Defective dentate nucleus GABA receptors in essential tremor

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The development of new treatments for essential tremor, the most frequent movement disorder, is limited by a poor understanding of its pathophysiology and the relative paucity of clinicopathological studies. Here, we report a post-mortem decrease in GABA\(_A\) (35\% reduction) and GABA\(_B\) (22–31\% reduction) receptors in the dentate nucleus of the cerebellum from individuals with essential tremor, compared with controls or individuals with Parkinson’s disease, as assessed by receptor-binding autoradiography. Concentrations of GABA\(_B\) receptors in the dentate nucleus were inversely correlated with the duration of essential tremor symptoms (\(r^2 = 0.44, P < 0.05\)), suggesting that the loss of GABA\(_B\) receptors follows the progression of the disease. In situ hybridization experiments also revealed a diminution of GABA\(_B\) receptor messenger RNA in essential tremor (\(\#27\%\)).

In contrast, no significant changes of GABA\(_A\) and GABA\(_B\) receptors (protein and messenger RNA), GluN2B receptors, cytochrome oxidase-1 or GABA concentrations were detected in molecular or granular layers of the cerebellar cortex. It is proposed that a decrease in GABA receptors in the dentate nucleus results in disinhibition of cerebellar pacemaker output activity, propagating along the cerebello-thalamo-cortical pathways to generate tremors. Correction of such defective cerebellar GABAergic drive could have a therapeutic effect in essential tremor.

Keywords: essential tremor; cerebellum; GABA receptors; dentate nucleus; deep cerebellar nuclei

Abbreviations: GABA = \(\gamma\)-aminobutyric acid

Introduction

More than 10 million Americans suffer from essential tremor, making it the most prevalent adult-onset movement disorder (Moghal et al., 1994; Louis and Ferreira, 2010). Essential tremor is characterized by a bilateral action tremor affecting predominantly the arms, the head and/or the voice (Rajput et al., 2004; Louis, 2005). Formerly regarded as benign, the symptoms of essential tremor evolve gradually and can become very disturbing for patients. Recent studies have emphasized the presence of...
abnormalities within γ-aminobutyric acid (GABA)-ergic Purkinje cells in a significant proportion of patients with essential tremor (Louis et al., 2007, 2009, 2010; Axelrad et al., 2008; Shill et al., 2008). Several lines of evidence dating back to the early 1970s suggest that cerebellar dysfunctions reverberating through the cerebello-thalamo-cortical pathway play a key role in essential tremor (Deuschl et al., 2000; McAuley and Marsden, 2000; Pinto et al., 2003; Elble and Deuschl, 2009; Schnitzler et al., 2009).

The standard pharmaceutical care of essential tremor has essentially not changed in the last 40 years and still relies on primidone and propranolol, which display moderate efficacy to decrease the amplitude of tremor in less than half of patients (Lyons et al., 2003; Louis et al., 2010; Deuschl et al., 2011). The small number of post-mortem studies performed on essential tremor subjects is likely linked to the limited therapeutic arsenal available today to treat essential tremor (Louis, 2005, 2009).

The fact that drugs acting on GABA A receptors, such as primidone, benzodiazepines or ethanol, decrease tremor amplitude suggests that altered GABAergic neurotransmission is involved in essential tremor. Although the exact location of this impaired GABAergic current is unknown, GABA dysfunction in the cerebellum has been suggested as an aetiologic factor in essential tremor (Deuschl et al., 2000; Kralic et al., 2005; Louis, 2005; Elble and Deuschl, 2009). More specifically, post-mortem studies indicate that cerebellar GABAergic Purkinje cells show increased numbers of axonal swellings and a reduction in numbers in a significant proportion of subjects with essential tremor (Louis et al., 2007, 2009; Axelrad et al., 2008; Shill et al., 2008). Low levels of GABA have been reported in the CSF of patients with essential tremor compared with controls (Mally and Baranyi, 1994; Mally et al., 1996). Moreover, toxins such as aflatoxin, penitrem A or harmaline have been proposed to induce tremor in rodents by interacting with GABA receptors (Cavanagh et al., 1998; Miwa, 2007). In addition, GABA A receptor α1 subunit knockout mice exhibit postural and kinetictremor, partly reproducing some features of essential tremor (Kralic et al., 2005). Finally, a recent PET study revealed decreased binding of 11C-flumazenil at the benzodiazepine receptor site of the GABA A receptor in several brain regions, supporting the possibility of a general GABAergic dysfunction in essential tremor (Boecker et al., 2010).

To characterize GABAergic neurotransmission within the cerebellum of patients with essential tremor, we have conducted post-mortem investigations of GABA A and GABA B receptors by autoradiography and in situ hybridization on horizontal cerebellar sections. Since the major GABAergic tract within the cerebellum connects Purkinje cells to deep cerebellar nuclei and all outgoing information leaving the cerebellum is funnelled through the deep cerebellar nuclei, it was essential to measure GABA receptor in this structure. However, deep cerebellar nuclei are both very small and irregularly shaped, and it is therefore technically challenging to measure their protein or messenger RNA content with available neuroimaging approaches or techniques requiring tissue homogenates. Autoradiography and in situ hybridization were thus selected because these techniques allow the 2D resolution necessary to quantify the expression of these receptors in the deep cerebellar nuclei, notably the dentate nucleus—the largest and one of the most important subparts of the deep cerebellar nuclei. A group of patients with essential tremor (n = 10) was compared with control (n = 16) and patients with Parkinson’s disease (n = 10), taking advantage of a clinicopathological study in which detailed clinical variables have been prospectively recorded by the same neurologists (A.H.R. and A.R.).

Materials and methods

Clinicopathological assessment of patients

Patients with essential tremor and Parkinson’s disease controls were all followed at the Movement Disorders Clinic Saskatchewan at 6- or 12-month intervals (Rajput et al., 2004, 2009). The Movement Disorders Clinic Saskatchewan has operated uninterrupted since 1968. Autopsy was restricted to those patients who have been assessed by the Movement Disorders Clinic Saskatchewan neurologists (A.H.R. and A.R.). The clinical diagnosis of essential tremor was made as described previously (Rajput et al., 2004). All cases with essential tremor had postural and/or kinetic tremor for several years. All cases with Parkinson’s disease had resting tremor: two were tremor-dominant cases and eight were classical (mixed) with approximately equal severity of bradykinesia, rigidity and tremor (Rajput et al., 2009). For this study, three main criteria were used to select subjects with essential tremor: the absence of neuropathologically confirmed Parkinson’s disease; the presence of dentate nucleus in the tissue block; and age and gender matching with the two other groups. The severity of tremor was recorded at each visit and was based on the visual assessment of tremor amplitude at any site [Unified Parkinson’s Disease Rating Scale (UPDRS III)] and the history of its impact on daily activities (Rajput et al., 2004). As essential tremor is often misdiagnosed as parkinsonian tremor (Rajput et al., 2004; Jain et al., 2006; Shahed and Jankovic, 2007; Minen and Louis, 2008; Benito-Leon et al., 2009; Adler et al., 2011), tissue from patients with Parkinson’s disease were included to identify changes that are specific for essential tremor (i.e. kinetic tremor versus resting tremor). Clinical data including the age and mode of onset, severity of the disease, duration of disease, response to treatment and adverse effects of treatment, were recorded prospectively after each clinical assessment. General information such as other diagnosis, sex, cause and age of death were also documented. All autopsies were done within 25 h of death. Table 1 shows a summary of relevant information available regarding the subjects involved in the study and more detailed information is provided in Supplementary Table 1. The maximal severity of tremor scores is given in Table 1 and Supplementary Table 1.

Tissue handling and processing

Details of autopsy procedures have been described previously (Rajput et al., 2004, 2009). One half of the brain was histologically examined by a neuropathologist using representative sections including: the cerebral cortex, hippocampus, caudate, lentiform nucleus, thalamus, mid-brain, pons, medulla and cerebellum with dentate nucleus. Standard stains including silver stain and α-synuclein, ubiquitin and tau immunostaining were used routinely as they became commercially available (Rajput et al., 2009). Neuropathologists produced detailed reports, which were provided to the family and discussed where appropriate. In some cases with essential tremor, cerebellar Purkinje...
cells were performed by a neuropathologist as reported previously (Raiput et al., 2011). The other half of the brain was frozen at –80°C and was cut by hand in the frontal plane into 2–3-mm thick slices. Coronal slices containing molecular layer of the cerebellar cortex, granular layer of the cerebellar cortex and dentate nucleus were cryostat-sectioned (20 μm), thaw-mounted onto SuperFrostPlus 75 × 50-mm slides (Brain Research Laboratories), desiccated overnight at 4°C, and stored at –80°C until assayed. For homogenate-based studies such as western immunoblotting or high-performance liquid chromatography, cerebellar cortex extracts (~100 mg) were homogenized and processed to generate a Tris-buffered saline-soluble fraction containing extracellular and cytosolic proteins, a detergent-soluble fraction containing membrane-bound proteins and a formic acid-soluble fraction containing detergent-insoluble proteins, as described in detail previously (Tremblay et al., 2007; Julien et al., 2008). Cerebellar pH was measured in 10 mg of tissue diluted into 10 volumes of unbuffered, deionized distilled water, as described previously (Kingsbury et al., 1995; Calon et al., 2003a; Julien et al., 2008). Indeed, tissue pH has been identified as one of the best markers to assess the degree of preservation of post-mortem brain tissue, as it is particularly sensitive to ante-mortem agonal phase and is a good marker of messenger RNA degradation (Kingsbury et al., 1995; Li et al., 2004; Lipka et al., 2006; Mexal et al., 2006; Vawter et al., 2006; Atz et al., 2007; Weis et al., 2007).

Western immunoblotting
For western immunoblotting, protein concentration was determined using bicinchoninic acid assays (Pierce). Equal amounts of protein per sample (20 μg of total protein per lane) were added to Laemmlli’s loading buffer, heated to 95°C for 5 min before loading, and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Proteins were electroblotted onto polyvinylidene fluoride membranes (Immobilon, Millipore) before blocking in 5% non-fat dry milk and 0.5% bovine serum albumin (Invitrogen) in phosphate-buffered saline containing 0.1% Tween for 1 h. Membranes were immunoblotted with appropriate primary and secondary antibodies followed by chemiluminescence reagents (Lumiglo reserve, KPL Inc.). Optical densities of bands were directly quantified using a KODAK Image Station 4000 MM Digital Imaging System (Molecular Imaging Software version 5.0.0.90). Optical densities were measured in the molecular layer of the cerebellar cortex, the granular layer of the cerebellar cortex and the dentate nucleus of the deep cerebellar nuclei.

Table 1 Selected characteristics of study volunteers and comparison of tissue quality between groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control</th>
<th>Parkinson’s disease</th>
<th>Essential tremor</th>
<th>Statistical comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>10</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>Men, ratio</td>
<td>6/16</td>
<td>2/10</td>
<td>2/10</td>
<td>Contingency 0.51</td>
</tr>
<tr>
<td>Age at death, mean (SD) (years)</td>
<td>76 (6)</td>
<td>82 (8)</td>
<td>84 (11)</td>
<td>ANOVA 0.08</td>
</tr>
<tr>
<td>Age of onset, mean (SD) (years)</td>
<td>–</td>
<td>63 (12)</td>
<td>54 (20)</td>
<td>–</td>
</tr>
<tr>
<td>Disease duration, mean (SD) (years)</td>
<td>–</td>
<td>19 (8)</td>
<td>30 (18)</td>
<td>–</td>
</tr>
<tr>
<td>Brain pH mean (SD)</td>
<td>6.14 (0.31)</td>
<td>6.46 (0.19)</td>
<td>6.36 (0.17)</td>
<td>ANOVA 0.06</td>
</tr>
<tr>
<td>Post-mortem interval, mean(SD) (h)</td>
<td>19 (6)</td>
<td>18 (6)</td>
<td>15 (8)</td>
<td>ANOVA 0.32</td>
</tr>
<tr>
<td>Brain weight, mean (SD) (g)</td>
<td>1249 (49)</td>
<td>1248 (150)</td>
<td>1178 (172)</td>
<td>ANOVA 0.49</td>
</tr>
<tr>
<td>Hoehn and Yahr scale, mean (SD)</td>
<td>–</td>
<td>3.5 (0.2)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tremor severity, UPDRS, mean (SD)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CO-1 messenger RNA DN-DCN, mean (SD)</td>
<td>525 (103)</td>
<td>562 (134)</td>
<td>584 (191)</td>
<td>ANOVA 0.68</td>
</tr>
<tr>
<td>CO-1 messenger RNA MolCtx, mean (SD)</td>
<td>433 (114)</td>
<td>437 (122)</td>
<td>490 (102)</td>
<td>ANOVA 0.51</td>
</tr>
<tr>
<td>CO-1 messenger RNA GraCtx, mean (SD)</td>
<td>872 (206)</td>
<td>931 (191)</td>
<td>1013 (159)</td>
<td>ANOVA 0.31</td>
</tr>
<tr>
<td>Poly-T messenger RNA MolCtx, mean (SD)</td>
<td>124 (119)</td>
<td>86 (33)</td>
<td>105 (72)</td>
<td>ANOVA 0.59</td>
</tr>
</tbody>
</table>

CO-1 = cytochrome oxidase-1; DN-DCN = dentate nucleus of the deep cerebellar nuclei; GraCtx = granular layer of the cerebellar cortex; MolCtx = molecular layer of the cerebellar cortex; UPDRS = Unified Parkinson’s Disease Rating Scale.

Receptor binding autoradiography
GABA_A receptor density was assessed with [3H]-flunitrazepam (GE-Healthcare; 87 Ci/mmol) allosteric binding to the α1- and γ2-interace (benzodiazepine site) of the GABA_A receptor on cerebellum sections, using a previously reported autoradiography technique (Calon et al., 2003a). Non-specific binding was determined in presence of 1-μM clonazepam. GABA_A receptor density was evaluated with [3H]-CGP 54626 (3-N-[1-(S)-(3,4-dichlorophenyl)-ethylamino]-2-(S)-hydroxypro pyl-P-(cyclo-hexylmethyl)-phosphinic acid; ANAWA; 40 Ci/mmol) binding to the antagonist sites of the GABA_A receptor using previously published procedures (Calon et al., 2001, 2003a). Non-specific binding was measured with 500 μM (–)-baclofen (Tocris Bioscience). N-(3-Methoxyphenyl)glycine, a potent and selective GABA_A receptor agonist, was used to verify the binding specificity of the GABA_A receptor binding sites.

N-Methyl-D-aspartate receptors containing GluN2B subunits (GluN2/B) were quantified using a high-affinity selective antagonist [3H]-Ro 25-6981 (F. Hoffmann-La Roche Ltd; 27.6 Ci/mmol; Mutel et al., 1998; Calon et al., 2003b). The [3H]-Ro 25-6981 is an ifenprodil derivative that has a high affinity for the GluN2B subunit (Mutel et al., 1998; Calon et al., 2003b). Non-specific binding was determined by adding 10 μM Ro 04-5595 hydrochloride (F. Hoffmann-La Roche Ltd) to the incubation buffer. Slide-mounted tissue sections were exposed to Kodak Biomax MR film (Carestream Health) along with standards ([3H]-micro-scales, GE Healthcare) during 2, 6 and 12 weeks for [3H]-flunitrazepam, [3H]-CGP 54626 and [3H]-Ro 25-6981, respectively. Macroscopic quantification of all autoradiograms was performed on a KODAK Image Station 4000 MM Digital Imaging System (Molecular Imaging Software version 5.0.0.90). Optical densities were measured in the molecular layer of the cerebellar cortex, the granular layer of the cerebellar cortex and the dentate nucleus of the deep cerebellar nuclei.
In situ hybridization

The general methodology for in situ hybridization has been fully described previously (Wisden and Morris, 1994; Calon et al., 2001; Julien et al., 2008, 2009). GABA<sub>B</sub> receptors contain an intrinsically ligand-gated chloride channel, formed by the pentameric assembly of multiple subunits. In situ hybridization, histochemistry and quantitative real-time polymerase chain reaction data show that deep cerebellar nuclei neurons mainly express the α1, α2, β2 and γ2 subunit messenger RNAs (Gambaramana et al., 1993; Rotter et al., 2000; Linnemann et al., 2006). Oligonucleotide probes for the human GABA<sub>A</sub> receptor subunits α1, α2, β2 and γ2 have thus been synthesized according to previous publications (Nicholson and Faull, 1996; Petri et al., 2002) and are summarized in Supplementary Table 2.

Metabotropic GABA<sub>B</sub> receptors are heterodimers consisting of GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits (Jones et al., 1998). In situ hybridization has been performed using oligonucleotide probes for GABA<sub>B1a+b</sub> and GABA<sub>B2a+b</sub> as described previously (Calon et al., 2001). Oligonucleotide sequences were specific for the coding region of the GABA<sub>B1a+b</sub> receptor messenger RNA (NM_001470.2; 1807–1763 and 2933–2884), whereas those specific to the GABA<sub>B2a+b</sub> receptor (NM_005458.6; 357–513 and 1315–1271), as described (Calon et al., 2001; Supplementary Table 2).

Subunit I of cytochrome c oxidase messenger RNA is encoded by the mitochondrial genome and is a classical marker of neuronal activity (Wong-Riley, 1989; Hirsch et al., 2000). Subunit I of cytochrome c oxidase oligonucleotide probes were chosen for their coding sequence within the human mitochondrial genome (NC_005089.1; 6268–6221, 6578–6534, 6854–6805 and 7441–7392) and are fully described in Supplementary Table 2. To further assess total messenger RNA content, in situ hybridization using a poly-T probe was performed to label poly-A tails, which are present on the vast majority of eukaryotic messenger RNAs (Julien et al., 2009).

Oligonucleotides were labeled with 33P-dATP (PerkinElmer) using a 3-terminal deoxyxynucleotidyltransferase enzyme kit (New England Biolabs). The reaction was carried out at 37°C for 60 min, and labelled oligonucleotides were purified using a QIAquick® Nucleotide Removal Kit (Qiagen). The purified probes were kept at −20°C until assayed the next day. Pre-hybridization and hybridization conditions were exactly as described (Julien et al., 2009). Slides were exposed to Kodak Biomax MR film for 28, 28, 7 and 2 days for GABA<sub>A</sub>, GABA<sub>B</sub>, poly-T and subunit I of cytochrome c oxidase probes, respectively, and macroscopic optical density quantification was performed using the KODAK Image Station. The hybridization signal value from a single section was obtained after subtracting the labelling from the white matter quantified in the same section. The final data from each individual were from the mean of four to eight tissue sections. Non-specific hybridization to tissue sections was found to be negligible, as determined by adding a 100-fold excess of unlabeled probes.

Amine quantification by high-performance liquid chromatography

GABA was measured by high-performance liquid chromatography (HPLC) coupled with UV detection. Supernatants from Tris-buffered saline fraction extracts of cerebellum were directly derivatized with the reagent dansyl chloride (Sigma-Aldrich) based on previously published methods with slight modifications (Saller and Czupryna, 1989; Calon et al., 1999). Briefly, 50 μl of dansyl chloride (1.2 mg/ml) and 50 μl of Tris-buffered saline extracts or standard solution, also in Tris-buffered saline, were mixed and then incubated for 30 min at 90°C. After a 10-min centrifugation at 9000 rpm (4°C), the supernatant was immediately injected into the chromatograph consisting of a Waters 717 plus autosampler automatic injector set at 6°C, a Waters 1525 binary pump equipped with an Atlantis dC18 (3 μl; 3.9 x 150 mm) column, and a Waters 2487 Dual L Absorbance detector (Waters limited). Absorbance was set at 337 nm and the sensitivity at 0.5 absorbance units full scale. The mobile phase consisted of a water-acetonitrile mixture (88.5–11.35% v/v) containing 0.15% (v/v) of phosphoric acid and was delivered at a rate of 0.8 ml/min. Samples were run in duplicates and peaks were identified and quantified using the Breeze software (Waters limited). HPLC concentration values were normalized to tissue weight.

Data and statistical analyses

Statistical comparisons of means between groups were performed using ANOVA when the homogeneity of variance was confirmed (P > 0.05 using Bartlett’s test). Log transformations of the data were used when needed to equalize variance and to provide more normally distributed measures. Post hoc tests were used to determine significant difference between groups (ANOVA: Newman–Keuls). Adjustments for age of death or cerebellar pH were performed using analysis of covariance (ANCOVA), when needed. Using the least squares method, coefficients of correlation and significance of the linear relationship between parameters were determined with a simple regression model. Adjustment for additional variables (age of death or cerebellar pH) was performed using partial correlation analyses. Likelihood ratio analysis of contingency tables using the Pearson’s method was used in the investigation of categorical data such as sex and apoE ε4 allele status, the statistical result being distributed as chi-squared. All statistical analyses were performed using JMP Statistical Analysis Software (version 8.0.2) and P < 0.05 were considered to be statistically significant.

Results

Prior to experiments, assessment of tissue quality was performed by measuring tissue pH, cytochrome oxidase (subunit I of cytochrome c oxidase) messenger RNA and total messenger RNA content in the cerebellum from our sample series. First, cerebellar pH was comparable between groups suggesting equivalent tissue quality between groups, although there was a trend for reduced brain pH in controls (Table 1). Overall, the general distribution of GABA<sub>A</sub> receptors within the cerebellum was in agreement with earlier analyses in human, monkey and rodent tissue sections (Bowery et al., 1987;
Dennis et al., 1988). Binding of $^{3}$H-CGP 54626 to GABA$_B$ receptors was readily detectable in the dentate nucleus (Fig. 2), but stronger in the cerebellar cortex, particularly in the molecular layer, conforming with previous autoradiographic studies in rodents, monkeys and humans (Bowery et al., 1987; Turgeon and Albin, 1993; Billinton et al., 1999; Calon et al., 2000). These patterns were also consistent with earlier immunohistochemical evaluation of GABA$_B$ receptors in human and rodent...
cerebellum (Margeta-Mitrovic et al., 1999; Billinton et al., 2000; Kulik et al., 2002).

The quantification of the specific binding of $^3$H-flunitrazepam and $^3$H-CGP 54626 revealed a significant decrease of GABA$_A$ (35% versus controls and 35% versus Parkinson’s disease) and GABA$_B$ (↓22% versus controls and ↓31% versus Parkinson’s disease) receptors in the dentate nucleus of patients with essential tremor, compared with the two other groups (Figs 1 and 2). The effects remained statistically significant after adjustment for covariates age of death and cerebellar pH using ANCOVA.

Figure 2 Decreased GABA$_B$ receptors in the dentate nucleus of individuals with essential tremor. (A) Representative autoradiograms of human cerebellum sections showing $^3$H-CGP 54626 to GABA$_B$ receptors in the dentate nucleus and the cerebellar cortex of individuals with essential tremor (ET) or Parkinson’s disease (PD), compared with controls (Ctrl). Specific binding of $^3$H-CGP 54626 to GABA$_B$ receptors in the dentate nucleus (B), molecular layer (D) and granular layer (E) of the cerebellar cortex. Correlation analyses between GABA$_A$ receptors and the duration of essential tremor symptoms showed an inverse relationship between the duration of essential tremor and GABA$_B$ receptors in the dentate nucleus (C). Each point represents an individual and the horizontal bar is the average ($n=9–15$ per group). Statistical comparisons were performed using an ANOVA followed by Newman–Keuls post hoc test or linear regression.
Interestingly, the concentrations of GABA$_A$ receptors in the dentate nucleus were inversely correlated [Pearson correlation coefficient (pairwise detection method) = 0.66, $r^2 = 0.44$, $P = 0.0365$] with the duration of essential tremor symptoms, indicating that the longer the clinical expression of the disease, the lower the levels of GABA$_A$ receptors (Fig. 2C). Adjustment for age of death or cerebellar pH using partial correlations had minimal effect on the significance of the correlation. Controlling for both age of death and cerebellar pH, a correlation of $-0.757$ ($P = 0.0402$) was obtained, as estimated by restricted maximum likelihood method. In contrast, no significant differences between groups were detected in either molecular or granular layers of the cerebellar cortex (Figs 1D and E and 2D and E). The absence of change in GABA$_A$ receptors in the cerebellar cortex was confirmed by western immunoblot (Supplementary Fig. 1). No difference in $[^3]$H-Ro 25-6981 specific binding to GluN2B subunits of aspartic acid receptors was detected in any subregion of the cerebellum, including the dentate nucleus, confirming that the decrease of GABA$_A$ receptor was specific (Supplementary Fig. 2).

We next ascertained whether the loss of GABA receptor in the dentate nucleus was associated with reduced messenger RNA transcription by performing in situ hybridization of subunits human GABA$_A$ receptor subunit $\alpha$1, $\alpha$2, $\beta$2 and $\gamma$2 (Supplementary Figs 3 and 4). Strong hybridization signals for $\alpha$1-, $\beta$2- and $\gamma$2 subunits were detected in the cerebellar cortex, mostly in the granular layer (Supplementary Fig. 3). The $\alpha$2 subunit displayed a different expression pattern being almost exclusively expressed in the Purkinje cells layer (Supplementary Figs 3 and 4). Overall, the distribution of GABA$_A$ receptor subunit messenger RNA is in agreement with previous work in the rat cerebellum (Laurie et al., 1992; Gambarana et al., 1993; Rotter et al., 2000; Linnemann et al., 2006). An increase of GABA$_A$ receptor subunit $\alpha$1 and $\beta$2 was detected in the cerebellar cortex of patients with Parkinson’s disease (Supplementary Fig. 4). However, the signal in the dentate nucleus was too weak to allow reliable quantification, except for $\alpha$1 (Supplementary Fig. 4, but was not different between groups (Supplementary Fig. 4).

In situ hybridization experiments targeting GABA$_B$(1a+b) and GABA$_B$(2) messenger RNA were performed in the same series of samples. The messenger RNA distribution of GABA$_B$(1a+b) and GABA$_B$(2) messenger RNA was stronger in the molecular layer of cerebellar cortex (Fig. 3A and B), as expected from previous studies in rodents (Billinton et al., 1999; Bischoff et al., 1999; Durkin et al., 1999; Clark et al., 2000; Liang et al., 2000). However, only GABA$_B$(1a+b) messenger RNA was detectable in the dentate nucleus (Fig. 3A). When comparing groups of patients, there was a significant decrease in the density of messenger RNA for GABA$_B$(1a+b) in the dentate nucleus from individuals with essential tremor, compared with controls without neurological impairment ($P < 0.01$) and to individuals suffering from Parkinson’s disease ($P < 0.05$; Fig. 3C). ANCOVA revealed that the changes of GABA$_B$(1a+b) messenger RNA remained significant after adjustment for age of death and cerebellar pH. No difference in GABA$_B$(1a+b) or GABA$_B$(2) subunit messenger RNA was seen in the cerebellar cortex (Fig. 3D and E). Inverse relationships between age of death and GABA$_B$(1a+b) messenger RNA were detected in the cerebellar cortex ($r^2 = 0.10$, $P < 0.05$) and the dentate nucleus ($r^2 = 0.15$, $P < 0.05$).

Finally, to probe whether the changes in receptor were associated with obvious changes in the production or release of the neurotransmitter GABA, we determined the post-mortem concentration of GABA in the cerebellar cortex by HPLC. However, no significant difference was detected (Fig. 4).

**Discussion**

To our knowledge, we report the first evidence of a statistically significant neurochemical alteration within cerebellar nuclei that distinguishes patients with essential tremor as a group from Parkinson’s disease and/or age-matched controls. The decrease in GABA receptors was present in patients who suffered tremors for years and as such, may represent a long-term neurochemical substrate of essential tremor. We propose that the disruption of the GABAergic input into the deep cerebellar nuclei contributes to the generation of oscillatory information conveyed to the thalamus and the motor cortex, expressed clinically as action tremor (Supplementary Fig. 5).

**What may have caused a reduction of GABA receptors?**

A first explanation is that the loss of GABA receptors is a consequence of a neurodegenerative process in the dentate nucleus, consistent with the correlation between the loss of GABA$_A$ receptor and with the progression of the disease. However, our data clearly show that the reduction of GABA receptor is specific: no alteration of N-methyl-D-aspartic acid receptors or subunit I of cytochrome c oxidase messenger RNA has been observed in the present sample series. Accordingly, post-mortem studies have not reported frank evidence of cell death in the deep cerebellar nuclei (Louis et al., 2007; Elble and Deuschl, 2009), although degeneration of the deep cerebellar nuclei has been observed in a subset of patients with essential tremor (Louis et al., 2006, 2007).

Secondly, a downregulation of dentate nucleus GABA$_A$ and GABA$_B$ receptors may result from an exaggerated GABAergic input from Purkinje cells, which project from the cerebellar cortex to the deep cerebellar nuclei. In classical pharmacology, a decrease in post-synaptic receptors is often a compensatory reaction to pre-synaptic overactivity. Thus, the decrease in GABA$_A$ and GABA$_B$ receptors could be a post-synaptic consequence of increased GABA input from Purkinje cells. However, although GABA concentration in the cerebellar cortex rather displayed a trend towards an increase, it was not statistically significant and thus does not confirm this viewpoint. Alternatively, the decrease of GABA receptors in the dentate nucleus could be a consequence of a loss of GABA receptor located on Purkinje cells axons. This hypothesis is consistent with reports of Purkinje cell loss, axonal swellings (torpedoes) and other abnormalities that have been observed in essential tremor (Louis et al., 2007; Axelrad et al., 2008; Shill et al., 2008). However, while only GABA$_A$ receptors are located pre-synaptically (see below), both GABA$_A$ and GABA$_B$
receptors were downregulated. In addition, the concomitant loss of GABA\textsubscript{A} receptor messenger RNA transcripts rather suggests that the loss of GABA receptors arises from a transcription decrease in dentate nucleus cells.

A third possibility is that the GABA\textsubscript{A} and GABA\textsubscript{B} receptor reduction per se plays a causative role in essential tremor by restricting the post-synaptic action of GABA released from Purkinje cell axons, thereby disinhibiting deep cerebellar nuclei neurons. The ensuing overactivity of deep cerebellar nuclei neurons would spread up through the cerebellar-thalamo-cortical circuit, possibly contributing to the generation of tremors (Supplementary Fig. 5).

Figure 3  Decreased GABA\textsubscript{B} receptor subunits messenger RNA in the dentate nucleus of individuals with essential tremor. The expression of GABA\textsub{B(1a + b)} (A) and GABA\textsub{B(2)} (B) receptor subunits was determined using in situ hybridization and quantified in the dentate nucleus (C) and the cerebellar cortex (E and F). Correlation analyses show an association between GABA\textsub{B(1a + b)} receptors messenger RNA and the duration of essential tremor symptoms in the dentate nucleus (D). Each point represents an individual and the horizontal bar is the average (n = 9–15 per group). Statistical comparisons were performed using an ANOVA followed by Newman–Keuls post hoc test (C, E and F) or linear regression (D).

How can a reduction of GABA receptors in the dentate nucleus play a role in essential tremor?

As mentioned in the ‘Introduction’ section, one of the most widely accepted hypotheses proposes that essential tremor is caused by central oscillators propagating within the cerebello-thalamo-cortical network (Deuschl et al., 2000; McAuley and Marsden, 2000; Pinto et al., 2003; Elble and Deuschl, 2009; Schnitzler et al., 2009). Virtually all output from the cerebellum originates from deep cerebellar nuclei neurons, which project their axons to various structures, including the ventral lateral nucleus of the thalamus forming a complex circuitry with cortico-thalamic afferents, thalamo-cortical projection neurons and thalamic GABAergic interneurons (Heck and Sultan, 2002; Ilinsky and Kultas-Ilinsky, 2002). Given the key position of the deep cerebellar nuclei in this circuitry, it is clear that the neurological information processed in the cerebellum has to interact with GABA receptors in deep cerebellar nuclei neurons before leaving the cerebellum. The deep cerebellar nuclei have been known to play a crucial role in the generation of tremor and experiments involving the local cooling or ablation of dentate nucleus cells induces oscillations resembling intention tremors in non-human primates (Growdon et al., 1967; Brooks et al., 1973; Goldberger and Growdon, 1973; Cooke and Thomas, 1976).
Electrophysiological studies show that all dentate nucleus cells are sensitive to GABAergic input from Purkinje cells (Uusisaari and Knopfel, 2008), whereas immunocytochemistry, autoradiography and in situ hybridization experiments in rodent and human tissue have detected both GABA A and GABA B receptors in the deep cerebellar nuclei (Kingsbury et al., 1980; Bowery et al., 1987; Chu et al., 1990; Albin et al., 1991; Gambarana et al., 1993; Turgeon and Albin, 1993). Electrophysiological recording of deep cerebellar nuclei neurons confirms that both GABA A and GABA B receptors mediate the inhibitory input coming from Purkinje cells (Ito et al., 1970; Mouginot and Gahwiler, 1996; Chen et al., 2005). In addition, GABA B receptors are present on the presynaptic terminals of Purkinje cells into the deep cerebellar nuclei (Mouginot and Gahwiler, 1996). Interestingly, electrophysiology data also indicate that deep cerebellar nuclei neurons belong to the family of single cell oscillators, with a pacemaker-like activity (Jahnsen, 1986; Linas, 1988; Linas and Muhlethaler, 1988; Mouginot and Gahwiler, 1996). Populations of neurons with an inherent tendency for spontaneous rhythmic discharge (i.e. pacemakers) have been identified in vertebrates and invertebrates (Ills and Pearson, 1969) and have long been suspected to be a driving force in essential tremor (DeLong, 1978). In the case of deep cerebellar nuclei neurons, it has been shown that their pacemaker-like activity is under tonic control by the GABAergic input from Purkinje cells (Mouginot and Gahwiler, 1996). Even more compelling is the demonstration that deep cerebellar nuclei neurons can impose their rhythmicity to their thalamic target neurons with highly regular patterns of activity (Pinault and Deschenes, 1992).

Therefore, a decrease of deep cerebellar nuclei GABA A and GABA B receptors in essential tremor, as reported here, is likely to have a critical impact on cerebellar output, as schematized in Supplementary Fig. 5. In this scheme, without the GABAergic inhibitory input from Purkinje cells, the pacemaker activity of dentate nucleus neurons would be the origin of the oscillatory patterns climbing the cerebello-thalamo-cortical pathways in essential tremor (Supplementary Fig. 5). In the normal setting, GABA released by axons originating from Purkinje cells interacts with GABA receptor to restrain the pacemaker activity of deep cerebellar nuclei neurons. It is suggested here that the lack of GABA receptors in the dentate nuclei leads to incorrect response to the GABAergic input from Purkinje cells. The ensuing disinhibition of dentate nuclei neurons would then facilitate the transmission of oscillatory discharge of information up to target neurons in the thalamus. Such faulty tremogenic information would then be conveyed from the thalamus to the primary motor, pre-motor and supplemental areas of the brain cortex (Supplementary Fig. 5). Ensuing oscillation in the 10 Hz range reaching the motor cortex could modulate descending motor pathways to limb muscle and thus drive essential tremor (McAuley and Marsden, 2000).

Interestingly, one of the most efficient treatments of essential tremor is deep brain stimulation of the ventral intermediate nucleus of the thalamus (Zesiewicz et al., 2005; Pahwa et al., 2006; Stani et al., 2009). The efficacy against tremor of the deep brain stimulation technique has been attributed to its capacity to disrupt pathological thalamic oscillatory activity, thereby stopping the propagation of tremor-inducing signal climbing the cerebello-thalamo-cortical system (Lozano et al., 2002; Kane et al., 2009). Therefore, a defect of deep cerebellar nuclei GABAergic receptor could play an upstream role in the generation of tremor.

The present results possibly explain the pharmacological efficacy of GABAergic drugs in essential tremor, and further support the hypothesis that GABAergic compounds specifically acting at the deep cerebellar nuclei level could be even more efficient. There is good evidence that tremor may be suppressed by substances that facilitate inhibitory neurotransmission mediated by GABA (Louis, 1999). For example, intrathecal pump releasing a GABA B receptor agonist baclofen has already been successfully used to decrease tremor in a case report (Weiss et al., 2003). Intrathalamic injection of GABA B receptor agonist led to tremor reduction (Pahapill et al., 1999). A tremolytic action of GABA B or GABA B receptor agonists has also been reported in animal models (Tariq et al., 2001; Paterson et al., 2009). Several clinical studies have been performed with GABA analogues, such as gabapentin, pregabalin or benzodiazepines showing some efficacy (Louis, 2005; Zesiewicz et al., 2007; Ferrara et al., 2009). An additional phase IV multi-site, prospective, double-blind, randomized, placebo-controlled, cross-over trial is currently being performed with pregabalin (ClinicalTrials.gov Identifier: NCT00584376). However, the complexity of using GABAergic treatments stems from the difficulty of selectively targeting one small nucleus like the deep cerebellar nuclei, which makes adverse effects practically unavoidable with current therapies. However, the fact that the dentate nuclei expresses GABA B (Ile + b), but not GABA B (2) subunits, suggests that GABA B receptors in the dentate nuclei may be specific to this nucleus. Indeed, the vast majority of GABA B receptor heterodimers contain both a GABA B (2) subunit and a GABA B (2) subunit, coexpressed by the same cells (Benke et al., 1999; Billinton et al., 1999; Bischoff et al., 1999; Durkin et al., 1999; Clark et al., 2000; Liang et al., 2000; Bettl and Tiao, 2006). In structures where only GABA B (1a + b) can be found, it has been proposed that an
unaligned subunit may couple to $\text{GABA}_B^{(1a+b)}$ to form a functional receptor (Bowery et al., 2002; Bowery, 2010; Marshall and Foord, 2010). If such is the case within the dentate nucleus, the $\text{GABA}_B$ receptor assembly might be unique there, which opens the door to tissue-specific pharmacological targeting. Then, hypothetically, an agonist specific for $\text{GABA}_B$ receptor subspecies present in the dentate nucleus could be used to re-establish the tonic inhibition on dentate nucleus neurons and reduce tremor.

Although we had access to a wide range of clinical data, some important information such as Braak (Alzheimer’s disease and Parkinson’s disease) scores, CERAD (Consortium to Establish a Registry for Alzheimer’s Disease) stages and ante-mortem cognitive performance, were not documented. Therefore, the fact that we were not able to link our observation on GABA receptors to Alzheimer’s disease-related neurodegeneration and symptoms is a limitation of our study. Finally, it is important to point out that the decrease in GABA receptors correlated with the progression of essential tremor, as patients who suffered for a longer time had lower levels of GABA receptors. This suggests that this GABAAergic defect is not an acute phenomenon but is established progressively during the evolution of the disease.

**Conclusion**

Owing to the small number of clinicopathological studies, our understanding of the pathophysiology of essential tremor has lagged behind other CNS diseases. The present investigation is probably the first to report a neurochemical difference in individuals with essential tremor, versus controls or patients with Parkinson’s disease, using human brain tissue. Since GABAAergic input into deep cerebellar nuclei neurons is critical for the regulation of its pacemaker activity extending through cerebello-thalamo-cortical networks, a reduction of GABA receptors may play an important role in the generation of tremor. Finally, our results suggest that GABA receptors within the deep cerebellar nuclei are potential drug targets in essential tremor.

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**Supplementary material**

Supplementary material is available at Brain online.

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