axioplan 2 microscope fit with Axiocam HR digital camera ( $\times 10$  objective lens). Between 15 and 50 pictures were taken by a clinically-blinded, trained technician in all areas where the molecular, granule and Purkinje cell layers were well stained, with preference given to areas in which the folia were straight rather than to peaks and troughs. Using a random digit table, 10 images were then randomly selected for analysis.

The same technician then counted Purkinje cell axonal features in all 10 sections; these features, including changes in both shape and connectivity (Fig. 1), have all been described in other studies of Purkinje cell axonal pathology (Chan-Palay, 1971; Dusart and Sotelo, 1994; Rossi *et al.*, 2006). Changes in axonal shape included thickened axonal profiles (axons at least double the width of other apparently normal axons) and torpedoes (ovoid axonal swellings). Changes in connectivity included axon recurrent collaterals (with at least a 90° turn back towards the Purkinje cell layer from their initial trajectory), the appearance of branching (any axon with at least one branch point; multiple bifurcations on the same axon were not separately counted), and terminal axonal sprouting (the presence of a frayed terminal axonal region, often with a kinky appearance). Arciform axons were axons that were more gradually curving back towards the Purkinje cell layer with a changing trajectory that was  $< 90^\circ$ . Mechanistically, their relation to axon recurrent collaterals is not clear. The technician also assessed the presence of these features in Purkinje cell axons with



**Figure 1** Calbindin immunohistochemistry on 100- $\mu$ m cerebellar neocortex sections,  $\times 100$ . (A) Normal control brain with thin visible axonal profiles. (B) Three torpedoes (arrow heads), two of which are on axons with recurrent collaterals (carets). Several thickened axonal profiles are shown (short arrows). (C) Terminal axonal sprouting (thick arrow). (D) Arciform axon profile; profile is also thickened. (E) Torpedoes (arrow heads) with axonal recurrent collaterals (carets) and axonal branching (long arrows and inset). (F) A visible portion of the recurrent collateral plexus at the Purkinje cell layer (boxed). (G) Thickened axonal profiles (short arrows).

torpedoes versus in Purkinje cell axons without torpedoes. The total length of the Purkinje cell layer was measured in all 10 images analysed. The raw counts of Purkinje cell axonal features were normalized to the total length of the Purkinje cell layer length. The length of the Purkinje cell layer with a visible recurrent collateral plexus was expressed as a percentage of the total Purkinje cell layer length.

## Statistical analyses

Statistical analyses were performed in SPSS (version 19.0). Clinical and post-mortem findings were compared in cases and controls using Student's *t*-tests, chi-square tests, and non-parametric tests (Mann-Whitney test), when required. In essential tremor cases, we also examined whether Purkinje cells with torpedoes were more likely to have additional axonal pathologies than Purkinje cells without torpedoes. Pearson's correlation coefficients were used to assess linear correlations, and Spearman's correlation coefficients for non-linear correlations. In one analysis, we stratified our data based on age of onset ( $>40$  and  $\leq 40$  years); this cut point was chosen *a priori* based on published data showing a bimodal distribution in essential tremor, with the trough located at  $\sim 40$  years of age (Louis and Dogu, 2007).

## Final sample

Among the 86 essential tremor brains, tissue was excluded on 31 essential tremor cases who had additional neurodegenerative disorders diagnosed on post-mortem examination (e.g. progressive supranuclear palsy), had Lewy bodies, or had other abnormalities (intranuclear inclusions), leaving 55 essential tremor cases. Calbindin immunohistochemistry was successful on 49 of 55 essential tremor cases and 39 controls; this was our final sample. Forty-one (83.7%) of 49 essential tremor cases and 31 (79.5%) of 39 controls post-dated an earlier

report (Louis *et al.*, 2007), which did not include the primary analyses we report here.

## Results

Cases and controls had similar brain weights, Braak Alzheimer's disease stage and CERAD plaque scores, but cases had a shorter post-mortem interval and a larger proportion were female (Table 1). While cases and controls were both of advanced age, cases were older than controls (Table 1). As expected, cases had higher torpedo counts [ $P < 0.001$  (LH&E) and  $P < 0.001$  (Bielschowsky)], lower Purkinje cell counts ( $P = 0.001$ ) and higher basket cell axonal plexus density ( $P = 0.015$ ) than controls (Table 1).

Essential tremor cases had more changes in axonal shape (thickened axonal profiles and torpedoes) than controls ( $P = 0.006$  and  $P = 0.038$ , respectively, Table 2, Figs 1 and 2). Cases also had more changes in nearly all measures of axonal connectivity than controls (Table 2, Figs 1 and 2); for five of seven comparisons,  $P < 0.05$ . For two comparisons, the *P*-values were 0.065 and 0.097. Some Purkinje cell axons showed changes in both shape and connectivity, but each type of change was counted independently.

Although cases and controls were both of advanced age, cases were older than controls; also, the two groups differed by gender (Table 1). In a sensitivity analysis, we age- and gender-matched cases and controls. This analysis resulted in a smaller number of cases ( $n = 35$ ) and controls ( $n = 21$ ) who did not differ to a significant degree by either age ( $84.6 \pm 5.6$  versus  $81.5 \pm 8.4$  years) or gender [19 (54.3%) female and 15 (46.9%) male]. Despite a

**Table 1 Clinical and post-mortem features of 49 essential tremor cases and 39 controls**

	ET Cases	Controls	Significance
Age (years)	87.8 ± 7.1	77.4 ± 12.0	<i>P</i> < 0.001 <sup>a</sup>
Female gender	33 (67.3)	16 (41.0)	<i>P</i> = 0.01 <sup>b</sup>
Tremor duration (years)	45.6 ± 23.3	Not applicable	Not applicable
Tremor onset (years)	41.9 ± 22.6 <sup>c</sup>	Not applicable	Not applicable
PMI (h)	3.0 ± 3.2 (2.3)	11.6 ± 13.4 (5.6)	<i>P</i> < 0.001 <sup>c</sup>
Brain weight (g)	1208 ± 153	1236 ± 197	<i>P</i> = 0.48 <sup>a</sup>
CERAD plaque score <sup>f</sup>			<i>P</i> = 0.45 <sup>b</sup>
0	16 (41.0)	14 (58.3)	
A	14 (35.9)	7 (29.2)	
B	7 (17.9)	3 (12.5)	
C	2 (5.1)	0 (0.0)	
Braak AD stage <sup>f</sup>			<i>P</i> = 1.00 <sup>d</sup>
0–4	35 (97.2)	29 (100)	
5 or 6	1 (2.8)	0 (0.0)	
Torpedo count (LH&E) <sup>f, g</sup>	13.8 ± 13.4 (9)	6.3 ± 8.6 (4)	<i>P</i> < 0.001 <sup>c</sup>
Torpedo count (Bielschowsky) <sup>f, g</sup>	25.0 ± 27.7 (15)	9.2 ± 9.5 (6)	<i>P</i> < 0.001 <sup>c</sup>
Purkinje cell count (LH&E) <sup>f, g</sup>	6.6 ± 1.7 (6.6)	9.9 ± 3.1 (11.6)	<i>P</i> < 0.001 <sup>c</sup>
Basket cell axonal plexus density <sup>f, g</sup>	2.0 ± 0.8 (2.0)	1.4 ± 0.7 (1.25)	<i>P</i> = 0.015 <sup>c</sup>

AD = Alzheimer's disease; CERAD = Consortium to Establish a Registry for Alzheimer's disease; PMI = post-mortem interval; ET = essential tremor.

All values are mean ± standard deviation (median) or number (percentage).

<sup>a</sup>Student's *t*-test.

<sup>b</sup>Chi-square test.

<sup>c</sup>Mann-Whitney test.

<sup>d</sup>Fisher's Exact Test.

<sup>e</sup>Age of onset was ≤ 65 years in all except five cases.

<sup>f</sup>Data not available on all essential tremor cases and all controls.

<sup>g</sup>In a multivariate logistic regression model (dependent variable = essential tremor case versus control), the case-control difference persisted after adjusted for age at death.

**Table 2 Axonal changes in 49 essential tremor cases and 39 controls**

	ET Cases	Controls	Significance
Changes in axonal shape			
Thickened axonal profiles	0.55 ± 0.71 (0.35)	0.35 ± 0.55 (0.17)	<i>P</i> = 0.006 <sup>a</sup>
Torpedoes	1.09 ± 0.74 (0.94)	0.76 ± 0.55 (0.57)	<i>P</i> = 0.038 <sup>a</sup>
Changes in axonal connectivity			
Axonal recurrent collaterals	0.96 ± 0.79 (0.74)	0.44 ± 0.49 (0.32)	<i>P</i> < 0.001 <sup>a</sup>
Axonal branching	0.33 ± 0.38 (0.23)	0.13 ± 0.19 (0.08)	<i>P</i> < 0.001 <sup>a</sup>
Terminal axonal sprouting	0.19 ± 0.22 (0.10)	0.07 ± 0.14 (0.00)	<i>P</i> < 0.001 <sup>a</sup>
Extent of Purkinje cell axon collateral plexus <sup>c</sup>	15.33 ± 9.71	11.95 ± 8.77	<i>P</i> = 0.097 <sup>b</sup>
Arciform axons	0.08 ± 0.11 (0.00)	0.04 ± 0.07 (0.00)	<i>P</i> = 0.065 <sup>a</sup>

ET = essential tremor.

All values are mean ± standard deviation (median).

<sup>a</sup>Mann-Whitney test.

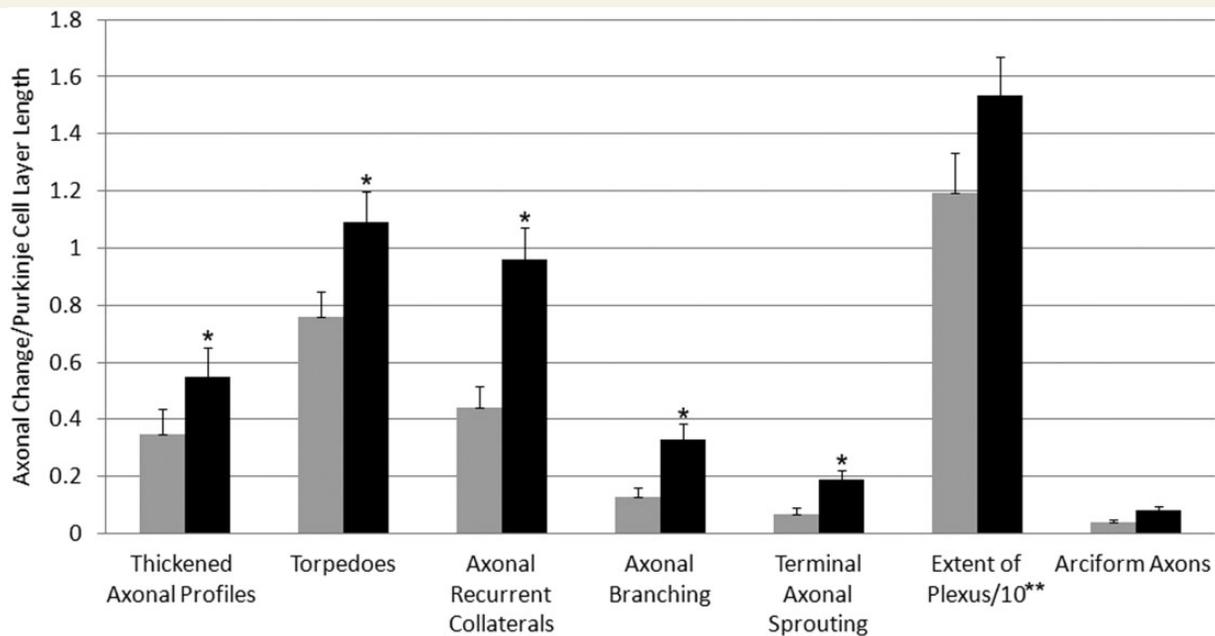
<sup>b</sup>Student's *t*-test.

<sup>c</sup>The extent of the Purkinje cell axon collateral plexus = (the length of the portion of the Purkinje cell layer with a visible recurrent collateral plexus/the length of the Purkinje cell layer) × 100. It is expressed as a percentage ± standard deviation.

~25% reduction in study power, the case-control differences persisted (Table 3); indeed, for five of seven comparisons, *P* < 0.05.

Three essential tremor cases were heavy ethanol users and three others had been exposed to medications known to cause cerebellar damage. Their axonal pathology was similar to that of the essential tremor cases without these exposures (though statistical comparisons were precluded due to the small sample size). Exclusion of these six essential tremor cases from the

analyses did not change any of the case-control differences presented in Tables 2 and 3 (data not shown), indicating that these exposures were not relevant to the current analyses. Furthermore, in essential tremor cases, beer, wine and liquor consumption did not correlate with any of our measures of axonal change; for example, the torpedo count did not correlate with daily beer consumption (Spearman's *r* = -0.01, *P* = 0.92), daily wine consumption (Spearman's *r* = 0.01,



**Figure 2** Comparison of axonal pathologies between essential tremor cases and controls. Bars represent one standard error. \* $P < 0.05$ . \*\*The extent of plexus is expressed as the per cent of the length of the Purkinje cell layer covered by a visible recurrent collateral plexus; it is divided by 10 for the purposes of graphical display.

**Table 3** Axonal changes in 35 essential tremor cases and 21 controls

	ET cases	Controls	Significance
Changes in axonal shape			
Thickened axonal profiles	0.42 ± 0.31 (0.35)	0.36 ± 0.53 (0.23)	$P = 0.056^a$
Torpedoes	1.11 ± 0.66 (0.99)	0.77 ± 0.51 (0.58)	$P = 0.025^a$
Changes in axonal connectivity			
Axonal recurrent collaterals	0.98 ± 0.74 (0.77)	0.43 ± 0.50 (0.28)	$P < 0.001^a$
Axonal branching	0.27 ± 0.22 (0.23)	0.12 ± 0.19 (0.08)	$P = 0.001^a$
Terminal axonal sprouting	0.15 ± 0.14 (0.10)	0.06 ± 0.12 (0.00)	$P < 0.001^a$
Extent of Purkinje cell axon collateral plexus <sup>c</sup>	16.00 ± 9.64	11.38 ± 8.38	$P = 0.04^b$
Arciform axons	0.08 ± 0.12 (0.00)	0.04 ± 0.07 (0.00)	$P = 0.12^a$

All values are mean ± standard deviation (median). ET = essential tremor.

<sup>a</sup>Mann-Whitney test.

<sup>b</sup>Student's *t*-test.

<sup>c</sup>The extent of the Purkinje cell axon collateral plexus = (the length of the portion of the Purkinje cell layer with a visible recurrent collateral plexus/the length of the Purkinje cell layer) × 100. It is expressed as a percentage ± standard deviation.

$P = 0.96$ ), or daily liquor consumption (Spearman's  $r = -0.11$ ,  $P = 0.59$ ).

In essential tremor cases, we examined whether Purkinje cells with torpedoes were more likely to have additional axonal changes (thickened profiles, recurrent collaterals, branching, terminal sprouting and arciform axons) than Purkinje cells without torpedoes (Table 4). In Purkinje cell axons with torpedoes, thickened axonal profiles, axonal recurrent collaterals and branched axons were increased 5.3-fold, 2.9-fold and 5.0-fold versus Purkinje cell axons without torpedoes ( $P$ -values  $< 0.001$ , Table 4). The number of Purkinje cells with arciform axons was approximately twice as high in Purkinje cells with torpedoes than in Purkinje cells without torpedoes, yet the difference was not statistically

significant (Table 4). Terminal sprouting was seen in a similar proportion of Purkinje cells with and without torpedoes (Table 4).

The correlations between each measure of axonal pathology were significant in nearly all comparisons (Table 5); the correlations between the number of recurrent collaterals and torpedoes, the number of recurrent collaterals and axonal branching, and axonal branching and terminal axonal sprouting were particularly robust (Spearman's  $r = 0.579$ ,  $P < 0.001$ , Spearman's  $r = 0.602$ ,  $P < 0.001$ , Spearman's  $r = 0.544$ ,  $P < 0.001$ , respectively, Table 5). The calbindin-based torpedo counts (Table 4) were correlated with both the LH&E-based torpedo counts (Spearman's  $r = 0.43$ ,  $P < 0.001$ ) and the Bielschowsky-based torpedo counts (Spearman's  $r = 0.41$ ,  $P = 0.002$ ) in paraffin sections (Table 1).

**Table 4 Association<sup>a</sup> between presence of a torpedo and presence of other axonal pathology**

	Purkinje cells with torpedoes	Purkinje cells without torpedoes	Significance
Percentage of Purkinje cells with a thickened axonal profile	21.6 ± 18.7	4.1 ± 8.7	$P < 0.001^b$
Percentage of Purkinje cells with an axonal recurrent collateral	21.4 ± 17.9	7.26 ± 6.9	$P < 0.001^b$
Percentage of Purkinje cells with axonal branching	12.68 ± 18.10	2.56 ± 4.25	$P < 0.001^b$
Percentage of Purkinje cells with a terminal axonal sprout	1.96 ± 7.72	1.84 ± 2.41	$P = 0.914^b$
Percentage of Purkinje cells with an arciform axon	1.35 ± 0.62	0.58 ± 0.13	$P = 0.216^b$

Values represent mean percentage ± standard deviation

<sup>a</sup>In individual Purkinje cells in 49 essential tremor cases.

<sup>b</sup>Paired sample *t*-test.

**Table 5 Correlations between measures of axonal pathology**

	Torpedoes	Recurrent collaterals	Axonal branching	Terminal axonal sprouting	Extent of Purkinje cell axon collateral plexus	Arciform axon
Thickened axonal profiles	$r = 0.423$ $P < 0.001$	$r = 0.405$ $P < 0.001$	$r = 0.477$ $P < 0.001$	$r = 0.489$ $P < 0.001$	$r = 0.384$ $P < 0.001$	$r = 0.267$ $P = 0.013$
Torpedoes		$r = 0.579$ $P < 0.001$	$r = 0.446$ $P < 0.001$	$r = 0.212$ $P = 0.050$	$r = 0.183$ $P = 0.092$	$r = 0.349$ $P = 0.001$
Recurrent collaterals			$r = 0.602$ $P < 0.001$	$r = 0.510$ $P < 0.001$	$r = 0.441$ $P < 0.001$	$r = 0.379$ $P < 0.001$
Axonal branching				$r = 0.544$ $P < 0.001$	$r = 0.386$ $P < 0.001$	$r = 0.301$ $P = 0.005$
Terminal axonal sprouting					$r = 0.434$ $P < 0.001$	$r = 0.226$ $P = 0.036$
Extent of Purkinje cell axon collateral plexus						$r = 0.264$ $P = 0.014$

*r* = Spearman's correlation coefficient

To examine the possible confounding effects of age, gender and post-mortem interval, we performed additional analyses. In our controls, there was no relationship between increasing age and any of the five measures of axonal pathology that differed between cases and controls (Spearman's *r*-values ranging from 0.007–0.21 and *P*-values ranging from 0.19–0.96). Similarly, there was no correlation in terms of these five measures and post-mortem interval (Spearman's *r*-values ranging from 0.04–0.26 and *P*-values ranging from 0.25–0.86). Gender was not associated with any of these five measures (Mann-Whitney test, *P*-values ranging from 0.19–0.81). Therefore, these variables could not have been confounders, and any case-control differences in these variables were not relevant to these analyses.

We stratified essential tremor cases into two groups based on age of onset [young age of onset ≤ 40 years (*n* = 22); older age of onset > 40 years (*n* = 24), information not available in three cases] and examined the measures of axonal pathology and their correlations with tremor duration in each stratum. All quantified changes in axonal connectivity were of similar magnitude in both age of onset strata (data not shown). For changes in axonal shape, thickened axonal profiles and axonal torpedoes were marginally higher in older onset versus younger onset cases (Mann-Whitney tests,  $P = 0.084$  and  $P = 0.069$ ). Regardless of these differences, thickened axonal profiles and

torpedoes in younger onset cases were still significantly increased versus controls (data not shown). In cases with younger age of onset, there was no correlation between any of these axonal measures and tremor duration, whereas in older onset cases there was a significant correlation between tremor duration and most measures of axonal pathology, including thickened axonal profiles, torpedoes, axonal recurrent collaterals, axonal branching, and terminal axonal sprouting (Table 6).

We also analysed whether any of the measures of axonal pathology in controls differed by source (Columbia controls versus Harvard controls), and they did not [all *P*-values > 0.615 except for axonal branching, in which the *P*-value for the comparison was 0.12 (Mann-Whitney tests)].

## Discussion

Recent post-mortem studies of essential tremor brains have revealed changes in the cerebellum, including the presence of torpedoes (Louis *et al.*, 2007), basket-cell process hypertrophy (Erickson-Davis *et al.*, 2010), Purkinje cell loss (Axelrad *et al.*, 2008; Shill *et al.*, 2008), and Bergmann gliosis (Louis *et al.*, 2006; Shill *et al.*, 2008). We have also demonstrated an increase in the number of LH&E and Bielschowsky-stained Purkinje cell

**Table 6** Correlations between axonal pathology and tremor duration in essential tremor cases stratified into two age of onset groups

	Age of onset ≤ 40	Age of onset > 40
Thickened axonal profiles	$r = -0.004$ $P = 0.986$	$r = 0.514$ $P = 0.010$
Torpedoes	$r = 0.069$ $P = 0.761$	$r = 0.535$ $P = 0.007$
Axonal recurrent collaterals	$r = -0.096$ $P = 0.672$	$r = 0.405$ $P = 0.050$
Axonal branching	$r = -0.073$ $P = 0.754$	$r = 0.459$ $P = 0.028$
Terminal axonal sprouting	$r = 0.209$ $P = 0.363$	$r = 0.628$ $P = 0.001$
Extent of Purkinje cell axon collateral plexus	$r = 0.042$ $P = 0.858$	$r = -0.028$ $P = 0.900$
Arciform axons	$r = -0.004$ $P = 0.986$	$r = 0.092$ $P = 0.677$

$r$  = Spearman's correlation coefficient

dendritic swellings in essential tremor cases than controls in a study of the dendritic compartment (Yu *et al.*, 2012). The axonal compartment is particularly susceptible to insult, given the metabolic challenge in maintaining its long structure, and it has increasingly become the focus of work on neuronal dysfunction (Kim *et al.*, 2012). Our detailed examination of the Purkinje cell axon has revealed a related series of more extensive axonal changes, further supporting the notion that the maintenance of Purkinje cell function is challenged in essential tremor.

In normal mammalian cerebellum, the main axon of the Purkinje cell courses in the parasagittal plane through the granule layer and into the white matter, synapsing in the deep cerebellar nuclei (Larsell and Jansen, 1972; Palay and Chan-Palay, 1974). Each axon has one, or occasionally multiple, thin recurrent collaterals that travel at an acute angle after the first node of Ranvier. They form the infraganglionic plexus in the upper granule cell and Purkinje cell layers, and the supraganglionic plexus in the lower molecular layer, though these collaterals are rarely visualized by calbindin immunostaining in thin paraffin sections. These collaterals ultimately synapse on Purkinje cell soma and dendrites, basket cell processes, Golgi cells, and Lugaro cells (Chan-Palay, 1971; Hawkes and Leclerc, 1989; O'Donoghue and Bishop, 1990).

The response of Purkinje cells to stress, not typical of that seen in most neurons, has been documented in axotomies and other human diseases of cerebellar dysfunction, and represents a partly degenerative and partly compensatory response, to a variety of cellular injuries (Ramon y Cajal, 1928; Rossi *et al.*, 1995b). This process involves the initial formation of torpedoes, hypertrophy of the initial neuritic segment and recurrent collateral system, and progressive atrophy of the distal axon. In contrast to most CNS neurons, injured Purkinje cells often still survive despite this profound inability to regenerate their severed axon (Rossi *et al.*, 1995a; Bravin *et al.*, 1997; Dusart *et al.*, 1999), and show a vigorous and progressive inclination towards sprouting both along the intracortical segment and the distal stump (Chan-Palay, 1971; Dusart *et al.*, 1999; Carulli *et al.*, 2004; Rossi *et al.*, 2006).

These axonal changes may represent the cell's attempt to access trophic factors by establishing additional connections with Purkinje cells or other granule layer neurons. The strong correlation seen in this study among the various Purkinje cell axon morphological changes within a brain, as well as their greater tendency to occur on cells with torpedoes, indicates that they are part of a related biological process.

A distinctive aspect of the injury response in Purkinje cells is that these axonal changes occur without a significant cell body reaction, which occurs in most injured CNS neurons and leads to upregulation of injury- and growth-associated genes that initially sustain compensatory or regenerative attempts, but eventually become regressive leading to atrophy or cell death (Rossi *et al.*, 1995a; Bravin *et al.*, 1997). Recent studies demonstrate that extrinsic cues from myelin-associated molecules, such as Nogo A, exert a constitutive retrograde inhibitory signal that dampens both this cell body response and the spontaneous inclination for axonal sprouting in Purkinje cells (Gianola and Rossi, 2004, 2005). Blocking Nogo A with neutralizing antibodies in cerebellum induces both injury/growth associated molecules and profuse sprouting along the intracortical segment of Purkinje cell axons in the recurrent collateral plexus (Zagrebelsky *et al.*, 1998; Foscarin *et al.*, 2009). Long-term injured Purkinje cell axons tend to lose myelin along their intracortical course, where some sprouting occurs (Gianola and Rossi, 2002). Altogether, these findings suggest that neurite-myelin interactions prevent aberrant growth of Purkinje cell axons and stabilize intracortical connectivity. Nogo A in myelin binds to a receptor complex in the underlying axonal membrane that includes Nogo receptor (NgR1), LINGO1, and p75 neurotrophin receptor or TROY, which then activates RhoA as a negative regulator for neurite outgrowth (Yamashita *et al.*, 2005). Interestingly, several genome-wide association studies have identified LINGO1 polymorphisms as a risk factor for essential tremor (Stefansson *et al.*, 2009). We have recently documented that LINGO1 expression is regionally increased in the cerebellum and is enriched in the pinceau structure around the Purkinje cell axon initial segment formed by distal processes of basket neurons (Kuo *et al.*, 2013). Furthermore, LINGO1 immunopositive pinceau more commonly showed a longer morphology in essential tremor cerebellum, and elongated pinceau strongly correlated with number of Purkinje cell torpedoes and basket plexus rating. These various myelin and LINGO1-related functions may provide important clues for understanding the regulation of Purkinje cell function and maintenance of axonal morphology, in both normal cerebellum and disease states such as essential tremor.

Although many of these Purkinje cell axonal changes can be seen in other disorders, the extent of these alterations, as well as the relationship between these pathological changes is likely to differ in essential tremor versus other cerebellar disorders. Axelrad *et al.*'s (2008) finding of reduced Purkinje cell linear density in essential tremor demonstrated a less severe reduction of Purkinje cells than seen in multiple system atrophy or spinocerebellar ataxias, where a greater number of Purkinje cells are lost (Bebin *et al.*, 1990; Kume *et al.*, 1991; Seidel *et al.*, 2012). Kuo *et al.* (2011) found that Purkinje cell heterotopias were elevated in essential tremor brains, but not in those of progressive supranuclear palsy, another disorder involving the cerebellum. In a

semi-quantitative analysis of basket-cell complexity, Erickson-Davis *et al.* (2010) found that the proportion of cases with higher complexity was significantly more in essential tremor than in cases of progressive supranuclear palsy, or Parkinson's disease and diffuse Lewy body disease. Additionally, Kuo *et al.* (2013) studied the basket cell–Purkinje cell interface; the distal basket cell axonal processes form a 'pinneau'-shaped structure around the Purkinje cell axonal initial segment. Essential tremor brains were distinctive in that a greater proportion of the pinneaus were of more extreme length (length >50  $\mu\text{m}$ ), while multiple system atrophy and spinocerebellar ataxia brains had a proportion similar to controls (Kuo *et al.*, 2013). These facts point to a related but possibly distinct process of cerebellar alterations in essential tremor; this requires additional exploration.

Although symptomatic loss of function in neurodegenerative diseases is frequently attributed to the loss of neurons, there may actually be events before this neuronal loss that result in disease manifestations. Studies in spinocerebellar ataxia 2 mouse models have shown that changes in gene expression and Purkinje cell firing actually coincide with the onset of motor behaviour changes, rather than the loss of neurons (Hansen *et al.*, 2013). It is likely that a unique manifestation of these subtle Purkinje cell changes is more closely linked with the onset of essential tremor. The changes in axonal connectivity found in this study, as well as Bergmann gliosis and basket cell hypertrophy demonstrated in previous post-mortem studies, indicate a broader rewiring of the cerebellum in the essential tremor brain (Erickson-Davis *et al.*, 2010; Kuo *et al.*, 2011) that may precede or coincide with neuronal loss.

Recent studies in essential tremor (Louis *et al.*, 2009a, 2012) have found that patients with an onset of symptoms later in life have more rapidly progressing symptoms and more degenerative or severe pathology. In a study examining data from a referral centre and the population, Louis and Dogu (2007) showed that age of diagnosis follows a bimodal distribution, with the trough located at ~40 years of age. We stratified our data based on the age of onset (>40 and  $\leq$ 40) and found that in the cases with older onset of symptoms, the extent of axonal pathology was correlated with the duration of the tremor, indicating that these changes are progressive from the point of tremor onset.

The higher proportion of females among our sample of essential tremor cases does not reflect a difference in the prevalence of essential tremor in males and females; the prevalence of essential tremor is similar across gender (Louis and Ferreira, 2010). Rather, it reflects the advanced age of the essential tremor sample and the longer lifespan of females than males. Indeed, in our sensitivity analysis, in which cases and controls were first age-matched, the difference in gender was no longer significant.

Although we were limited in our ability to rigidly match for gender, post-mortem interval and age for the entire sample, further analyses showed that this did not significantly affect our results. These analyses also included a sensitivity analysis that resulted in an age- and gender-matched sample; the case-control differences persisted even with a 25% reduction in sample size. This study provides the largest reports to date on human post-mortem tissue in essential tremor, enabling the detection of case-control differences in the axonal pathologies under consideration. This detailed quantitative analysis of Purkinje cell axons in the

essential tremor cerebellum expands the current base of information available detailing the response of these unique cells to injury. Our study documents that this response/plasticity may be a feature of the Purkinje cell response in essential tremor, and may additionally contribute to disease pathogenesis. Additional study of these morphological changes in patients with other cerebellar degenerations and in patients with other tremor disorders (e.g. Parkinson's disease) would be of further interest. In addition, though study of the relationship between changes in the Purkinje cell axonal compartment and those in the dendritic compartment would be of interest, given the high density of calbindin-labelled Purkinje cell dendrites in the molecular layer and the tendency to stain overlapping Purkinje cell dendritic arbors, it is often difficult to determine which arbor belongs to which particular Purkinje cell body/axon.

In summary, changes associated with Purkinje cell injury—torpedoes, fusiform axonal thickenings, increased axonal collateralization, branching, and sprouting—are visible in the essential tremor cerebellum to a much greater extent than that seen in well-characterized control tissue. Additionally, these changes are highly correlated and the presence of multiple abnormalities on one axon shaft is extremely common, indicating a related and possibly progressive biological process, as the cell attempts to reorganize in order to preserve connections and prevent cell death. Since this response has been documented in other instances, knowledge of the general mechanism by which these axonal changes occur and the relative extent of these changes in essential tremor as compared with other cerebellar disorders would be helpful in discerning the causes of essential tremor as well as whether this plasticity of the Purkinje cell axon is partially neuroprotective or ultimately ineffective at slowing further cellular changes.

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