Published in final edited form as:
Neurotoxicology. 2013 September ; 38: 131–135. doi:10.1016/j.neuro.2013.07.002.

Elevated Brain Harmane (1-methyl-9H-pyrido[3,4-b]indole) in Essential Tremor Cases vs. Controls

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Abstract

Background—Harmane (1-methyl-9H-pyrido[3,4-β]indole), a potent neurotoxin that has tremor-producing properties in animal models, is present in many foods; Although we have demonstrated a difference in tissue harmane concentrations in ET cases vs. controls, all work to date has involved blood samples.

Objectives—We quantified harmane concentrations in human cerebellum, a brain region of particular pathogenic interest in essential tremor (ET), comparing ET to control brains.

Methods—Cerebellar cortex was snap frozen and stored at -80ºC in aliquots for biochemical analyses. Harmane concentration was assessed using high performance liquid chromatography.

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Conflict of Interest Statement
The authors declare that there are no conflicts of interest.

Statistical Analyses: The statistical analyses were conducted by Dr. Louis and Dr. Liu.

Results—Geometric mean brain harmane concentrations (adjusted for postmortem interval [PMI] and freezer time) were higher in ET cases than controls: 1.0824 (95% confidence interval = 0.9405 – 1.2457) vs. 0.8037 (0.6967 – 0.9272), p = 0.004. Geometric mean of brain harmane concentrations (adjusting for PMI and freezer time) was highest in ET cases who reported other relatives with tremor (1.2005 [0.8712 – 1.6541]), intermediate in ET cases without family history (1.0312 ([0.8879 – 1.1976]), and both were significantly higher than controls (p= 0.02).

Conclusions—This study provides additional evidence of a possible etiological importance of this toxin in some cases of the human disease ET.

Keywords

brain; epidemiology; essential tremor; harmane; pathology; toxicant

1. Introduction

The β-carboline alkaloids are a group of neurotoxins that produce tremor. Laboratory animals injected with high doses acutely exhibit action tremor that shares several clinical features with the human disease essential tremor (ET) (Fuentes and Longo, 1971; Zetler et al., 1972), and human volunteers exposed to high doses display action tremor (Lewin, 1928). ET is among the most prevalent neurological diseases, yet there is little understanding of its environmental epidemiology, despite the widespread acknowledgement that many cases are sporadic (Benito-Leon et al., 2003; Benito-Leon and Louis, 2006).

Harmane (1-methyl-9H-pyrido[3,4-*b*]indole) is among the most potent tremor-producing β-carboline alkaloids (McKenna, 1996). It is very lipid soluble (Zetler et al., 1972), and broadly distributed within the rat brain (Anderson et al., 2006; Matsubara et al., 1993; Moncrieff, 1989). Brain concentrations are several fold higher than those in the blood both in exposed (i.e., harmane-injected) laboratory animals and in control animals as well (Anderson et al., 2006; Zetler et al., 1972). Although harmane is produced endogenously, it is also present in the diet (especially in meats but also in many plant-derived foods) and exogenous exposure is thought to be the main source of bodily exposure to harmane (Pfau and Skog, 2004).

In 2000, we had hypothesized that this neurotoxin could play a role in the etiology of ET, and in 2002, demonstrated that blood harmane concentration was elevated in an initial sample of 100 ET patients compared with 100 controls (Louis et al., 2002). In 2008, in a new sample of 150 ET cases and 135 controls, we replicated this finding (Louis et al., 2008). In 2012, we showed that blood concentrations were elevated in ET cases in a case-control study in Spain (Louis et al., 2013a). We also demonstrated that blood harmane concentration was elevated in ET cases compared to controls when reassessed at a second time point several years later, indicating what seems to be a stable association between this environmental toxin and ET (Louis et al., 2012). Although we have demonstrated a difference in tissue harmane concentrations in ET cases vs. controls, all of our work to date has involved blood samples. Blood concentrations of harmane, however, reflect the kinetic aspect of the toxin in the body. It would be more interesting to know the presence of the toxin in brain, the principal organ of interest, as ET is a brain disease. In 2003, we established the Essential Tremor Centralized Brain Repository (ETCBR) at Columbia University (Louis et al., 2005a), a centralized NIH-funded national repository for the procurement and study of ET brains. Frozen cerebellar tissue was collected in a standardized manner in ET cases and controls. This brain region of particular interest in ET due to the presence of degenerative changes (Purkinje cell loss and Purkinje cell axonal swellings [torpedoes]) on adequately powered postmortem studies (Louis, 2010; Louis et al., 2007). We are in the position now for the first time, to quantify harmane concentrations in human

brain tissue, comparing ET to control brains. We hypothesized that harmane concentrations would be elevated in ET brains compared to control brains.

2. Material and Methods

2.1 Cases and Controls

This study was conducted at the ETCBR, New York Brain Bank (NYBB), Columbia University Medical Center (CUMC). All ET cases were diagnosed by their treating neurologist and the ET diagnosis was confirmed using ETCBR criteria by a second neurologist specializing in movement disorders (E.D.L.) (Louis et al., 2007). ETCBR criteria were as follows: (i) bilateral action tremor of the arms for 5 or more years with a diagnosis of ET during life, (ii) either head tremor or action tremor of at least one arm that was moderate or severe (i.e. arm tremor resulted in difficulty with two or more activities of daily living or required medication) and (iii) action tremor was not the result of other movement disorders (e.g. dystonia, Parkinsonism, ataxic disorders), hyperthyroidism, other medical conditions or medications (Louis et al., 2007).

Age-matched control brains were normal elderly control subjects from the NYBB, derived from the Alzheimer's Disease Research Center and the Washington Heights Inwood Columbia Aging Project; they were free of clinical diagnoses of Alzheimer's disease (AD), ET, or Parkinson's disease (PD) and without neuropathological diagnoses of neurodegenerative disease (Louis et al., 2007). The NYBB operates under approval of the Institutional Review Board of CUMC.

2.2 Clinical Evaluation

During life, demographic and clinical data were collected using a series of semi-structured questionnaires (Louis et al., 2007). Data on lifetime exposure to medications known to cause cerebellar damage (e.g., lithium, diphenylhydantoin, chemotherapeutic agents) were collected. Heavy ethanol use was defined as consumption of an average of four or more standard drinks (15 ml of absolute ethanol) per day for a man, or three or more per day for a woman, at any point in their lives (Harasymiw and Bean, 2001; Louis et al., 2007). Most ET cases also underwent a standardized, videotaped neurological examination, which included an assessment of postural tremor (sustained arm extension), five tests of kinetic tremor (pouring, drinking, using spoon, finger-nose-finger maneuver, and drawing spirals), and head and voice tremors (Louis et al., 2005b).

2.3 Neuropathological Assessment

As previously described, all brains underwent a complete neuropathological assessment by a senior neuropathologist (J.P.G.V.) at the NYBB (Louis et al., 2007). Each brain had a standardized measurement of brain weight (grams), postmortem interval (PMI 1, hours between death and placement of brain in a cold room or upon ice; PMI 2, hours between death and freezing of brain samples), Braak and Braak AD staging for neurofibrillary tangles (Braak and Braak, 1997; Braak et al., 2006), and Consortium to Establish a Registry for AD (CERAD) ratings for neuritic plaques (Mirra, 1997). As described, Lewy pathology was assessed using alpha-synuclein immunohistochemistry in the brain regions examined in our standardized neuropathological assessment (Louis et al., 2011; Louis et al., 2007).

A standard 3 × 20 × 25 mm parasagittal, formalin-fixed, tissue block was harvested from the neocerebellum (Louis et al., 2007; Louis et al., 2006a); the block included the cerebellar cortex, white matter and dentate nucleus. A senior neuropathologist, blinded to all clinical information, counted and averaged Purkinje cells in fifteen 100× fields (LH&E) (Louis et al., 2006a).

Cerebellar cortex was snap frozen in liquid nitrogen, ground using a mortar and pestle, and stored at -80ºC in 1.5 - 1.8 g aliquot vials for biochemical analyses. One 1.5 - 1.8 g aliquot of frozen cerebellar tissue was be shipped on dry ice to the Zheng Laboratory at Purdue University, West Lafayette, Indiana. Using this tissue, brain harmane concentrations were quantified.

2.4 Quantification of Brain Harmane Concentration

In 2008, we demonstrated the feasibility of detecting harmane in frozen cerebellar tissue samples; we shipped one aliquot (1.5 - 1.8 g) of frozen cerebellar tissue (six ET cases and three age matched-controls) on dry ice to Purdue University. Brain harmane concentrations were quantified using high performance liquid chromatography (HPLC). We demonstrated the following: (1) Harmane was detected in all nine samples both on an initial HPLC run and on a second confirmatory run. (2) Results were reproducible (correlation [r] between the results of the first and second HPLC run = 0.82, p = 0.01). (3) Brain concentrations were 2.5× higher than the mean blood concentrations we had been observing in ET cases in general. (4) The mean brain harmane concentration in cases was 53% higher than that of controls (7.6 ± 0.26 g^{-10}/g vs. 4.9 ± 0.2 g^{-10}/g, p = 0.055 even in this small pilot sample) and the median was 38% higher (7.0 vs. 5.1 g^{-10}/g, p = 0.095).

For the current analyses, brain harmane concentrations were assessed blinded to demographic data and diagnosis. Samples are run in three batches, containing the same ratio of case to control samples in each batch. Triplicate samples of each frozen sample were prepared as follows. The samples were homogenized in a buffer (1:4, g:ml) containing 20 mM Tris, pH 7.5, 5 mM EGTA, 1% TritonX-100, 0.1% SDS, and protease inhibitor cocktail (Calbiochem, San Diego, CA) on ice. The homogenate was then mixed with 10 ml of 1M NaOH. Following vortex for 10 sec, the samples were placed on a horizontal rotator and shaken at room temperature for 30 min. The extraction solution consisting of methyl-t-butyl ether + ethyl acetate (98:2, v/v) was added to the tube and the extraction carried out by shaking the samples at room temperature for 45 min. After centrifugation, the upper organic phase was transferred to another tube. The above extraction procedure was repeated two more times. The combined organic phase was then evaporated under nitrogen in the hood to dryness. The samples were reconstructed in methanol. After another centrifugation, the supernatant was transferred to HPLC autosampler microvials and sealed for HPLC analysis. Harmane was separated and quantified by HPLC with a fluorescence detector at an excitation wavelength of 300 nm and an emission wavelength of 435 nm.

2.5 Sample Size

Numerous studies (Anderson et al., 2006; Fekkes and Bode, 1993; Ho et al., 1970; Kuhn et al., 1996) have demonstrated that concentrations of β-carboline alkaloids, including harmane, are substantially higher and therefore easier to detect in the brain than in the plasma; indeed, our pilot data demonstrated that the brain harmane concentrations were 2.5× higher than the mean blood concentrations we had been observing in ET cases in general. One other issue is that we realized that the number of cases would be greater than the number of available controls. Precise age-matching was not feasible due to the advanced age of many of the ET cases; however, age was not associated with brain harmane concentration and hence, was not a confounder. Using our pilot data (mean brain harmane concentration in 6 ET cases = 7.6 ± 0.26 g^{-10}/g vs. 4.9 ± 0.2 g^{-10}/g in 3 controls) to calculate the effect size, we estimated that 70 ET cases and 25 controls would provide greater than 99% power to detect the estimated effect size (assuming a two sided test with alpha = 0.05).

2.6 Statistical Analyses

Statistical analyses were performed in SPSS (Version 19.0) and SAS (version 9.2). The empirical distribution of the brain harmane concentration was positively skewed (one-sample Kolmogorov-Smirnov test, $z = 2.91$, $p < 0.001$) and required log transformation (one-sample Kolmogorov-Smirnov test, $z = 1.28$, $p = 0.08$). There are several issues that relate to the stability and potential for degradation of harmane in tissue; hence, postmortem interval and length of time (in days) that the brain specimen was in frozen storage (freezer time) was noted for each sample and considered *a priori* as covariates in the analyses. As freezer time had a skewed distribution, it was logarithmically transformed to reduce the impact of skewness in the application of parametric methods.

We present the geometric mean values and 95% confidence interval for the brain harmane concentration. We used linear models, regressing the harmane variable on variables of PMI and freezer time to obtain a covariate-adjusted harmane variable. We also present geometric mean values of harmane after having adjusted for these confounders, testing whether there was a case-control difference using the Satterthwaite method for t-test, which accounts for unequal variance between comparison groups. In a final analysis, we stratified ET cases into those who reported one or more relative with tremor vs. those who did not, and in a Kruskal-Wallis test, looked for a difference among three groups (ET cases with family history, ET cases without family history, controls). The ratio of group geometric means was derived from parameters in the regression model for covariate adjusted harmane with non-constant variance.

3. Results

There were 70 ET cases and 27 controls. Cases were on average 9.8 years older than controls, with both groups being of advanced age (Table 1). The two groups did not differ by gender (Table 1) or brain weight. The PMI was longer in ET cases than controls, as was the freezer time. The CERAD score was marginally higher in ET cases (Table 1).

Brain harmane concentration was not correlated with age (Spearman $r = 0.01$, $p = 0.90$, $n = 97$), CERAD score (Spearman's $r = -0.07$, $p = 0.48$), Braak AD stage (Spearman's $r = 0.09$. $p = 0.39$), or brain weight (Spearman's $r = -0.003$, $p = 0.98$). Brain harmane concentration was correlated with PMI (for PMI 1, Spearman's $r = 0.29$, $p = 0.006$; for PMI 2, Spearman's $r = -0.20$, $p = 0.046$). Brain harmane was associated with freezer time at marginal significance (Spearman $r = 0.19$, $p = 0.06$). Brain harmane was not associated with gender.

Brain harmane concentrations were higher in cases than controls in analyses that adjusted for PMI and freezer time (Table 2), with the ratio of geometric means = 1.3468, suggesting that the mean concentration was approximately 35% higher in ET cases than controls after adjusted for PMI and freezer time.

There was no association between brain harmane concentration and the counts of the number of Purkinje cells on LH&E (Spearman's $r = 0.09$, $p = 0.43$).

In a final analysis, we stratified ET cases into those who reported one or more relative with tremor vs. those who did not, and in a Kruskal-Wallis test, looked for a group difference (ET cases with family history, ET cases without family history, controls). Brain harmane concentrations (adjusting for PMI and freezer time) were highest in ET cases who reported other relatives with tremor, intermediate in ET cases without family history, and lowest in controls (Table 3, $p = 0.054$). The ratios of geometric means for cases with and without family history compared to controls were 1.49 ($p = 0.02$) and 1.28 ($p = 0.02$), respectively.

4. Discussion

Harmane is a β-carboline alkaloid that has been linked to ET (Louis et al., 2013a, Louis et al., 2008, Louis et al., 2002), making this potent neurotoxin of etiological interest in this disease. Prior studies have demonstrated higher blood concentrations of this neurotoxin in ET cases than controls (Louis et al., 2013a, Louis et al., 2008, Louis et al., 2002) but this is the first study to demonstrate that the concentration of this neurotoxin was higher in the cerebellum of ET cases than controls.

In earlier studies, we demonstrated that blood harmane concentrations seemed to be highest among ET cases with a family history of ET (Louis et al., 2013a). In the current study, brain harmane concentrations were highest in ET cases who reported other relatives with tremor. The higher concentrations in familial ET cases suggests that the mechanism for this elevated concentrations may be at least partly genetic and/or metabolic (i.e., possibly some combination of an inherited tendency for decreased metabolism in the setting of increased exposure). ET itself is a highly familial disorder.

A growing literature on the environmental epidemiology of ET is emerging (Fabrizio et al., 2007; Jiménez-Jiménez et al., 2007; Prakash et al., 2006; Salemi et al., 1998), focusing on the role of a number of toxins in the emergence of tremor, with studies examining the role of β-carboline alkaloids, lead, and other toxins (Dogu et al., 2007; Louis et al., 2006b).

In the selection of possible toxic causes of ET for investigation, the β-carboline alkaloids are an obvious choice. The β-carboline alkaloids are a group of naturally occurring chemicals that include harmane, norharman, harmine, harmaline, and others (McKenna, 1996; Sakai, 1995; Zetler et al., 1972). β-carboline alkaloids are a type of heterocyclic amine because they are made up of several five- and six-ringed (i.e., cyclic) structures, which contain an amine (i.e., nitrogen) group (De Meester, 1995). There is a structural similarity to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which has a two-ring structure, and which has served as one of the main animal models for PD (Langston et al., 1984; Smeyne and Jackson-Lewis, 2005). Like MPTP, β-carboline alkaloids are highly neurotoxic, and it has been known for approximately 100 years that administration of β-carboline alkaloids to a wide variety of laboratory animals produces action tremor that resembles ET (Zetler et al., 1972).

In animal studies, harmane is a highly lipid soluble (Zetler et al., 1972), and broadly distributed within the rat brain (Anderson et al., 2006; Matsubara et al., 1993; Moncrieff, 1989). We did not measure harmane concentration in the inferior olivary nucleus, which is involved in the generation of tremor in the harmaline model of tremor (Martin and Handforth, 2006), but it would have been interesting to have examined this brain region as well. Conventional and detailed histopathological studies, however, have not revealed any abnormalities in the ET inferior olivary nucleus (Louis et al., 2013b; Louis et al., 2007).

These results should be interpreted with appropriate caution. First, in ET cases, we did not detect a correlation between brain harmane concentration and number of Purkinje cells. Second, a 35% increase in brain harmane concentration is of only moderate magnitude. With these caveats being said, it is important to point out that exposure to harmane (mainly through diet) in humans takes the form of a chronic, low dose exposure rather than an acute, high dose exposure, as occurs in routine animal toxicity studies. These are different scenarios and they might not involve similar patho-mechanisms. For example, low dose, chronic exposure could result in Purkinje cell dysfunction rather than Purkinje cell death.

This study had limitations. First, cases and controls could not be matched on age, yet brain harmane concentration was not associated with age, indicating that it could not account for

the case-control difference which we observed. Second, our analysis of brain tissue was restricted to samples of the cerebellum as other regions were not routinely available. In future studies it would be useful to compare the regional distribution of harmane in different brain regions and, more specifically, in different regions of the cerebellum (e.g., hemispheres vs. vermis). Moreover, it would be interesting to see the metabolic profiles of β-carboline derivatives in brain regions. The study also had several strengths. First, it is the only study to have directly examined brain harmane concentration in carefully diagnosed ET and in human brain tissue more generally. We focused on the primary tissue of interest (brain rather than blood). The sample size, which required many years to compile accrue, was also sufficiently large for our purposes.

5. Conclusions

This study provides additional evidence of a possible etiological importance of this toxin in some cases of the human disease ET. These data require confirmation. Also, further work is required on the mechanisms whereby chronic and low level exposure to harmane could contribute to the development of tremor in humans.

Acknowledgments

None.

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HIGHLIGHTS

- Environmental correlates for essential tremor (ET) are largely unexplored.
- Harmane (1-methyl-9H-pyrido[3,4-b]indole) is a potent tremor-producing toxin.
- Brain (cerebellar) harmane levels were quantified by high performance liquid chromatography.
- Brain harmane levels were elevated in ET cases vs. controls, and highest in familial ET cases.
- This study provides additional evidence of a possible etiological importance of this toxin in ET.

Table 1

Characteristics of study subjects

	ET (N = 70)	Controls (N = 27)	Statistical Test
Age at death (years)	86.0 ± 6.6	76.2 ± 15.9	t = 3.37, p = 0.002
Female gender	43 (61.4)	13 (48.1)	X^2 = 1.41, p = 0.24
Family history of tremor	22 (31.4)	0 (0)	Fisher's p = 0.005
Age of onset of tremor (years)	40.5 ± 22.6 [range = 4 −85]	Not applicable	Not applicable
PMI 1 (hours)	3.5 ± 3.3 [median = 2.8]	6.0 ± 5.0 [median = 4.3]	Mann-Whitney = 2.85, p = 0.004
PMI 2 (hours)	25.3 ± 10.1	18.3 ± 8.9	t = 3.14, p = 0.002
Freezer time (years)	2.4 ± 1.9 [median = 1.7]	6.9 ± 2.75 [median = 7.9]	Mann-Whitney = 173.0, p < 0.001
Brain Weight (grams)	1179 ± 192	1233 ± 157	t = 1.33, p = 0.19
Braak AD Score			Fisher's p = 0.005
0 – 4	52 (89.7)	22 (88.0)	
5 or 6	6 (10.3)	3 (12.0)	
Braak PD Score >0	0 (0)	0 (0)	X^2 = 0.00, p = 1.00
CERAD Score			X^2 = 1.80, p = 0.05
0	21 (30.0)	15 (55.6)	
A	28 (40.0)	9 (33.3)	
B	11 (15.7)	3 (11.1)	
C	10 (14.3)	0 (0.0)	

Values are means ± standard deviation or number (percent).

AD = Alzheimer's Disease, CERAD = Consortium to Establish a Registry for AD, PD = Parkinson's disease, PMI = postmortem interval.

Table 2

Brain Harmane Concentrations in Study Subjects

	ET (n=70) Geometric mean (95% CI)		Controls (n=27) Geometric mean (95% CI)	P-value
Unadjusted	0.9950 (0.8578, 1.1541)		0.9108 (0.7933,1.0456)	0.38
Unadjusted[a]	1.0025 (0.8630 – 1.1645)		0.8938 (0.7716 – 1.0353)	0.27
Covariate Adjusted[a,b]	1.0824 (0.9405 – 1.2457)		0.8037 (0.6967 – 0.9272)	0.004
	ET + FH (n = 23)	ET – FH (n=47)	Controls (n = 27)	
Unadjusted	1.0456 (0.7568, 1.4446)	0.9711 (0.8243, 1.1440)	0.9108 (0.7933,1.0456)	0.98
Unadjusted[a]	1.0731 (0.7680 – 1.4994)	0.9711 (0.8243 – 1.1440)	0.8938 (0.7716 – 1.0353)	0.96
Covariate Adjusted[a,b]	1.2005 (0.8712 – 1.6541)	1.0312 (0.8879 – 1.1976)	0.8037 (0.6967 – 0.9272)	0.054

FH = family history of tremor.

[a]Two controls with missing data on freezer time and one ET case with family history and missing data on PMI were excluded from the analysis.

[b]Adjustment for PMI and freezer time.

p-value from t-test for difference between two groups and Kruskal-Wallis test for difference among three groups.