Morphological and morphometric changes in the Purkinje cells of patients with essential tremor

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Abstract. Essential tremor (ET) is a progressive neurological syndrome characterised by involuntary tremors of the hands or arms, head, jaw and voice. The pathophysiology of ET is not clearly understood yet. However, previous studies have reported several changes in the brain of patients with ET. One of the brain areas extensively investigated is the cerebellum. In the present study, a morphometric analysis of Purkinje cells in patients with ET and ET-plus was performed, and subsequently compared with normal controls using the Golgi silver staining method and 3D neuronal reconstruction. Substantial morphological changes were uncovered in the Purkinje cells of patients with ET compared with normal controls, including a decreased dendritic length and field density, an overall loss of terminal branches and a decreased density of dendritic spines.

Introduction

Essential tremor (ET) is a chronic progressive neurological syndrome characterised by involuntary tremors of the hands or arms, and progressively of the head, jaw and voice (1-4). ET can present with heterogeneous clinical phenotypes, and some patients manifest extensive and complex deficits (5-10). ET is often considered a hereditary benign condition, with an isolated tremor and no further neurological signs (11); however, it is clearly defined in the new classification of tremors that ET is a syndrome that may have multiple aetiologies (5). The new diagnostic criteria for ET require the occurrence of isolated action tremor syndrome of the bilateral upper limbs with a minimum duration of 3 years, and/or tremors in the head, voice and lower limbs and mild neurological signs, such as dystonia, ataxia and/or Parkinsonism. Soft neurological deficits, such as mild memory impairment and impaired tandem gait, are also accepted; however, such patients are now diagnosed with ET-plus syndrome (5).

Numerous studies have been conducted to clarify the pathological, neuroimaging, physiological and clinical features of ET; however, the pathophysiological mechanism is not clearly understood (6-48). The cerebellum is one of the brain structures that has been extensively investigated at the macroscopic and microscopic levels (6-8). A number of studies have reported different levels of structural and functional alterations in the cerebellum of ETs. For example, neuroimaging studies have revealed a reduction in the volume of the cerebellar vermis with marked atrophy, atrophy of the cerebellar cortex, white matter changes in multiple cerebellar areas, and overactivity of the deep cerebellar nuclei and the cerebellar cortex and their connections (11-29). Meanwhile, additional studies have failed to identify any significant differences in the cerebellum between patients with ET and normal controls (NCs) (11,15,16,28-40). Neuropathological studies have also reported heterogeneous findings, with a decrease in the linear density of Purkinje cells, alterations in dendritic arborisation, axonal torpedoes, heterotopic Purkinje cells, hypertrophy of

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the basket cells and decreased climbing fibre-Purkinje cell synaptic density (29,44-50).

The present study investigated the morphological and morphometric changes in Purkinje cells in ET and ET-plus brains compared with age-matched control brains.

Materials and methods

Patients with ET and NC samples. In the present study, the morphology of the dendritic arborisation of Purkinje cells from different parts of the cerebellum was analysed in 12 patients with ET (7 males and 5 females) and 15 individuals (8 males and 7 females) with no history of neurological conditions, who died accidentally and were used as NC samples (Table I). The mean age was 61.3±6.4 years (range, 50-74 years) and 65.6±6.0 years (range, 50-74 years) for patients with ET and NCs, respectively. Three parts were excised from the cerebellum: The first from the flocculonodular lobe or lobule X, the second from the anterior lobe (lobule IV) and the third from the posterior lobe (lobule crus I). These parts were selected as representative parts of the three functional divisions of the cerebellum. All the brain tissues were obtained from the Laboratory of Forensic Medicine and Toxicology of the Aristotle University of Thessaloniki between January 2009 and December 2016. For each brain, written informed consent was obtained from the relatives of the deceased. The research was performed in full accordance with the Greek Democracy legislation (v. 2472/1997, 2819/2000, 2915/2001, 3235/2004, 3471/2006) and the Committee for Research Deontology Principles of the Aristotle University of Thessaloniki (ethical approval no. 12/2/4431/2019) (51). The average time from death to autopsy for all individuals was 8±2.9 h. All patients in the ET group were clinically diagnosed with ET. Their history and neurological examination were retrospectively reviewed, and they were divided into three groups: ET with head and arm tremor (ET-h; n=5); ET with arms tremor only (ET-a; n=4); and ET-plus (n=3). An independent neuropathologist who was blinded to the medical history examined the brain. After the autopsy, the brains were immersed at 18˚C in a 10% solution of formaldehyde for 25 days (52). Subsequently, small parts of the anterior, posterior and flocculonodular lobes were excised and used for Golgi staining at 120 μm-thick sections, as described in our previous work (52), and studied with an Axiostar plus Carl Zeiss light microscope at a standard (magnification, x400). Golgi method stains the whole dendritic field and makes the tracing of cells possible in a 3-dimensional manner.

Table I. Demographic features of patients and NCs.

A, Patients with ET

Case	Age, years	Sex	Diagnosis
1	57	M	ET-h
2	68	M	ET-a
3	50	M	ET-a
4	62	F	ET-h
5	63	M	ET-plus
6	61	F	ET-a
7	74	F	ET-plus
8	55	M	ET-h
9	63	M	ET-plus
10	62	F	ET-h
11	59	F	ET-a
12	54	M	ET-a
Average/total	61.3±6.4	7M/5F	

B, NCs

Case	Age, years	Sex	Diagnosis
1	67	M	NA
2	59	F	NA
3	69	F	NA
4	72	F	NA
5	70	M	NA
6	62	M	NA
7	68	M	NA
8	62	F	NA
9	63	M	NA
10	74	F	NA
11	71	M	NA
12	69	F	NA
13	66	F	NA
14	50	M	NA
15	63	M	NA
Average/total	65.6±6.0	8M/7F	

Average values are presented as the mean ± SD. M, male; F, female; ET, essential tremor; ET-a, ET with arm tremor only; ET-h, ET with head and arm tremor; NC, normal control; NA, not applicable.

Cell selection criteria. Based on the criteria suggested by Jacobs *et al* (53), 25 Purkinje cells were selected from each brain. To avoid experimental bias, all selected cells were randomly pooled and were assigned serial numbers, and every third cell was then chosen.

Neuronal tracing and dendritic quantification. Neuronal tracing and dendritic measurements were performed as described in our previous study (52) using a motorised XYZ microscope stage system (MLS203/MZS500-E; ThorLabs, Inc.), with the movement on the Z-axis being controlled by the MZS500-E-Z-Axis Piezo Stage and Controller Kit, with the aim of the APC software provided by Thorlabs, Inc. with a JogStep of 1 μm a Travel Range of 250 μm. The video captures were analysed in digital image sequences of 200 serial pictures. They were then imported into neuromantic application with the help of which the cells were traced and quantified along x-, y- and z-coordinates (52). Neuronal tracing was performed using a semi-automatic form by two different investigators. All the measurements were used for statistical analysis. The quantitative evaluation of the dendritic trees was based on the method suggested by Uylings *et al* (54).

Table II. ANOVA analysis of Purkinje cell properties.

A, Vermis

Parameter	ET-a	ET-h	ET-plus	NC	F-value
Total dendritic length, μm	9,487.73±551.06	6,361.28±661.17[a]	9,388.75±486.88[b]	9,507±1053.13[c]	922.1
Number of terminals	273.84±14.36	199.86±27.04[d]	276.12±14.64[c]	288.94±16.66[c,e,f]	915.43
Branch length, μm	562.9±33.79	513.25±35.51	570.56±39.05[a,c]	587.35±37.68[a,c]	151.89
Branch order	26.38±1.19	20.24±2.48	26.61±1.37	27.04±0.88	816.06
Spines	9.73±0.67	7.87±0.71[a]	9.84±0.68[g]	9.37±0.93[f,g]	290.99

B, Hemispheres

Parameter	ET-a	ET-h	ET-plus	NC	F-value
Total dendritic length, μm	7,889.4±1,387.22[g,h]	7,794.89±1,017.91[b,c]	9,407.22±642.27[a,b]	10,757.3±1,666.24[a,c,f]	257.33
Number of terminals	202.72±23.98	219.01±17.73[d]	306.86±23.04[d]	325.69±14.39[d,h]	1,871.33
Branch length, μm	531.66±31.72	538.44±31.54[a]	594.69±36.65[d,g]	597.62±36.54[d,g]	214.29
Branch order	22.96±1.66	24.64±1.58[d]	28.41±1.75[a,c]	30.61±1.62[a,c,e]	890.35
Spines	7.75±0.67	7.4±0.49[d]	9.76±0.66[a]	9.98±0.68[a]	902.82

[a]P<0.001 vs. ET-a; [b]P<0.01 vs. ET-h; [c]P<0.0001 vs. ET-h; [d]P<0.0001 vs. ET-a; [e]P<0.01 vs. ET-a; [f]P<0.001 vs. ET-plus; [g]P<0.001 vs. ET-h; [h]P<0.0001 vs. ET-plus. Data are presented as the mean ± SD. ET, essential tremor; ET-a, ET with arm tremor only; ET-h, ET with head and arm tremor; NC, normal control; vs., versus.

Dendritic measures and Sholl's analysis. Neuromantic automatically provides all the measurements for the total length of dendritic trees, total number of dendritic segments and terminal branches, total dendritic area and total dendritic volume, which were analysed further in the statistical analysis. The tracings were additionally analysed using the Fiji (version 2017; Fiji) and Simple Neurite Tracer plugin on ImageJ (v.1.8; National Institutes of Health) based on Sholl's method of concentric spheres centred on the cell's soma at intervals of 10 μm (55).

Spine counts. Spine counts were performed for 500 images. Images were captured with an AxioCam HR (Zeiss GmbH), at a magnification of x1,000, with an Axiostar plus photomicroscope. All the visible spines were measured on the three segments of the dendritic field: The first segment, 20-30 μm in length at a distance of 50 μm from the cell soma; the second segment, 20-30 μm in length within 150 μm; and the third segment, 20-30 μm within 250 μm from the cell body.

Purkinje cell density. The linear density of Purkinje cells was measured in Nissl-stained specimens on 30 randomly selected images (magnification, x20), using ImageJ (v.1.8; National Institutes of Health) and the cell counter plugin (56).

Statistical analysis. RStudio (version, 1.4.1717) with R (version, 4.1.1) was used for the statistical analysis and plotting of graphs (57). All data was stored in comma-separated value files and presented for statistical analysis as data frames. One-way ANOVA followed by Tukey's post hoc test was used to determine whether significant differences existed across the independent parameters of Purkinje cells among the four groups. ANOVA mixed-effects model followed by Tukey's post hoc test was used for the statistics of Sholl's analysis, based on an R Script. Pearson's correlation test was performed to identify any correlation between the autolysis time and Purkinje cell dendritic complexity variables. P<0.05 was considered to indicate a statistically significant difference.

Results

Total dendritic length. The total dendritic length of Purkinje cells from the cerebellar vermis was significantly lower in the ET-h group compared with the ET-a, ET-plus and NC groups (Table II). No significant difference was identified between the ET-a, ET-plus and NC groups (Fig. 1A). However, the total dendritic length of Purkinje cells in the cerebellar hemispheres was significantly decreased in the ET-a group compared with ET-plus and NC groups (Fig. 1B). Similarly, the difference was statistically significant between the ET-h, ET-plus and NC groups, and between the ET-plus and NC groups. No significant differences were found between the ET-a and ET-h groups (Fig. 1B).

Terminal branches. The total number of terminal branches of Purkinje cells from the cerebellar vermis was significantly lower in the ET-h group compared with the ET-a, ET-plus and NC groups. No significant difference was found between the ET-a and ET-plus groups, but there was a statistically significant difference between the ET-a and NC groups, and between the ET-plus and NC groups (Figs. 1C and 2 and Table II). Purkinje cells from the cerebellar hemispheres from the ET-a group had significantly fewer terminal branches than

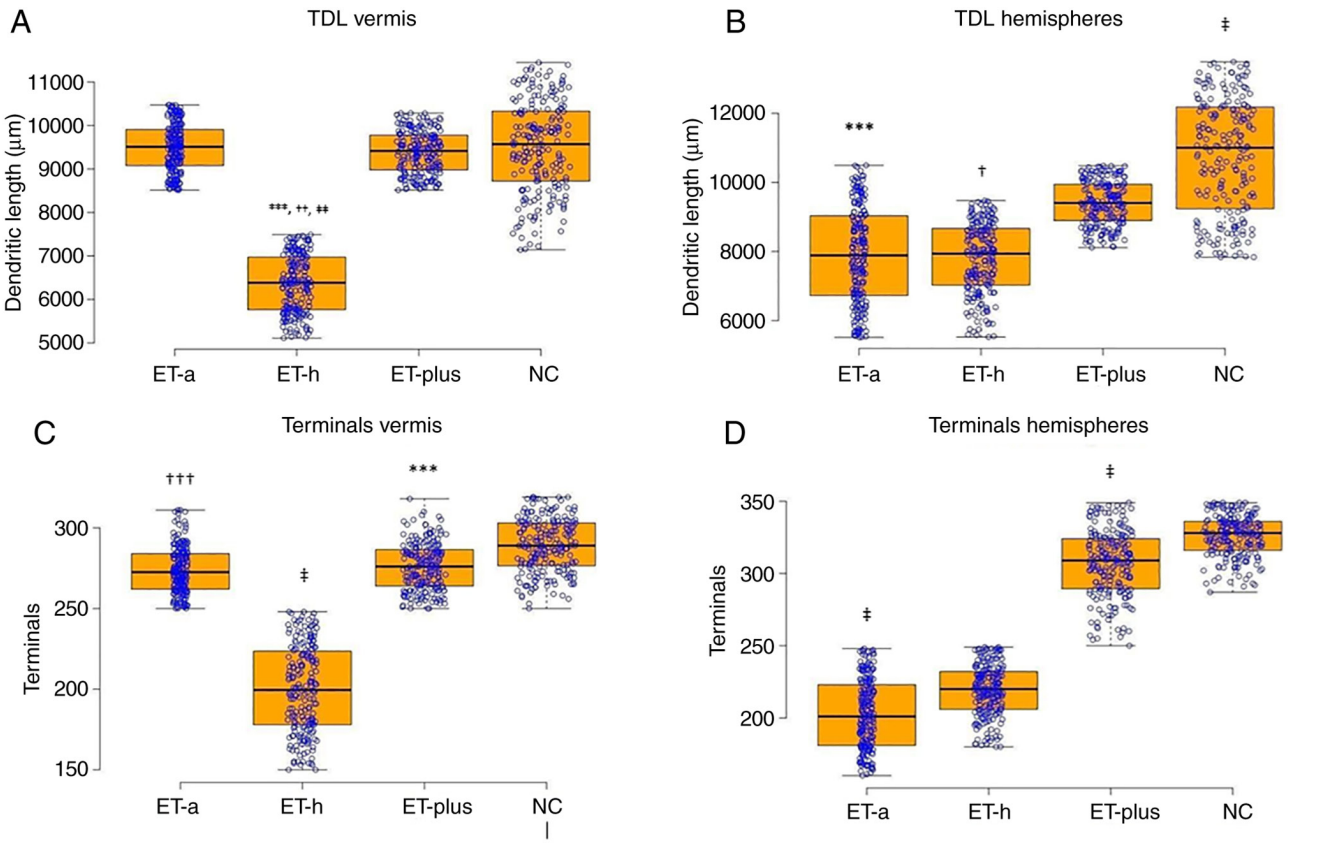

Figure 1. TDL of Purkinje cells from (A) the cerebellar vermis and (B) the cerebellar hemispheres. Total number of terminal branches of the Purkinje cells from (C) the cerebellar vermis and (D) the hemispheres. Results are presented as boxplot charts and the statistical significance in ANOVA test was expressed as symbols (A) ***P<0.001 ET-h vs. ET-a; ††P<0.003 ET-h vs. ET-plus; ‡‡P<0.00001 ET-h vs. NC; (B) ***P<0.001 ET-a vs. ET-plus, NC; ‡P<0.0001 NC vs. ET-plus, ET-h; †P<0.002 ET-h vs. ET-plus; (C) ‡P<0.0001 ET-h vs. ET-a, ET-plus, NC; †††P<0.005 ET-a vs. NC; ***P<0.001 ET-plus vs. NC; (D) ‡P<0.0001 ET-a vs. ET-plus, ET-h, NC, ET-plus vs. NC. TDL, total dendritic length; ET, essential tremor; ET-a, ET with arm tremor only; ET-h, ET with head and arm tremor; NC, normal control.

the ET-plus, NC and the ET-h groups (Fig. 1D). In addition, the ET-plus group displayed a significantly lower number of terminal branches compared with the NC group (Fig. 1D).

Branch length and branch order. The mean branch length (Fig. 3A and Table II) and the maximum branch order of the cerebellar vermis were both significantly decreased in the ET-h group compared with the ET-plus and NC groups. The ET-a group also indicated a significantly lesser branch length and maximum branch order than the ET-plus and NC groups. The ET-plus group demonstrated a decreased branch length and branch order compared with the NC group. Purkinje cells from the cerebellar hemispheres exhibited a decreased branch length in the ET-a group compared with the other groups, and in the ET-h group compared with the ET-plus and NC groups. No statistically significant difference was observed between the ET-plus and NC groups (Fig. 3B). The maximum branch order was similarly decreased in the ET-h group compared with the other groups, in the ET-a group compared with the ET-plus and NC groups, and in the ET-plus group compared with the NC group.

Dendritic spines. The dendritic spine density was reduced in the ET-h group compared with the ET-a, ET-plus and NC groups in Purkinje cells from the cerebellar vermis; however, no significant difference was found between the ET-a and ET-plus groups. The latter displayed notably more dendritic spines compared with Purkinje cells from the NC group (Figs. 4A and 5 and Table II). Meanwhile, Purkinje cells from the cerebellar hemispheres demonstrated a significant difference between the ET-h and ET-a groups, between the ET-plus and NC groups, between the ET-a and ET-plus, and between the ET-a and NC groups (Figs. 4B, 5 and Table II). Moreover, Pearson's correlation test did not reveal a significant correlation between autolysis time and dendritic and spinal measurements.

Sholl's analysis. Sholl's concentric circle analysis revealed significant restriction of the dendritic field of Purkinje cells from the cerebellar vermis and cerebellar hemispheres in the ET-h and ET-a groups compared with the ET-plus and NC groups for distances >120 μm from the cell soma (Fig. 6A and B). The areas under the curve also confirmed the significance of the differences in the overall dendritic field density (Fig. 6C).

Discussion

Multiple studies have reported neuropathological changes in ET, with morphological and morphometric alterations of Purkinje cells and Lewy bodies in the locus coeruleus; however, whether these changes are the characteristic features

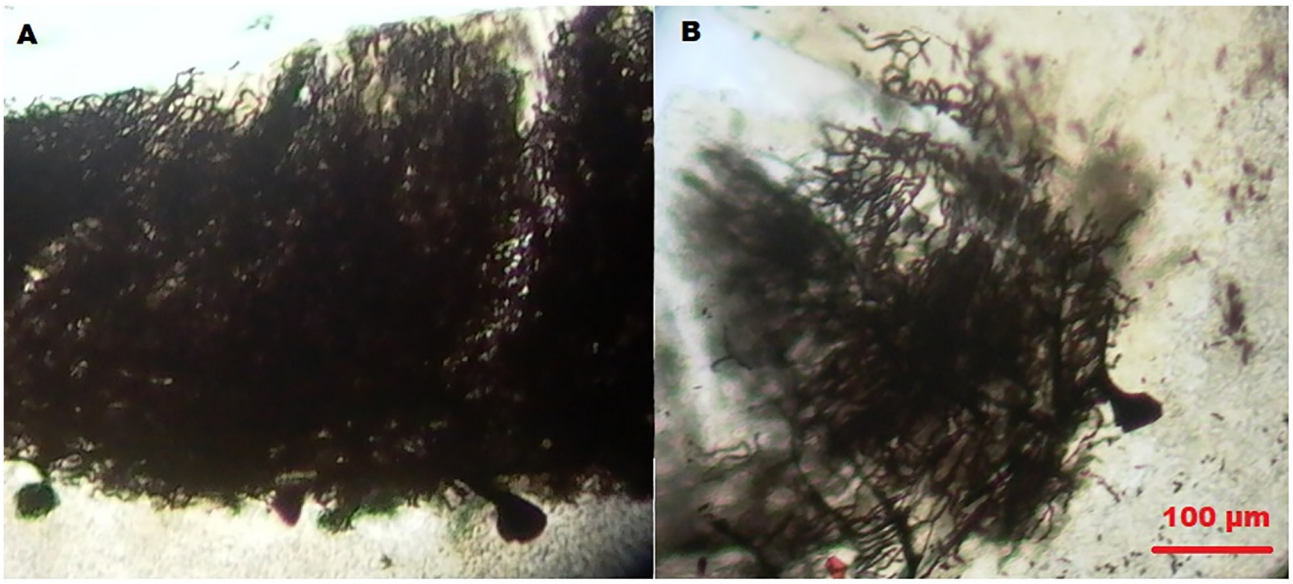

Figure 2. Representative images of Purkinje cells from the cerebellum from (A) a control and (B) a patient with essential tremor from the ET-h group, generated by the Golgi method. Magnification, x100. Scale bar, 100 μm.

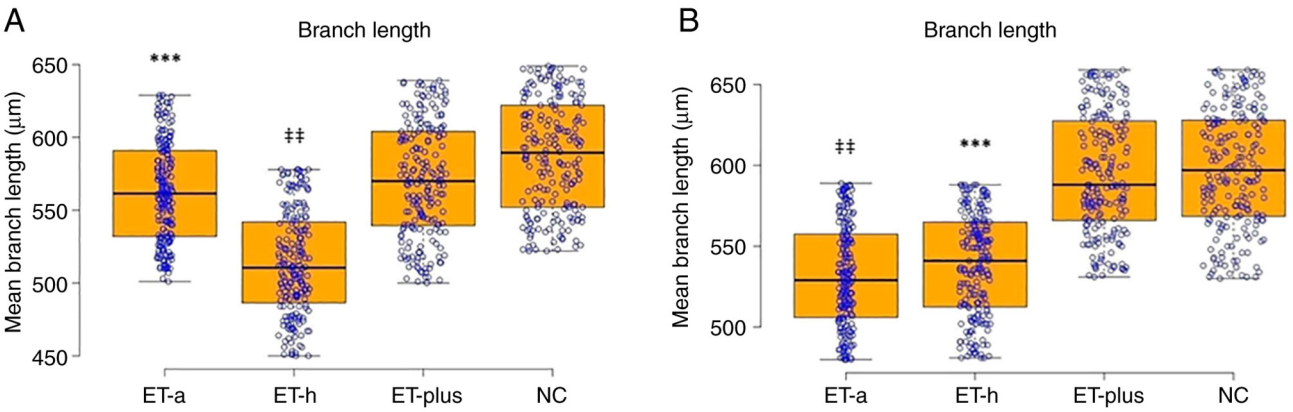

Figure 3. Mean branch length of Purkinje cells from (A) the vermis and (B) the cerebellar hemispheres (B). Results are presented as boxplot charts and the statistical significance in ANOVA test is expressed as symbols. (A) ‡‡P<0.00001 ET-h vs. ET-plus, NC; ***P<0.001 ET-a vs. ET-plus, NC; (B) ‡‡P<0.00001 ET-a vs. ET-plus, NC; ***P<0.001 ET-h vs. ET-a, ET-plus, NC. ET, essential tremor; ET-a, ET with arm tremor only; ET-h, ET with head and arm tremor; NC, normal control.

of the condition is unclear (15,16,11-49). One possible explanation may be that different types of essential tremor exhibit different pathological mechanisms and neuropathological backgrounds.

Two previous studies demonstrated a significant reduction in the linear Purkinje cell density in ET, and greater distances between single Purkinje cell bodies (28,29); however, another study failed to confirm these findings (58). Further studies reported increased numbers of heterotopic Purkinje cells in ET, up to three times compared with controls (59), and certain morphological changes, with a substantial loss of dendritic spines, total dendritic length and restriction of the dendritic fields (44).

The most common neuropathological feature of ET is axonal changes, known as axonal torpedoes; they are focal swellings of Purkinje cell axons containing an accumulation of hyperphosphorylated neurofilaments and disrupted organelles, and can also be found in spinocerebellar ataxias (46,47). Additional changes, which could potentially explain the pathophysiology of ET, are also referred to as the basket cell morphology (47) and climbing fibre-Purkinje cells' synaptic density (49).

Patients with ET may have tremors of the hands, head, voice and rarely the jaw. According to the 2016 classification of ET, patients who develop soft neurological symptoms, such as mild rigidity, balance impairment or mild memory impairment are classified as experiencing ET-plus (5). However, whether ET-plus or ET are different entities remains controversial.

In the present study, significant morphological changes were found in Purkinje cells, including a decreased dendritic length and field density, an overall loss of terminal branches, and a decreased density of dendritic spines in ET patients compared with controls. Furthermore, ET patients were divided into three groups based on the revised criteria for ET. The results indicated that the ET-h group exhibited significant

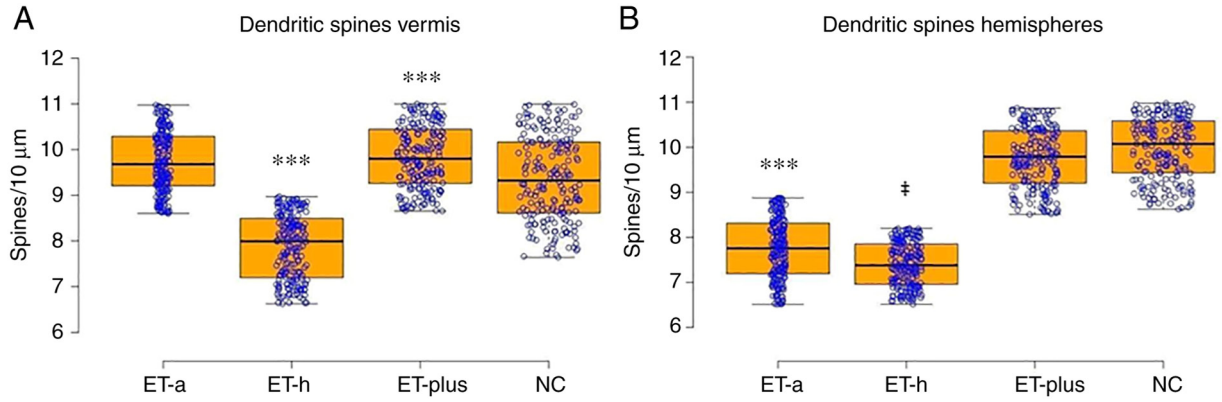

Figure 4. Dendritic spine density on Purkinje cells from the vermis (A), and the cerebellar hemispheres (B). Results are presented as boxplot charts and the statistical significance in ANOVA test is expressed as symbols (A) ***P<0.001 ET-h vs. ET-a, ET-plus, NC, ET-plus vs. NC; (B) ‡P<0.0001 ET-h vs. ET-a; ***P<0.001 ET-a vs. ET-plus, NC. ET, essential tremor; ET-a, ET with arm tremor only; ET-h, ET with head and arm tremor; NC, normal control.

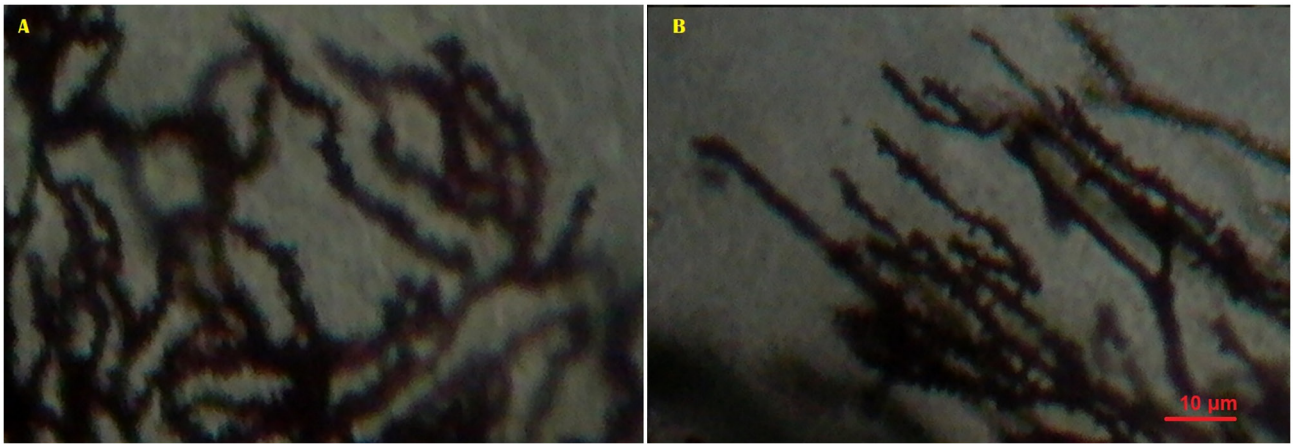

Figure 5. Representative images showing the dendritic spines in dendrites from Purkinje cells from (A) a control and (B) a patient with essential tremor, generated by the Golgi method, Magnification, x1,000. Scale bar, 10 μm.

changes in both the cerebellar vermis and hemispheres, while the ET-a group displayed significant differences only in Purkinje cells from the cerebellar hemispheres compared with controls. Meanwhile, Purkinje cells from patients with ET-plus demonstrated only minor changes. The significant loss of dendritic spines and terminal branches, which are the most plastic components of the dendritic field (52), could lead to a substantial decrease in the synaptic contacts of Purkinje cells. This could be of significant importance in the pathophysiology of ET.

The flocculonodular lobe of the cerebellum corresponds to the vestibulocerebellum and is functionally related to head movements, while the anterior and posterior lobes of the hemisphere are related to limb movements and movement scheduling (60,61). Different Purkinje cell pathologies were identified in the different groups of the study, which could explain the different symptoms in each patient group. Patients with ET-a did not exhibit significant pathology in the Purkinje cells of the cerebellar vermis. However, patients with ET-a exhibited pathology in the cerebellar hemispheres, which are functionally related to Purkinje cells. Patients with ET-h exhibited pathology in the Purkinje cells of the cerebellar hemispheres and vermis. Patients with ET-plus did not display any significant pathology in the Purkinje cells of the cerebellum, which may suggest that this condition has a different pathophysiological background; however, whether these changes are primary and degenerative, or are instead compensatory, remains unclear.

The heterogeneous findings among the different groups of ET could correspond to clinical heterogeneity. Therefore, it can be assumed that ET-plus syndrome is different from ET syndrome in terms of not only pathology and physiopathology, but also clinical aspects. Although the cause of ET remains unknown, the morphological changes found in Purkinje cells could be the structural background of ET symptomatology. Differences in the Purkinje cell pathology between ET-h and ET-a also reflected the differences in the clinical presentation of ET.

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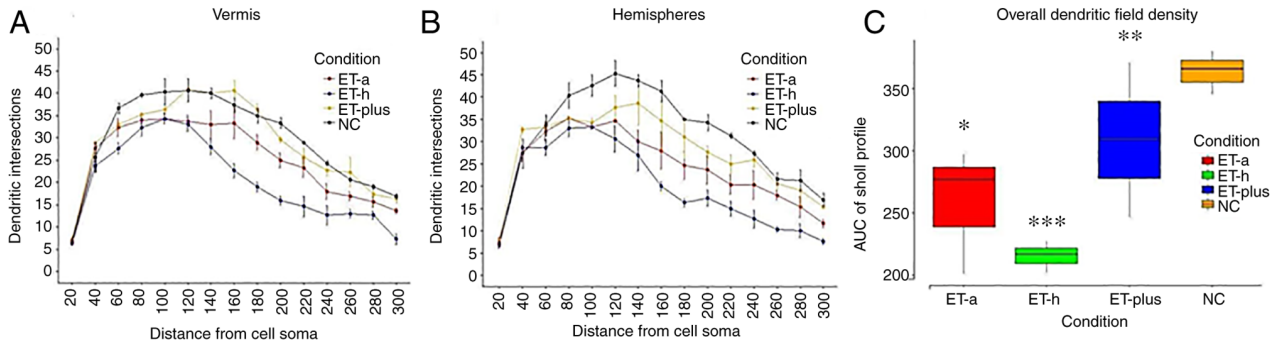

Figure 6. Sholl's analysis of Purkinje cells from (A) the vermis and (B) the cerebellar hemispheres, and (C) AUC analysis confirming the significant difference in the overall dendritic field density. Results are presented as line and boxplot charts and the statistical significance in ANOVA test was expressed as symbols (C) ***P<0.001 ET-h vs. NC; *P<0.05 ET-a vs. ET-h, ET-plus vs. NC; **P<0.01 ET-plus vs. ET-h. ET, essential tremor; ET-a, ET with arm tremor only; ET-h, ET with head and arm tremor; NC, normal control; AUC, area under the curve.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

IM made substantial contributions to conception and design of the study. DK, FP, SC and EK contributed to data acquisition and analysis. IM, DK, SNN, VC, AC, CT, IMB and SJB contributed to data interpretation and preparation of the manuscript. IM, VC, CT, IMB and SJB supervised the study and critically reviewed the manuscript. IM, DK, FP, SC, EK, AC, VC, CT and SJB confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The research was performed in full accordance with the Greek Democracy legislation (v. 2472/1997, 2819/2000, 2915/2001, 3235/2004, 3471/2006) and the Committee for Research Deontology Principles of the Aristotle University of Thessaloniki. The ethical approval number of this study was 12/2/4431/2019. For each brain, written informed consent was obtained from the relatives of the deceased.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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