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DETECTION OF BMAA IN THE HUMAN CENTRAL NERVOUS SYSTEM

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Abstract—Amyotrophic lateral sclerosis (ALS) is an extremely devastating neurodegenerative disease with an obscure etiology. The amino acid β-*N*-methyl-L-alanine (BMAA) produced by globally widespread phytoplankton has been implicated in the etiology of human motor neuron diseases. BMAA was recently proven to be present in Baltic Sea food webs, ranging from plankton to larger Baltic Sea organisms, some serving as important food items (fish) for humans. To test whether exposure to BMAA in a Baltic Sea setting is reflected in humans, blood and cerebrospinal fluid (CSF) from individuals suffering from ALS were analyzed, together with sex- and age-matched individuals not inflicted with ALS. Ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) and multiple reaction monitoring (MRM), in conjunction with diagnostic transitions revealed BMAA in three (12%) of the totally 25 Swedish individuals tested, with no preference for those suffering from ALS. The three BMAA-positive samples were all retrieved from the CSF, while BMAA was not detected in the blood. The data show that BMAA, potentially originating from Baltic Sea phytoplankton, may reach the human central nervous system, but does not lend support to the notion that BMAA is resident specifically in ALS-patients. However, while dietary exposure to BMAA may be intermittent and, if so, difficult to detect, our data provide the first demonstration of BMAA in the central nervous system of human individuals *ante mortem* quantified with UHPLC–MS/MS, and therefore calls for extended research efforts.

Key words: neurotoxin, BMAA, neurodegeneration, ALS, CSF, blood.

INTRODUCTION

The environmental non-protein amino acid β-*N*-methyl-L-alanine (BMAA) was initially reported in tropical cycad plants (Vega and Bell, 1967, see also Marler et al., 2010; Snyder and Marler, 2011). BMAA has gained increased attention in recent years as it was found to be produced by prokaryotic cyanobacteria, including cyanobacteria living in symbiosis with cycads (Cox et al., 2003, 2005; Bergman et al., 2008), as well as globally widespread free-living cyanobacteria (Cox et al., 2005; Esterhuizen and Downing, 2008; Metcalf et al., 2008; Faassen et al., 2009; Jiao et al., 2014). The known producers of BMAA have recently expanded to include also photoautotrophic eukaryotes such as diatoms (Jiang et al., 2014a) and dinoflagellates (Lage et al., 2014). BMAA producers thus include numerous phytoplanktonic organisms that constitute fundamental primary producers at the base of elaborate aquatic food webs.

Despite the widespread occurrence of these photosynthetic microorganisms in nature, the cellular and ecological role of BMAA in these organisms remains enigmatic. The neurotoxic effects and mechanisms of action of BMAA have been extensively studied in eukaryotes (*in vitro* and *in vivo*) using both animal test systems (reviewed in Karamyan and Speth, 2008; Chiu et al., 2011) and various human cell lines (Chiu et al., 2012; Lee and McGeer, 2012; Dunlop et al., 2013; Muñoz-Saez et al., 2013; Okle et al., 2013). There is considerable evidence for BMAA being a crucial factor in the pathophysiology of the neurodegenerative disease amyotrophic lateral sclerosis Parkinsonism dementia complex (ALS-PDC). This disease was first observed among the native Chamorro population of Guam (in Western Pacific Ocean) and claimed to be caused by ingestion of BMAA containing food such as cycad flour and flying foxes, the latter feeding on cycad seeds (see e.g., Spencer et al., 1987; Banack et al., 2006). ALS-PDC has many clinical and neuropathological features in common with the neurodegenerative diseases amyotrophic lateral sclerosis (ALS), Parkinson's and Alzheimer's (AD), which are common in all parts of the world. Consequently, BMAA is now thought to play a possible role also in the otherwise unclear etiology of these diseases. Perhaps the most notable observation that suggests a link between BMAA and human neurodegeneration is the claimed consistent presence of BMAA in the central nervous system (CNS) of patients deceased from ALS-PDC (Guam), and from those with AD and ALS (USA and Canada), while BMAA is not detected in controls or in patients suffering from Huntington's disease, a disorder

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Abbreviations: AD, Alzheimer's disease; AEG, N-(2-aminoethyl) glycine; ALS, amyotrophic lateral sclerosis; ALS-PDC, amyotrophic lateral sclerosis Parkinsonism dementia complex; AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; BMAA, β-*N*-methyl-L-alanine; CNS, central nervous system; CSF, cerebrospinal fluid; D_3, deuterated; DAB, 2-4-diaminobutyric acid hydrochloride; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; *S*/*N*, signal-to-noise ratio; UHPLC–MS/MS, ultra high performance liquid chromatography–tandem mass spectrometry.

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with a known direct genetic cause (Cox et al., 2003; Murch et al., 2004a,b; Pablo et al., 2009). However, as other studies analyzing similar human CNS based sample sets have failed to detect BMAA in either patients or matched controls (Montine et al., 2005; Kushnir and Bergquist, 2009; Snyder et al., 2009a,b, 2010; Combes et al., 2014), the occurrence of BMAA in the human CNS is still under debate as well as the observed presence of BMAA in patients with neurodegenerative disorders.

The first evidence of the presence of BMAA at different levels in aquatic food webs was found in the brackish Baltic Sea (Jonasson et al., 2010). Hence, surrounding inhabitants (today ~180 million people in nine countries) may be exposed to BMAA-amended staple food items derived from this body of water, such as shellfish and fish. The increase in the already massive summer blooms of cyanobacteria (Kahru and Elmgren, 2014) through eutrophication suggests enhanced BMAA production and human exposure. Similar BMAA transfer routes have been confirmed by studies in other geographic regions and include both marine and limnic ecosystems (USA and China) (Brand et al., 2010; Christensen et al., 2012; Mondo et al., 2012; Jiao et al., 2014; Al-Sammak et al., 2014). Hence, BMAA in the environment may represent a public health concern should the connection between environmental BMAA and neurodegeneration be proven.

To test whether exposure to BMAA in a Baltic Sea setting is reflected in humans, blood and cerebrospinal fluid (CSF) from individuals suffering from ALS, together with sex- and age-matched individuals not inflicted with ALS (referred to as non-ALS individuals), were analyzed for BMAA using ultra high performance liquid chromatography-mass spectrometry (UHPLC-MS/MS).

EXPERIMENTAL PROCEDURES

Chemicals

BMAA (L-BMAA hydrochloride B-107) was purchased from Sigma–Aldrich (Steinheim, Germany), L-2-4-diaminobutyric acid hydrochloride (DAB) was from Fluka (#32830; Buchs, Switzerland) and N-(2-aminoethyl) glycine (AEG) from Tokyo Chemical Industry (A1153; Tokyo, Japan). Deuterated (D_3) BMAA was synthesized and kindly provided by Dr. K. Bogar (Department of Organic Chemistry, Stockholm University) and stored as stock solution at −20 °C (760 ng/ml). All reagents used were of high/highest analytical grade.

Material and sample treatment

Cerebrospinal fluid (CSF) was obtained from 12 (Swedish) ALS-patients as well as 13 age- and gender-matched non-ALS individuals (controls). Whole blood and plasma were collected from 10 of the ALS-patients and seven of the controls. CSF and blood were sampled within the same day from the individual patients and controls. ALS-patients were diagnosed according to the revised El Escorial criteria (Brooks et al., 2000) at the Department of Neurology, (Karolinska University Hospital, Sweden). Ethical permission for the study was obtained from the Stockholm County regional ethics committee. Samples, including CSF, whole blood and plasma, were blinded and the identity was not revealed until the material was fully analyzed and BMAA-negative and -positive samples identified. Samples were stored at −20 °C and the majority was analyzed within 2 years after collection (a few within 5–7 years). The extraction procedure for CSF, whole blood and plasma was adapted from Jiang et al. (2014a) and adjusted to the requirements of each different material in the study as given below.

CSF. A volume of 3-ml CSF was sonicated for 2 min at 70% efficiency using a Sonopuls, Model HD 2070 (Bandelin Electronic, Berlin, Germany) and the material was kept cool in an ice/methanol bath to prevent protein degradation. The cell solution was centrifuged at 4100×*g* for 30 min at 4 °C. The supernatant was transferred into a new tube with twice the supernatant volume of cold acetone added. The sample was precipitated at −20 °C overnight and centrifuged at 4100×*g* for 30 min at −4 °C. Even though acetone is a generally efficient agent for protein precipitation (Sedgwick et al., 1991), small soluble peptides and protein fragments may escape precipitation. Therefore, the free fraction was hydrolyzed just as the protein fraction. For hydrolyzation 600-µl 6 M HCl was added to both the protein pellet and the (dried) supernatant, and the samples were hydrolyzed overnight (22 h) at 110 °C. The hydrolyzed samples were lyophilized (CoolSafe, SCANVAC, Lynge, Denmark) before resuspension in 100-µl borate buffer (Waters AccQ-Tag Ultra kit, Waters, Milford, MA, USA) and 20-µl derivatization agent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Waters AccQ-Tag Ultra kit, Waters, Milford, MA, USA) was added. Due to scarce material, only one replicate of each individual was analyzed.

Whole blood and plasma. Aliquots of 100-µl whole blood/plasma mixed with 400-µl dH_2O (HPLC-grade) were prepared in triplicate. The total dry weight of 100 µl was approximately 23.3 mg ± 0.6 for whole blood and 9.3 mg ± 0.1 for plasma, and corresponded to an average protein pellet dry weight of approximately 18.2 mg ± 1.6 for whole blood and 7.3 mg ± 0.5 for plasma. Lysis of the red blood cells was achieved by freeze–thawing in liquid nitrogen (×1), however, no lysis procedure was performed for plasma. Cell debris was removed by centrifugating (2570×*g*) for 30 min at 4 °C. The supernatant was transferred to a new microtube with 1000 µl (1:2) acetone added to precipitate proteins at −20 °C overnight. Next, the sample was centrifuged at 20,160×*g*, −4 °C for 30 min in order to separate free amino acids from precipitated proteins. Before further procedures, both supernatant and the protein pellet were dried in a speedvac (miVac centrifugal vacuum concentrator, Genevac, Ipswich, UK) and in a freeze dryer, respectively. The dry weight of the protein pellet was then recorded. As with the CSF, both the dried supernatant and the protein pellet were hydrolyzed (500 µl 6 M HCl; 20 h; 110 °C) and freeze-dried overnight. This was followed by reconstitution in 500 µl

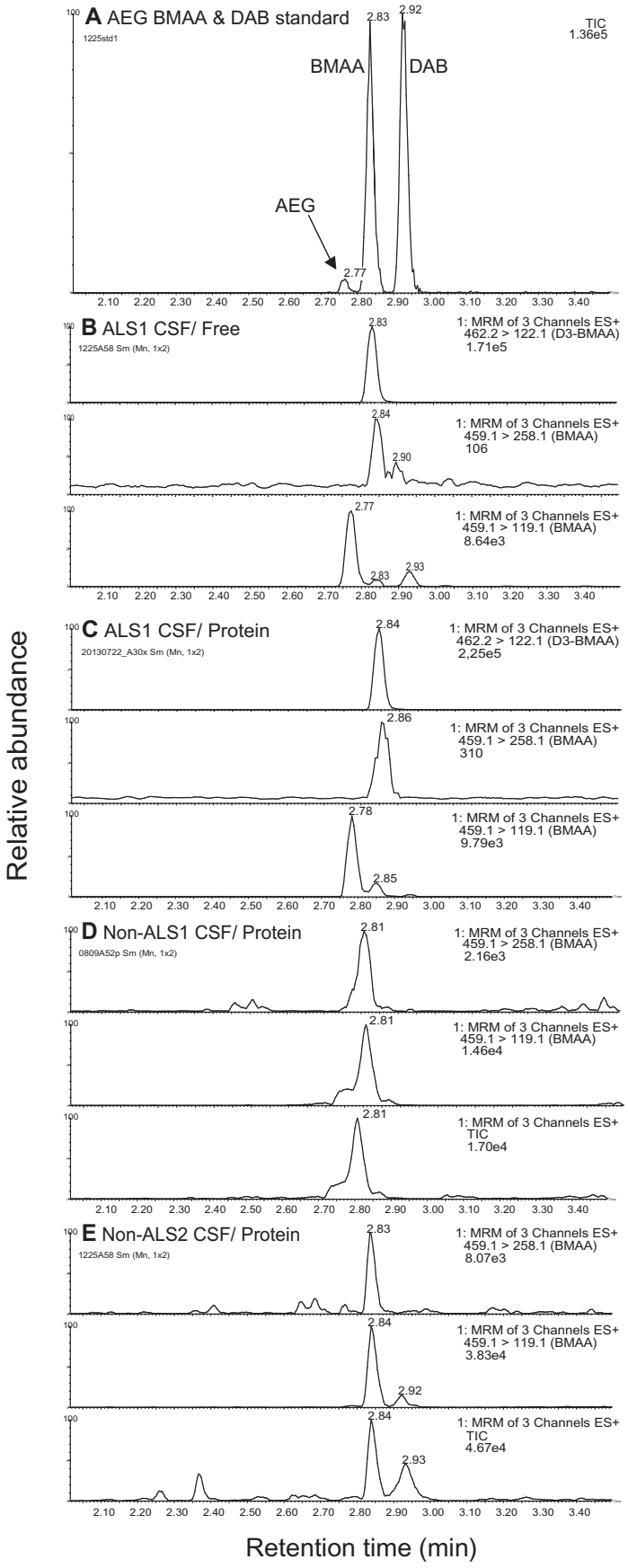
20% methanol. Particulate matter was removed by spin-filtration (Ultrafree centrifugal filters, 0.22 μm, Merck Millipore, Cork, Ireland or nanosep MF 0.2 μm, Pall Life Sciences, Dreieich, Germany). The clear solution was transferred to a microtube and lyophilized. The derivatization procedure for the free hydrolyzed fraction was the same as for CSF. The hydrolyzed protein fraction, on the other hand, went through sequential dilutions before derivatization. The dried sample was reconstituted in 15–20-μl 20 mM HCl to a concentration corresponding to approximately 0.4-mg hydrolyzed protein/μl for whole blood, and 0.28 mg/μl for plasma. All samples were diluted in two steps to reach a final concentration of 0.025-mg hydrolyzed protein/μl. From this solution, 20 μl was used for the derivatization reaction, equaling 0.5-mg hydrolyzed protein. When only diluting the samples in borate buffer (Waters AccQ-Tag Ultra kit, Waters, Milford, MA, USA), the pH of the final derivatization solution of a majority of the samples was below the pH (8.2–9.7) recommended for an optimal derivatization reaction (Cohen and Michaud, 1993). Therefore, pH was raised in all samples during dilution. The two-step dilution procedure was initiated by dilution in borate buffer and 2 M NaOH, yielding approximately 0.04-mg hydrolyzed protein/μl. NaOH was added in a volume corresponding to approximately 8.3 and 7.2 μmol NaOH/mg hydrolyzed protein in whole blood and plasma, respectively. In the second dilution step the samples were diluted in borate buffer to a concentration of 0.025-mg hydrolyzed protein/μl, and the final solution used for the derivatization reaction. The addition of NaOH during dilution resulted in a pH increase of 0.5–1 units, compared to dilutions made in borate buffer only. Accordingly, the pH was 8–9 (measured with pH-indicator strips due to small sample volumes, pH 0–14, MColorpHast™, Merck, Darnstadt, Germany) prior to derivatization. For derivatization, 20 μl of NaOH/borate-containing 0.025 mg/μl-solution (corresponding to a total amount of 0.5-mg hydrolyzed protein) was added to 80-μl borate buffer and 20-μl AQC.

UHPLC and mass spectrometry

BMAA analyses were performed using a Xevo triple quadrupole mass spectrometer connected to an Acquity ultra performance liquid chromatography (UPLC) instrument (Waters, Milford, MA, USA). Separation was performed on an Acquity UPLC®BEH C18 (1.7-μm particle size, 2.1 × 100-mm column; Waters, Milford, MA, USA) at 55 °C and a gradient elution (solvent A, 0.01% formic acid, 0.05% ammonia in water, solvent B, MeOH with 0.01% formic acid), delivered at a flow rate of 600 μl/min. The gradient was as follows 0.0 min, 0.1% B; 0.54 min, 0.1% B (curve 6); 4.00 min, 55.0% B (curve 5); 4.10 min, 100% B (curve 7); 5.10 min, 100% B (curve 6); 5.20 min, 0.1% B (curve 6) and 6.20 min, 0.1% B (curve 6). Ion source parameters were: capillary voltage 4 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 550 °C, desolvation gas flow 1000 L/h and collision gas flow 0.15 ml/min. To minimize contamination of the interface the flow was diverted to waste between 0 min and 1.2 min, data were recorded from 1.2 min to 3.5 min, and thereafter the flow was again diverted to waste. Electrospray ionization (ESI) was employed and the mass spectrometer was set to positive ion mode using multiple reaction monitoring (MRM) of five different transitions. The transitions used were (459.18 > 119.1) which is common for AEG, BMAA and DAB; (459.18 > 258.1) which is the specific fragment for BMAA; (459.18 > 188.1) specific for DAB (Spáčil et al., 2010), (459.18 > 214.1) specific for AEG (Banack et al., 2011) and (462.2 > 122.1) specific for D_3-BMAA (Fig. 1). Collision energy was 26 V for all transitions. Standard solutions containing BMAA, DAB and AEG were injected repeatedly during analysis of each sample set to ensure separation, and to monitor the retention time of each compound throughout the analysis. Injection volume was 10 μl for CSF and 20 μl for whole blood and plasma samples. To ensure that no carry-over between injections occurred and to improve column life-length, approximately every seventh sample was followed by a column wash, an equilibration step and the injection of a blank sample. Smoothing was performed on all mass spectra using the MassLynx v4.1 (Waters, Milford, MA, USA) built-in mean algorithm, with either 1 × 2 or 2 × 2 for CSF samples and whole blood/plasma samples respectively.

Detection criteria for BMAA. The minimum criteria for acceptance of a peak were signal-to-noise ratio (S/N) $\geqslant$ 4 and a minimum peak width of 2 s. To ensure correct assignment of BMAA, retention time as well as both the qualifier (459.18 > 258.1) and quantifier fragment (459.18 > 119.1) were considered. Instrument limit of detection (LOD) for BMAA was determined as the lowest concentration with peaks of [119.1] m/z and [258.1] m/z with (S/N $\geqslant$ 4), at the correct retention time (±5% compared to a BMAA standard in the same sample set), and was 3.6 fmol or 0.42 pg on-column. The criteria for determining LOD in sample matrices were the same as for instrument LOD, but with the additional requirement that the ratio (119.1/258.1) was correct within ±20% (relative standard deviation; RSD) compared to the mean ratio (5.4), calculated from BMAA in borate buffer (see standard curve Fig. A.1A (Appendix)). The limit of quantification (LOQ) in sample matrix was set at the concentration where (in addition to fulfilling the criteria for LOD) the area of [119.1] m/z and [258.1] m/z and their ratio did not vary more than ±20% (RSD) in between the replicates. When using less than three replicates for the standard curve (as for the CSF matrix where $n = 2$) the criteria for LOQ in matrix, as stated above, were not applicable. Instead, S/N $\geqslant$ 30 were required for both the [119.1] m/z and [258.1] m/z fragments, as this normally resulted in replicates keeping within %RSD ± 20 of the 119/258 ratio.

Calibration curve CSF. A standard curve with five BMAA concentration levels ($n = 2$ for each concentration) was constructed using a human CSF sample previously determined as negative for BMAA (Fig. A.1B (Appendix)). LOD and LOQ in the hydrolyzed protein fraction was determined to 0.12 pmol (14.3 pg)


A AEG BMAA & DAB standard
1225std1
TIC
1.36e5
BMAA
DAB
AEG
2.83
2.92
2.77
B ALS1 CSF/ Free
1225A58 Sm (Mn, 1x2)
1: MRM of 3 Channels ES+
462.2 > 122.1 (D3-BMAA)
1.71e5
1: MRM of 3 Channels ES+
459.1 > 258.1 (BMAA)
106
1: MRM of 3 Channels ES+
459.1 > 119.1 (BMAA)
8.64e3
C ALS1 CSF/ Protein
20130722_A30x Sm (Mn, 1x2)
1: MRM of 3 Channels ES+
462.2 > 122.1 (D3-BMAA)
2,25e5
1: MRM of 3 Channels ES+
459.1 > 258.1 (BMAA)
310
1: MRM of 3 Channels ES+
459.1 > 119.1 (BMAA)
9.79e3
D Non-ALS1 CSF/ Protein
0809A52p Sm (Mn, 1x2)
1: MRM of 3 Channels ES+
459.1 > 258.1 (BMAA)
2.16e3
1: MRM of 3 Channels ES+
459.1 > 119.1 (BMAA)
1.46e4
1: MRM of 3 Channels ES+
TIC
1.70e4
E Non-ALS2 CSF/ Protein
1225A58 Sm (Mn, 1x2)
1: MRM of 3 Channels ES+
459.1 > 258.1 (BMAA)
8.07e3
1: MRM of 3 Channels ES+
459.1 > 119.1 (BMAA)
3.83e4
1: MRM of 3 Channels ES+
TIC
4.67e4
2.10 2.20 2.30 2.40 2.50 2.60 2.70 2.80 2.90 3.00 3.10 3.20 3.30 3.40
Relative abundance
Retention time (min)

BMAA on-column (equals 57 pg BMAA/ml CSF) and the standard curve was linear up to 1.7 pmol (197 pg) and $r^2 = 0.96$ (Table A.1; Fig. A.1B (Appendix)).

Calibration curve whole blood. A standard curve with six BMAA concentrations ($n = 5$) was made from the whole-blood protein fraction of a sample previously determined as negative for BMAA (Fig. A.1C (Appendix)). Also, a standard curve in the free hydrolyzed fraction of the same sample was prepared with six concentrations of BMAA ($n = 4$). The LOD was 0.75 pmol (89 pg) BMAA on-column (equals 171 ng BMAA/ml whole blood) for the protein fraction and 7.5 pmol (892 pg) BMAA on-column (equals 53 ng BMAA/ml whole blood) for the free hydrolyzed fraction. LOQ was determined to 7.5 pmol (892 pg) BMAA on-column (equals 1.7 µg BMAA/ml whole blood) for the protein fraction and 18.9 pmol (2.2 ng) BMAA on-column (equals 134 ng BMAA/ml whole blood) for the free hydrolyzed fraction (Table A.1 (Appendix)). The linear range was between 0.75 and 43 pmol on-column ($r^2 = 0.99$) for the protein fraction. For the free hydrolyzed fraction only two concentrations of the standard curve reached the criteria for BMAA detection, hence no curve was established. The calibration curves in whole blood were also applied for plasma samples.

Quantification method in CSF. For quantification of BMAA in human samples, 43-pmol D_3-BMAA was added as internal standard to each sample prior to the acid hydrolysis and recovery was determined and used to compensate for sample loss during work-up. As D_3-BMAA is highly soluble and will follow the free fraction during the sample work-up it was added to the samples prior to acid hydrolysis, and can only account for losses during and after hydrolysis.

RESULTS

UHPLC–MS/MS was used to analyze the presence of the neurotoxin BMAA in human samples from Sweden (Fig. 1). Out of the 25 individuals tested, BMAA was detected in three (12%), while not in the remaining 22 individuals (88%) (Table 1). Notably, only one BMAA-positive sample derived from an ALS-patient (ALS1), while the other two were from non-ALS individuals (non-ALS1 and non-ALS2; controls).

Table 1. BMAA (β-*N*-methyl-L-alanine) in cerebrospinal fluid (CSF) in Swedish ALS patients and non-ALS individuals. The ALS patients and non-ALS individuals were age- and gender-matched. The concentrations given represent pg BMAA per ml CSF in hydrolyzed free or protein fraction.

Sample	Age (year)	Gender	CSF pg/ml ($n = 1$)	
			Free fraction	Protein fraction
ALS-patients				
ALS1	68	M	78.0	96.6
ALS2	63	F	ND	ND
ALS3	78	F	ND	ND
ALS4	58	M	ND	ND
ALS5	46	M	ND	ND
ALS6	79	F	ND	ND
ALS7	69	M	ND	ND
ALS8	85	M	ND	ND
ALS9	66	F	ND	ND
ALS10	71	M	ND	ND
ALS11	61	M	ND	ND
ALS12	59	M	ND	ND
Non-ALS individuals				
non-ALS1	31	M	ND	234.5*
non-ALS2	42	M	ND	540.9*
non-ALS3	76	M	ND	ND
non-ALS4	65	F	ND	ND
non-ALS5	32	M	ND	ND
non-ALS6	22	M	ND	ND
non-ALS7	68	F	ND	ND
non-ALS8	35	F	ND	ND
non-ALS9	69	M	ND	ND
non-ALS10	81	M	ND	ND
non-ALS11	60	M	ND	ND
non-ALS12	71	M	ND	ND
non-ALS13	71	M	ND	ND

ND: not detected (concentration < LOD/LOQ); LOD: limit of detection; LOQ: limit of quantification.

* Quantification was made without correction for internal standard (D_3-BMAA), see Result section.

The three BMAA-positive samples were retrieved from the human CSF, with the highest concentration found in one control/non-ALS individual (non-ALS2) (Table 1). BMAA was detected in the hydrolyzed protein fraction of all three individuals, but also in the free hydrolyzed fraction of the ALS-patient (ALS1) (Table 1; Fig. 1). By using D_3-BMAA as an internal standard, BMAA was confidently quantified in the CSF of the ALS patient (ALS1; Table 1). The hydrolyzed free and protein

Fig. 1. UHPLC–MS/MS chromatograms illustrating the presence of BMAA (β-*N*-methyl-L-alanine) in human cerebrospinal fluid. Chromatograms showing the multiple reaction monitoring (MRM) transitions and total ion count (TIC) for the BMAA-positive samples found. The y-axis represents relative abundance and the x-axis is retention time in minutes. (A) Elution patterns for standard solutions of the isomers AEG (N-(*2*-aminoethyl) glycine), BMAA (β-*N*-methyl-L-alanine) and DAB (L-2-4-diaminobutyric acid). (B) BMAA in the free fraction of cerebrospinal fluid (CSF) from a Swedish ALS patient (ALS1) showing the MRM transitions 459.1 > 258.1 (middle panel, Rt 2.84 min) and 459.1 > 119.1 (bottom panel Rt 2.83 min), as well as the transition for deuterated BMAA (D_3-BMAA) at 462.2 > 122.1 (top panel, Rt 2.83 min) used as internal standard. The MRM transition 459.1 > 119.1 for AEG is apparent at retention time 2.77 min and DAB at 2.93 min (bottom panel). (C) BMAA in the CSF protein fraction of a Swedish ALS patient (ALS1) showing the MRM transitions 459.1 > 258.1 (middle panel, Rt 2.86 min) and 459.1 > 119.1 (bottom panel Rt 2.85 min), as well as the transition for deuterated BMAA (D_3-BMAA) at 462.2 > 122.1 (top panel, Rt 2.84 min) used as internal standard. The MRM transition 459.1 > 119.1 for AEG is apparent at retention time 2.78 min (bottom panel). (D) BMAA in the CSF protein fraction of a Swedish individual not diagnosed with ALS (non-ALS1) showing the MRM transitions 459.1 > 258.1 (top panel, Rt 2.81 min) and 459.1 > 119.1 (middle panel, Rt 2.81 min) as well as the TIC (bottom panel). (E) BMAA in the CSF protein fraction of a Swedish individual not diagnosed with ALS (non-ALS2) showing the MRM transitions 459.1 > 258.1 (top panel, Rt 2.83 min) and 459.1 > 119.1 (middle panel, Rt 2.84 min), as well as the TIC (bottom panel). The MRM transition 459.1 > 119.1 for DAB is apparent at retention time 2.92 min (middle panel) and in the TIC (bottom panel, Rt 2.93 min).

Table 2. Summary of BMAA (β-*N*-methyl-L-alanine) analyses performed in human material. Given are the number and types of samples analyzed, analytical method, LOD/LOQ in sample matrix and BMAA results. Data set No.1–5 refers to examinations of *post mortem* stored brains; data set No. 6–7 refers to *ante mortem* cerebrospinal fluid **(**CSF) and blood.

Data set No.	Human samples and their geographical origin	Tissue/fluid	Fraction analyzed	Method of analysis	LOD/LOQ (in sample matrix)	Number of BMAA positive samples/total samples	BMAA concentrations	Reference
1	*Guam* a. 6 ALS-PDC b. 2 ctrl *Canada* c. 2 AD d. 13 ctrl	Superior frontal gyrus	(α) Free (β) Free and protein	(α, β) HPLC-FLD* MS verification[1] AQC-derivatization	(α) Not given (β) Not clearly stated[2]	(α) a. Not given[3] b. No results given c. Not given[3] d. 0/13 (β) a. 6/6 b. 1/2 c. 2/2 d. 0/13	(α) a.6 μg/g (average) b. Not given c. 6.6 μg/g (average) d. – (β) a. 3.3–1190 μg/g b. 48–82 μg/g c. 3.4 and 264 μg/g d. –	Cox et al. (2003) (α) Murch et al. (2004a) [4] (β)
2	*Canada* a. 7 AD b. 1 ctrl	Frontal cortex, temporal cortex, parahippocampal gyrus, caudate, cerebellum	Protein	HPLC-FLD* MS verification[1] AQC-derivatization	Not given	a. 6/7 b. 0/1	a. 25.9–235.6 μg/g b. –	Murch et al. (2004b)[5]
3	*Guam* a. 8 PDC b. 2 ctrl *USA* c. 5 AD d. 5 ctrl	(γ) Frontal cortex, temporal cortex, cerebellum, middle frontal gyrus (δ, ε) Frontal cortex, temporal cortex (ζ) Frontal cortex, temporal cortex,	(γ) Free (δ, ε) Free and protein (ζ) Free	(γ) HPLC-FLD* FMOC-derivatization (δ, ε) GC–MS*, IS, ECF-derivatization* (ζ) GC × GC-TOFMS*, TMS-derivatization	(γ) 1 pmol/ not given (δ, ε) Free fraction: 0.1/0.2 μg/g, protein fraction: 5/10 μg/g (ζ) 0.7 ppb/1 ppb (4.5 ng/g)	(γ,δ,ε,ζ) 0/20	(γ,δ,ε,ζ) –	Montine et al. (2005) (γ) Snyder et al. (2009a)[6] (δ) Snyder et al. (2009b) (ε) Snyder et al. (2010) (ζ)
4	*USA* a. 13 ALS b. 8 HD c 12 AD d. 12 ctrl	Frontal cortex, temporal cortex, caudate, spinal cord	Protein	HPLC-FLD* MS/MS verification[1] AQC-derivatization	2/10 ng on-column	a. 13/13 b. 1/8 c. 12/12 d. 2/12	a. 31–256 μg/g b. 11 μg/g c. 10–228 μg/g d. 39 and 45 μg/g	Pablo et al. (2009)
5	*France* 2 ALS	Frontal lobe, hippocampus	Free	LC–MS/MS* underivatized	Not clearly stated/20 ng/g	0/2	–	Combes et al. (2014)

6	*Sweden* a. 12 ALS b. 10 ctrl	CSF, serum, plasma	Free	LC–MS/MS*, IS, iTRAQ-derivatization	Not given/0.3 µg/ml	a. 0/12 b. 0/10	–	Kushnir and Bergquist (2009)
7	*Sweden* a. 12 ALS b. 13 ctrl	CSF, whole blood, plasma	Free[7] and protein	UHPLC–MS/MS*, IS[8], AQC-derivatization	57/57 pg/ml (CSF protein fraction), 0.17/1.7 µg/ml (whole-blood protein fraction), 0.05/ 0.13 µg/ml (whole-blood free)	a. 1/12 b. 2/13	a. 78–97 pg/ml b. 235–541 pg/ml[9]	Present study

LOD: limit of detection; LOQ: limit of quantification; ALS-PDC: amyotrophic lateral sclerosis Parkinsonism dementia complex; ctrl: control; AD: Alzheimer's disease; HPLC-FLD: high performance liquid chromatography-fluorescence detection; MS: mass spectrometry; AQC: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; FMOC: 9-fluorenylmethylchloroformate; GC–MS: gas chromatography–mass spectrometry; IS: internal standard; ECF: ethyl chloroformate; GC × GC TOFMS: two-dimensional gas chromatography coupled to time-of-flight mass spectrometry; TMS: trimethylsilation reagent; ALS: amyotrophic lateral sclerosis; HD: Huntington's disease; LC–MS/MS: liquid chromatography–tandem mass spectrometry; iTRAQ: isobaric tag for relative and absolute quantification; UHPLC: ultra high performance liquid chromatography.

* BMAA quantifications were based on this method.

1 Number of verified samples not clearly stated.

2 Reports LOD/LOQ = 0.0001/0.013 µmol, matrix/buffer not given.

3 Only mean value of the BMAA concentrations given.

4 Reports methods and result details of the free BMAA fraction of Cox et al. (2003).

5 Includes results of Cox et al. (2003) and Murch et al. (2004a) and a discussion thereof.

6 Short notes on results of Snyder et al. (2009b).

7 The free fraction was hydrolyzed.

8 IS not used for all samples.

9 Concentrations not corrected with IS.

fractions of patient ALS1 contained 78.0 and 96.6 pg BMAA/ml CSF, respectively. The BMAA concentrations in the hydrolyzed protein fractions of the non-ALS1 and non-ALS2 samples were above the LOQ. However, these were analyzed prior to the availability of D_3-BMAA to our research group and could therefore not be quantified as accurately as sample ALS1. However, by assuming 100% recovery to avoid overestimations, the minimum BMAA concentrations were calculated to be 234.5 and 540.9 pg BMAA/ml CFS in the protein fraction of sample non-ALS1 and non-ALS2, respectively. Hence, the BMAA concentrations in the CSF protein fractions of these control individuals were approximately 2–6 times higher than that found in the CSF protein fraction of the ALS1 patient (Fig. 1; Table 1). The fact that BMAA was not present in 88% of the human CSF samples suggests that these were devoid of BMAA, or that the BMAA concentrations were below the LOD/LOQ of the analytical method used here.

BMAA was not detected in whole blood or plasma in any of the 25 individuals tested, irrespective of whether the hydrolyzed protein or free fractions were analyzed (Table A.2 (Appendix)). It cannot be excluded that BMAA-positive samples escaped detection due to the relatively high LOD (on-column) for whole blood compared to CSF, being 6 and 63 times higher for the hydrolyzed protein and free fraction, respectively.

DISCUSSION

Today there is general agreement that capacity to biosynthesize the neurotoxic BMAA is a trait held by globally widespread phytoplankton, although reported concentrations vary considerably (Faassen et al., 2012; Faassen, 2014). As for other cyanobacterial toxins, little is known about the role of BMAA in these organisms (Downing et al., 2011, 2015; Scott et al., 2014). Diverging interpretations of the effects of BMAA in other organisms also exist (Spencer et al., 1987; Perry et al., 1989; Matsuoka et al., 1993; Cruz-Aguado et al., 2006; Chiu et al., 2012; Lee and McGeer, 2012).

Our results show for the first time that BMAA, previously reported to be present in food webs of a brackish water body in a temperate climate (the Baltic Sea; see Jonasson et al., 2010), can be recovered from humans potentially exposed to Baltic Sea BMAA-'contaminated' food items (fish, shellfish). Although BMAA was detected in only a few of the 25 individuals tested (12%), the data presented here support the hypothesis that BMAA in the Baltic Sea environment may bioaccumulate in aquatic organisms and reach humans, thereby potentially constituting a threat to human health (Jonasson et al., 2010). The risk of human BMAA exposure in the Baltic Sea area is further corroborated by a recent study reporting the presence of BMAA in shellfish and fishes (locally caught and imported) sold in Swedish food markets (Jiang et al., 2014b). Our findings are also noteworthy in view of the documented enhanced Baltic Sea cyanobacterial blooms in recent decades (Kahru and Elmgren, 2014), known to produce BMAA (Jonasson et al., 2010); and perhaps particularly in view of the reported increase

in ALS incidence rates in Sweden from 1991 to 2005, the reason for which is unknown (Fang et al., 2009).

The present observations are, to the best of our knowledge, the first to demonstrate the occurrence of BMAA in human individuals *ante mortem* (see Table 1), and to quantify BMAA in human material using UHPLC–MS/MS. The BMAA was found in the CNS, more specifically in the CSF, while no BMAA was observed in the blood (whole blood and plasma) of the tested individuals.

BMAA was present in the CSF protein fraction or in peptides of the free CSF fraction and was released only after acid hydrolysis. The synthesis of these proteins/peptides and their association to BMAA most likely occurred in the CNS (Xie et al., 2013), although some may have been transported into the CNS (Ghasemzadeh et al., 2008). The CSF is an extension of the extracellular space of the CNS and is known to reflect its contents with regard to many low molecular weight substances (Saunders et al., 1999). Free BMAA have the ability to pass the blood–brain barrier, even though the transport is relatively ineffective despite possibly making use of the large neutral amino acid carrier (Duncan et al., 1991; Smith et al., 1992) or cerebral capillary transfer (Xie et al., 2013). Association/incorporation of BMAA into proteins has been suggested in animal studies (Karlsson et al., 2009a, 2014), and the ability to misincorporate BMAA during protein biosynthesis was recently demonstrated in human cells (Dunlop et al., 2013) as well as in a cell-free protein expression system (Glover et al., 2014). These findings support the proposed BMAA-protein bioaccumulation mechanism (Murch et al., 2004b).

The absence of BMAA in the free amino acid fraction (non-hydrolyzed) of the CSF may indeed indicate that most BMAA ends up as associated with, or incorporated into, proteins in human organs. Alternatively, the BMAA concentrations in free form were below our LOD. Furthermore, free BMAA in the human CNS could potentially escape detection due to a short half-life, known to be less than one day in the CNS of rats (Duncan et al., 1991; Smith et al., 1992). The reported rapid binding of BMAA to specific regions of the brain in neonatal mice also implies that free BMAA perhaps reside in the CSF for only a short time span (Karlsson et al., 2009b). In addition, free BMAA in the plasma and brain of BMAA-injected mice steadily decreased (within days), while the protein-associated BMAA levels in the brain reached a plateau (within hours) and accounted for most of the BMAA detected one week later (Xie et al., 2013). Due to the variety of proteins in the human CNS with different localizations, functions, and half-lives, the persistence of protein-associated BMAA in the CNS might vary considerably. However, both free and protein-associated BMAA (single subcutaneous injection) were cleared from the rat brain within months (Karlsson et al., 2014). In addition, the dietary exposure to BMAA most likely fluctuates over time.

The lack of BMAA in whole blood/plasma in the 25 individuals tested here (free and protein fraction) could be a consequence of a more rapid clearance of BMAA from the blood than from the CNS, as previously reported (Xie et al., 2013). Or alternatively, it may be due to the higher detection limit of BMAA in the blood than in CSF for our methodological approach (Table A.1 (Appendix)).

As seen in Table 2, some earlier studies detected BMAA in the brain of the majority of tested patients that had deceased from ALS-PDC, AD and ALS (Cox et al., 2003; Murch et al., 2004a,b; Pablo et al., 2009); while in other studies no BMAA was found in the brains of patients deceased from the same disorders (Montine et al., 2005; Snyder et al., 2009a,b, 2010; Combes et al., 2014). Likewise, BMAA was not detected in the CSF (or blood) in a similar study comprising 12 Swedish ALS patients and 10 controls, using an approach with a slightly higher detection limit than in the present study (Kushnir and Bergquist, 2009; Table 2; Table A.1 (Appendix)). Hence, our data, with BMAA found in 12% of the tested individuals *ante mortem* fall between the previous reports. It clearly confirms the presence of BMAA in the human CNS, although the data do not support the idea of BMAA being resident specifically in ALS patients. The low number of BMAA positives prevents valid generalizations regarding the distribution of BMAA between ALS patients and controls. Due to the 'liquid' nature of CSF, the BMAA concentrations detected in our study (pg/ml) are not directly comparable to the BMAA concentrations reported for *post mortem* brain material (μg/g). Discrepancies between the different studies (Table 2) may be explained by actual differences in the patient cohorts examined: geographic and cultural differences, life strategies, variations in the overall genetic susceptibility or by the organs examined (including *post* and *ante mortem*), but perhaps more likely by differences in the analytical approaches used (ours being one of only three studies using MS/MS for quantification).

Even though our study does not support previous findings of BMAA prevailing in the CNS of patients suffering from specific neurodegenerative diseases, the data do not rule out BMAA as a potential environmental risk factor in the pathogenetic process underlying neurodegenerative disorders. Rather, the fact that BMAA, by now a proven neurotoxin, can be retrieved from the CNS of human individuals is alarming. As indicated by the rare occurrence of ALS (approximately 2.5 cases/100,000 inhabitants yearly in Sweden) (Fang et al., 2009), BMAA may not act as a stand-alone etiological risk factor but perhaps as a potentiating agent in concert with other environmental factors. Hence, the finding of BMAA in the human CSF, including in one ALS patient, stresses the need for continued investigations in regard to the role of BMAA in the terminal neurodegenerative disease ALS, as this could open a potential avenue for effective treatments and cures.

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APPENDIX

Table A.1. Limit of detection (LOD) and limit of quantification (LOQ) of β-*N*-methyl-L-alanine (BMAA) in human matrices. Both free and protein fractions were hydrolyzed.

Matrix	Limit of detection (LOD)	Limit of quantification (LOQ)
CSF protein fraction	57 pg BMAA/ml CSF (0.12 pmol on-column)	57 pg BMAA/ml CSF (0.12 pmol on-column)
Whole-blood protein fraction	171 ng BMAA/ml whole blood (0.75 pmol on-column)	1.7 μg BMAA/ml whole blood (7.5 pmol on-column)
Whole-blood free fraction	53 ng BMAA/ml whole blood (7.5 pmol on-column)	134 ng BMAA/ml whole blood (18.9 pmol on-column)

CSF: cerebrospinal fluid.

Table A.2. BMAA (β-*N*-methyl-L-alanine) in cerebrospinal fluid (CSF), whole blood and plasma from Swedish ALS patients and non-ALS individuals. Values represent pg BMAA per ml CSF in hydrolyzed free or protein fractions.

Sample	CSF pg/ml (n = 1)		Whole blood (n = 3)		Plasma (n = 3)	
	Free fraction	Protein fraction	Free fraction	Protein fraction	Free fraction	Protein fraction
ALS-patients						
ALS1	78.0	96.6	ND	ND	ND	ND
ALS2	ND	ND	ND	ND	ND	ND
ALS3	ND	ND	–	–	–	–
ALS4	ND	ND	ND	ND	ND	ND
ALS5	ND	ND	ND	ND	ND	ND
ALS6	ND	ND	–	–	–	–
ALS7	ND	ND	ND	ND	ND	ND
ALS8	ND	ND	ND	ND	ND	ND
ALS9	ND	ND	ND	ND	ND	ND
ALS10	ND	ND	ND	ND	ND	ND
ALS11	ND	ND	ND	ND	ND	ND
ALS12	ND	ND	ND	ND	ND	ND
Non-ALS individuals						
non-ALS1	ND	234.5*	ND	ND	ND	ND
non-ALS2	ND	540.9*	ND	ND	ND	ND
non-ALS3	ND	ND	–	–	–	–
non-ALS4	ND	ND	–	–	–	–
non-ALS5	ND	ND	ND	ND	ND	ND
non-ALS6	ND	ND	–	–	–	–
non-ALS7	ND	ND	–	–	–	–
non-ALS8	ND	ND	–	–	–	–
non-ALS9	ND	ND	ND	ND	ND	ND
non-ALS10	ND	ND	ND	ND	ND	ND
non-ALS11	ND	ND	ND	ND	ND	ND
non-ALS12	ND	ND	–	–	–	–
non-ALS13	ND	ND	ND	ND	ND	ND

ND: not detected (concentration < LOD/LOQ); dash: not analyzed.
LOD: limit of detection; LOQ: limit of quantification.
* Quantification was made without correction for internal standard (D_3-BMAA), see Result section.

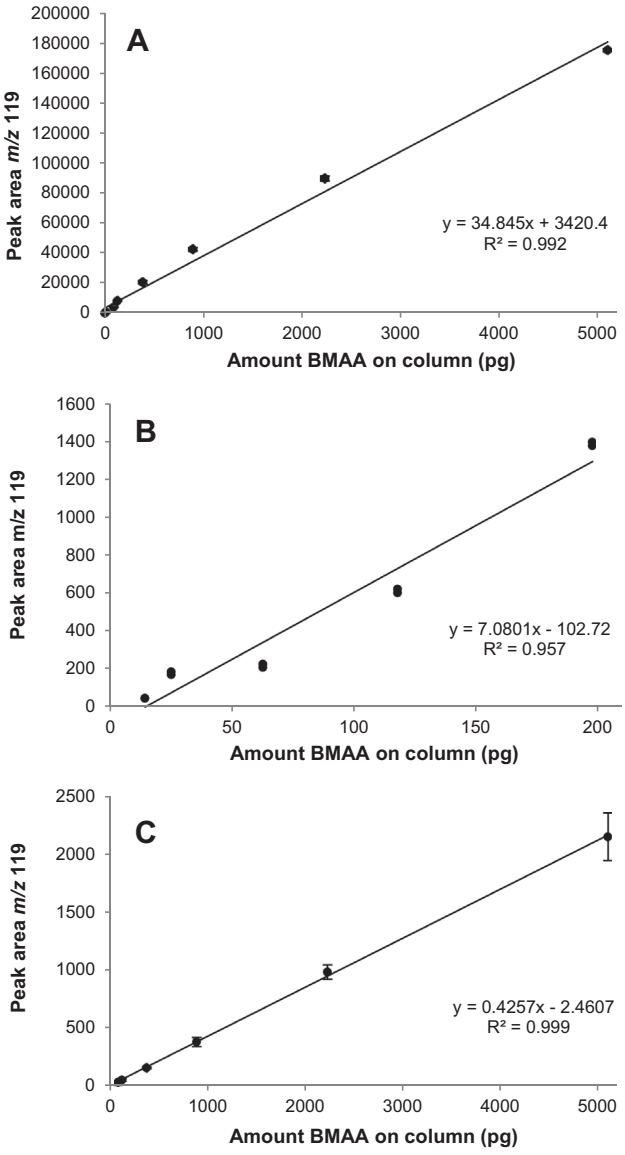

Fig. A.1. BMAA (β-*N*-methyl-L-alanine) calibration curves in buffer and protein fraction of cerebrospinal fluid (CSF) and whole blood. (A) Calibration curve in borate buffer (0.64–5094 pg BMAA on column, n = 5). (B) Calibration curve (14.3–197 pg BMAA on column, n = 2) obtained by spiking BMAA into hydrolyzed protein extracts of human cerebrospinal fluid (CSF). (C) Calibration curve (89–5094 pg BMAA on column, n = 5) obtained by spiking BMAA into hydrolyzed protein extracts of human whole blood. Points in graph A and C represent mean values with error bars denoting standard deviation. In graph B, each point represents the peak area (m/z 119) of the specific replicate.

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