Analysis of BMAA enantiomers in cycads, cyanobacteria and mammals: *in vivo* formation and toxicity of D-BMAA

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Abstract

Chronic dietary exposure to the cyanobacterial toxin BMAA triggers neuropathology in non-human primates, providing support for the theory that BMAA causes a fatal neurodegenerative illness among the indigenous Chamorro people of Guam. However, since there are two stereoisomers of BMAA, it is important to know if both can occur in nature, and if so, what role they might play in disease causation. As a first step, we analysed both BMAA enantiomers in cyanobacteria, cycads and in mammals orally dosed with L-BMAA, to determine if enantiomeric changes could occur *in vivo*. BMAA in cyanobacteria and cycads was found only as the L-enantiomer. However, while the L-enantiomer in mammals was little changed after digestion, we detected a small pool of D-BMAA in the liver (12.5%) of mice and in the blood plasma of vervets (3.6%), possibly as a result of racemisation. Chiral analysis of cerebrospinal fluid (CSF) of vervets and hindbrain of mice showed that the free BMAA in the central nervous system was the D-enantiomer. *In vitro* toxicity investigations with D-BMAA showed toxicity, mediated through AMPA rather than NMDA receptors. These findings raise important considerations concerning the neurotoxicity of BMAA and its relationship to neurodegenerative disease.

Keywords: chiral, enantiomer, neurodegenerative disease, ALS/PDC, Alzheimer's, neurotoxicity

Introduction

β-*N*-Methylamino-L-alanine (BMAA) is a neurotoxic amino acid produced by cyanobacteria, potentially also produced by other microorganisms such as diatoms (Jiang et al. 2014; Réveillon et al. 2016). Cyanobacteria of the genus *Nostoc*, are harboured in specialised coralloid roots of cycads, where they live in symbiosis (Banack and Cox, 2003). Human consumption of foods derived from cycads and animals that feed on cycads has been implicated in the development of Amyotrophic Lateral Sclerosis/ Parkinsonism Dementia Complex (ALS/PDC) among the Chamorro people of Guam (Whiting, 1963; Cox et al. 2003; Murch et al. 2004; Banack et al. 2006). BMAA has been shown to cause neurological damage *in vitro* (Rao et al. 2006; Lobner 2009) and *in vivo* (Spencer et al. 1987; Karamyan and Speth 2008; Nunn 2009; de Munck et al. 2013; Karlsson et al. 2015; Cox et al. 2016a).

Cyanobacteria produce a wide range of toxic compounds, including hepatotoxins and neurotoxins (Codd et al. 2005). When isolated and grown as free-living organisms, the symbiotic cyanobacteria from cycads can produce BMAA, which accumulates in cycads (Cox et al. 2003). Furthermore, analysis of a wide range of non-symbiotic free-living cyanobacteria suggests that BMAA production can occur worldwide (Cox et al. 2005; Metcalf et al. 2008; Esterhuizen and Downing, 2008; Spacil et al. 2010).

Vega and Bell (1967) and Vega et al. (1968) crystallised the L- enantiomer of BMAA from an alcohol extract of *Cycas micronesica* Hill [Cycadaceae] seeds. At that time, no D-BMAA was isolated and it was concluded that D-BMAA was not present in cycads. Furthermore, although L-BMAA has been shown to be toxic in a range of test systems,

the limited research into the toxicity of synthetic D-BMAA suggested that it has little or no toxicity (Vega et al. 1968; Polsky et al. 1972; Nunn et al. 1987). The implications of exposure to BMAA from the environment and its association with human disease are considered to be a result of exposure to the L- enantiomer of BMAA.

A variety of analytical methods are available for the analysis of BMAA in a range of matrices (Cox et al. 2003, 2005; Metcalf et al. 2008; Pablo et al. 2009) including HPLC-FLD, LC-MS, LC-MS/MS, GC-MS and amino acid analyser (e.g. Banack et al. 2007; Esterhuizen and Downing 2008). However, these methods do not discriminate between the L- and D- enantiomers of BMAA and at present little information is available as to which enantiomers of BMAA occur naturally. An HPLC method employing pre-derivatisation of L- and D-BMAA as *o*-phthaldialdehyde derivatives worked well, successfully separating synthetic standards (Euerby et al. 1989). In order to provide useful information on the presence of this amino acid in environmental and clinical materials, chiral methods may become important to understand whether D-BMAA exists in nature, and also whether this can be related to toxicity events and human disease.

Racemisation and enantiomeric changes to amino acids is a well-recognised phenomenon, with alterations of pH, time, and temperature affecting this process to differing degrees (Friedman 2010). As the Chamorro people of Guam consume BMAA in free and protein-bound forms, derived from cycads (Cheng and Banack 2009), few if any studies have considered the potential for enantiomeric changes to this amino acid *in vivo*.

The occurrence of D-amino acids has been well documented in the bacterial Domain, with D-amino acids known to occur in secondary metabolites and peptides, including cyanobacterial toxins (Codd et al. 2005). In the mammalian brain, D-amino acids also occur, with D-serine and D-aspartic acid being produced by racemases that change the enantiomeric configuration of the L-amino acid. Such D-amino acids play important roles in mammalian neurochemistry (Kim et al. 2010; Baumgart and Rodriguez-Crespo 2008; Krashia et al. 2016). Although D- amino acids are important in neurochemistry, their occurrence and the presence of enzymes involved in their racemisation and metabolism can be found in other tissues. The presence of D-glutamate in the heart (Ariyoshi et al. 2017), and D-aspartate and D-serine in tissues such as the testes, kidney, skin and skeletal system have been documented (Guevara and Mani, 2016; Ito et al. 2016).

The purpose of this study was to develop a method and to analyse naturally-occurring enantiomers of BMAA and to determine whether any enantiomeric changes could occur during digestion in the mammalian gut after oral exposure. Furthermore, testing of D-BMAA in *in vitro* toxicity systems was carried out. Such information may prove useful in understanding the fate and the potential toxicities of BMAA and its relationship to neurological disease.

Materials and Methods

Collection of cycads, growth of cyanobacteria and BMAA extraction

Cycad seeds from *Cycas micronesica* Hill were collected at the Montgomery Botanical Garden, Coral Gables, Florida and transported to the Brain Chemistry Labs for

extraction. The fleshy sarcotesta was removed and the rigid sclerotesta was cut to expose the gametophyte. The gametophyte was removed and cut into small pieces for amino acid extraction. Free amino acids were extracted three times using 0.1 M trichloroacetic acid (TCA) with ultrasonication at room temperature at a concentration of 61 mg dry wt. per ml. The TCA extract was ultrafiltered (0.22 µm Millipore Ultrafree MC), dried and stored at 4 °C until analysed. An isolate of *Nostoc* sp., isolated from a *Cycas micronesica* coralloid root, was maintained in BG11 medium supplemented with sodium nitrate for 4 years at 25ºC and with cool white fluorescent light at 25µE m^{-2} s^{-1}, with periodic sub-culturing and freeze-drying of biomass. A collection underwent extraction in 0.1 M TCA with ultrasonication at room temperature, prior to ultrafiltration and storage at 4 °C.

BMAA administration, plasma sampling and experimental design

In order to obtain samples for chiral testing, a limited number of samples were available from two independent studies investigating the toxicology, metabolism and organ distribution of BMAA. For mice studies, animals were sacrificed, allowing samples of brain and liver to be obtained for analysis, whereas vervets were not sacrificed. Consequently, post-digestion in vervets, samples were obtained from blood plasma and for the central nervous system, CSF was collected. Although different samples were obtained from test organisms, they do represent collections post digestion and in the central nervous system for both species.

Mice were bred and maintained under standard conditions (14 hr light:10 hr dark, 18-23° C temp and 40-60% humidity, acidified H_20, sterilized pine shaving bedding and

cages changed weekly) in the Research Animal Facility at The Jackson Laboratory. The nutritionally complete 6% fat NIH 31-based 5K52 diet (LabDiet, St Louis, MO) supplemented with or without L-BMAA was provided *ad libitum* in a 6-month chronic dosing experiment (25 mg/kg/day) in C57BL/6J background mice (JR#004435, The Jackson Laboratory). The pre-ground 5K52 diet was mixed with a 1g/100ml (1%) stock solution of L-BMAA to a final concentration of 20.83 mg per 100 grams of diet, formed into patties and dehydrated overnight in a low-heat dehydrating oven (45°C) and stored at –20 °C until use. Diet was supplied to the mice (4 per cage) in the food hopper and was replaced weekly. Dosing was based on a short dietary exposure study published by Cruz-Aguado *et al*. (2006).

Animal care was in accordance with the National Research Council, *Guide for the Care and Use of Laboratory Animals* (1996), and approved by the Institutional Animal Care and Use Committee (protocol #01006). Mice were euthanized by cervical dislocation, and liver and hindbrain were excised and stored at -80ºC. For extraction, once thawed, subsamples of the hindbrain and liver from a BMAA-dosed mouse were extracted with 100µl 0.1M TCA (Sigma, St. Louis, MO). The extract was dried in a Speedvac (Thermo Savant Speedvac Plus, SC250DDA), and the residue resuspended with 50µl 2mM $CuSO_4$ in Millipore DirectQ (DQ) water or 100µl 2mM $CuSO_4$ in DQ water for liver and hindbrain extracts, respectively. The aqueous copper sulphate extracts were directly injected into the chiral HPLC system and fractions collected. The collected fractions were derivatised with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) and analysed by UPLC-MS/MS.

Vervets (*Chlorocebus sabaeus*) obtained from the Behavioural Science Foundation (BSF) in St. Kitts, West Indies were fed L-BMAA. Animal care was in accordance with the National Research Council, *Guide for the Care and Use of Laboratory Animals* (1996), and approved by the Institutional Animal Care and Use Committee.

The subjects used in this study were three adult male vervets (4.5 kg) that were housed in outdoor social groups. Each outdoor enclosure measured approximately 30 m^2 and contained perches and play structures. Subjects were always housed in social groups except during sampling. Vervets were maintained on a modified diet, routinely fed two days of primate chow (Teklad NIB primate diet, Madison, WI, USA) per week and fresh produce only on other days (n=5). Slight variation occurred at times when produce was limited. L-BMAA was applied as a powder (70 mg/kg body weight) to a hollowed-out piece of fruit, and offered to the animals. No control animals were included and prior exposure to BMAA was considered to be minimal, and based on the dose of BMAA administered, considered to be unlikely to influence the concentrations in dosed animals. Animals were orally dosed every day and the dosing took place over 10 days with CSF and plasma collected under ketamine HCl (10 mg/kg) anaesthesia at 240 and 244 hr, respectively. CSF was collected directly into cryovials and frozen and blood was collected in heparinised tubes which were centrifuged, prior to removal of the plasma and stored frozen. Vervets were not sacrificed for this study. One hundred microliters of the plasma and CSF from a BMAA-dosed vervet were dried in a Speedvac, prior to resuspension to the same volume with 2mM aqueous copper sulphate and fraction collection by chiral HPLC. The collected fractions were derivatised with AQC and analysed by UPLC-MS/MS.

Analysis of BMAA by UPLC-UV-MS

BMAA was detected in the TCA extracts by comparison with a triple crystallised L-BMAA standard (P. B. Nunn), a DL-BMAA mixture from Thesis chemistry (Cambridge, Ontario, Canada) and an L-BMAA standard (Sigma B-107, St. Louis, MO) after derivatisation with AQC by UPLC-UV/MS using the methods in Banack et al. (2007). Extracts found to contain BMAA according to retention time and molecular weight were separated by chiral HPLC.

Chiral HPLC analysis and fractionation of TCA extracts

Chiral separation of L- and D- BMAA was achieved using a Chirex 3126 chiral HPLC column (Phenomenex; 250 × 4.6 mm i.d.) with mobile phases of 2 mM $CuSO_4$ in water (eluent A) and 2 mM $CuSO_4$ in 15% (v/v) aqueous acetonitrile (eluent B) using a Waters 717 autosampler and Waters 1525 binary pump. A gradient elution was used: 100% A to 70% B from 0-13 min, curve 10, followed by a wash from 13-16 min of 70% B before returning to 100% A at 17 minutes, with a total run time of 35 minutes to condition the column prior to the next injection. Flow was maintained at 1.2 ml/min throughout. Detection was performed using a 2487 UV detector (Waters) at a wavelength of 254 nm. The gradient was tested using a mix of 17 standard amino acids (Pierce #NCI0180) plus the L-BMAA isomers/enantiomers, L-2,4-diaminobutyric acid (L-DAB; #32830, Sigma, St. Louis, MO), N-(2-aminoethyl)glycine (AEG; A1153 TCI America; Banack *et al*., 2012), D-BMAA (P.B. Wyatt) and D-DAB (P.B. Nunn).

Based on the amount of BMAA in the cycad TCA extract, a 1/50 dilution with 2 mM $CuSO_4$ in water was prepared and separated by chiral HPLC, in order to not overload

the chiral column. Fractions corresponding to the retention time of L-BMAA, between L- and D-BMAA, and D-BMAA were collected by hand (approx. 1ml per fraction). Collected cycad fractions were analysed by UPLC-MS/MS after derivatisation with AQC. Separately, after drying in the Speedvac, the residue of each fraction was reconstituted with 100 µl 20 mM HCl and derivatised with AQC for UPLC-MS/MS analysis.

The TCA extract from the cyanobacterium was dried, resuspended to the same volume with 2 mM $CuSO_4$ in water, and injected directly onto the chiral HPLC column. Nine separate blank injections (2mM $CuSO_4$ in water) and nine cyanobacterial extract injections, each of 10 µl, were performed and the fractions collected by hand (approx. 1ml per fraction). The fractions were freeze-dried and each resuspended in 100 µl 20 mM HCl. The nine chiral fractions from the blank and cyanobacterial injections were respectively pooled, to allow concentration of the free BMAA fractionated from the TCA extract, in comparison with a set of blank injections. The copper present in the pooled extracts was precipitated by addition of 10 µl 12 M NaOH for the cyanobacterial extract and the blank, to simplify the extract containing a low amount of BMAA for subsequent analysis. The suspensions were centrifuged to remove copper and the supernatants transferred to clean microcentrifuge tubes. To each cleared supernatant, 10 µl 12 M HCl were added to neutralise the sodium hydroxide and the solutions again freeze-dried. After drying, the residue was resuspended with 100µl 20mM HCl and the solutions were derivatised with AQC. Subsequently, the derivatised amino acids of both the blank and the cyanobacterial TCA chiral fractions were dried and resuspended in DQ water equivalent to a 3× concentration step for UPLC-MS/MS analysis.

UPLC-MS/MS analysis of BMAA

AQC-derivatised samples of TCA extracts or fractions after collection from the chiral HPLC were run on a triple quadrupole UPLC-MS/MS system (Thermo Scientific Finnigan TSQ Quantum UltraAM, San Jose, CA) after separation with a Waters Acquity-UPLC system with a Binary Solvent Manager, Sample Manager and a Waters AccQTag Ultra column (part# 186003837, 2.1x100 mm) at 55 °C. Separation was achieved using gradient elution at 0.65 ml/min with 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B): 0.0 min= 99.1%A; 0.5 min= 99.1%A curve 6; 2 min= 95%A curve 6; 3 min= 95%A curve 6; 5.5 min= 90%A curve 8; 6 min= 15%A curve 6; 6.5 min =15%A curve 6; 6.6 min=99.1%A curve 6; 8 min =99.1%A curve 6. Nitrogen gas was supplied to the heated electrospray ionisation (HESI) probe with a nebulising pressure of 40 psi and a vaporiser temperature of 400 °C. The mass spectrometer was operated under the following conditions: the capillary temperature was set at 270 °C, capillary offset of 35, tube lens offset of 110, aux gas pressure of 35, spray voltage 3500V, source collision energy of 0, and multiplier voltage of -1719V. A divert valve was used during equilibration and cleaning phases of the gradient. The second quadrupole was pressurised to 1.0 mtorr with 100% argon. The precursor ion was set for *m/z* 459 which excludes in the first quadrupole ions of all other masses from analysis. During BMAA analysis of cycad extracts, collision-induced dissociation (CID) was achieved in the second quadrupole using the following transitions: *m/z* 459 to *m/z* 119 CE 21; *m/z* 459 to *m/z* 289 CE 17; *m/z* 459 to *m/z* 171 CE 38. The resultant four product ions (*m/z* 119, 289, 171, 188) were scanned by the third quadrupole, subsequently detected, and their relative abundances quantified in comparison with AQC-derivatised N-(2-aminoethyl)glycine (AEG), L-BMAA and L-2,4-diaminobutyric acid (L-DAB) standards.

For cyanobacterial extracts, the precursor ion of *m/z* 459 was monitored for CID daughter ions of *m*/*z* 171, 289, 119, 258 (BMAA, CE 21eV), 214 (AEG, CE 35eV) and 188 (DAB, CE 38eV), with the latter three being qualifier ions for the different isomers. The quantification of L- and D-BMAA was carried out using L-BMAA standards in water using the transition 459>171. LOD (48 fmol) and LOQ (0.48 pmol) were calculated using the EPA Method Detection Level (Betz et al. 2011; Banack et al. 2014).

In vitro toxicity assessment of D-BMAA

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE, USA). Mixed cortical cell cultures containing neurons and astrocytes were prepared from foetal (15-16 day gestation) mice as previously described (Lobner, 2000). The mice were handled in accordance with a protocol approved by the institutional animal care committee. All experiments were performed on mixed cultures 13-15 days *in vitro* (DIV). Toxicity was induced by exposure to BMAA for 24 hours in media as for plating except without serum. All exposure media contained 26 mM $NaHCO_3$, as it has been shown previously that HCO_3^- is required for expression of NMDA receptor-mediated BMAA toxicity (Weiss and Choi, 1988). Cell death was assessed by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 hours after the beginning of the insult. Blank LDH levels were subtracted from insult LDH values and results normalized to 100% neuronal death caused by 500 μM NMDA. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Koh and Choi, 1987; Lobner, 2000). In none of the conditions in the studies presented was there any evidence of glial cell death observed (assessed by

trypan blue staining). Therefore, results are presented as percent neuronal death, determined by subtracting blank LDH release levels from experimental condition LDH release and dividing by the total LDH release levels induced by exposure to NMDA and then expressing as a percentage. Cells were not counted when plating as astrocytes are present which are dividing cells, so initial plating is not necessarily correlated with final cell numbers. All compounds were made up in water at a stock concentration 1000 x the final concentration, therefore vehicle controls were not believed to be necessary. Blank LDH levels mean LDH release in cultures without drug treatment.

Results

Using chiral HPLC, L- and D-BMAA enantiomer standards could be clearly distinguished from standard amino acids and the BMAA isomers, the achiral *N*-(2-aminoethyl)glycine (retention time 2 minutes), D-2,4-diaminobutyric acid (retention time 4 mins), which roughly co-eluted with L-2,4-diaminobutyric acid (Fig. 1A). The difference in retention time between the two BMAA enantiomers was approximately 1 minute, with L-BMAA eluting from the column before D-BMAA (Fig. 1B). UPLC-UV/MS and MS/MS analysis showed that the TCA extract from seeds of *Cycas micronesica* contained BMAA. Analysis of a TCA extract from the cycad gametophyte by chiral HPLC indicated peaks with the same retention time as L-BMAA, but not for D-BMAA (Fig. 1C). As the sensitivity of the chiral method was approximately 100 times less than standard amino acid analysis techniques employed for BMAA, the chiral HPLC method was developed and used as a sample preparation technique to a) remove interfering compounds in the analysis of BMAA, b) to separate and determine what enantiomers of BMAA are present

and c) as a multiple fractionation tool to collect enantiomers (Fig. 2). The cycad extract (Fig. 1C) was fractionated into 3 fractions from 7.5 to 8.2 minutes, 8.3 to 8.7 minutes and 8.8 to 9.4 minutes by chiral HPLC. These fractions were then analysed by UPLC-MS/MS to confirm the presence of BMAA enantiomers. In the L-BMAA fraction a peak at 4.95 minutes was observed in the fractionated cycad, consistent with the BMAA standards (Fig. 3A). The peak collected between L- and D-BMAA showed a trace amount of BMAA and although a peak was observed in the third fraction, analysis of the ratios of daughter ions indicated that D-BMAA was not present or was below limits of detection (Fig. 3B, Table 1). In comparison with a BMAA standard, retention times and daughter ions were consistent for BMAA in the first two fractions. The presence of trace amounts of BMAA in the DL- fraction was considered to be carry-over from the collection of the L-enantiomer fraction. Although slight shifts in retention time were observed from day to day, no significant retention time shifts were noted. Before fractionation, the chiral HPLC system was calibrated with fresh L- and D-BMAA standards and these time windows were used for the fractionation of unknown samples. Therefore, we do not expect to witness cross-contamination of L- and D- BMAA in fractions.

As the concentration of BMAA in cycads is much greater than that in cyanobacteria, the chiral method was used to collect multiple fractions with retention times corresponding to those of L- and D-BMAA from a symbiotic *Nostoc* TCA extract, in comparison with an identical set of blank injections. Samples were purified and concentrated and analysed by LC-MS/MS after derivatisation with AQC. UPLC-MS/MS analysis of blank fractions showed no BMAA enantiomers to be present, whereas the presence of L-BMAA was

confirmed in the TCA extract from the *Nostoc* symbiont (Fig. 3C, Table 1). Based on the variation in daughter ion ratios for cycads and cyanobateria (Table 1), only L-BMAA was confirmed with the ion ratios for D-BMAA being outside that expected for synthetic standards.

Chiral analysis was performed on tissues from animals dosed with L-BMAA. Chiral HPLC analysis of the stock L-BMAA showed the preparation administered was exclusively the L-enantiomer for both the BMAA administered to mice and vervets (this study, Cox et al., 2016b). Analysis of the free BMAA in these tissues showed that the mouse liver and the vervet plasma was predominantly the L-enantiomer (Fig. 4, Table 2), showing little, if any, racemization of L-BMAA occurs before its appearance in serum or liver. In contrast, figure 5 shows that the majority of BMAA determined by UPLC-MS/MS analysis of the chiral fractions in brain or CSF was the D-enantiomer (also Table 2). As enantiomeric mixtures were observed, the percentage of L-BMAA and D-BMAA in each extract was assessed. Using the peak areas from the MS/MS chromatograms to calculate the amount of BMAA in each enantiomeric fraction, the liver and plasma contained predominantly L-BMAA (plasma, 96.4%; liver, 87.5%), whereas only D-BMAA was detected in the hindbrain and CSF. A further comparison of the amounts of BMAA in the various chiral fractions from unspiked tissues was carried out with 2.23 nmol/mg wet wt. of L-BMAA versus 0.35 nmol/mg wet wt. of D-BMAA in the mouse liver and 0.62 nmol/mg wet wt. of D-BMAA in the mouse hindbrain. In vervet tissues, 20.3 μmol/μl of L-BMAA and 464 nmol/μl of D-BMAA were recorded in vervet plasma, versus 176 nmol/μl of D-BMAA in CSF.

As the potential long-term toxicity of D-BMAA is little understood or not known, *in vitro* experiments were carried out with D-BMAA. Both enantiomers of BMAA were shown to cause neuronal cell death in a dose-dependent manner (Figure 6 A,B), with significant neuronal death occurring at mM concentrations. When neuronal cell death was measured in the presence of MK801 (Figure 6 C,D), this NMDA receptor agonist prevented neuronal cell death in the presence of L-BMAA but not in the presence of D-BMAA. When neurons were incubated with D-BMAA (Figure 6 E), neuronal cell death could be prevented using the AMPA receptor agonist NBQX, but not the calcium permeable AMPA receptor agonist NASPM.

Discussion

The discovery of BMAA and its toxic properties more than four decades ago have inspired a wealth of scientific research. To date, only the L-BMAA enantiomer has been crystallized from cycad extracts and the synthetically produced D-BMAA enantiomer has been considered non-toxic (Vega and Bell 1967; Vega et al. 1968, Polsky et al. 1972; Nunn et al. 1987; Nunn 2009). The finding that BMAA is produced by cyanobacteria (Cox et al. 2003; 2005), raises the question of the possible presence of the D-BMAA enantiomer in cyanobacteria, particularly because cyanobacteria are well-known to produce and incorporate D-amino acids into other compounds (Codd et al. 2005). For example, D-alanine and D-glutamic acid are common components of the cyanobacterial heptapeptide microcystins (Codd et al. 2005). In order to understand the environmental occurrence of BMAA and to better understand the relationship between cycads and their cyanobacterial symbionts, we developed a chiral HPLC method to determine which BMAA enantiomers occur in cycads and to determine whether small

concentrations of previously undetected D-BMAA may be present (Vega and Bell 1967; Vega et al. 1968). Although D-BMAA was not observed in the cycad extract in the original work of Vega and Bell (1967) and Vega et al. (1968), its absence may have been due to the sample preparation methods used. If D-BMAA was present in the cycad extracts, the likelihood of confirming its presence would be increased if chiral HPLC was used. Since exposure to BMAA has been implicated in the development of Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC) on Guam (Banack and Cox 2003; Murch et al. 2004; Cox et al. 2016a) and BMAA has been identified in the brains of ALS and Alzheimer's patients in the USA and Canada (Murch et al. 2004; Pablo et al. 2009), a greater understanding of L- and D-BMAA enantiomers might shed further light on disease aetiology.

Fractionation of a TCA extract from *Nostoc* isolated from Guamanian cycad coralloid root and a TCA extract of cycad gametophyte tissue did not indicate the presence of D-BMAA, whereas the L-enantiomer was positively identified using the chiral method described herein. The use of chiral HPLC as a fractionation tool had certain advantages due to the fact that multiple fractions could be iteratively collected and combined to increase the potential concentration of BMAA for later mass spectrometric analysis. Furthermore, this fractionation method also removed interfering compounds from the sample matrix, thus simplifying the mass spectrometric analysis and providing separation of BMAA enantiomers from the structural isomers L-2,4-DAB (Banack et al. 2010), D-2,4-DAB and the achiral AEG (Banack et al. 2012). This is largely because only two approximate 1 minute windows were collected resulting in a large number of compounds being retained by the chiral column or eluting at a different

time which would not be present in the fractions during subsequent analysis. The chiral method shown here will be useful for investigating which free BMAA enantiomers exist in a wide range of plant, cyanobacterial and clinical materials.

Amyotrophic Lateral Sclerosis/ Parkinsonism Dementia Complex (ALS/PDC) among the Chamorro people has been linked to high concentrations of BMAA in their diet (Banack et al. 2006; Bradley and Mash 2009; Banack and Murch 2009; Cox et al. 2016a). In mammals, food is processed within the mouth and stomach, with the potential to alter the enantiomeric ratio of L- and D-amino acids due to changes in pH and temperature (Friedman 2010), although no changes were noticed with L- or D-BMAA stored in HCl (data not shown). Since the traditional Chamorro diet included BMAA-containing washed cycad flour and flying foxes (Banack and Cox 2003; Banack et al. 2006; Cheng and Banack 2009), there is a possibility that BMAA enantiomers present in their food may be transformed to a racemic mixture during digestion. Analysis of mouse liver and vervet plasma indicated that after oral administration and digestion of L-BMAA, the majority of this amino acid was still present as the L-enantiomer. In contrast, D-BMAA was the only free enantiomer found in the central nervous system of both mice and vervets. Since, the D-enantiomer has not previously been described in nature, then a mechanism for the interconversion between BMAA enantiomers in mammals might exist. Within the mammalian brain, enzymes such as serine racemase [EC.5.1.1.18] are present that racemise L-serine to D-serine, which is then used as a neurotransmitter (Baumgart and Rodriguez-Crespo 2008). A number of amino acids are known to affect the activity of this enzyme (Dunlop and Neidle 2005) and BMAA may be a substrate for or affect the activity of this enzyme. If BMAA were shown to interfere with serine

racemase, it could alter the delicate balance of amino acids in the central nervous system. Furthermore, incorporation of L-BMAA into proteins can be blocked by L-serine, suggesting that these two amino acids may have metabolic as well as structural similarities (Dunlop et al. 2013).

A further consideration is whether L- or D-BMAA is actively transported into the brain. Our analyses indicate that the L-enantiomer is found in the circulatory system whereas the D-enantiomer is the free enantiomer in the central nervous system. The transportation and uptake of BMAA into the brain is considered to occur through the L1 transport system for large neutral amino acids, and although inefficient, BMAA was observed to accumulate in the brain (Duncan et al. 1991; Xie et al. 2013). This uptake occurs as a result of the essentially uncharged nature of BMAA at pH 7.4 (Nunn and Ponnusamy 2009), along with leucine, valine, methionine, histidine, isoleucine, tryptophan, phenylalanine and threonine (Bradley 2009). It has been previously demonstrated that the toxic effects of BMAA can be inhibited by leucine in *Drosophila* (Zhou et al. 2009) and in rats (Smith et al. 1992) which supports the suggestion that BMAA may be transported across the blood brain barrier by the L1 transport system. Work by Liu et al. (2009) has discovered a second transport system (X_c^-) which is also inhibited by BMAA, and it is possible that BMAA may also cross cell membranes using this system. Therefore, it would seem unlikely that the D-enantiomer would be actively transported, as evidenced by the fact that serine racemase is present within the central nervous system to convert L- to D- serine. This reasoning, along with the data showing L-BMAA in the blood plasma and liver *versus* D-BMAA in the brain and CSF, suggests that the free D-BMAA is produced within the brain itself.

A second racemase in the brain, aspartate racemase [EC.5.1.1.13] produces D-aspartic acid, which is an endogenous ligand for NMDA receptors (Kim et al. 2010). The presence of a number of racemases and D-BMAA in the brains and CSF of mice and vervets, respectively, raises a further question concerning what other non-protein amino acids may be converted from their L- to D- enantiomers, which may or may not cause adverse effects. Furthermore, it is possible that other racemases, in addition to serine- and aspartate racemase may exist in the mammalian brain, having the potential to racemise L- to D-BMAA.

Based on the preliminary *in vitro* toxicological studies carried out here D-BMAA does appear to have some neurotoxicity, although via a different mechanism to that observed with L-BMAA. Further studies examining the toxicity of D-BMAA are required to determine whether enantiomeric changes to BMAA result in neurological deficits. Chiral HPLC analysis of human brains known to contain BMAA may shed some light on which of these scenarios is more likely. A third possibility is that deficiencies in the serine racemase enzyme itself may constitute a risk factor for neurodegeneration.

The finding that L-BMAA can be converted from the L- to D- enantiomer raises interesting questions concerning the etiology of sporadic ALS, and further studies to determine the molecular mechanisms which interconvert this amino acid may shed new light on the causes of ALS and its association with neurotoxic amino acids.

Compliance with ethical standards

Conflict of interest: the authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and the appropriate institutional reviews and permissions were granted before the study was conducted.

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Figure 1. Chiral separation of L-BMAA, D-BMAA and L-2,4-diaminobutyric acid (L-DAB) from a standard mixture of amino acids. (A) chiral separation of a L- and D- BMAA standard (solid line) from a mixture of standard amino acids and L-2,4 DAB (hatched line). (B) comparison of a DL-BMAA standard (solid line) with a L-BMAA standard (hatched line). (C) A DL-BMAA standard (hatched line) compared with chiral HPLC analysis of a TCA extract from cycads (solid line).

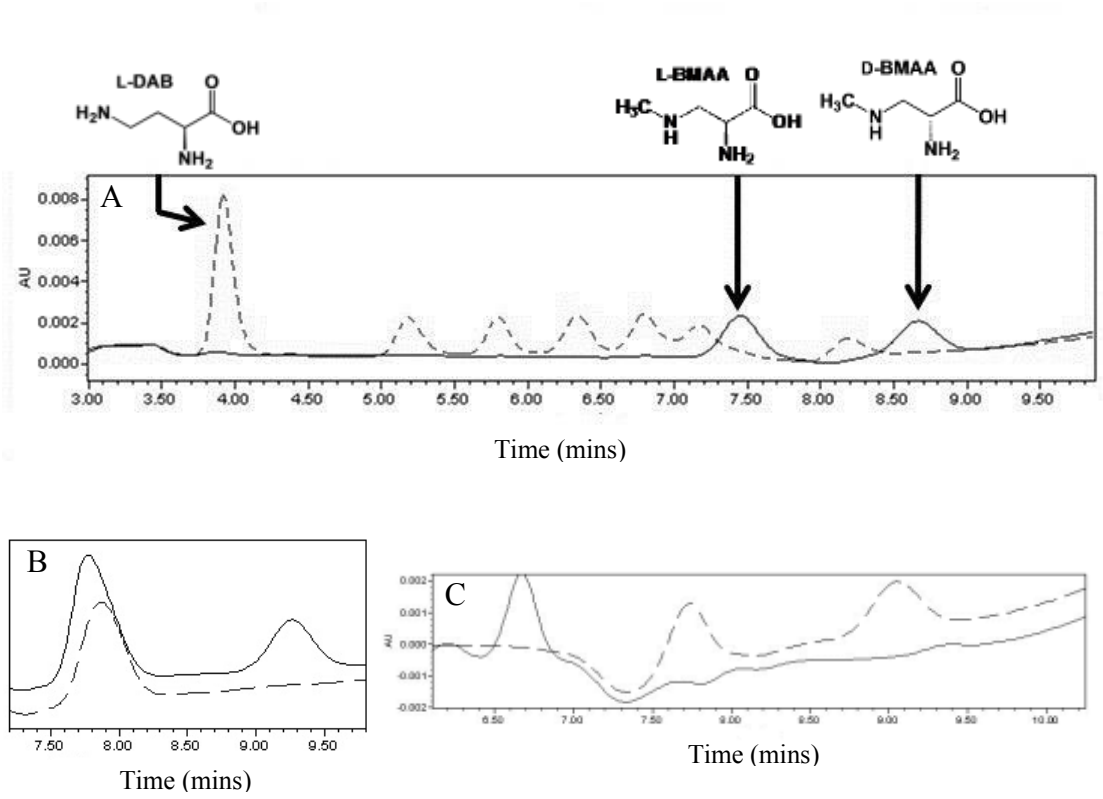

Figure 2. Schematic overview of the chiral fractionation procedure.

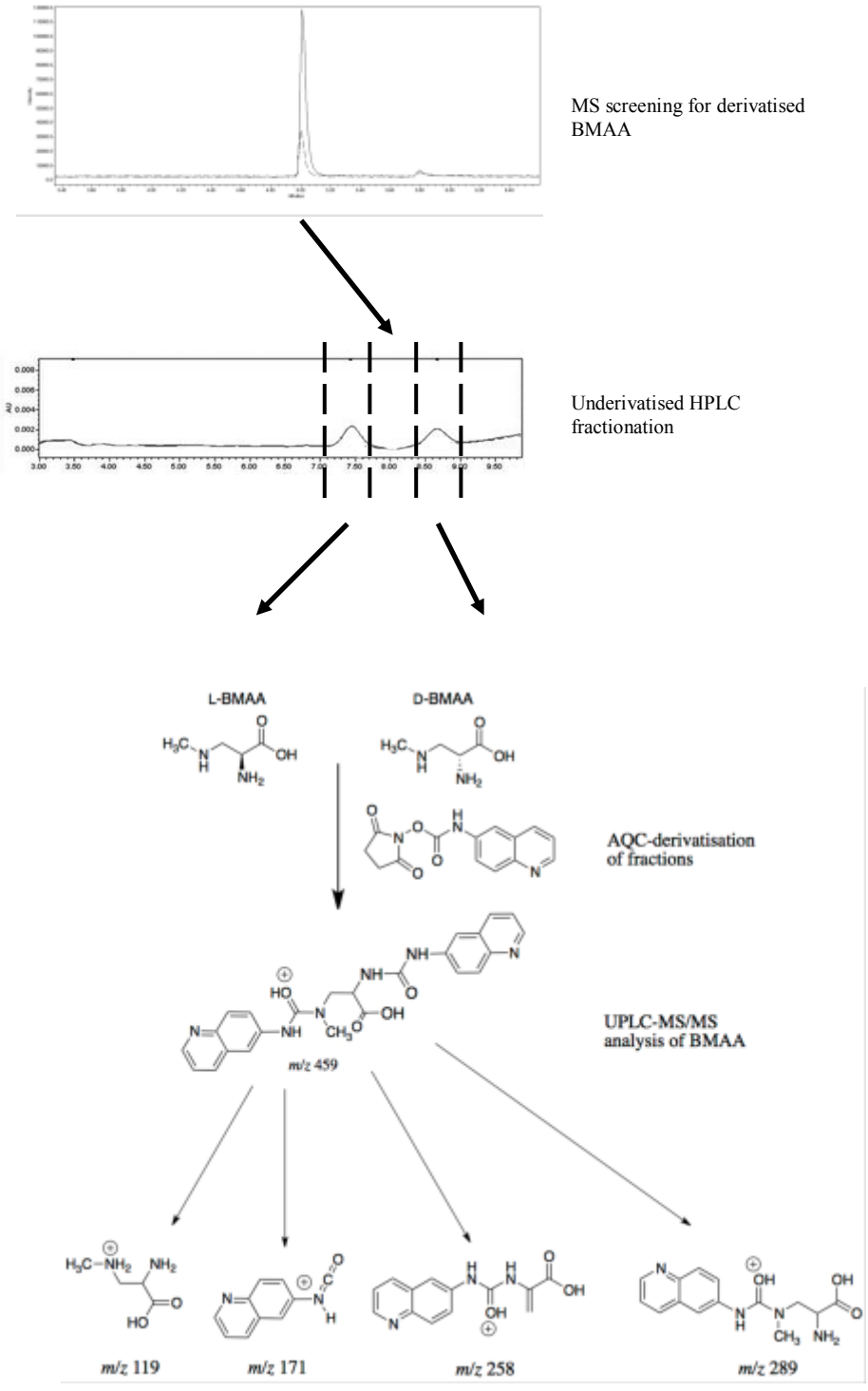

Figure 3. UPLC-MS/MS analysis of AQC-derivatised fractions obtained by chiral HPLC for the presence of L- and D-BMAA in cycads and cyanobacteria. (A) cycad L-BMAA fraction; (B) cycad D-BMAA fraction; (C) cyanobacteria L-BMAA fraction; (D) cyanobacteria D-BMAA fraction. Grey area shows the retention time of BMAA and the CID daughter ions isolated from a parent mass of *m/z* 459.

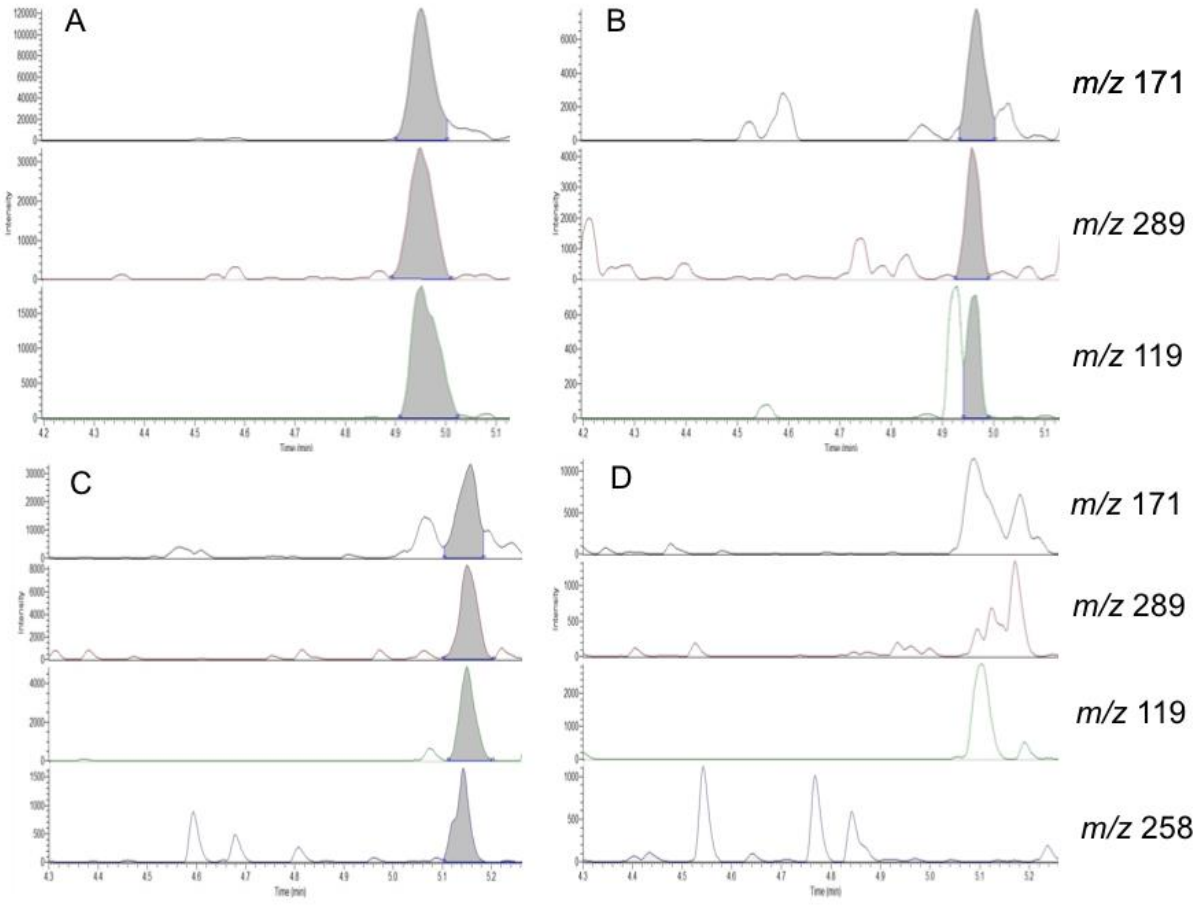

Figure 4. UPLC-MS/MS analysis of chiral HPLC fractions from mouse liver and vervet plasma. A, mouse liver L-BMAA fraction; B, mouse liver D-BMAA fraction; C, vervet plasma L-BMAA fraction; D, vervet plasma D-BMAA fraction. Grey areas show the retention time of BMAA, with daughter ions isolated from a parent mass of *m/z* 459.

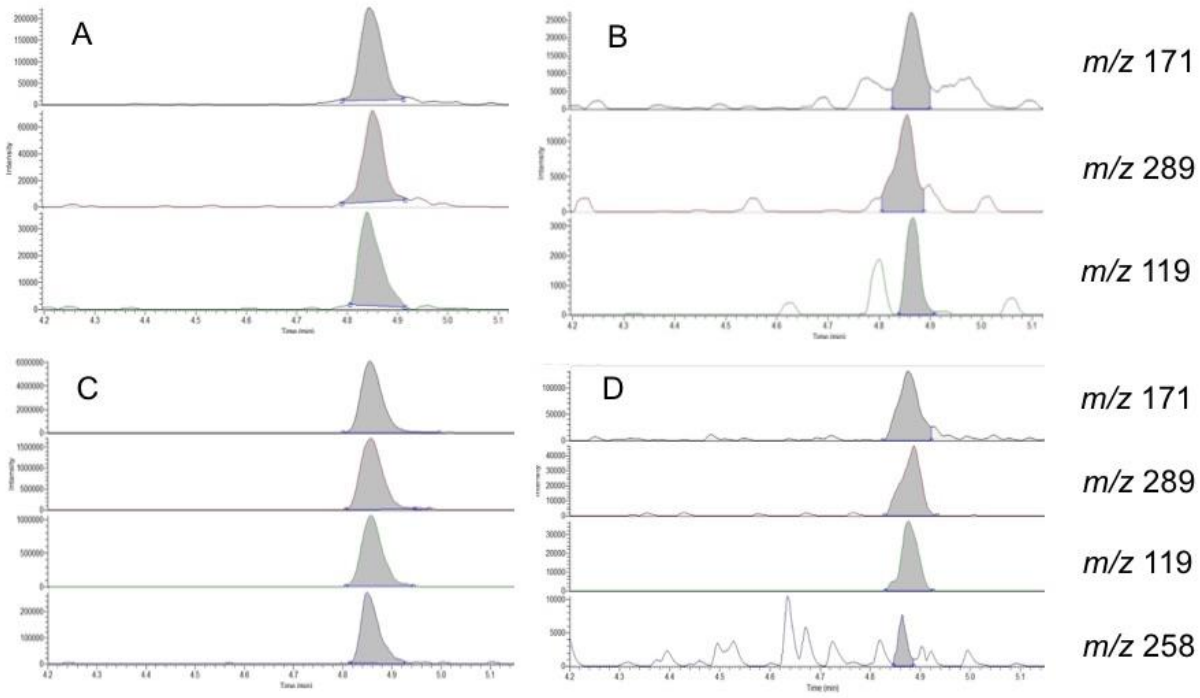

Figure 5. UPLC-MS/MS analysis of free BMAA in mouse hindbrain and vervet CSF. A, mouse hindbrain L-BMAA fraction; B, mouse hindbrain D-BMAA fraction; C, vervet CSF L-BMAA fraction; D, vervet CSF D-BMAA fraction. Grey areas show the retention time of BMAA, with daughter ions isolated from a parent mass of 459.

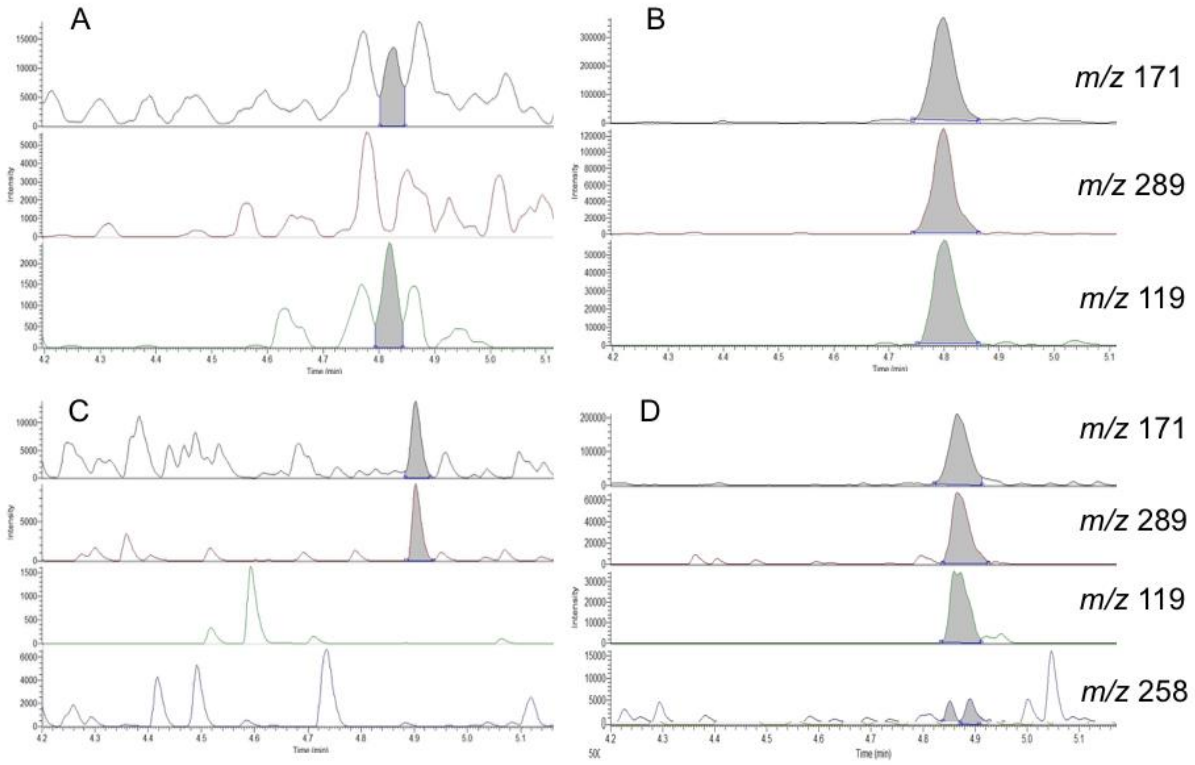

Figure 6. Toxicity assessment of L- and D- BMAA in an *in vitro* LDH release assay for neuronal cell death. A, effect of L-BMAA on LDH release; B, effect of D-BMAA on LDH release; C, effect of 3mM L-BMAA on LDH release in the presence of MK-801 (10 μM); D, effect of 1mM D-BMAA on LDH release in the presence of MK-801 (10 μM); E, effect of 1mM D-BMAA on LDH-release in the presence of NBQX (10 μM) and NASPM (10 μM). Bars show % neuronal death (mean + SEM, n = 8-16). * Indicates significant difference, $P < 0.05$ (one-way ANOVA followed by the Bonferroni *t*-test). N values represent individual wells on a plate, n = 8-16 indicate 2-4 independent experiments (cultures prepared from different sets of embryos).

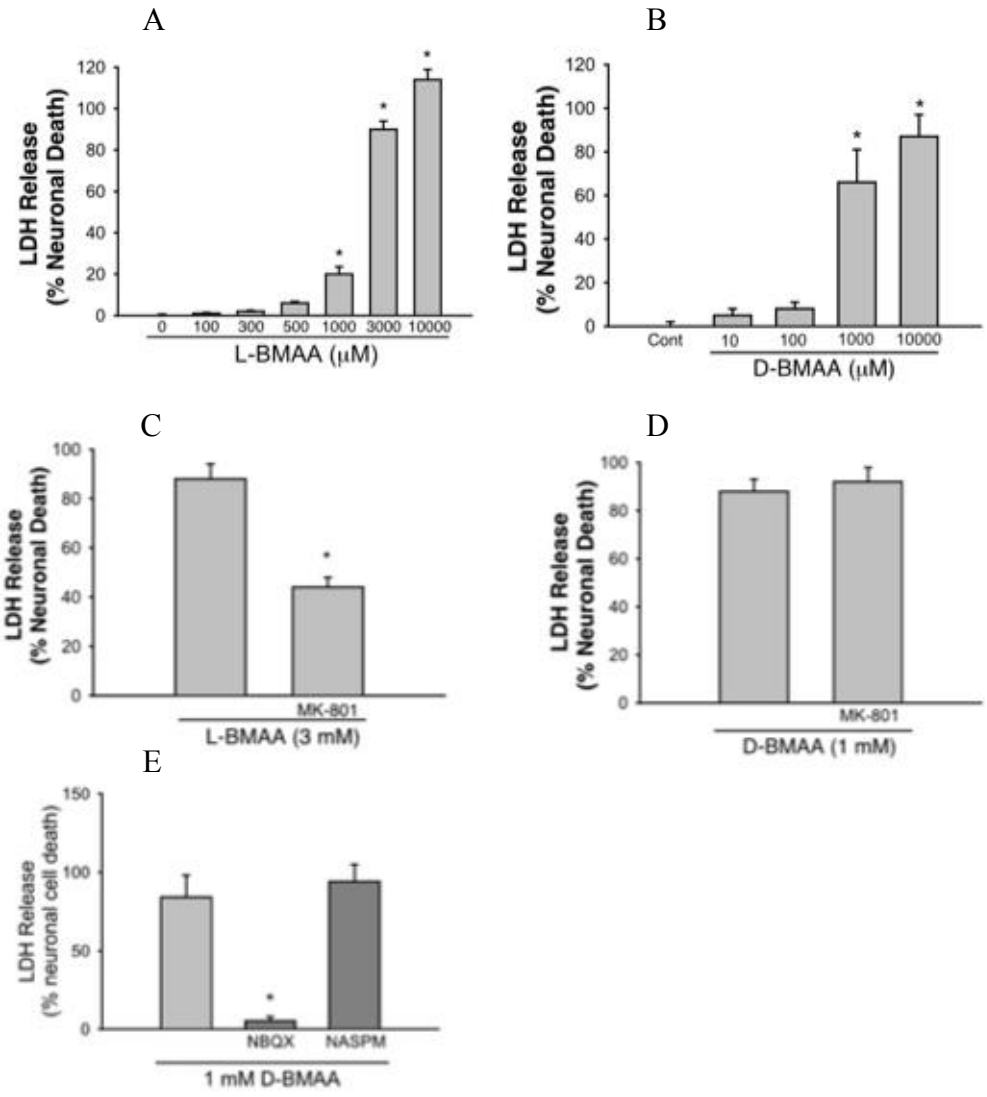
A
LDH Release
(% Neuronal Death)
120
100
80
60
40
20
0
0
100
300
500
1000
3000
10000
L-BMAA (μM)
B
LDH Release
(% Neuronal Death)
120
100
80
60
40
20
0
Cont
10
100
1000
10000
D-BMAA (μM)
C
LDH Release
(% Neuronal Death)
100
80
60
40
20
0
MK-801
L-BMAA (3 mM)
D
LDH Release
(% Neuronal Death)
100
80
60
40
20
0
MK-801
D-BMAA (1 mM)
E
LDH Release
(% neuronal cell death)
150
100
50
0
NBQX
NASPM
1 mM D-BMAA

Table 1. Daughter ion ratios for AQC-derivatised BMAA isolated from fractionation of an underivatised extract from cycads and cyanobacteria after selection for parent ion at *m/z* 459 in comparison with a BMAA standard.

Daughter ion	Daughter ion ratios (Cycad)		
m/z	BMAA std	L-BMAA fraction	D-BMAA fraction
119	15.6	17.2	16.5
171	100	100	100
188	0	0	0.2
289	33.4	28.8	54.8

	Daughter ion ratios (Cyanobacteria)				
Daughter ion *m/z*	BMAA std	Blank inj* L-BMAA	*Nostoc* L-BMAA	Blank inj* D-BMAA	*Nostoc* D-BMAA
119	18.45	0.66	12.5	8.49	0.8
171	100	100	100	100	100
188	0.32	0.87	0.86	0.35	0.19
214	0.03	16.79	0.51	2.89	1.63
258	2.06	1.4	3.34	1.4	0.3
289	28.25	0.7	23.35	0.7	20.52

* An equivalent set of blank injections and chiral fractionations were carried out to compare to the cyanobacterial TCA extract chiral fractionation.

Table 2. BMAA daughter ion ratios for L- and D-BMAA fraction windows collected from mouse (liver, hind brain) and vervet tissues (plasma, cerebral spinal fluid) from animals dosed with L-BMAA in comparison with BMAA standards.

Daughter ion	BMAA std	Mouse liver		Mouse hindbrain	
m/z		L- fraction	D-fraction	L-fraction	D-fraction
119	15.1	15.1	10.3	12.5	15.8
171	100	100	100	100	100
289	31.1	29.6	42.2	32.5	31.0
		Vervet plasma		Vervet CSF	
		L-fraction	D-fraction	L-fraction	D-fraction
119	17.6	17.3	25.8	0.6	17.0
171	100	100	100	100	100
214	0	0.1	2.2	7.0	0.4
258	6.2	4.0	3.3	10.3	1.9
289	31.2	29.5	32.8	2.4	30.2