Abnormal climbing fibre-Purkinje cell synaptic connections in the essential tremor cerebellum

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Structural changes in Purkinje cells have been identified in the essential tremor cerebellum, although the mechanisms that underlie these changes remain poorly understood. Climbing fibres provide one of the major excitatory inputs to Purkinje cells, and climbing fibre-Purkinje cell connections are essential for normal cerebellar-mediated motor control. The distribution of climbing fibre-Purkinje cell synapses on Purkinje cell dendrites is dynamically regulated and may be altered in disease states.

The aim of the present study was to examine the density and distribution of climbing fibre-Purkinje cell synapses using post-mortem cerebellar tissue of essential tremor cases and controls. Using vesicular glutamate transporter type 2 immunohistochemistry, we labelled climbing fibre-Purkinje cell synapses of 12 essential tremor cases and 13 age-matched controls from the New York Brain Bank. Normally, climbing fibres form synapses mainly on the thick, proximal Purkinje cell dendrites in the inner portion of the molecular layer, whereas parallel fibres form synapses on the thin, distal Purkinje cell spiny branchlets. We observed that, compared with controls, essential tremor cases had decreased climbing fibre-Purkinje cell synaptic density, more climbing fibres extending to the outer portion of the molecular layer, and more climbing fibre-Purkinje cell synapses on the thin Purkinje cell spiny branchlets. Interestingly, in essential tremor, the increased distribution of climbing fibre-Purkinje cell synapses on the thin Purkinje cell branchlets was inversely associated with clinical tremor severity, indicating a close relationship between the altered distribution of climbing fibre-Purkinje cell connections and tremor. These findings suggest that abnormal climbing fibre-Purkinje cell connections could be of importance in the pathogenesis of essential tremor.

Keywords: essential tremor; climbing fibre; Purkinje cell; synapse; VGlut2
Abbreviation: VGlut = vesicular glutamate transporter

Introduction

Essential tremor is among the most prevalent neurological disorders and the most common cause of abnormal tremor (Benito-Leon et al., 2003; Louis and Ferreira, 2010). The most prominent clinical feature of essential tremor is kinetic tremor of the arms; neck, voice and other tremors may also be present (Louis, 2001). Neuroimaging studies indicate decreased cerebellar metabolism and mild cerebellar atrophy in essential tremor (Louis et al., 2002; Quattrone et al., 2008; Cerasa et al., 2009). In post-mortem studies of essential tremor, structural...
changes in the cerebellum have been identified, including Purkinje cell loss in some studies (Louis et al., 2013), heterotopic Purkinje cells (Kuo et al., 2011a), Purkinje cell axonal torpedoes and related axonal changes (Babij et al., 2013), and basket cell axonal alterations (Erickson-Davis et al., 2010; Kuo et al., 2013), indicating that Purkinje cell degeneration may be central to pathogenesis of essential tremor (Louis, 2009; Grimaldi and Manto, 2013).

Purkinje cells receive two types of excitatory inputs, which form glutamatergic synapses. One is from granule cells; their axon terminals form parallel fibre-Purkinje cell synapses, which specifically express vesicular glutamate transporter type 1 (VGlut1, now known as SLC17A7). The other is from climbing fibres, which form climbing fibre-Purkinje cell synapses, which specifically express VGlut2 (now known as SLC17A6) (Fremeau et al., 2001). Stimulation of parallel fibres triggers short duration action potentials of Purkinje cells whereas stimulation of climbing fibres causes long duration action potentials with complex spikes and calcium influx in Purkinje cells (Eccles et al., 1966; Thach, 1967). The distribution of parallel fibre-Purkinje cell synapses and climbing fibre-Purkinje cell synapses on Purkinje cell dendrites is critically regulated. Climbing fibres form synapses predominantly on the thick, proximal Purkinje cell dendrites whereas parallel fibres form synapses on the thin, terminal Purkinje cell branchlets, characterized by the branchlet diameter <1 µm and often with numerous dendritic spines (Palay and Chan-Palay, 1974). A small percentage of climbing fibre synapses are also normally present on the Purkinje cell spiny branchlets (Ichikawa et al., 2002). Normally, climbing fibres form synapses on Purkinje cell dendrites in the inner 80% of the molecular layer, whereas the outer 20% of the molecular layer is predominantly parallel fibre-Purkinje cell synaptic territory (Ichikawa et al., 2002; Miyazaki et al., 2010). Thus, climbing fibres and parallel fibres have their respective ‘innervation territory’ on the Purkinje cell dendritic arbor, which is important for the proper physiological function of Purkinje cells (Watanabe, 2008). Defective climbing fibre-Purkinje cell connections can occur in the setting of Purkinje cell degeneration in various forms of spinocerebellar ataxias (Koeppen, et al., 2013) and/or in the presence of persistent abnormal activity of climbing fibres (Cheng et al., 2013; Helmich et al., 2013). In fact, it has been postulated that the hyperexcitability of the inferior olivary nucleus could lead to the synchronized firing of Purkinje cells, dentate nucleus, and thalamus, perhaps leading to tremor in essential tremor (Cheng et al., 2013; Helmich et al., 2013).

We previously reported a normal parallel fibre density in essential tremor (Kuo et al., 2011b). In the present study, we now turn our focus to the climbing fibre-Purkinje cell interface, including climbing fibre-Purkinje cell synaptic density and innervation territory in the cerebellar cortex, comparing cases with essential tremor to control subjects.

**Materials and methods**

**Subjects and tissue processing**

We selected 13 non-diseased control brains and 12 age-matched cases with essential tremor from the Essential Tremor Centralized Brain Repository in the New York Brain Bank, Columbia University Medical Centre. Cerebellar cortical tissue was available for immunohistochemistry on these 12 cases with essential tremor and 13 control subjects. One essential tremor case could not be labelled with dual immunofluorescence with calbindin and VGlut2. Therefore, for the dual immunofluorescence portion of the study, 11 cases with essential tremor and 13 controls were included. Ten of these essential tremor cases and six of the control subjects have been assessed for Purkinje cell numbers and Purkinje cell axonal torpedoes in our previous study (Babij et al., 2013).

As detailed previously, the clinical diagnosis of essential tremor was first made by treating physicians, and then confirmed by an Essential Tremor Centralized Brain Repository neurologist (E.D.L.) using data from clinical questionnaires, medical records, videotaped neurological examination, and assessment of standardized Archimedes spirals (Louis et al., 2005; Babij et al., 2013). The neurologist (E.D.L.) reviewed all videotaped neurological examinations and rated the severity of postural and kinetic (pouring, drinking, using spoon, drawing spirals, finger-nose-finger) arm tremors (ratings = 0–3), resulting in a total tremor score [range = 0 to 36 (maximum)] (Louis et al., 2014). A 31-item tremor disability questionnaire was used to assess the functional impact of tremor, with the tremor-related disability score ranging from 0 (no disability) to 100 (completely disabled) (Wendt et al., 2000). 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., 2004). The medicine used by each patient was recorded (Supplementary Table 1). The age-matched control brains were from individuals followed at the Alzheimer’s disease Research Centre, and the Washington Heights Inwood Columbia Aging Project at Columbia University. They were followed prospectively with serial neurological examinations, and were clinically free of Alzheimer’s disease, essential tremor, Parkinson’s disease, Lewy body dementia, or progressive supranuclear palsy.

All brains received ratings of neurofibillary tangles using Braak and Braak staging (Braak and Braak, 1997; Braak et al., 2006), and Consortium to Establish a Registry for Alzheimer’s disease ratings for neuritic plaques (Mirra, 1997). Post-mortem interval was the number of hours between death and placement of the brain in a cold room or on ice. We excluded essential tremor cases with brainstem (dorsal vagal nucleus, locus ceruleus, substantia nigra) Lewy body pathology on α-synuclein stained sections (Louis et al., 2007).

A standard 3 × 20 × 25 mm parasagittal neocerebellar block was obtained from a 0.3-cm thick parasagittal slice located 1 cm from the cerebellar midline, which corresponded to the anterior and posterior quadrangular lobules in the anterior lobe of the cerebellar cortex (Vonsattel et al., 2008; Apps and Hawkes, 2009). Paraffin sections (7-µm thick) were stained with Luxol Fast Blue haematoxylin and eosin. We quantified Purkinje cell axonal torpedoes, basket plexus rating and heterotopic Purkinje cell counts using a modified Bielschowsky silver technique as previously described (Erickson-Davis et al., 2010; Kuo et al., 2011a; Babij et al., 2013).
anti-VGlut2 antibody (1:250) (Koeppen et al., 2011, 2013) at 4°C for 48 h followed by incubation with biotinylated anti-rabbit IgG (Vector Labs, 15 μg/ml), and the signals were amplified by avidin/biotinylated complex (Vector Labs). The sections were developed with 3,3′-diaminobenzidine precipitation. Images were acquired with bright field microscopy (Zeiss AxioPlan 2 with AxioCam HR digital camera). We tested the immunohistochemical specificity of VGlut2 antibody with VGlut2 peptide blockade (Abcore, AC21-2970). VGlut2 peptide and VGlut2 antibody at 10:1 ratio or VGlut2 antibody alone were incubated at 4°C for 24 h and were used as the primary antibody for immunohistochemistry.

Immunofluorescence studies were performed with dual staining of monoclonal mouse anti-CalbindinD28k (Sigma-Aldrich, 1:1000) or mouse monoclonal anti-synaptophysin antibody (Abcam, 46 μg/ml) and polyclonal rabbit anti-VGlut2 antibody (1:250) (Koeppen et al., 2011, 2013), with secondary Alexa Fluor® 594 goat anti-mouse IgG and Alexa Fluor® 488 donkey anti-rabbit IgG antibody (Life Technologies, both 20 μg/ml). All immunofluorescence images were taken using confocal laser scanning microscopy (Leica TSC SP2 two photon microscope).

Assessment of VGlut2 synaptic density

A trained rater (C.Y.L.) performed imaging acquisition and all readout quantification without the knowledge of the diagnosis of each subject. We chose the superior border of the cerebellar cortical section as the starting point and we moved the objective along the molecular layer to identify each individual field in a sequential order. We used a random digit table to select the field for image acquisition. We selected 25 fields in the cerebellar cortex in each subject and quantified the total number of visualized VGlut2 puncta in a ×400 field directly above the Purkinje cell layer. We also traced the total visualized climbing fibre length in the same ×400 field with NeuriteTracer (Neuron), a plugin of ImageJ (Pool et al., 2008). VGlut2 synaptic density was defined as the total number of visualized VGlut2 puncta divided by the total traced climbing fibre length in the ×400 field. Our pilot study of five cases with essential tremor and five controls (each subject with 25 images) indicated that the rater (C.Y.L.) had high test-retest reliability (Pearson’s correlation coefficient between two ratings performed 2 weeks apart = 0.96, P < 0.001) and high inter-rater reliability (Pearson’s correlation coefficient between C.Y.L. and S.H.K. = 0.96, P < 0.001) for VGlut2 synaptic density.

Assessment of climbing fibres in the outer portion of the molecular layer of the cerebellar cortex

To assess the distribution of climbing fibres across the height of the molecular layer, we investigated the percentage of climbing fibres extending into the outer 20% of the molecular layer. We randomly imaged 15 × 200 fields in each subject, and imported images into Image J. We first measured the total thickness of the molecular layer and then drew a line at the border between the outer 20% and inner 80% of the molecular layer in the cerebellar cortex. We quantified climbing fibres extending above this line into the outer 20% of the molecular layer. We calculated (i) the number of climbing fibres in the outer 20% of the molecular layer in a given field; and (ii) the percentage of climbing fibres in the outer 20% of the molecular layer in a given field (climbing fibres in outer 20% of the molecular layer/total climbing fibres linear arrays in the field × 100). We also measured the molecular layer thickness at the centre of the each image.

Assessment of the distribution of VGlut2 synapses on Purkinje cell dendrites

We quantified (i) the total number of climbing fibre-Purkinje cell synapses; (ii) the number of climbing fibre-Purkinje cell synapses on the Purkinje cell spiny branchlets, identified by the presence of numerous dendritic spines along the dendrites (Ichikawa et al., 2002); and (iii) the number of climbing fibre-Purkinje cell synapses on the Purkinje cell terminal spiny branchlets, which have <1 μm in diameter with numerous spines (Fox and Barnard, 1957; Palay and Chan-Palay, 1974). We performed a pilot study on five cases with essential tremor and five controls to reconstruct the dendritic arbor from the base of the molecular layer to the pial surface of four Purkinje cells per subject. The number of VGlut2 puncta on terminal Purkinje cell spiny branchlets, defined either by the presence of numerous dendritic spines or by the branchlet diameter <1 μm, was divided by the total number of VGlut2 puncta to determine percentage of climbing fibre synapses in the spiny branchlet domain. We compared this method to another method that randomly selected 25 fields in the molecular layer in each subject (×63 objective and ×5 digital zoom). We found that the two methods were highly correlated (for the percentage of VGlut2 puncta on the Purkinje cell spiny branchlets, Pearson’s r = 0.95, P < 0.001; for the percentage of VGlut2 puncta on Purkinje cell branchlets <1 μm in diameter, Pearson’s r = 0.95, P < 0.001). Therefore, moving forward, we used the more efficient method of random field selection. In each subject, we randomly chose fields based on the presence of VGlut2 puncta (green channel) without the visualization of the corresponding calbindin immunostaining (red channel). Once the field was chosen, we acquired the images of both green and red channels and merged two channels to obtain composite images. We analysed the composite images and quantified the percentage of VGlut2 puncta synapses on the Purkinje cell spiny branchlets as identified by the Purkinje cell branchlet morphology and, in a separate analysis, by the Purkinje cell dendritic diameter <1 μm.

Diameter of VGlut2 synaptic puncta

We also measured the diameter of all VGlut2 puncta in the images that had been used for the analysis of VGlut2 puncta distributions on Purkinje cell dendrites. For each field, the diameters of all VGlut2 puncta were measured (5–10 puncta/field); therefore, the diameters of ~150–200 VGlut2 puncta were measured on each subject.

Statistical analyses

Analyses were performed in SPSS (v 20). Demographic and clinical characteristics of essential tremor cases and controls were compared using Student t-tests and chi-square tests. Our primary outcome measures (i.e. (i) VGlut2 synaptic density on Purkinje cell dendritic shafts; (ii) the number of climbing fibres in the outer 20% of the molecular layer in a given field; (iii) the percentage of the climbing fibres in the outer 20% of the molecular layer in a given field; (iv) the percentage of VGlut2 puncta on Purkinje cell spiny branchlets; (vi) the percentage of VGlut2 puncta on Purkinje cell branchlets <1 μm in diameter; and (vi) the diameter of VGlut2 puncta) all followed a normal statistical distribution (Kolmogorov-Smirnov test), as did our clinical measures (total tremor score and tremor related disability score); therefore, we used parametric tests [Student t-test, Pearson’s correlation coefficient (r)] to assess these measures.
We also used Pearson’s correlation coefficients (r) to investigate the association between VGlut2 puncta density and distributions, and total tremor scores and tremor-related disability scores in essential tremor cases. The association between Braak Alzheimer’s disease stage and two of the primary outcome measures (i.e. the percentage of VGlut2 puncta on Purkinje cell spiny branchlets and the percentage of VGlut2 puncta on Purkinje cell branchlets <1 μm in diameter) was also evaluated using Pearson’s correlation coefficients.

Results

Essential tremor cases (n = 12) and controls (n = 13) were similar in age, gender, brain weight, post-mortem interval, and CERAD ratings for neuritic plaques, but essential tremor cases had a higher Braak Alzheimer’s disease score. Consistent with our previous study (Louis et al., 2007), cases with essential tremor had a lower Purkinje cell count than controls (Table 1). The mean number of Purkinje cell axonal torpedoes in cases with essential tremor was approximately twice of that in the controls, consistent with our previous study (Babij et al., 2013), but the difference was not statistically significant due to the small sample size. Removal of one outlying control, who had been exposed to chemotherapeutic agents, further increased the case-control difference (24.7 ± 25.2 versus 10.6 ± 10.3, t = 1.73, P = 0.10). There was an inverse trend between Purkinje cell numbers and Purkinje cell axonal torpedo counts in cases with essential tremor and control subjects (Pearson’s r = −0.44, P = 0.077).

VGlut2 immunohistochemistry showed a punctate pattern organized in linear arrays in the molecular layer and also labelled the glomeruli in the granule cell layer, consistent with previous studies using this antibody (Fig. 1A) (Koeppen et al., 2011, 2013) and another study of post-mortem human cerebellar cortex using a different VGlut2 antibody (Hills et al., 2013). Pre-incubation VGlut2 antibody with VGlut2 peptide completely abolished the VGlut2 signals in the cerebellum, thereby indicating the specificity of this antibody (Fig. 1B and C). Dual immunofluorescence labeling revealed that VGlut2 prominently labelled the climbing fibre-Purkinje cell synapses along the Purkinje cell dendritic arbor (Fig. 1D–F). VGlut2 puncta in the molecular layer also co-localized with synaptophysin, confirming that VGlut2 puncta were presynaptic climbing fibre terminals (Fig. 1G–I).

We first quantified the linear density of VGlut2 puncta on Purkinje cell dendritic shafts. Cases with essential tremor had a 33% reduction in VGlut2 synaptic density versus controls (25.88 ± 1.45 VGlut2 puncta/100 μm of climbing fibre length in essential tremor cases versus 33.46 ± 4.33 VGlut2 puncta/100 μm of climbing fibre length in controls, P < 0.001) (Fig. 2A–C).

We next examined the distribution of climbing fibres across the height of the molecular layer. In cases with essential tremor, the number of climbing fibres extending into the outer 20% of molecular layer in each ×200 field was 1.9-fold higher than in the control subjects (2.39 ± 0.77 in essential tremor cases versus 1.27 ± 0.45 in controls, P < 0.001) (Fig. 3A–C). We also found that a 1.9-fold higher percentage of total climbing fibres in each ×200 field extended into the outer 20% of the molecular layer in cases with essential tremor versus controls (28% ± 7% in essential tremor cases versus 15% ± 5% in controls, P < 0.001) (Fig. 3D). We did not observe any differences in the molecular layer thickness between cases with essential tremor and control subjects (347.52 ± 23.65 μm in essential tremor cases versus 337.08 ± 27.30 μm in controls, P = 0.642) (Fig. 3E).

Normally, climbing fibres predominantly form synapses on the Purkinje cell dendritic shafts (Palay and Chan-Palay, 1974). In pathological conditions, an abnormal increased innervation of climbing fibres onto Purkinje cell spiny branchlets may occur (Ichikawa et al., 2002; Miyazaki et al., 2010). To further investigate the phenomenon of climbing fibres extending into the parallel fibre territory, we performed dual immunofluorescence to label both VGlut2 and calbindin to visualize the relationship between climbing fibre terminals and Purkinje cell dendrites (Fig. 4A–C). In stitched images spanning the molecular layer to reconstruct the Purkinje cell dendritic arbor in essential tremor, we visualized that most climbing fibres formed synapses on the thick, proximal Purkinje cell dendrites; occasionally, we also observed that climbing fibres formed synapses on thin, Purkinje cell spiny branchlets (Fig 4D, arrows). Higher magnification images also revealed that the VGlut2 puncta were distributed mainly on Purkinje cell dendritic shafts (Fig. 4E) but sometimes also on Purkinje cell spiny branchlets (Fig. 4F) forming synaptic contact directly with Purkinje cell dendritic spines (Fig. 4G and H). The percentage of VGlut2 puncta on the Purkinje cell spiny branchlets was 1.7-fold higher in cases with essential tremor than control subjects (42% ± 5% in essential tremor cases versus 25% ± 3% in controls, P = 0.006) (Fig. 4I). Similarly, the percentage of VGlut2 puncta on thin Purkinje cell branchlets <1 μm in diameter was 2.2-fold higher in cases with essential tremor than control subjects.

**Table 1** Demographic characteristics of cases with essential tremor and control subjects

<table>
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<th>Essential tremor cases</th>
<th>Controls</th>
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<tr>
<td>n</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Age (years)</td>
<td>84.2 ± 5.2</td>
<td>81.3 ± 7.0</td>
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<tr>
<td>Female gender</td>
<td>4 (33%)</td>
<td>6 (46%)</td>
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<tr>
<td>Brain weight (g)</td>
<td>1215.4 ± 134.0</td>
<td>1197.5 ± 134.1</td>
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<tr>
<td>Post-mortem interval (h)</td>
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<td>9.9 ± 12.7</td>
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<tr>
<td>0</td>
<td>5 (41.7%)</td>
<td>7 (53.8%)</td>
</tr>
<tr>
<td>A</td>
<td>3 (25.0%)</td>
<td>4 (30.8%)</td>
</tr>
<tr>
<td>B</td>
<td>3 (25.0%)</td>
<td>2 (15.4%)</td>
</tr>
<tr>
<td>C</td>
<td>1 (8.3%)</td>
<td>0 (0.0%)</td>
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<tr>
<td>Braak Alzheimer’s disease stage</td>
<td>4.7 ± 1.0**</td>
<td>2.6 ± 1.6</td>
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<tr>
<td>Purkinje cell count</td>
<td>8.8 ± 1.1*</td>
<td>10.3 ± 2.1</td>
</tr>
<tr>
<td>Torpedo count</td>
<td>24.7 ± 25.2</td>
<td>13.0 ± 12.0</td>
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<tr>
<td>Basket plexus rating</td>
<td>2.2 ± 0.9</td>
<td>1.9 ± 0.4</td>
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<tr>
<td>Heterotopic Purkinje cell count</td>
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<td>Total Tremor Score</td>
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<td>Tremor Related Disability Score</td>
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Mean ± SD and frequency (%) are reported.

*P = 0.05, **P = 0.001 compared to controls.

CERAD = Consortium to Establish a Registry for Alzheimer’s disease.
(26% ± 12% in essential tremor cases versus 12% ± 7% in controls, P = 0.003) (Fig. 4I). Finally, we measured the diameter of VGlut2 puncta and found that the VGlut2 puncta diameter was similar in essential tremor cases and controls (0.91 ± 0.17 μm in essential tremor cases versus 0.87 ± 0.10 μm in controls, P = 0.54) (Fig. 4K).

Interestingly, VGlut2 puncta density was similar among cases with essential tremor, with ~25 VGlut2 puncta/100 μm climbing fibres regardless of the tremor severity (correlation between VGlut2 puncta density and total tremor scores, r = −0.02, P = 0.96) and was significantly lower than that in control subjects (Fig. 5A). In addition, we found that in cases with essential tremor, the percentage of VGlut2 puncta on Purkinje cell branchlets <1 μm in diameter strongly and inversely correlated with total tremor scores (r = −0.88, P = 0.004) (Fig. 5B) and tremor-related disability scores (r = −0.64, P = 0.033). Although cases with
Discussion

Because proper Purkinje cell physiology is heavily dependent on climbing fibre-Purkinje cell connections, abnormal climbing fibre-Purkinje cell connections could lead to dysfunctional motor control and possibly clinical tremor and/or ataxia in essential tremor. In this study, we found abnormal climbing fibre-Purkinje cell connections in essential tremor cerebellar cortex, including decreased VGlut2 synaptic density, and abnormal extension of climbing fibres into the outer 20% of the molecular layer (a predominant parallel fibre territory) versus control brains, and increased synaptic contacts of climbing fibres on spiny branchlets of Purkinje cells in essential tremor. The potential importance of our findings for Purkinje cell physiology in essential tremor is further supported by the robust correlations we observed between the clinical tremor scores and the abnormal climbing fibre-Purkinje cell synapses on distal, thin Purkinje cells dendritic branchlets.

The mechanism of abnormal climbing fibre-Purkinje cell synaptic connections in essential tremor remains to be elucidated. Climbing fibre synaptic density and distribution is highly plastic and is dynamically regulated in the context of Purkinje cell degeneration. In mouse models of spinocerebellar ataxias, expression of mutant proteins specifically in Purkinje cells causes Purkinje cell degeneration and abnormal climbing fibre-Purkinje cell connections including: changes in the climbing fibre-Purkinje cell synaptic territory, abnormal climbing fibre-Purkinje cell synapses on Purkinje cell soma, and defective climbing fibre-Purkinje cell synaptic transmission (Shuvaev et al., 2011; Ebner et al., 2013; Furrer et al., 2013); therefore, the abnormal climbing fibre-Purkinje cell synaptic connections in essential tremor could be secondary to Purkinje cell degeneration. Purkinje cells play an active role in regulating climbing fibre-Purkinje cell synaptic connections. Purkinje cells express two molecules, glutamate receptor δ2 (GluR δ2) and P/Q type voltage-dependent calcium channel, Ca_{2,1}, which can critically regulate the climbing fibre and parallel fibre territories in the Purkinje cell dendritic arbor. Purkinje cell-specific GluRδ2 knockout mice have abnormal extension of climbing fibres into the outer molecular layer parallel fibre territory and excessive climbing fibre synapses on the Purkinje cell spiny branchlets (Hashimoto et al., 2001; Ichikawa et al., 2002; Miyazaki et al., 2010), also two of our main findings in essential tremor cerebellum. In contrast, Purkinje cell-specific Ca_{2,1} knockout mice have a decreased climbing fibre territory and an expanded parallel fibre territory in their Purkinje cell dendritic arbor (Miyazaki et al., 2012). Recently, disabled homologue 2-interacting protein (DAB2IP) has also been identified as an additional molecule to regulate parallel fibre-Purkinje cell and climbing fibre-Purkinje cell synaptic distributions, and mice with DAB2IP knockdown had decreased parallel fibre-Purkinje cell synaptic density and increased climbing fibre-Purkinje cell synaptic density (Qiao et al., 2013). Interestingly, GluR δ2 knockout mice have cerebellar ataxia and 10 Hz rhythmic oscillations in the eyes (Yoshida et al., 2004). Intriguingly, an autoantibody against GluRδ2 has been reported to cause acute cerebellar ataxia and also hand tremor in humans (Shihara et al., 2007). Further investigations are warranted to elucidate the parallel fibre-Purkinje cell synaptic distributions and also the role of GluRδ2, Ca_{2,1}, and DAB2IP in essential tremor.
Alternatively, long-standing hyperexcitability in the olivocerebellar loops can also lead to abnormal climbing fibre-Purkinje cell connections, as climbing fibre-Purkinje cell synaptic density and distribution on Purkinje cell dendrites could be regulated by climbing fibre activity. Inhibition of cerebellar electric activity by tetradotoxin infusion leads to climbing fibre regression and decreased climbing fibre-Purkinje cell synaptic density, whereas the subsequent withdrawal of tetradotoxin results in the re-establishment of climbing fibre-Purkinje

**Figure 3** Increased climbing fibres in the outer portion of the molecular layer in the essential tremor cerebellar cortex. The dotted line indicates the border between the outer 20% and inner 80% of the molecular layer (A and B). Representative cerebellar sections of a control (A) and a case with essential tremor (B) labelled with anti-VGlut2 antibodies are shown. The square areas are shown at higher magnification, to the right of each panel, and demonstrated that an essential tremor case had an increased number of climbing fibres passing the dotted line as compared to a control subject. Cases with essential tremor, when compared to controls, had a significantly increased number of climbing fibres extending into the outer 20% of the molecular layer in randomly selected ×200 fields (C) and significantly increased percentage of total climbing fibre profiles extending into the outer 20% of the molecular layer (D). We did not observe any differences in the thickness of the molecular layer between cases with essential tremor and control subjects (E). CF = climbing fibre; ET = essential tremor.
Figure 4 Increased climbing fibre synaptic puncta on the thin Purkinje cell spiny branchlets in essential tremor cerebellar cortex. Dual immunofluorescence with anti-VGlut2 (Alexa Fluor® 488, green) and anti-calbindin D28k antibody (Alexa Fluor® 594, red) of cerebellar sections (A–H). VGlut2 puncta followed the climbing fibres and were distributed over Purkinje cell dendrites (A–C). Higher magnification of stitched images revealed the relationship of VGlut2 puncta and Purkinje cell dendrites in a case with essential tremor. Most of the VGlut2 puncta were distributed on the thick Purkinje cell dendritic shafts and occasionally on the Purkinje cell spiny branchlets (arrows) (D). Representative images showed that VGlut2 puncta were distributed over the thick Purkinje cell dendrites (E) and occasionally on the Purkinje cell spiny branchlets (F). Higher magnification images revealed that VGlut2 puncta form synaptic connections with Purkinje cell spiny branchlets (G and H). In cases with essential tremor, a significantly higher percentage of VGlut2 puncta synapsed on the Purkinje cell spiny branchlets (I) and a significantly higher percentage of VGlut2 puncta synapsed on Purkinje cell branchlets <1 μm in diameter (J). The diameter of VGlut2 puncta is similar between essential tremor cases and controls (K). PC = Purkinje cell; ET = essential tremor.
Purkinje cell degeneration and/or persistent long-standing tremor (tremor (Fig. 5B) suggests that in severe essential tremor, continued puncta on distal branchlets and total tremor scores in essential controls. Third, the strong negative correlation between VGlut2 cell distal branchlets in cases with essential tremor compared with climbing fibre-Purkinje cell synapses preferentially on the Purkinje essential tremor. Second, we found an abnormal distribution of enhance susceptibility to essential tremor or might be the result of decreased climbing fibre-Purkinje cell synaptic connections might that in control subjects (Fig. 5A). This finding suggests that (with essential tremor had a similar VGlut2 density to one another (Cesa and Strata, 2005, 2009; Cesa et al., 2007). Other factors such as environmental toxin exposures and trauma also cause abnormal climbing fibre distributions (Letellier et al., 2008; Pierce et al., 2011).

The consequences of abnormal climbing fibre-Purkinje cell connections are not entirely clear, but it is possible that abnormal climbing fibre-Purkinje cell synaptic transmission causes defective Purkinje cell physiology and subsequently leads to tremor and/or ataxia. Both climbing fibres and Purkinje cells can drive motor learning (Nguyen-Vu et al., 2013), and the abnormal climbing fibre-Purkinje cell connections we now observe along with previously reported Purkinje cell loss (Louis et al., 2013) and Purkinje cell axonal pathology (Babij et al., 2013) could jointly contribute to defective motor control in essential tremor. The strong correlation we observed between clinical tremor severity and aberrant climbing fibre-Purkinje cell synaptic connections supports this notion. In Parkinson’s disease, the synaptic changes in the medium spiny neurons that result from dopamine depletion can cause aberrant synaptic connections that predispose patients to develop clinical dyskinesia (Zhang et al., 2013). In essential tremor, the mislocalization of climbing fibre-Purkinje cell synapses in the Purkinje cell spiny branchlets may cause disturbed calcium homeostasis within the distal Purkinje cell dendritic compartment (Hashimoto et al., 2001), which increases vulnerability of Purkinje cells to excitotoxic cell death (Stemmer et al., 2005).

We made three significant observations. First, most of the cases with essential tremor had a similar VGlut2 density to one another (~25/100 μm of climbing fibre length), and this was lower than that in control subjects (Fig. 5A). This finding suggests that decreased climbing fibre-Purkinje cell synaptic connections might enhance susceptibility to essential tremor or might be the result of essential tremor. Second, we found an abnormal distribution of climbing fibre-Purkinje cell synapses preferentially on the Purkinje cell distal branchlets in cases with essential tremor compared with controls. Third, the strong negative correlation between VGlut2 puncta on distal branchlets and total tremor scores in essential tremor (Fig. 5B) suggests that in severe essential tremor, continued Purkinje cell degeneration and/or persistent long-standing tremor seems to cause preferential pruning of distal branches of Purkinje cells and climbing fibres, obscuring and/or reversing the pattern that is seen in mild essential tremor.

This study had limitations. First, the number of studied subjects was small. Despite this, we found significant case-control differences as well as robust clinicopathological correlations. Second, we do not know the specificity of these abnormal climbing fibre-Purkinje cell synaptic connections in cerebellar degenerative disorders, and thus studies on climbing fibre-Purkinje cell synaptic pathology in other cerebellar degenerative diseases such as spinocerebellar ataxias would provide further insights.

In conclusion, we have found abnormal climbing fibre-Purkinje cell synaptic connections in the essential tremor cerebellum. Future investigations on the mechanism of abnormal climbing fibre-Purkinje cell synaptic connections in essential tremor may help with the development of novel therapeutic targets for essential tremor.

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Supplementary material
Supplementary material is available at Brain online.

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