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Excitatory Amino acid transporter expression in the essential tremor dentate nucleus and cerebellar cortex: A postmortem study

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ABSTRACT

Background: Genome-wide association studies have revealed a link between essential tremor (ET) and the gene *SLC1A2*, which encodes excitatory amino acid transporter type 2 (EAAT2). We explored EAAT biology in ET by quantifying EAAT2 and EAAT1 levels in the cerebellar dentate nucleus, and expanded our prior analysis of EAAT2 levels in the cerebellar cortex.
Objective: To quantify EAAT2 and EAAT1 levels in the cerebellar dentate nucleus and cerebellar cortex of ET cases *vs.* controls.
Methods: We used immunohistochemistry to quantify EAAT2 and EAAT1 levels in the dentate nucleus of a discovery cohort of 16 ET cases and 16 controls. Furthermore, we quantified EAAT2 levels in the dentate nucleus in a replicate cohort (61 ET cases, 25 controls). Cortical EAAT2 levels in all 77 ET cases and 41 controls were quantified.
Results: In the discovery cohort, dentate EAAT2 levels were 1.5-fold higher in 16 ET cases *vs.* 16 controls ($p = 0.007$), but EAAT1 levels did not differ significantly ($p = 0.279$). Dentate EAAT2 levels were 1.3-fold higher in 61 ET cases *vs.* 25 controls in the replicate cohort ($p = 0.022$). Cerebellar cortical EAAT2 levels were 20% and 40% lower in ET cases *vs.* controls in the discovery and the replicate cohorts (respective p values $= 0.045$ and < 0.001).
Conclusion: EAAT2 expression is enhanced in the ET dentate nucleus, in contrast to differentially reduced EAAT2 levels in the ET cerebellar cortex, which might reflect a compensatory mechanism to maintain excitation-inhibition balance in cerebellar nuclei.

1. Introduction

Essential tremor (ET) is one of the most common neurological disorders; disease prevalence increases with advancing age, reaching values in excess of 20% among persons in their 90s [1]. A growing body of clinical, physiological and postmortem evidence now links it with an abnormal cerebellum [2]. Indeed, a number of postmortem features distinguish ET from similarly-aged control brains, including (1) increased numbers of torpedoes and related Purkinje cell (PC) axonal pathologies [3,4], (2) increased heterotopic displacement of PCs [5], (3) abnormal climbing fiber (CF)-PC connections [6,7], and (4) a dense and elongated basket cell plexus surrounding the PCs [8,9]. In addition, there is a loss of PCs in several [3,10] although not all studies [11,12].

A polymorphism in the gene *solute carrier family 1, member 2* (*SLC1A2*) has recently been associated in many [13–15] although not all studies with ET [16]. Hence, the biological links between *SLC1A2* and ET are of significant current interest. The *SLC1A2* gene encodes excitatory amino acid transporter 2 (EAAT2), which belongs to a family of excitatory amino-acid transporters (EAATs) [17].

Abbreviation: AD, Alzheimer's disease; CERAD, the Consortium to establish a Registry for Alzheimer's disease; GFAP, glial fibrillary acidic protein.

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Astrocytes express both EAAT1 and EAAT2, and these transporters regulate extracellular glutamate levels in the brain. Excessive excitatory neurotransmission has been postulated as a mechanism for tremor in animal models of tremor, although there is no empiric evidence that this occurs in ET [18]. We previously reported that the levels of EAAT2, but not EAAT1, were reduced in the cerebellar cortex in 16 ET cases *vs*. 13 controls [19]. In theory, this reduction could be associated with a decreased capacity of astrocytes to clear extracellular glutamate, thereby enhancing the vulnerability of PCs to excitotoxic injury.

Aside from the cerebellar cortex, another brain region implicated in ET is the dentate nucleus. Structural and functional changes of the dentate nucleus in ET have been reported in imaging studies [20]. The dentate nucleus receives strong inhibitory input from PCs, which provide the sole neuronal output from the cerebellar cortex; it also receives excitatory inputs from collateral branches of mossy fibers and CF collaterals [21]. Long-standing rhythmic firing of CF excitatory inputs on PCs and, to a lesser degree, on dentate nuclei via CF collaterals, has been postulated as a pathogenic mechanism in animal models of tremor [22]. *In vivo* analyses in animal studies have demonstrated that intrinsic firing rates of cerebellar nuclear cells are modulated during movements [21], with effectiveness of inhibitory versus excitatory inputs varying during specific motor behaviors. Therefore, the proper timing of excitatory neurotransmission regulated by EAATs in the dentate nucleus could be important in ET. Extending our previous study [19], we now assess the expression levels of EAAT2 and EAAT1 in the dentate nucleus of ET cases *vs*. controls, and significantly expand and replicate our analysis of EAAT2 in the cerebellar cortex in a far larger cohort of ET cases and controls.

2. Methods

2.1. Brain repository and study subjects

All ET brains were from the Essential Tremor Centralized Brain Repository (ETCBR), New York Brain Bank (NYBB), Columbia University, New York. The clinical diagnosis of ET was initially assigned by treating neurologists. Cases were assessed by a series of semi-structured clinical questionnaires, including Archimedes spirals and the information regarding family history and tremor characteristics. Finally, the diagnosis of ET was confirmed by an ETCBR study neurologist (EDL) using medical records, a detailed, videotaped, neurological assessment, and ETCBR diagnostic criteria [3]. The severity of tremor was rated using the total tremor score, based on the severity of postural and kinetic arm tremors (range = 0–36).

Most of the control brains were obtained from the NYBB (n = 28, 10 in the discovery cohort and 18 in the replicate cohort) and were from individuals followed at the Alzheimer disease (AD) Research Center or the Washington Heights Inwood Columbia Aging Project at Columbia University. They were followed prospectively with serial neurological examinations, and were clinically free of AD, ET, Parkinson's disease (PD), Lewy body dementia, or progressive supranuclear palsy. Thirteen controls were from the Harvard Brain Tissue Resource Center (McLean Hospital, Belmont, MA) (6 in the discovery cohort and 7 in the replicate cohort). EAAT2 immunostaining intensity in the cerebellar cortex of 8 of the current controls and 10 of the current ET cases was reported previously [19].

All study subjects signed informed consent approved by these University Ethics Boards. We performed a power analysis, using data collected in our prior study of cerebellar cortical EAAT2 levels by immunohistochemistry [19]. We found that, with the sample size of 13 in each group, we had a power of 0.90 to detect case-control differences of the magnitude detected previously. To be conservative, we chose 16. We chose controls who had available paraffin-embedded sections of the dentate nucleus and were >70 years old at the time of death, thereby allowing us to frequency-match them to ET cases by age at death. For our replicate cohort, we identified all of the remaining ET cases and controls in the brain bank with available dentate nucleus in paraffin-embedded sections; there were 61 such ET cases and 25 controls.

2.2. Neuropathological assessment

All ET and control brains had a complete neuropathological assessment at the NYBB and Harvard Brain Bank, as described previously [2]. The postmortem interval (PMI), the time from death to the time of brain placed in −80 °C, in each case was recorded. We did not include ET cases with Lewy body pathology (α-synuclein staining) or dentate nucleus degeneration [2].

A standard 3 × 20 × 25 mm parasagittal neocerebellar block, including dentate nucleus, was obtained from a 0.3-cm-thick parasagittal slice located 1 cm from the cerebellar midline. Paraffin sections (7 μm thick) were stained with Luxol fast blue hematoxylin and eosin (LH&E), and PC counts and torpedo counts were quantified as described previously [2].

2.3. Dentate nucleus immunohistochemistry

Seven-μm-thick paraffin-embedded cerebellar sections were incubated with guinea pig anti-EAAT2 antibody (Millipore, ab1783, 1:200) or mouse anti-glial fibrillary acidic protein (GFAP) antibody (Sigma, G3893, 1:100) at 4 °C for 24 h after antigen retrieval in Trilogy (Cell Marque) for 60 min, 100 °C. The sections were incubated with goat anti-mouse (Fisher Scientific, 1:200) or anti-guinea pig IgG biotin-conjugated secondary antibody (Millipore, 1:200), respectively, followed by 3,3′-diaminobenzidine (DAB) precipitation. These antibodies have been validated previously [19].

Whole slide scanning was performed with a 20× lens (Leica SCN400 scanner) and a trained and clinically-blinded technician (GCK) traced the outline of the dentate nucleus and measured the average intensity of EAATs immunoreactivity/μm^2 of dentate area with Leica Biosystems Tissue IA Software, version 2.0. EAAT2 immunoreactivity was similarly quantified in the cerebellar cortex, as previously described [19]. The EAAT immunoreactivity was normalized to controls, for which EAAT immunoreactivity was set to be 1.00.

GFAP immunohistochemistry was used to assess whether increased astrocytes in the dentate nucleus could account for increases in EAAT2 levels. Due to the lack of a distinctive border between white matter and the dentate nucleus in many cases and dense astrocyte processes normally present in white matter, the GFAP immunostained sections were not suitable for quantitative image analysis as performed for EAAT proteins. Therefore, we developed a semi-quantitative GFAP rating scale. A senior neuropathologist (PLF), blinded to clinical information, evaluated the GFAP-positive process and cell body densities in the dentate nucleus in each ET case and control. The following semi-quantitative scale was used: 1 (few discernible processes, paler overall staining than adjacent white matter); 2 (mild to moderate processes, rare to sparse cell body staining); 3 (moderate number of processes, mild cell body staining), 4 (dense processes and moderate to severe cell body staining). For each ET case and control, eight microscopic images (4 each at 50× and 100× magnification) were acquired in randomly selected areas spanning the dentate nucleus, and each image was assigned a 1–4 rating.

For dual immunofluorescence studies, we immunostained paraffin-embedded sections with anti-EAAT2 antibodies and mouse anti-glutamine synthetase (BD Transduction, 610518, 1:300) or mouse anti-GFAP. The secondary antibodies were goat anti-

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