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Cellular Density in the Cerebellar Molecular Layer in Essential Tremor, Spinocerebellar Ataxia, and Controls

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Abstract

Background—It would be useful to identify additional postmortem markers of Purkinje cell loss in essential tremor (ET). In hereditary cerebellar ataxia, Purkinje cell loss has been reported to result in a secondary increase in the density of the remaining cell populations in the cerebellar molecular layer. However, this phenomenon has not been studied in ET. We quantified cerebellar molecular layer cellular density in 15 ET cases, 15 controls, and 7 spinocerebellar ataxia (SCA) cases (2:2:1 ratio).

Methods—A standard neocerebellar tissue block was stained with Luxol fast blue Hematoxylin & Eosin. Within 5 selected fields, cell soma (e.g., stellate, basket, and glial cell bodies) were counted. Cellular density was the number of cells/cm².

Results—The Purkinje cell count differed across the three groups ($p < 0.001$), with the highest counts in controls, intermediate counts in ET cases and lowest counts in SCA cases. ET cases and controls had similar molecular layer cellular density ($p = 0.79$) but SCA cases had higher values than both groups ($p < 0.01$). A robust inverse correlation between Purkinje cell count and molecular layer cellular density (i.e., brains with more Purkinje cell loss had higher molecular layer cellular density), observed in SCA and controls ($r = -0.55$, $p = 0.008$), was not observed in ET cases.

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Discussion—Although Purkinje cell counts were reduced in ET cases compared to controls, an increase in molecular layer cellular density was not evident in ET. The increase in molecular layer cellular density, observed in SCA cases, may require a more marked loss of PCs than occurs in ET.

Keywords

Essential tremor; Spinocerebellar ataxia; Purkinje cell; Neurodegeneration; Cerebellum; Molecular layer

Introduction

Essential tremor (ET) is widely acknowledged to be one of the most common neurological disorders among adults [1]. There is growing clinical and neuroimaging evidence implicating cerebellar dysfunction in the pathogenesis of ET [2–4]. With recent efforts to perform detailed postmortem neuropathological examinations, a deeper understanding of the anatomic pathology of ET is also emerging. In ET brains, changes occur in the cerebellum [5, 6]. These changes include an abundance of axonal swellings (torpedoes) and other axonal changes (i.e., increased recurrent collaterals and axonal branching), dendritic swellings, changes in the basket cell axonal processes, and a loss of Purkinje cells in some studies [5].

Along with the dense Purkinje cell dendritic arbor, the molecular layer of the cerebellum comprises several cell populations (i.e., predominantly stellate and basket neurons and some glial cells as well). In patients with spinocerebellar ataxia (SCA), loss of Purkinje cells and pruning of the rich Purkinje cell dendritic tree are thought to result in a crowding (i.e., increased density) of remaining cellular populations in the molecular layer [7]. In our postmortem studies of ET, we have been interested in Purkinje cell loss as well as potential markers of such loss. In this study, we asked whether an increased molecular layer cellular density might be present in ET. The current study utilized postmortem tissue from 15 ET cases and 15 age-matched controls as well as 7 available SCA cases (2:2:1 ratio) to quantify the density of cells in the molecular layer of the cerebellum. Our *a priori* hypothesis was that an increased cellular density would be observed in both ET and SCA in comparison with controls.

Methods

This study was conducted at the Essential Tremor Centralized Brain Repository (ETCBR) at the New York Brain Bank (NYBB), Columbia University. The ETCBR is a centralized repository for the prospective collection of brains from ET patients in the United States. Participants signed an informed consent form approved by the Columbia University Medical Center Internal Review Board.

ET diagnoses were assigned using three sequential methods, as described in detail [5]. Every six months, ET cases completed a follow-up telephone questionnaire, which included a series of screening questions for Parkinson's disease (PD) and dystonia. A follow-up in-person evaluation was performed if any screening question was positive for PD or dystonia. During life, demographic and clinical data were collected using semi-structured

questionnaires; heavy ethanol use was identified previously as consumption, on average, of four or more standard drinks (15 ml of absolute ethanol) per day for a man, or three or more per day for a woman, at any time over the course of their lives. Data on lifetime exposure to medications known to lead to cerebellar damage (e.g., lithium, diphenylhydantoin) were collected on ET cases.

Control brains were normal, elderly control subjects from the NYBB, derived from the Alzheimer's Disease Research Center and the Washington Heights Inwood Columbia Aging Project; they were free of clinical diagnoses of Alzheimer's disease (AD), ET, or PD and had no neuropathological diagnoses of neurodegenerative disease. The NYBB operates under approval of the IRB of Columbia University Medical Center.

SCA cases were diagnosed during life based on clinical features (i.e., ataxia and other cerebellar signs) and confirmed by quantification of CAG repeat expansions. Tissue from seven cases with clinical and postmortem diagnoses of SCA (1 SCA-7 and 6 SCA-1 [these six were from Albany, NY - see acknowledgements]) were available for these analyses.

As previously described, all ET and control brains had a complete neuropathological assessment at the NYBB [5]. Brains had standardized measurements of brain weight (grams), postmortem interval (PMI, hours between death and placement of brain in a cold room or upon ice), Braak and Braak AD staging for neurofibrillary tangles [8], Braak PD staging [9], and Consortium to Establish a Registry for AD (CERAD) ratings for neuritic plaques [10]. As described [5, 11], a standard $3 \times 20 \times 25$ mm parasagittal, formalin-fixed, tissue block was harvested from the neocerebellum; 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 and averaged Purkinje cells in fifteen 100x fields in one Luxol fast blue Hematoxylin & Eosin (LH&E) stained section.

From the LH&E stained section, fields were selected for additional analyses. Our pilot study of 5 ET cases and 5 controls compared cellular density counts from 20 vs. 10 vs. 5 fields, finding similar results (correlation coefficient for 5 vs. 10 fields = 0.99, $p < 0.001$; correlation coefficient for 5 vs. 20 fields = 0.98, $p < 0.001$); hence, 5 fields were counted in all subsequent slides. Potential fields were identified using a 20x objective lens by a trained technician (R.J.L.), who was blinded to all clinical information. Within each field a box was drawn in the molecular layer, starting just above the Purkinje cell layer and extending to the pial surface, with a standard width to encompass the microscopic field. The area, height, and length of the box were measured. Within each box, the cell soma (e.g., stellate cell, basket cell and glial cell bodies) were counted. Blood vessels and endothelial cells were not counted. The density was computed as the number of counted cells per centimeters squared. We also measured the height of the molecular layer (in μm) from the base of the Purkinje cell layer to the pial surface.

The technician was trained for two weeks by a senior neuropathologist (P.L.F.) to perform these counts until their agreement was substantial. Our pilot study of 5 ET cases and 5 controls also indicated that the technician had high test-retest reliability (correlation coefficient between two ratings performed 2 weeks apart = 0.93, $p < 0.001$).

Our pre-study sample size calculation, which used our pilot data on 5 ET cases and 5 controls, determined that a sample size of 15 ET cases and 15 controls would power the study to detect as little as a 20% difference between the two groups (assuming power = 80%, $\alpha = 0.05$, and a two-sided test). As we expected the pathological changes in the cerebellum to be more marked in SCA, we calculated that a smaller number of such cases ($n = 7$) would be adequate to detect a 20% or higher difference when compared to controls. Hence, the final sample size calculation required 15 ET cases, 15 controls and 7 SCA cases. Using a random digit table, 15 ET cases were selected from the ETCBR data bank, and 15 controls were age-matched to these 15 ET cases.

Statistical analyses were performed in SPSS (version 21.0). Clinical and postmortem findings were compared across all three diagnostic groups (controls, ET cases, SCA) using analysis of variance (ANOVA) and chi-square tests (χ^2) or nonparametric tests (e.g., Kruskal-Wallis test) when appropriate. If there were differences across the three groups, in post hoc tests, we compared two groups at a time. We assessed the correlation between Purkinje cell counts and molecular layer cellular density using a Pearson's correlation coefficient (r).

Results

There were 15 ET cases, 15 controls and 7 SCA cases. ET cases and controls were similar in age, but as expected, SCA cases were younger than both (Table 1). The groups did not differ significantly by gender. ET cases and controls had similar brain weights, although PMI was shorter in ET cases than controls (Table 1). The Braak and CERAD scores in cases and controls were similar (Table 1). None of the ET cases were heavy ethanol users nor had any been exposed to medications known to lead to cerebellar damage. None of the case or control brains had brainstem or cortical Lewy bodies.

The Purkinje cell count differed across the three groups (Table 1, ANOVA $p < 0.001$), with the highest counts in controls, intermediate counts in ET cases and lowest counts in SCA cases. There was considerable overlap in Purkinje cell counts between ET and controls but also some overlap between ET and SCA (Figure 1).

The molecular layer cellular density differed across the three groups (Table 1, ANOVA $p = 0.01$); post hoc tests revealed that ET cases and controls had similar values but SCA cases had higher values than both of these groups (Table 1 and Figure 1). We also measured the median height of the molecular layer (in μm) and this was similar in controls and ET cases but lower in SCA cases (295.0 μm [controls], 307.0 μm [ET] and 248.3 μm [SCA]), a difference that was marginally significant (Kruskal-Wallis test = 0.086).

In controls, there was no correlation between molecular layer cellular density and age (Pearson's $r = -0.19$, $p = 0.51$) or PMI (Pearson's $r = -0.33$, $p = 0.28$); hence neither age nor PMI were considered as sources of confounding.

There was a robust inverse correlation between Purkinje cell counts and molecular layer cellular density (i.e., brains with more Purkinje cell loss had higher molecular layer cellular density), Pearson's $r = -0.41$, $p = 0.01$ (Figure 1). Interestingly, however, for the ET cases,

the correlation was not negative ($r = 0.09$). When ET cases were removed from the analysis, the correlation between Purkinje cell counts and molecular layer cellular density became even more robust (Pearson's $r = -0.55$, $p = 0.008$).

Discussion

Recent postmortem studies have noted degenerative changes within the cerebellum of patients with ET, with evidence that many of these changes involve the Purkinje cells [5, 11,12]. Therefore, Purkinje cell loss and potential markers of such loss in ET are of particular interest. To our knowledge, no prior studies have quantified molecular layer cellular density in ET cases.

In the current study, molecular layer cellular density was significantly higher in SCA cases than in ET cases and controls. In SCA and controls, there was a robust inverse correlation between Purkinje cell counts and molecular layer cellular density (i.e., brains with more Purkinje cell loss had higher molecular layer cellular density). The presumed basis for these findings is either a loss of Purkinje cells and/or pruning of the rich Purkinje cell dendritic tree in the setting of neurodegeneration. As there was no correlation between molecular layer cellular density and age, the age difference between SCA and controls is not likely to have explained our results.

A second finding was that in ET, while there was a reduction in Purkinje cell counts, the molecular layer cellular density was similar to that of controls, and there was no discernible correlation between Purkinje cell counts and molecular layer cellular density. One possible explanation for this finding is that the degree of Purkinje cell loss in ET, which is less than that seen in SCA, does not result in a significant change in molecular layer cellular density. In other words, it is possible that there is a threshold effect, and more extensive Purkinje cell loss might be required before changes in molecular layer cellular density become evident. In favor of this interpretation is that, as seen in Figure 1D, there was one SCA cases whose Purkinje cell loss was in the ET range. The molecular layer neuronal density of that SCA case was also more within the range of that seen in ET.

A third finding was that while the PC counts in ET and controls overlapped to some extent with one another, the PC counts in ET and SCA overlapped as well. Hence, in this respect, ET cases were intermediate between normal controls and SCA cases. This suggests that ET cases and SCA cases have different patterns of degeneration.

A goal of this study was to identify/develop clinically useful tissue-based biomarkers for ET. The development of diagnostic biomarkers (either pre- or post-mortem) for ET is important, as these are lacking. One important clinical implication of the present findings is that molecular layer neuronal density does not seem to be useful biological marker for the diagnosis of ET on postmortem examination.

This study had limitations. [11] Although cases and controls were carefully matched by age, given the younger age of onset of SCA, age-matching was not feasible. However, there was no correlation between molecular layer cellular density and age, so that this age difference could not have served as a confounder. Also, we recognize that increased cell density in the

molecular layer could reflect both neuronal as well as glial cell populations. The current study did not attempt to distinguish the two. Yet our prior study did not detect significant differences in numbers of Bergman glia between ET cases and controls [11] nor have our immunohistochemical studies using glial fibrillary acidic protein (GFAP) revealed ET case-control differences [12]. The study also had several strengths. First, all counts were performed by a clinically- and diagnostically-blinded technician who performed identical analyses across the three diagnostic groups. Second, the research question we addressed has not been posed before.

In summary, although Purkinje cell counts were reduced in ET cases compared to controls, an increase in molecular layer cellular density was not evident in ET cases. The increase in molecular layer cellular density, observed in SCA cases, may require a more significant loss of PC than occurs in ET.

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References

1. Benito-León J. Essential tremor: one of the most common neurodegenerative diseases? *Neuroepidemiology*. 2011; 36(2):77–78.
2. Bares M, Lungu OV, Husárová I, Gescheidt T. Predictive motor timing performance dissociates between early diseases of the cerebellum and Parkinson's disease. *Cerebellum*. 2010; 9(1):124–135. [PubMed: 19851820]
3. Gitchel GT, Wetzel PA, Baron MS. Slowed saccades and increased square wave jerks in essential tremor. *Tremor Other Hyperkinet Mov (N Y)*. 2013;3.
4. Benito-León J, Alvarez-Linera J, Hernández-Tamames JA, Alonso-Navarro H, Jiménez-Jiménez FJ, Louis ED. Brain structural changes in essential tremor: voxel-based morphometry at 3-Tesla. *J Neurol Sci*. 2009; 287(1-2):138–142. [PubMed: 19717167]
5. Babij R, Lee M, Cortés E, Vonsattel JP, Faust PL, Louis ED. Purkinje cell axonal anatomy: quantifying morphometric changes in essential tremor versus control brains. *Brain*. 2013; 136(Pt 10):3051–3061. [PubMed: 24030953]
6. Grimaldi G, Manto M. Is essential tremor a Purkinjopathy? The role of the cerebellar cortex in its pathogenesis. *Mov Disord*. 2013; 28(13):1759–1761. [PubMed: 24114851]
7. Fratkin, JD.; Vig, PJS. Neuropathology of degenerative ataxias. In: Subramony, SH.; Dürr, A., editors. *Ataxic Disorders, a volume of the Handbook of Clinical Neurology*. Amsterdam: Elsevier Science; 2012. p. 111-125.
8. Braak H, Alafuzoff I, Arzberger T, Kretschmar H, Del Tredici K. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. *Acta Neuropathol*. 2006; 112(4):389–404. [PubMed: 16906426]

9. Braak H, Del Tredici K, Rüb U, de Vos RA, Jansen Steur EN, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging*. 2003; 24(2):197–211. [PubMed: 12498954]
10. Mirra SS. The CERAD neuropathology protocol and consensus recommendations for the postmortem diagnosis of Alzheimer's disease: a commentary. *Neurobiol Aging*. 1997; 18(4 Suppl):S91–S94. [PubMed: 9330994]
11. Louis ED, Faust PL, Vonsattel JP, Honig LS, Rajput A, Robinson CA, et al. Neuropathological changes in essential tremor: 33 cases compared with 21 controls. *Brain*. 2007; 130(Pt 12):3297–3307. [PubMed: 18025031]
12. Kuo SH, Tang G, Louis ED, Ma K, Babij R, Balabat MS, et al. Lingo-1 expression is increased in essential tremor cerebellum and is present in the basket cell pinceau. *Acta Neuropathol*. 2013; 125(6):879–889. [PubMed: 23543187]

- It would be useful to identify postmortem markers of Purkinje cell loss in ET.
- Purkinje cell loss may result in higher cerebellar molecular layer cell density.
- ET cases and controls had similar cell density, but SCA cases had higher density.
- Increased cell density may require more marked Purkinje cell loss than occurs in ET.

Figure 1.

A – C. Molecular layer of the cerebellar cortex showing similar cellular density in a control (A) and an ET case (B), and higher density in an SCA case (C). Luxol fast blue Hematoxylin & Eosin stained section, 20x magnification. D. Purkinje cell count by molecular layer cellular density in controls (blue circles), ET cases (red squares) and SCA cases (green diamonds).

Table 1

Clinical and pathological characteristics of subjects

Characteristic	Controls (n = 15)	ET (n = 15)	SCA (n = 7)	Significance
Age (years) ¹	82.6 ± 8.0	85.2 ± 4.9	56.0 ± 10.0	p < 0.001 ^a
Female gender	4 (26.7)	9 (60.0)	3 (42.9)	p = 0.18 ^b
Brain weight (grams)	1216 ± 100	1213 ± 196	1209 ± 174	p = 0.73 ^a
Postmortem interval (hours)	Median = 3.8	Median = 3.0	Median = 18.0	p = 0.003 ^c
Braak AD Score ^d			Not available	p = 1.00 ^b
0 – 4	14 (100)	13 (93.9)		
5 – 6	0 (0)	1 (7.1)		
CERAD Score ^d			Not available	p = 0.20 ^b
0	9 (64.3)	9 (64.3)		
A	4 (28.6)	2 (14.3)		
B	1 (7.1)	0 (0.0)		
C	0 (0.0)	3 (21.4)		
Purkinje cell count ²	11.3 ± 1.5	8.9 ± 1.3	5.7 ± 1.9	p < 0.001 ^a
Molecular layer cellular density ³	0.45 ± 0.12	0.43 ± 0.07	0.61 ± 0.11	p = 0.01 ^a

All values are mean ± standard deviation or numbers (percentages) unless specified otherwise. For Braak AD scores and CERAD scores, data were not available on 1–2 subjects.

^a Analysis of variance (ANOVA).

^b Chi-square test or Fisher's Exact test.

^c Mann-Whitney test.

^d Data were incomplete in 1 or 2 subjects.

¹ Post hoc Tukey test: ET vs. controls p = 0.60, SCA vs. controls p < 0.001, ET vs. SCA p < 0.001.

² Post hoc Tukey test: ET vs. controls p = 0.001, SCA vs. controls p < 0.001, ET vs. SCA p < 0.001.

³ Post hoc Tukey test: ET vs. controls p = 0.79, SCA vs. controls p = 0.004, ET vs. SCA p = 0.01.