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Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease

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Objective – The aim of this study was to screen for and quantify the neurotoxic amino acid β-*N*-methylamino-L-alanine (BMAA) in a cohort of autopsy specimens taken from Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and non-neurological controls. BMAA is produced by cyanobacteria found in a variety of freshwater, marine, and terrestrial habitats. The possibility of geographically broad human exposure to BMAA had been suggested by the discovery of BMAA in brain tissues of Chamorro patients with ALS/Parkinsonism dementia complex from Guam and more recently in AD patients from North America. These observations warranted an independent study of possible BMAA exposures outside of the Guam ecosystem. *Methods* – Postmortem brain specimens were taken from neuropathologically confirmed cases of 13 ALS, 12 AD, 8 HD patients, and 12 age-matched non-neurological controls. BMAA was quantified using a validated fluorescent HPLC method previously used to detect BMAA in patients from Guam. Tandem mass spectrometric (MS) analysis was carried out to confirm the identification of BMAA in neurological specimens. *Results* – We detected and quantified BMAA in neuroproteins from postmortem brain tissue of patients from the United States who died with sporadic AD and ALS but not HD. Incidental detections observed in two out of the 24 regions were analyzed from the controls. The concentrations of BMAA were below what had been reported previously in Chamarro ALS/ Parkinsonism dementia complex patients, but demonstrated a twofold range across disease and regional brain area comparisons. The presence of BMAA in these patients was confirmed by triple quadrupole liquid chromatography/mass spectrometry/mass spectrometry. *Conclusions* – The occurrence of BMAA in North American ALS and AD patients suggests the possibility of a gene/environment interaction, with BMAA triggering neurodegeneration in vulnerable individuals.

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Introduction

β-*N*-methylamino-L-alanine (BMAA), a neurotoxic amino acid produced by cyanobacteria (1) was originally isolated from cycad seeds used by the Chamorro people of Guam to prepare tortillas and soups (2–5). Early researchers suggested links of BMAA to amyotrophic lateral sclerosis (ALS)/Parkinsonism dementia complex (PDC) in Guam (6). The BMAA hypothesis was soon rejected because of reported low BMAA concentrations in cycad flour. However, only free BMAA in cycad flour was analyzed rather than protein-bound BMAA, even though the inclusion of other unusual amino acids into proteins had been well characterized (7–9). Recent discoveries have re-invigorated the interest in BMAA: (i) BMAA is produced by cyanobacteria, including endosymbiotic species of *Nostoc* found in cycad roots; (ii) BMAA occurs in far higher amounts in

cycad flour than was previously suspected because of its occurrence in the protein fraction; (iii) BMAA can be biomagnified in animals which forage on cycad seeds and are subsequently consumed by the Chamorro people; (iv) diverse taxa of cyanobacteria produce BMAA, and (v) BMAA has been detected in blinded studies of brain tissues of North American patients with Alzheimer's disease (AD) but not healthy controls (1, 2, 5, 10–12).

β-*N*-methylamino-L-alanine has been artificially incorporated in synthetic polypeptides (13). Endogenous protein-bound BMAA may serve as a potential reservoir for repeated human exposures (14). We tested frozen brain samples at the University of Miami/NPF (National Parkinson Foundation) Brain Endowment Bank from blindly selected cohorts of patients who died with ALS, AD, Huntington's disease (HD), and from controls, for BMAA using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) as a fluorescent amino acid tag (2). The HPLC analyses at the University of Miami were carried out with a validated fluorescent HPLC method, used to detect BMAA in brain tissues from Guamanian patients, but were performed independently of previous investigators.

Material and methods

Case selection

Archived brain tissue from 12 demented (aged 66–90 years) and 12 non-demented cases (aged 46–87 years) was obtained from the University of Miami/NPF Brain Endowment Bank™ Miami, FL, USA (Table 1). These cases were compared with a well-characterized cohort of ALS (n = 13) and HD (n = 8; aged 47–55) patients. Two neurologists (S. P. and W. G. B.) completed an independent retrospective chart review to confirm diagnostic classification. Sufficient numbers of neuritic plaques and neurofibrillary tangles to fulfill diagnostic criteria for AD were present in the demented patients studied. All but one case had advanced Braak stage IV or V neuropathological changes (15). AD cases were not included if there was evidence of concomitant pathology, including infarcts, diffuse Lewy bodies, or Pick's disease. Two patients had evidence of degeneration of the substantia nigra without Lewy bodies. Patients with HD had marked degeneration of the caudate and putamen consistent with the clinical diagnosis and family history. The non-demented control cases showed no evidence of neurological disease and were free from neuropathological changes except for small numbers of senile plaques in one case (aged 85 years). Archived brain tissues of 13 cases of ALS (ages 55 to 83 years) were selected for BMAA analysis (Table 1). All had pathological evidence of degeneration of anterior horn cells in the spinal cord and gliosis of the corticospinal tracts, indicating combined lower and upper motor neuron degeneration.

High pressure liquid chromatography

Quantitation of BMAA was performed on post-mortem, unfixed tissues by a modification of previously validated methods (2, 10, 16). Regional samples of gray matter were taken for analysis from the frontal cortex (Brodmann area 10) and temporal polar cortex (Brodmann area 38). The caudate and spinal cord was taken from HD and ALS cases for analysis, respectively. A blind analysis was carried out in which the assays were conducted with individual diagnostic information kept unknown until the sample measurements were essentially complete. All cases were analyzed at least twice in the selected brain regions assayed in duplicate. Briefly, frozen-pulverized (Model# 36903-10; Cole-Parmer, Quebec, Canada) brain tissues (1:10 w/v) were homogenized twice in 0.1 M trichloroacetic acid and centrifuged at 15,800 *g* for 10 min to remove free amino acids. After the second wash and centrifugation, the supernatant was aspirated and discarded. The pellet was hydrolyzed overnight in 6 M HCl (1:10 w/v) at 110°C (10,14,17). Particulate matter was removed in 500 µl aliquots by ultrafiltration (Ultrafree-MC; Millipore, Billerica, MA, USA) at 15,800 *g*. The extract was freeze-dried in a lyophilizer (Virtis Benchtop 2K, Camden, NJ, USA). The lyophilized residue was resuspended in 100 µl borate buffer (0.24 M sodium tetraborate, pH 9.0). Samples (20 µl) and standards were derivatized with AQC using the AccQ-Fluor reagent (WAT052880 Lot. No.: 011188, 013824, and 061561; Waters Corp, Millford, MA, USA). BMAA was separated from the protein amino acids by reverse-phase elution (Waters Nova-Pak C18 column, 3.9 × 300 mm using 140 mM sodium acetate, 5.6 mM triethylamine, pH 5.7 (mobile phase A), and 52% acetonitrile in water (mobile phase B) at 37°C. The elution gradient (30 min) was as follows: time 0 = 75% A; 2 min = 75% A (curve 6); 17 min = 63% A (curve 7); 18.5 min = 100% B (curve 6); 23.5 min = 100% B (curve 6); 25 min = 75% A (curve 6); 30 min = 75% A. Samples were run in duplicate followed by an AQC blank (containing borate buffer and AQC tag) to ensure that there is no carryover between samples. BMAA was quantified by detection of the AQC fluorescent tag (Waters 2475 Multi λ-Fluorescence Detector) with excitation at 250 nm and emission

Table 1 Clinical characteristics of ALS, AD, HD, and non-neurological control subjects

Disease	Gender	Age at death (years)	Age at disease onset (years)	Onset symptom	PMI (h)	Neuropathology
ALS1	Male	55	53	Bulbar (dysphagia, dysarthria)	18.0	Moderate bilateral loss of anterior horn cells and gliosis in cervical and thoracic cord
ALS2	Male	59	52	N/A	6.3	Neurons slightly shrunken. Bilateral loss of anterior horn cells with gliosis. Focal degeneration of corticospinal tract
ALS3	Male	65	62	Upper limb weakness	11.0	Several neuritic plaques. Prominent bilateral loss of anterior horn cells associated with gliosis
ALS4	Female	65	57	Upper limb weakness	7.0	Degeneration of anterior horn cells
ALS5	Male	66	62	Lower limb weakness	11.5	Severe bilateral loss of anterior horn cells and gliosis in cervical and thoracic cord
ALS6	Female	67	64	Bulbar (dysarthria)	12.0	Severe bilateral loss of anterior horn cells and gliosis in cervical and thoracic cord
ALS7	Female	67	64	Lower limb weakness	11.0	Severe bilateral loss of anterior horn cells and gliosis in cervical and thoracic cord
ALS8	Male	70	64	Diffuse fasciculations and lower limb weakness	9.0	Neurofibrillary tangles in the frontal cortex. Severe bilateral loss of anterior horn cells and gliosis in cervical and thoracic cord
ALS9	Male	70	68	Bulbar (dysarthria)	5.0	Corticospinal tract degeneration. Severe bilateral loss of anterior horn cells and gliosis in cervical and thoracic cord
ALS10	Female	74	63	Upper limb weakness	12.0	Marked bilateral loss of anterior horn cells and gliosis in cervical and thoracic cord
ALS11	Male	77	80	Lower limb weakness	23.0	Scattered neurofibrillary tangles and white matter gliosis. Corticospinal tract degeneration. Marked bilateral loss of anterior horn cells and mild gliosis in cervical and thoracic cord
ALS12	Male	79	74	Lower limb weakness	7.0	Lateral corticospinal tract degeneration. Marked bilateral loss of anterior horn cells and mild gliosis in cervical and thoracic cord
ALS13	Male	83	79	Upper limb weakness	20.0	Marked bilateral loss of anterior horn cells and gliosis in cervical and thoracic cord
AD1	Male	66	58	Cognitive decline	5.0	Diffuse neuritic plaques and neurofibrillary tangles in cortex and hippocampus
AD2	Female	67	58	Cognitive decline	9.5	Diffuse neuritic plaques and neurofibrillary tangles in cortex and hippocampus
AD3	Male	67	Preclinical	None	10.0	Diffuse neuritic plaques and neurofibrillary tangles in cortex and hippocampus
AD4	Male	76	69	Cognitive decline	4.0	Diffuse neuritic plaques and neurofibrillary tangles in cortex and hippocampus Substantia nigra degeneration
AD5	Male	80	72	Cognitive decline	6.0	Diffuse neuritic plaques and neurofibrillary tangles in cortex and hippocampus Basoganglionic gliosis
AD6	Female	81	78	Cognitive decline	5.0	Diffuse neuritic plaques and neurofibrillary tangles in cortex and hippocampus Granulovacuolar degeneration of the hippocampus Diffuse cerebral ischemic lesions
AD7	Male	81	73	Cognitive decline	5.0	Diffuse neuritic plaques and neurofibrillary tangles in cortex and hippocampus Substantia nigra degeneration.
AD8	Male	81	78	Cognitive decline	3.0	Mild to moderate number of neuritic plaques and neurofibrillary tangles in the cortex, amygdala and hippocampus Focal gliosis in anterior periventricular white matter
AD9	Female	84	78	Cognitive decline	22.0	Neurofibrillary tangles and plaques in the cerebral cortex and hippocampus Thalamic CVA
AD10	Female	87	85	Cognitive decline	8.0	Many senile plaques and some neurofibrillary tangles in cerebral cortex Senile plaques and neurofibrillary tangles in hippocampus and amygdala
AD11	Male	87	75	Cognitive decline	9.5	Numerous neuritic plaques and neurofibrillary tangles in cortex and hippocampus Mild cerebral atherosclerosis
AD12	Female	90	84	Cognitive decline	9.0	Diffuse neuritic plaques and neurofibrillary tangles in cortex and hippocampus Diffuse atherosclerotic changes

Table 1 (*Continued*)

Disease	Gender	Age at death (years)	Age at disease onset (years)	Onset symptom	PMI (h)	Neuropathology
HD1	Male	47	34	Depression and incoordination	8.0	Striatal degeneration and atrophy of caudate nucleus
HD2	Male	54	44	Loss of motor control	12.0	Gliosis in caudate and putamen with moderate loss of small neurons
HD3	Male	59	45	Changes in personality and incoordination	4.5	Bilateral degeneration of basal ganglia
HD4	Female	62	41	Chorea and depression	12.5	Bilateral degeneration of basal ganglia
HD5	Female	66	46	Loss of motor control	14.0	Marked striatal degeneration
HD6	Male	69	55	Chorea and personality changes	6.8	Caudate and putamen show severe neuronal loss and fibrillary astrocytosis
HD7	Male	77	60	Distinct chorea	16.0	Neurodegenerative of basal ganglia
HD8	Female	85	46	Chorea and incoordination	12.5	Degeneration and gliosis in basal ganglia. Mild age-related senile plaque counts
Control1	Male	46	N/A	N/A	21.0	Normal brain. Entire brain appeared unremarkable
Control2	Male	54	N/A	N/A	23.0	Focal cerebral atherosclerosis with non-specific degeneration of white matter
Control3	Male	67	N/A	N/A	22.0	Normal brain. Entire brain appeared unremarkable
Control4	Female	68	N/A	N/A	24.5	Normal brain. Entire brain appeared unremarkable
Control5	Male	68	N/A	N/A	16.0	Normal brain. Entire brain appeared unremarkable
Control6	Male	70	N/A	N/A	8.5	Normal brain. Entire brain appeared unremarkable
Control7	Female	78	N/A	N/A	21.0	Normal brain. Entire brain appeared unremarkable
Control8	Male	81	N/A	N/A	21.0	Normal brain. Entire brain appeared unremarkable
Control9	Female	83	N/A	N/A	5.5	Normal brain. Entire brain appeared unremarkable
Control10	Male	85	N/A	N/A	4.5	Normal brain. Entire brain appeared unremarkable
Control11	Female	85	N/A	N/A	4.0	Few age-related senile plaques. Focal changes consistent with aging process
Control12	Male	87	N/A	N/A	21.0	Normal brain. Entire brain appeared unremarkable

AD, Alzheimer's disease; ALD, amyotrophic lateral sclerosis; NFTs, neurofibrillary tangles; N/A, not applicable; CVA, Cerebrovascular accident; HD, Huntington disease; PMI, postmortem interval.

at 395 nm. Experimental brain samples were compared with standard spiked brain matrix containing commercial (Sigma B-107; >95% purity; Sigma, St Louis, MO, USA) or authentic standards of BMAA (99% purity). The percentage of recovery of BMAA was >96%.

LC/MS/MS confirmation of BMAA peaks

Identification of a BMAA peak detected by reverse-phase HPLC was verified by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) using product ion mode in a triple quadrupole system. The frozen (unfixed) brain sample was hydrolyzed for 18 h in 6 N HCl at 110°C (17) and then dried to remove HCl in a Thermo-Savant SC250DDA Speed Vac Plus (Waltham, MA, USA). The sample was reconstituted in dilute HCl (20 mM) and derivatized with AQC, which increased the molecular weight of the BMAA analyte from 118 to 458. To increase the quantity of analyte injected into the LC/MS/MS, the BMAA peak was repeatedly collected (20 times) from 60 µl injections using a Waters 1525 binary HPLC pump and a Waters 717 Auto-sampler, with separation through a Waters 3.9 × 300 mm Nova-Pak C18, 4 µm particle column (Lot No.: 11143603410254) at 37°C. Individual compounds were eluted from the column with a gradient elution of 140 mM sodium acetate buffer, 5.6 mM triethylamine, pH 5.7 (mobile phase A) and 52% acetonitrile (mobile phase B) with a flow rate of 1.0 ml/min. The collected peaks were processed to complete dryness in a Thermo-Savant SC250DDA Speed Vac Plus with a Savant refrigerator trap RVT 4104. The first set of 10 collected peaks were then reconstituted in 50 µl HPLC grade water, heated to 55°C, vortexed, and then added to the second set of 10 collected peaks, which were then heated and vortexed in a similar fashion. These samples were combined (0.5 ml) and then cooled and spun through a Millipore Ultrafree-MC 0.22 µm filter to remove sodium acetate crystals. The filtrate was then concentrated in the Speed Vac, removed before complete dryness (90 µl), warmed to 55°C, and injected into the triple quadrupole system (Thermo model; TSQ Quantum Discovery Max, San Jose, CA, USA). Separation was achieved by liquid chromatography (Thermo model Surveyor LC, TSQ Quantum Discovery Max) using a Thermo

Hypersil GOLD 100 × 2.1 mm, 3 μm particle column at 0.28 ml/min using the eluents of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B) with the following gradient: time 0 = 100% A, 2 min = 100% A, 16 min = 80% A, 20 min = 2% A, 22 min = 2% A, 24 min = 81% A, 26 min = 81% A, 27 min = 100% A, 31 min = 100% A. Nitrogen gas was supplied to the heated electrospray ionization (ESI) probe with a nebulizing pressure of 25 psi and a vaporizing temperature of 300°C. The mass spectrometer was operated in the positive ESI mode under the following conditions: nebulizing pressure of 25 psi, vaporizing temperature of 300°C, capillary temperature set at 280°C, capillary offset of 35, tube lens offset of 94, source collision energy of 5 V. The protonated molecular ion of double derivatized BMAA (*m/z* 459) was used as the precursor ion for single reaction monitoring analysis. Three transitions were monitored: 459 to 119 V (collision energy of 19 V), 459 to 171 V (collision energy of 32 V), 459 to 289 V (collision energy of 16 V). The ratios of these three product ions were compared with the ratios of the product ions created by injection of AQC-derivatized pure BMAA standard (synthesized and then triple crystallized by Peter Nunn, University of Portsmouth, Hampshire, UK, P012DJ) into the triple quadrupole LC/MS/MS under the same conditions.

Results

We detected BMAA in significant concentrations in blinded brain tissues from 13 American ALS and 12 AD patients, but generally not in HD patients or age-matched non-neurological controls (Table 1, see Fig. 1 for a representative chromatogram). Our limit of detection for BMAA in human brain matrix was 2 ng on column, approximately 20-fold higher than that of a BMAA standard assayed in borate buffer (Fig. 2). Standard curves were obtained by assay of brain matrix spiked with serial dilutions of BMAA (n = 3 assayed in duplicate). The linear dynamic range for BMAA is shown in Fig. 2A. Linear regression of the data gave an R^2 value of 0.987 ($P < 0.0001$). The lower limit of quantification in human brain matrix was 10 ng (Fig. 2B). Recovery of BMAA was approximately 95%. Separate control runs were carried out to test the effects of acid hydrolysis at high temperature (6 N HCl, 110°C) on the recovery of BMAA spiked into control brain matrix. These test runs demonstrated no difference in BMAA quantities by comparing analyte concentrations before or after acid hydrolysis (Fig. S1).

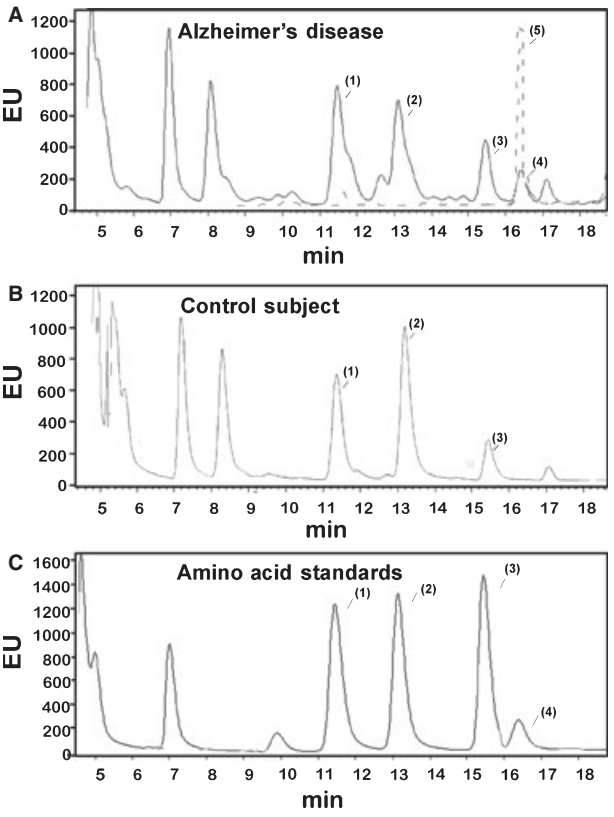
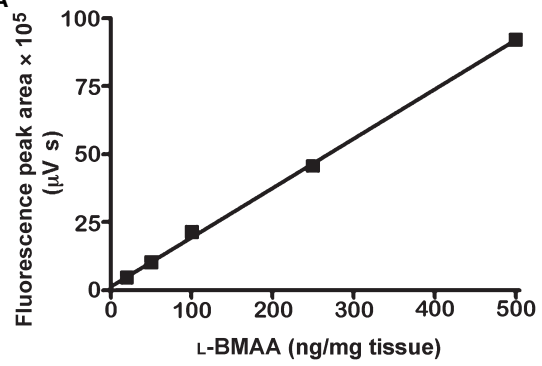


Figure 1. HPLC identification of β-*N*-methylamino-L-alanine (BMAA) in human brain. A) Representative chromatogram of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC)-tagged amino acids in protein-bound extract of the frontal cortex of an 81-year-male Caucasian with Alzheimer's disease. Peaks indicated by numbers are the amino acids closest to BMAA: L-tyrosine (1), L-valine (2), and L-methionine (3). The sample chromatographic peak of AQC-BMAA (4) at 16.4 min was confirmed by comparison to a co-run AQC standard of L-BMAA (dashed line, 5). (B) Derivitized amino acids in human brain tissue taken from an aged-matched non-neurological controls (male, 81 years). Peaks indicated by number are L-tyrosine (1), L-valine (2), and L-methionine (3). No chromatographic peak was observed at the retention time corresponding to BMAA. (C) Separation of the derivatized amino acid standards is optimized on a C18 column with complete resolution in less than 30 min. Numbered peaks are the same as in panels A and B.

We found varying concentrations of BMAA in neuroproteins from the frontal and temporal cortices of all the ALS and AD patients (Table 2, left column), ranging from 31 to 256 μg/g in brain tissue samples from American ALS patients [134 ± 12.8 μg/g (mean ± SEM) and 10 to 228 μg/g in American AD patients (111 ± 14.6 μg/g)]. BMAA was measured in the spinal cord specimens available for four cases (ALS6, 87 ± 3 μg/g; ALS9, 47 ± 3 μg/g; ALS11, 166 ± 11 μg/g; ALS13, 197 ± 10 μg/g). The levels are comparable with quantification of BMAA in protein-bound fractions previously reported in Canadian AD patients (Table 2, right column; 95 ± 32 μg/g).

A



B

	LOD (ng) (% CV)	% Δ	LOQ (ng) (% CV)	% Δ	Recovery (%)
Brain	2 (13.3)	7.0	10 (15.3)	8.0	95.4 ± 3.6
Buffer	0.1 (12.7)	10.2	2 (8.7)	6.0	—

Figure 2. Calibration of the HPLC assay of β-*N*-methylamino-L-alanine (BMAA) with fluorometric detection. A) Hydrolyzed protein matrix from a non-neurological control (84-year-old, male, Caucasian) brain specimen was spiked with increasing amounts of L-BMAA. Neutralized samples derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) were analyzed by HPLC without further treatment. The area of the BMAA peak was measured in every case. Each point represents an average of three runs assayed in duplicate. The response was linear up to 500 ng/mg, $R^2 = 0.987$). B) Validation parameters and results summary from case study of human postmortem brain. The limit of detection (LOD) and quantitation (LOQ) were validated in HCl extracts of brain protein and compared with buffer blanks to ensure specificity and sensitivity of the quantitation limits. Recovery was determined in control brain extracts spiked with BMAA prior to acid hydrolysis and compared with the same extract with BMAA added immediately before derivatization with AQC as described in the Materials and methods. % CV, Percent Coefficient of variation; %Δ, Percent deviation of the mean from target value.

BMAA levels in affected Chamorros on Guam were about fivefold higher (Table 2, right column; mean: 627 ± 141 μg/g) as compared with the results shown here for sporadic ALS and AD patients. In contrast, we failed to detect BMAA in the caudate of HD patients, with one testing positive with levels at the lower level of detection (Table 2). Low levels of BMAA were detected in two controls who had no evidence of age-related senile plaque counts (Table 2, left columns 5 and 6). A previous report of BMAA in a single asymptomatic Chamorro patient (11) is shown for comparison in Table 2 (right column).

Identity of the HPLC BMAA peak from brain tissue taken from an ALS patient was verified using triple quadrupole LC/MS/MS (Fig. 3). We applied tandem MS in the product ion mode to determine the amino acid with a high degree of sensitivity and selectivity, which results because the first mass filter only transmits a small ion population, and thus minimizes the overall chemical background (18). Designed to detect low molecular weight molecules in a complex physiological matrix, the application of triple quadrupole LC/MS/MS has four checks to verify the identity of the BMAA peak: (i) a specific single parent mass [molecular weight (MW): 459] is selected in the first quadrupole with all other masses excluded from the second quadrupole; (ii) the column retention time of the peak is unique; (iii) collision-induced dissociation of product ions in the second quadrupole must be all detected in the third quadrupole; (iv) the product ion ratios must match repetitive runs of the standard injected at a similar concentration within ±5% variation. In the analysis of a representative ALS frontal cortex sample, all four of these LC/MS/MS checks were verified with ratios of the product ions (MW: 289, 171, 119) of BMAA matching the daughter ion ratios of the derivatized standard (Fig. 3). This confirms and extends the HPLC results shown for the selected disease cohorts of subjects.

Discussion

We found that protein-bound BMAA is measurable in postmortem brain tissue from American patients who died with ALS or AD. We detected significant levels of BMAA in 49 out of 50 samples from 12 AD and 13 ALS patients, but in only 1 out of 16 samples from 8 HD patients and 2 out of 24 samples from 12 non-neurological disease controls (Table 2, left column). As cyanobacteria are widespread in freshwater, marine, and terrestrial ecosystems, this finding suggests that cyanobacterial neurotoxins may be an environmental factor for certain sporadic neurodegenerative diseases in vulnerable individuals. The negative screen for BMAA in HD, an autosomal-dominant, progressive neurodegenerative disorder suggests that BMAA is not an endogenous artifact of neurodegeneration.

The presence of both free and protein-bound BMAA in Chamorro patients who died from ALS/PDC supports the hypothesis that dietary exposure to biomagnified BMAA is linked to neurodegeneration (2, 19). Although similar ecological biomagnification has not been reported from North America, exposure to cyanobacterially contaminated water in genetically vulnerable individuals may result in the BMAA levels which

Table 2 Comparison of BMAA concentrations in frontal and temporal cortices from ALS, AD, HD, and non-neurological control subjects

Diagnosis	Nationality	Age at death (year)	Gender	BMAA (μg/g) frontal cortex	BMAA (μg/g) temporal cortex	BMAA (μg/g) caudate	BMAA (μg/g) spinal cord	Diagnosis	Nationality	Age at death (year)	BMAA (μg/g)
ALS1	USA	55	Male	91 ± 8	87 ± 11			ALS[1]	Chamorro	68	610 (SFG)
ALS2	USA	59	Male	185 ± 16	61 ± 9			PDC[1]	Chamorro	60	149 (SFG)
ALS3	USA	65	Male	158 ± 12	112 ± 8			PDC[1]	Chamorro	60	1190 (SFG)
ALS4	USA	65	Female	135 ± 21	205 ± 23			PDC[1]	Chamorro	67	433 (SFG)
ALS5	USA	66	Male	102 ± 7	63 ± 9			PDC[1]	Chamorro	69	644 (SFG)
ALS6	USA	67	Female	86 ± 5	77 ± 11		87 ± 3	PDC[1]	Chamorro	77	736 (SFG)
ALS7	USA	67	Female	118 ± 10	57 ± 8						
ALS8	USA	70	Male	225 ± 27	161 ± 12						
ALS9	USA	70	Male	256 ± 37	221 ± 26		47 ± 3				
ALS10	USA	74	Female	238 ± 10	187 ± 21						
ALS11	USA	77	Male	182 ± 11	189 ± 22		166 ± 11				
ALS12	USA	79	Male	89 ± 7	31 ± 8						
ALS13	USA	83	Male	115 ± 8	52 ± 7		197 ± 10				
AD1	USA	66	Male	ND	85 ± 10			AD[1]	Canadian	–	220 (SFG)
AD2	USA	67	Female	182 ± 15	140 + 9			AD[1]	Canadian	–	264 (SFG)
AD3	USA	67	Male	24 ± 3	18 ± 4			AD[2]	Canadian	91	46 (FC)
AD4	USA	76	Male	41 ± 7	10 ± 3			AD[2]	Canadian	75	26 (FC)
AD5	USA	80	Male	191 ± 6	157 ± 3			AD[2]	Canadian	86	41 (FC)
AD6	USA	81	Female	217 ± 14	180 ± 16			AD[2]	Canadian	82	53 (TC)
AD7	USA	81	Male	228 ± 10	98 ± 14			AD[2]	Canadian	84	171 (TC)
AD8	USA	81	Male	172 ± 13	158 ± 19			AD[2]	Canadian	79	34 (PG)
AD9	USA	84	Female	56 ± 6	40 ± 5			AD[2]	Canadian	67	ND
AD10	USA	87	Female	155 ± 21	114 ± 1						
AD11	USA	87	Male	188 ± 10	101 ± 8						
AD12	USA	90	Female	61 ± 8	46 ± 6						
HD1	USA	47	Male	ND		ND					
HD2	USA	54	Male	ND		ND					
HD3	USA	59	Male	ND		ND					
HD4	USA	62	Female	ND		11 ± 1					
HD5	USA	66	Female	ND		ND					
HD6	USA	69	Male	ND		ND					
HD7	USA	77	Male	ND		ND					
HD8	USA	85	Female	ND		ND					
Control1	USA	46	Male	ND	ND			Control[1]	Chamorro	41	82 (SFG)
Control2	USA	54	Male	ND	ND			Control[1]	Chamorro	61	ND
Control3	USA	67	Male	ND	ND			Control[1]	Canadian	39	ND
Control4	USA	68	Female	ND	ND			Control[1]	Canadian	62	ND
Control5	USA	68	Male	36 ± 6	ND			Control[1]	Canadian	69	ND
Control6	USA	70	Male	ND	ND			Control[1]	Canadian	80	ND
Control7	USA	78	Female	ND	ND			Control[1]	Canadian	60	ND
Control8	USA	81	Male	ND	ND			Control[1]	Canadian	86	ND
Control9	USA	83	Female	ND	ND			Control[1]	Canadian	89	ND
Control10	USA	85	Male	ND	ND			Control[1]	Canadian	76	ND
Control11	USA	85	Female	ND	45 ± 8			Control[1]	Canadian	89	ND
Control12	USA	87	Male	ND	ND			Control[1]	Canadian	71	ND
								Control[1]	Canadian	80	ND
								Control[1]	Canadian	87	ND
								Control[1]	Canadian	85	ND
								Control[2]	Canadian	–	ND

ALS, amyotrophic lateral sclerosis; AD, Alzheimer's disease; BMAA, β-*N*-methylamino-L-alanine HD, Huntington disease; PDC, Parkinsonian dementia complex; ND, non-detectable; SFG, superior frontal gyrus; TC, temporal cortex; FC, frontal cortex.

[1]*Acta Neurol Scand* 2004;110:267–9 and [2]*Proc Natl Acad Sci USA* 2004;101:12228–31.

Values represent the mean ± SD for two duplicate determinations

we detected in American ALS and AD patients. Recent analysis of 12 water supplies in the United Kingdom indicates that BMAA may be common in cyanobacterially contaminated waters, and may co-occur with other known cyanotoxins (20). BMAA is known to be excitotoxic at concentrations as low as 10–30 μM at glutamate receptors (21–23) and induces a neurological disorder in

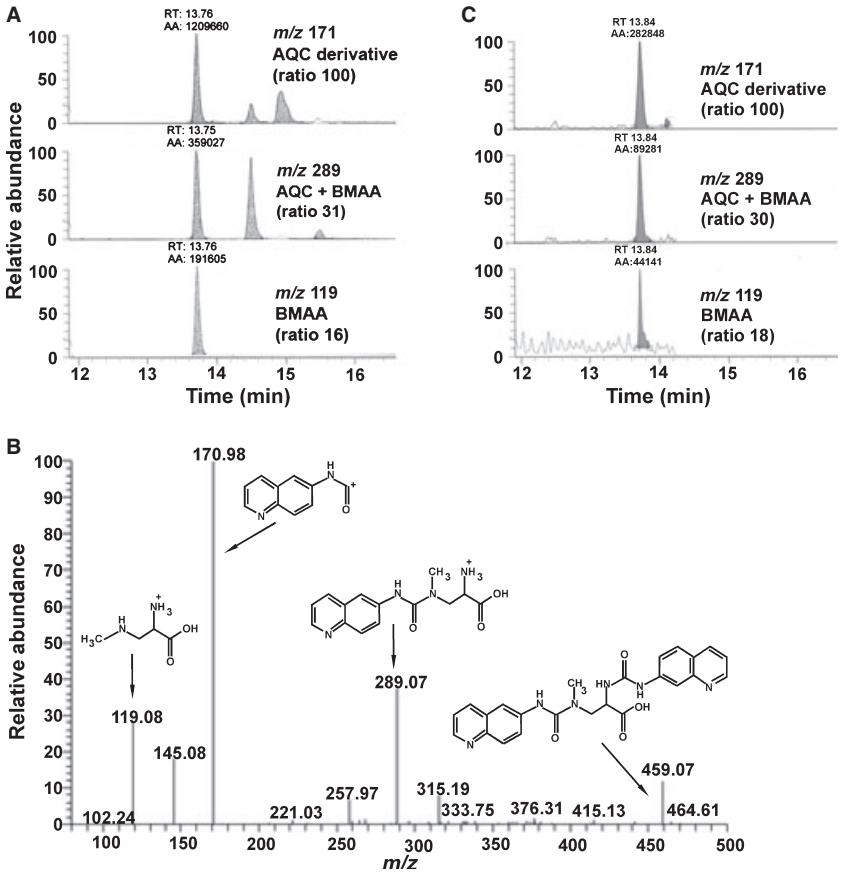


Figure 3. LC/MS/MS identification and verification of L-BMAA in postmortem brain matrix from an ALS patient. (A) Triple quadrupole LC/MS/MS verification of BMAA standard. Ion chromatograms of product ion from collision induced dissociations of *m/z* 459. The chromatography of the three major ions produced are: (1) protonated AQC derivative fragment (*m/z* 171), the quantitation ion, (2) protonated-BMAA AQC fragment (*m/z* 289), the first qualifier ion, and (3) protonated-BMAA fragment (*m/z* 119), the second qualifier ion; (B) Full product ion scan of an injection of AQC derivatized BMAA (217 fmole) in LC/MS/MS. Spectrum shows the dissociation of *m/z* 459 at 20 V. (C) Triple quadrupole LC/MS/MS verification of BMAA in a North American ALS patient. Ion chromatograms are the same as in panel A.

monkeys, which includes motor and extrapyramidal degeneration (6).

β-*N*-methylamino-L-alanine occurs as a free and protein-bound amino acid in cyanobacteria, cycads, animals that forage on cycads, and in the brain tissues of Chamorro ALS/PDC patients, Canadian AD patients, and as shown here in American ALS and AD but not in HD patients (5, 11, 24). Previous studies have demonstrated that BMAA can cross the blood-brain barrier following intravenous or oral administration (25). Several mechanisms of BMAA neurotoxicity have been proposed (22, 23). BMAA has been found to selectively kill motor neurons at concentrations as low as 30 μM by α-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor activation (22) and can potentiate injury to motor neurons from other neurotoxins at concentrations as low as 10 μM (23). The possible incorporation of BMAA into neuronal proteins may produce conformational changes, leading to increased protein aggregation. One hypothesis suggests that protein-bound BMAA may function as an endogenous neurotoxic reservoir (14). Within brain tissues, the slow release of free-BMAA may cause recurrent damage to motor neurons and other cell types over years or even decades. This hypothesis has been advanced to explain the observed long latency period for onset of ALS/PDC (11, 14). Cumulative exposures to BMAA may occur over the life span with normal aging, accounting for the occasional detection of BMAA in the brains of non-neurological controls.

The cycad hypothesis of BMAA as the cause of Guamanian ALS/PDC has been a source of dispute since its initial proposal (26–29), but it is now known that there are multiple inputs of BMAA into the Chamorro diet including the protein component of cycad flour, flying foxes, and possibly feral deer and pigs which forage on

cycad seeds (5, 19). The possibility of hazardous exposures of genetically vulnerable individuals to BMAA in regions other than Guam is supported by the results shown here. Assays of brain tissues from American patients with ALS and AD demonstrate concentrations of BMAA that are considerably higher than in control brains, although lower than those in autopsy cases from Guam.

An important goal of this study was to provide independent replication of the presence of BMAA in the brains of patients with specific neurodegenerative illnesses using an independent cohort of patients based on two different assay methods. The results from postmortem brain tissue validate and extend previous findings of BMAA in Canadian AD patients and provide the first evidence for BMAA exposures in a group of sporadic ALS patients. Taken together, these observations demonstrate that detection of BMAA in human brain tissue samples in Western populations is a reproducible finding. Montine et al. (28) failed to detect BMAA in brain homogenates from controls, AD patients, or Chamorros with PDC, but their method was based on an older fluorenyl methylchloroformate technique that lacks sensitivity. In addition, these investigators did not measure protein-bound BMAA in brain extracts nor did they provide sufficient details of analytical procedure or extraction to allow independent replication of a negative finding (30).

In this study, BMAA was quantified in protein-bound fractions from brain tissue using the validated fluorescence HPLC method as previously described (2, 11). HPLC peaks were confirmed by triple quadrupole LC/MS/MS. It has been estimated that there is a 60- to 130-fold greater quantity of BMAA in the protein-bound fraction than is recovered from the free amino acid pool (14). BMAA levels in human postmortem brain are approximately two orders of magnitude below the concentration range of glutamate in the total protein fraction (31). This range would suggest a potential rate of misincorporation of $<1\%$ if BMAA substituted for glutamate in brain (10–30 mg/100 g fresh brain).

The results shown here for American AD and ALS patients from the Atlantic southeast compared with Canadian AD patients from the Pacific northwest suggest that exposure to BMAA may be widespread. Cyanobacterially contaminated water supplies and/or entry into the marine food web are potential sources of human BMAA exposures in North America. This finding taken together with the recent report of BMAA in cyanobacterially contaminated water supplies in the United Kingdom (20) suggests that replication in independent cohorts of ALS and AD cases from Europe or elsewhere may be a timely research question. Marine and freshwater cyanobacterial blooms and the entry of BMAA into these ecosystems and food webs which may contribute to sporadic neurodegenerative diseases occurring outside of Guam have important implications for global health that warrant further study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. HPLC method validation and separation of β-*N*-methylamino-L-alanine (BMAA) in acid-hydrolyzed postmortem human brain matrix. A) Full-range (24–35 min) chromatograms of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatized, acid-hydrolyzed non-neurological human brain tissue. Identifiable peaks of AQC-derivatized L-valine (26.8 min) and L-methionine (28.1 min) are evident with and without spiked BMAA. Dashed area is shown expanded below. B) Identification of BMAA (retention time = 29.7 min) across experimental condition. (A, purple) Synthetic BMAA standard alone (no brain tissue), (B, green) control human brain tissue spiked with BMAA prior to acid hydrolysis, and (C, pink) control hydrolyzed human brain tissue spiked with BMAA prior to derivatization. Note the absence of BMAA in (trace D, orange) control human brain tissue without added BMAA and E (blue) AQC blank (no tissue extract). The standard and co-elution amino acid peaks were obtained in 60 min according to the method of Murch et al. (14) [*Proc Natl Acad Sci USA* 2004;101(33):12228–31].

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