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BLOOD HARMANE (1-METHYL-9H-PYRIDO[3,4-*b*]INDOLE) CONCENTRATIONS IN ESSENTIAL TREMOR: REPEAT OBSERVATION IN CASES AND CONTROLS IN NEW YORK

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Essential tremor (ET) is a widespread late-life neurological disease. Genetic and environmental factors are likely to play important etiological roles. Harmane (1-methyl-9H-pyrido[3,4-*b*]indole) is a potent tremor-producing neurotoxin. Previously, elevated blood harmane concentrations were demonstrated in ET cases compared to controls, but these observations have all been cross-sectional, assessing each subject at only one time point. Thus, no one has ever repeat-assayed blood harmane in the same subjects twice. Whether the observed case-control difference persists at a second time point, years later, is unknown. The current goal was to reassess a sample of our ET cases and controls to determine whether blood harmane concentration remained elevated in ET at a second time point. Blood harmane concentrations were quantified by a well-established high-performance liquid chromatography method in 63 ET cases and 70 controls. A mean of approximately 6 yr elapsed between the initial and this subsequent blood harmane determination. The mean log blood harmane concentration was significantly higher in cases than controls ($0.30 \pm 0.61 \text{ g}^{-10}/\text{ml}$ versus $0.08 \pm 0.55 \text{ g}^{-10}/\text{ml}$), and the median value in cases was double that of controls: $0.22 \text{ g}^{-10}/\text{ml}$ versus $0.11 \text{ g}^{-10}/\text{ml}$. The log blood harmane concentration was highest in cases with a family history of ET. 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.

Essential tremor (ET) is a widespread, predominantly late-life neurological disease that affects 4% of individuals aged 40 yr and older,

and an estimated 20% or more of individuals in their 90s and older (Benito-Leon et al. 2003; Dogu et al. 2003; Louis and Ferreira

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2010; Louis et al. 2009). As such, it is one of the most common neurological diseases (Louis et al. 1998; 2010). In addition to action tremor, which may range from mild to severe, ET patients may exhibit other neuropsychiatric signs, including gait ataxia and incoordination (Louis et al. 2010; Rao et al. 2011; Singer et al. 1994) and various levels of cognitive impairment (Benito-Leon et al. 2006). Both genetic (Gulcher et al. 1997; Higgins et al. 1997) and nongenetic (environmental) factors (Jiménez-Jiménez et al. 2007; Louis 2001; 2008; Salemi et al. 1998) are likely to play a role in disease etiology.

The β -carboline alkaloids are a group of neurotoxins that produce tremor. Laboratory animals injected with high doses acutely exhibit action tremor that resembles ET (Fuentes et al. 1971; Zetler et al. 1972). Human volunteers exposed to high doses display a coarse, reversible action tremor (Lewin 1928).

Harmane (1-methyl-9H-pyrido[3,4-*b*]indole) is among the most potent tremor-producing β -carboline alkaloids; 38 mg/kg of subcutaneously administered harmane produces tremor in mice (McKenna 1996). Harmane is also lipid soluble (Zetler et al. 1972), and broadly distributed within the rat brain (Anderson et al. 2006; Matsubara et al. 1993; Moncrieff 1989). Indeed, brain concentrations are severalfold higher than those in the blood in both control lab animals as well as harmane-injected laboratory animals (Anderson et al. 2006; Zetler et al. 1972). Although harmane is produced endogenously, it is also present in the diet and exogenous exposure is postulated to be the main source of bodily exposure to harmane (Pfau et al. 2004). Humans may be exposed to low levels of harmane through a variety of foods, including vegetables and meat, with concentrations in animal protein considered to be among the highest. Harmane and other β -carboline alkaloids are found in particularly high nanograms per gram concentrations in beef, chicken, and pork, and cooking leads to furthermore increased concentrations (Louis et al. 2008). The formation of β -carboline alkaloids in

cooked meat is a function of cooking temperature and time, with β -carboline alkaloid concentrations increasing most rapidly with time at higher temperatures (Louis et al. 2008). Pan frying and grill/barbequing produce the highest concentrations of β -carboline alkaloids (Louis et al. 2008).

In 2000, it was proposed that this neurotoxin could play a role in the etiology of ET, and in 2002 it was demonstrated that blood harmane concentration was elevated in 100 ET patients compared with 100 controls ("sample 1 at time point 1") (Louis et al. 2002). In 2008, Louis et al. demonstrated that blood harmane concentration was elevated in a new sample of 150 ET cases and 135 controls ("sample 2 at time point 1"). The 2000 and 2008 studies enrolled completely different individuals; there was no overlap and no one was assessed twice. Thus, in our studies, the finding of an elevated harmane concentration remains a cross-sectional observation, sampling blood from each individual (a combined sample of 250 ET cases and 235 controls) at only a single time point (time point 1), thereby raising the possibility that the finding is spurious. Since no one has ever repeat-assayed blood harmane in the same subject twice, it is not known whether the case-control difference (observed at time point 1) persists at a second time point now many years later. Thus, the current goal (2009–2011) was to reassess a subsample of our ET cases and controls (i.e., "sample 1 and 2 at time point 2") to determine whether blood harmane concentration is still elevated in ET.

METHODS

Participants

Our initial published sample of cases and controls was enrolled between 2000 and 2007 (Louis et al. 2002; 2008). Beginning in 2009, a subsample of the participants initially enrolled between 2000 and 2007 was reenrolled. Sixty-three cases and 70 controls have been reenrolled thus far.

Initial Recruitment (2000–2007, Time Point 1)

As described in two prior studies of blood harmane in ET (Louis et al. 2002; 2008), between 2000 and 2007, 250 ET cases were enrolled in a study of the environmental epidemiology of ET at Columbia University Medical Center (CUMC). The large majority of cases were derived from two sources: (1) a computerized billing database of ET patients at the Neurological Institute of New York, CUMC, and (2) advertisements to members of the International Essential Tremor Foundation (Louis et al. 2008). All cases lived within 2 h driving distance of CUMC in New York, New Jersey, and Connecticut. The case-finding process included screening questionnaires for alternative neurological diagnoses and, when available, an examination of clinical records (E.D.L.); patients with diagnoses of dystonia, Parkinson's disease (PD), or spinocerebellar ataxia were excluded. Control subjects ($n = 235$) were recruited during the same time period (2000–2007). Controls, identified using random digit telephone dialing within a defined set of telephone area codes that were represented by the ET cases (e.g., 212, 201, 203, 516, 718, 914) within the New York Metropolitan area, were frequency-matched to cases based on gender, race, and current age (5-yr intervals). The CUMC Internal Review Board approved of all study procedures; written informed consent was obtained upon enrollment.

Subsequent Recruitment (2009–2011, Time Point 2)

As described earlier, 250 ET cases and 235 controls were enrolled between 2000 and 2007 (samples 1 and 2 at time point 1). In April 2009, follow-up assessments of ET cases and controls began (samples 1 and 2, time point 2), starting with the oldest because these had the highest likelihood of loss to follow-up due to mortality. Cases were frequency matched to controls based on age and gender. All cases and controls signed informed consent approved by

the CUMC Internal Review Board. Sixty-three cases and 70 controls have been reenrolled thus far. Aside from age, these 63 ET cases and 70 controls were similar in demographic and clinical features (gender, race, education, body mass index, smoking habits, and medical comorbidity) to the base sample of 250 ET cases and 235 controls from which they were drawn. As expected, they had aged during the intervening time between initial and subsequent assessments.

Clinical Evaluation

At the time of the initial (time point 1) and subsequent (time point 2) evaluations, all case and control subjects were evaluated in person by a trained tester who administered clinical questionnaires and performed a videotaped examination (Louis et al. 2002; 2008). Most evaluations required significant travel either to the subjects' homes or vice versa, with subjects coming in to the medical center. Therefore, evaluations were performed in the late morning or early afternoon, making fasting blood harmane concentrations impractical. Some data suggest that plasma concentrations of harmane do not change significantly during the day; indeed, in one study (Rommelspacher et al. 1991), human subjects ingested food or ethanol, and plasma harmane concentrations were measured hourly for 8 h. The concentration remained stable.

The trained tester collected demographic, clinical and family history information using a structured questionnaire. ET cases and controls were classified as having a family history of tremor if they reported at least one first-degree relative with tremor (i.e., ET or any unspecified tremor), and were classified as having a family history of ET if they reported at least one first-degree relative with ET. Current smoking status was assessed in each subject, as was past cigarette use, which allowed us to calculate cigarette pack-years. Medical comorbidity was assessed using the Cumulative Illness Rating Scale, in which the severity of medical problems (0 [none]–3 [severe]) was rated in 14 body

systems (e.g., cardiac, respiratory, renal) and a Cumulative Illness Rating Scale score was assigned (range = 0–42 [maximal comorbidity]) to each subject (Linn et al. 1968).

Weight and height were assessed using a balance scale designed for field surveys (Scale-Tronix 5600, White Plains, NY) and a movable anthropometer (GPM Martin Type, Pfister Inc, Carlstadt, NJ), and body mass index (weight/height²) was calculated and expressed as kilograms per square meter.

The tester videotaped a tremor examination in all subjects (Louis et al. 1997; 2002), and each of 12 videotaped action tremor items was rated by Dr. Louis, resulting in a total tremor score (range = 0–36 [maximum]) (Louis et al. 2001). These ratings were assigned blinded to data on blood harmane concentration. The diagnosis of ET was confirmed by Dr. Louis using published diagnostic criteria (moderate or greater amplitude tremor during three activities or a head tremor in the absence of PD, dystonia, or another neurological disorder) (Louis et al. 1997). None of the cases or control subjects had PD or dystonia.

Blood Harmane Concentrations

As at time point 1, at time point 2, phlebotomy was performed. At each time point, blood concentrations of harmane were measured blinded to all demographic, clinical, and diagnostic information; 63 ET cases and 70 controls had phlebotomy at both time points 1 and 2, and it is their time point 2 blood harmane concentration that is the focus of these analyses.

Harmane concentrations in blood were quantified by a well-established high-performance liquid chromatography (HPLC) method in this group and used in our previous studies (Louis et al. 2002; 2008; Zheng et al. 2000). In short, one volume (9–12 ml) of whole blood was mixed with half a volume (5–6 ml) of 1 M NaOH. Following vortex for 30 s, the samples were placed on a horizontal rotator and shaken at room temperature for 30 min. An aliquot (15 ml) of the extraction solution consisting of ethyl acetate and methyl-*t*-butyl

ether (2:98, v:v) was added to the tube. The tube was then vigorously shaken by hand for 1–2 min, followed by shaking on a horizontal rotor at room temperature for 45 min. After centrifugation at 3000 × g for 10 min, the upper organic phase was separated. The extraction procedure was repeated two additional times. The organic phase was combined and evaporated under nitrogen to dryness. The samples were reconstructed in 0.25 ml methanol. After centrifugation at 3000 × g for 10 min, the supernatant was transferred to autosampler vials with sealed caps for HPLC analysis.

A Waters model 2695XE complete HPLC system including autosampler, temperature control module, seal wash, and degasser, and a Waters model 2475 multichannel fluorescent detector were used for separation and quantification. Separation was accomplished using an ion-interaction, reversed-phase Econosphere C₁₈ column (ODS2, 5 μm, 250 × 4.6 mm) attached to a Spherisorb guard column (ODS2, 5 μm, 10 × 4.6 mm). Both analytical and guard columns were purchased from Alltech (Deerfield IL). An isocratic mobile phase consisted of 17.5 mM potassium phosphate buffer, pH 6.5 (equal molar concentration of both monobasic and dibasic potassium salts) and methanol (30:70, v:v). A 50-μl aliquot of sample extracts was injected and the separation performed at room temperature at a flow rate of 1 ml/min. The detector was set at an excitation wavelength of 300 nm and an emission wavelength of 435 nm. A Dell Windows-based computer equipped with Waters data analysis package was used to collect and analyze the data. The identity of harmane on HPLC chromatographs previously has been clarified (Guan et al. 2001; Zheng et al. 2000). The intraday precision, measured as a coefficient of variation at 25 ng/ml, was 6.7% for harmane. The interday precision was 7.3% for harmane (Zheng et al. 2000).

Statistical Analyses

Statistical analyses were performed in SPSS (Version 19.0). Chi-squared tests (χ^2) and

Fisher's exact tests were used to analyze proportions, and Student's *t*-tests were used to examine group differences in continuous variables. Pearson (*r*) correlation coefficients were used to assess correlations between continuous variables.

The empirical distribution of harmane was positively skewed. Therefore, harmane concentrations were logarithmically transformed; the log-transformed blood harmane values were normally distributed. Case-control differences in log blood harmane concentrations were assessed using Student's *t*-tests. In confirmatory analyses, a nonparametric (Mann-Whitney *U*) test also was performed on harmane data that were not logarithmically transformed. Log blood harmane concentrations were also stratified based on the median concentration ($0.25 \text{ g}^{-10}/\text{ml}$) and then based on tertiles, and cases and controls were compared with respect to proportion within each stratum. The total tremor score was also stratified into a high score (≥ 25) vs. low (< 25) score category, as in prior studies (Louis et al. 2008).

To assess the null hypothesis that blood harmane concentration was not a predictor of diagnostic group (ET vs. control), logistic regression analysis was performed using diagnostic group as the outcome and log blood harmane concentration as the primary independent variable, resulting in odds ratios (OR) with 95% confidence intervals (CI). As in prior studies (Louis et al. 2002; 2008), a number of potential confounders were considered (age in years, gender, race, years of education, body mass index, Cumulative Illness Rating scale score, current cigarette smoker, ever cigarette smoker, cigarette pack-years among current cigarette smokers, cigarette pack-years among ever cigarette smokers, number of cigarettes smoked on the day of the evaluation, answered "yes" to the question "have you had something to drink today," and answered "yes" to the question "have you had something to eat today"), and were included in the adjusted logistic regression analyses if they were associated with either ET or blood harmane concentration in the current data set or were consistently associated with blood harmane

concentration in prior publications (Louis et al. 2002).

In three analyses, which tested for trend, log blood harmane concentration was the dependent variable in a linear regression analysis. In the first analysis, the ordinal distributed independent variable was as follows: ET cases with a family history of tremor, ET cases without a family history of tremor, controls. In the second analysis, the independent variable was: ET cases with a family history of ET, ET cases without a family history of ET, controls. In the third analysis, the independent variable was: ET cases with high total tremor score (≥ 25), ET cases with lower total tremor score, controls.

A correlation coefficient was used to assess the association between initial and subsequent log blood harmane concentration. Then, in a linear regression model in which subsequent log blood harmane concentration was the dependent variable and initial log blood harmane concentration was the independent variable, the role of confounding factors at the time of the subsequent visit was assessed (see list of confounders given earlier).

RESULTS

The 63 ET cases and 70 controls had phlebotomy at both time points 1 and 2, and it is their time point 2 blood harmane concentrations that are the focus of these analyses.

The 63 ET cases and 70 controls were similar with respect to demographic and clinical variables, including age, gender, race, education, body mass index, smoking habits, and medical comorbidity (Table 1). A numerically larger but not significant percentage of controls had something to drink on the morning of the evaluation but a similar percentage had something to eat (Table 1). ET cases had a mean age of tremor onset of 42.1 ± 21.6 yr, and their mean tremor duration was 30.7 ± 18.8 yr. Forty (63.5%) ET cases had a family history of tremor and 16 (25.4%) had a family history of ET. Thirty-one (49.2%) ET cases took daily medication to treat tremor. The mean total tremor score in ET cases was 21.7 ± 5.4 .

TABLE 1. Characteristics of 63 ET Cases Versus 70 Controls

Characteristic	ET cases (N = 63)	Controls (N = 70)	Significance
Age (yr)	71.7 ± 11.6	70.0 ± 10.0	$t = .89, p = .38$
Female gender	31 (49.2)	43 (61.4)	$\chi^2 = 2.01, p = .16$
Non-Hispanic white race	59 (93.7)	63 (90.0)	$\chi^2 = .58, p = .45$
Years of education	15.8 ± 2.4	16.3 ± 2.2	$t = 1.11, p = 0.27$
Body mass index (kg/m ²)	25.1 ± 4.3	25.9 ± 4.7	$t = 1.02, p = 0.31$
Current cigarette smoker	4 (6.3)	5 (7.1)	Fisher's exact test $p = 1.00$
Pack-years (among current cigarette smokers)	12.0 ± 8.5	35.6 ± 17.3	$t = 1.77, p = .14$
Ever cigarette smoker	34 (54.0)	35 (50.0)	$\chi^2 = .21, p = .65$
Pack-years (among ever cigarette smokers)	14.5 ± 16.5	22.6 ± 23.7	$t = 1.61, p = .11$
Number of cigarettes smoked on the day of the evaluation	0.08 ± 0.50	0.09 ± 0.50	$t = .09, p = .93$
Answered "yes" to the question "have you had something to eat today"	39 (67.2)	53 (76.8)	$\chi^2 = 1.45, p = .23$
Answered "yes" to the question "have you had something to drink today."	32 (50.8)	46 (65.7)	$\chi^2 = 3.04, p = .08$
Cumulative Illness Rating Scale score	7.1 ± 3.1	6.2 ± 3.7	$t = 1.44, p = .15$

Note. Values are mean ± standard deviation, or numbers (percentages).

A mean of 5.8 ± 2.4 (range = 1.6–10.1) yr had elapsed between the initial and the subsequent blood harmene determinations, and this was similar in cases and controls (5.5 ± 2.4 vs. 6.1 ± 2.4 yr).

Using our control sample, the correlates of log blood harmene concentration were assessed. Log blood harmene concentration was higher in women than men (0.19 ± 0.49 g⁻¹⁰/ml vs. -0.11 ± 0.60 g⁻¹⁰/ml). However, log blood harmene concentration was not associated with age in years ($r = -0.16$), years of education ($r = -0.005$), body mass index ($r = -0.14$) or Cumulative Illness Rating Scale score ($r = -0.05$). Log blood harmene did not differ by race (0.08 ± 0.57 g⁻¹⁰/ml for non-Hispanic whites vs. -0.004 ± 0.33 g⁻¹⁰/ml in others). There was no difference between current cigarette smokers and nonsmokers (-0.26 ± 0.73 g⁻¹⁰/ml vs. 0.10 ± 0.53 g⁻¹⁰/ml, respectively), between ever cigarette smokers and never cigarette smokers (0.11 ± 0.50 g⁻¹⁰/ml vs. 0.04 ± 0.60 g⁻¹⁰/ml, respectively), and no association with number of pack years among current cigarette smokers ($r = -0.64$), number of pack years among ever cigarette smokers ($r = -0.04$), or number of cigarettes smoked on the day of the evaluation ($r = -0.09$). Controls

who had answered "yes" to the question "have you had something to drink today" had significantly higher log blood harmene concentrations than did those who had answered "no": 0.18 ± 0.53 g⁻¹⁰/ml versus -0.13 ± 0.55 g⁻¹⁰/ml, respectively. Controls who had answered "yes" to the question "have you had something to eat today" had numerically higher log blood harmene concentrations than those who had answered "no": 0.40 ± 0.53 g⁻¹⁰/ml versus 0.14 ± 0.69 g⁻¹⁰/ml, respectively.

The mean log blood harmene concentration was severalfold significantly higher in our 63 cases than 70 controls (0.30 ± 0.61 g⁻¹⁰/ml vs. 0.08 ± 0.55 g⁻¹⁰/ml) (Figure 1). In a confirmatory analysis, a nonparametric (Mann-Whitney) test also was performed on harmene data that were not logarithmically transformed, and the median blood harmene value in cases was twice as high as that of controls: 0.22 g⁻¹⁰/ml versus 0.11 g⁻¹⁰/ml. Strata were created based on the median log blood harmene concentration (0.25 g⁻¹⁰/ml); a significantly larger percent of cases versus controls (39 or 61.9% vs. 28 or 40%) had a high log blood harmene concentration based on this median split. Strata were also created based on the tertiles of log blood harmene concentration, and 27 (42.9%) cases versus 17 (24.3)

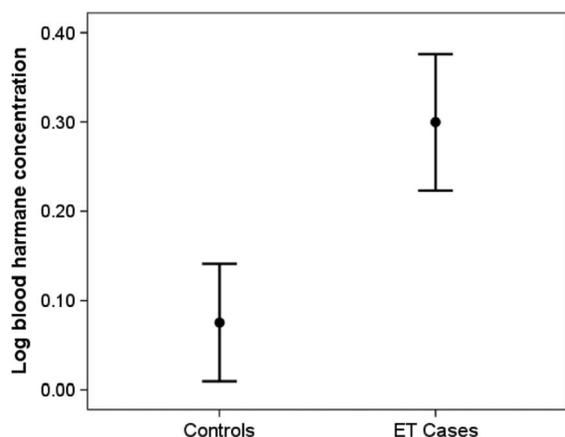


FIGURE 1. The mean log blood harmane concentration was severalfold higher in cases than controls. The mean \pm 1 standard error is shown for each value.

controls were in the highest log blood harmane tertile ($>0.43 \text{ g}^{-10}/\text{ml}$), a difference that was significant.

In an unadjusted logistic regression analysis, log blood harmane concentration was significantly associated with the outcome (diagnosis of ET vs. control) (OR = 1.98, 95% CI = 1.07–3.68) (i.e., for every doubling of the harmane concentration, the odds of ET increased by 98%). In a logistic regression analysis that adjusted for age in years, gender, non-Hispanic white race, years of education, current smoker, ever cigarette smoker, number of cigarette pack years, number of cigarettes smoked on the day of the evaluation, answered “yes” to the question “have you had something to eat today,” and answered “yes” to the question “have you had something to drink today,” the OR increased to 2.72, 95% CI = 1.34–5.55, indicating that these factors did not account for the observed association between log blood harmane concentration and diagnosis, and may indeed have enhanced the association.

After stratifying by family history, and the log blood harmane concentration was highest in ET cases with a family history of ET (Table 2 and Figure 2). The log blood harmane concentration was highest in the 15 ET cases with high total tremor scores (≥ 25), intermediate in 48 ET cases with lower total tremor scores and lowest in the 70 controls ($0.39 \pm 0.54 \text{ g}^{-10}/\text{ml}$ vs. $0.22 \pm 0.59 \text{ g}^{-10}/\text{ml}$ vs. $0.08 \pm 0.55 \text{ g}^{-10}/\text{ml}$),

TABLE 2. Log Blood Harmane Concentration by Group

Group	Log blood harmane concentration	Significance
Controls	0.08 ± 0.55	Beta = 0.12, $p = .03^a$
ET cases with no family history of tremor	0.28 ± 0.75	
ET cases with family history of tremor	0.31 ± 0.40	Beta = 0.16, $p = .027^b$
Controls	0.08 ± 0.55	
ET cases with no family history of ET	0.28 ± 0.60	
ET cases with family history of ET	0.36 ± 0.63	

^aTest for trend by treating log blood harmane concentration as the dependent variable in a linear regression analysis, and examining the association with an ordinal distributed independent variable (ET cases with a family history of tremor, ET cases without a family history of tremor, controls).

^bTest for trend by treating log blood harmane concentration as the dependent variable in a linear regression analysis, and examining the association with an ordinal distributed independent variable (ET cases with a family history of ET, ET cases without a family history of ET, controls).

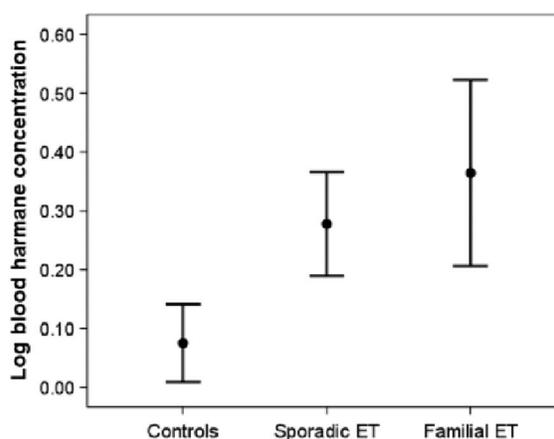


FIGURE 2. The log blood harmane concentration was highest in ET cases with a family history of ET, intermediate in sporadic ET cases, and lowest in controls. The mean \pm 1 standard error is shown for each value.

a trend that was significant. ET cases who took daily medication to treat tremor had a log blood harmane concentration that was similar to ET cases who did not take such medication ($0.26 \pm 0.66 \text{ g}^{-10}/\text{ml}$ vs. $0.34 \pm 0.56 \text{ g}^{-10}/\text{ml}$).

Strata were also created based on the tertiles of log blood harmane concentration; 1 case (1.6%) was in the lowest tertile at time point 1 and the highest tertile at time point 2,

and 9 cases (14.3%) were in the highest tertile at time point 1 and the lowest tertile at time point 2; otherwise, the time point 1 and 2 values were similar. Yet there was not a significant linear correlation between the initial (time point 1) log blood harmane concentration and the subsequent (time point 2) log blood harmane concentration. In a linear regression model in which subsequent log blood harmane concentration was the dependent variable and initial log blood harmane concentration was the independent variable, the role of confounding factors was assessed. Confounding factors that were associated significantly with subsequent log blood harmane concentration in the final model were fewer years of education, non-Hispanic white race, number of cigarettes smoked on the day of the subsequent evaluation, and having answered "yes" to the question "have you had something to drink today." In the final model, there was no correlation between the initial and subsequent log blood harmane concentrations.

DISCUSSION

In this study, blood harmane concentration was elevated in ET cases compared to controls reassessed at a second time point many years later, indicating what seems to be a stable association between this environmental toxin and ET.

β -Carboline alkaloids such as harmane are an obvious choice for investigation in the search for possible toxic environmental causes of ET. These toxins are structurally similar to MPTP, a neurotoxin that serves as one of the main animal models for PD (Langston et al. 1984; Smeyne et al. 2005). For more than 100 years, it has been known that the administration of β -carboline alkaloids to a broad range of laboratory animals (e.g., mice, cats, monkeys) produces a form of action tremor that shares clinical and drug-response features with ET (Cross et al. 1993; Du et al. 1997; Fuentes et al. 1971; Milner et al. 1995; Rappaport et al. 1984; Sinton et al. 1989; Trouvin et al. 1987). Indeed, β -carboline alkaloid administration is

the main animal model for ET, and new pharmacotherapies are tested using exposed animals (Handforth et al. 2001; Martin et al. 2005; 2006).

In our earlier studies, blood harmane concentrations seemed to be highest both among ET cases with a family history of ET and in ET cases with more marked tremor (Louis et al. 2008). These results were observed again in cases resampled at a second time point. The higher concentration in familial ET cases suggests that the mechanism for this elevated concentration may be at least partly genetic. ET itself is a highly familial disorder. Many kindreds with autosomal dominant inheritance of ET have been described, and linkage has been demonstrated to regions on chromosomes 2p, 3q, and 6p, although at present the genes that are responsible for ET have not been identified (Deng et al. 2007).

There was no correlation between the initial log blood harmane concentration and the subsequent log blood harmane concentration. This may reflect differences in dietary structure over time. Other lifestyle factors (number of cigarettes smoked on the day of the evaluation, consumption of food or liquids on the day of the evaluation) may have influenced the continuity of blood harmane concentrations over time as well. Finally, harmane in the body is known to metabolize to harmine in the liver (Guan et al. 2001), and changes in subjects' metabolic activities over the time period of this study, due to either endogenous or extraneous induction/inhibition of the harmane metabolic pathways, may also contribute to the variation of blood harmane concentrations in each individual participating in this study.

This study had limitations. Fasting blood harmane concentrations were not assessed. Therefore, it was difficult to assess the extent to which case-control differences reflect a difference in dietary intake of harmane. While a similar proportion of cases and controls had had something to eat on the morning of the evaluation, a larger proportion of controls had had something to drink on the morning of the evaluation, yet consideration of the possible

confounding effects of these data in our models only served to increase the magnitude of the association between log blood harmane concentration and ET diagnosis. Also, neither liver function, nor variability in the cytochrome P-450 system, nor renal function was assessed to see whether factors that might influence the metabolism of harmane differed between cases and controls. This study also had several strengths. To our knowledge, this is the only repeat-measure study of the association between blood levels of this toxin and ET. Blood harmane levels were measured in the same laboratory using identical analytic methods at both time points. Participants were reevaluated up to 10 yr after their initial assessment, and all blood harmane determinations were performed blinded to clinical information and vice versa. In the analyses, the potential confounding effects of multiple relevant covariates were assessed. Finally, data on tremor severity and familial occurrence of tremor complemented the primary data on case versus control levels of blood harmane.

In summary, both genetic and environmental factors are likely to play a role in the etiology of ET. The contribution of environmental risk factors to disease etiology has been examined in detail in epidemiological studies of PD as well as Alzheimer's disease and amyotrophic lateral sclerosis (Baldereschi et al. 2008; Dick 2006; Gorell et al. 1997; 1998; Morahan et al. 2007; Racette et al. 2001; Shcherbatykh et al. 2007). Yet the study of such toxins in ET lags far behind. It is commonly stated that approximately 50% of ET cases are sporadic (Lambert et al. 1999; Louis et al. 1996). With a population prevalence for ET of 4% (age 40 yr and older) (Dogu et al. 2003), this suggests that approximately 2% of the population aged ≥ 40 yr has a nonfamilial form of ET, yet the environmental determinants for this tremor are only just beginning to be explored. Blood harmane has been associated with ET in cross-sectional studies. The present study reveals a persistent case-control difference after a multi-year lapse, indicating what seems to be a stable association between this environmental toxin and ET.

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