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Abstract

There is evidence that the environmental toxin β-N-methylamino-L-alanine (L-BMAA) may be involved in neurodegenerative diseases. However, a number of controversies exist regarding L-BMAA, one of

which is the possibility that when assaying for L-BMAA, its isomers are being detected instead. There are at least four isomers of BMAA that are known to occur: L-BMAA, β-N-methylamino-D-alanine (D-BMAA), 2,4-diaminobutyric acid (DAB), and N-(2-aminoethyl)glycine (AEG). The fact that isomers of BMAA exist in nature also leads to the possibility that they are involved in toxicity. We set out to determine both the potency and the mechanism of toxicity of L-BMAA, D-BMAA, DAB, asnd AEG using primary cortical cultures. The results were surprising with the following order of potency of toxicity: AEG > DAB > D-BMAA > L-BMAA. These results suggest that AEG may be an overlooked neurotoxin. We found that AEG induced toxicity through mGluR5 receptors and induction of oxidative stress. While the potential role of L-BMAA in neurodegenerative diseases has been emphasized, other isomers of L-BMAA, particularly AEG, are actually more potent toxins, and could therefore potentially contribute to neurodegenerative diseases.

Keywords

β-N-Methylamino-L-alanine, Neurotoxin, Oxidative stress, Cyanobacteria

1. Introduction

L-BMAA is a non-protein amino acid neurotoxin produced by cyanobacteria that was first implicated in the neurodegenerative disease amyotrophic lateral sclerosis/Parkinson's Dementia Complex (ALS/PDC) on Guam (Cox et al., 2003). Since these initial reports, a number of studies have suggested that L-BMAA may be involved in neurodegenerative diseases, not only on Guam, but also throughout the world. First, cyanobacteria widely distributed around the world have been shown to produce L-BMAA (Cox et al., 2005; Esterhuizen and Downing, 2008; Metcalf et al., 2008). Second, L-BMAA is biomagnified in systems not only on Guam (Cox et al., 2003), but also in the Baltic Sea (Jonasson et al., 2010), the Gulf of Mexico (Brand et al., 2010), France (Réveillon et al., 2014), and China (Li et al., 2019). Third, L-BMAA can become protein-associated which allows it to build up in tissue and provides a mechanism for slow release (Murch et al., 2004). Fourth, L-BMAA is found not only in brain samples of ALS/PDC patients in Guam, but also in the brains of Alzheimer's disease and ALS patients from North America (Pablo et al., 2009). Fifth, clusters of ALS patients have been found around cyanobacteria producing lakes (Caller et al., 2009), with aerosolization of L-BMAA being a possible route of exposure (Stommel et al., 2013). These results suggest that L-BMAA may be of concern not only for people on select pacific islands, but may represent a wider health concern.

However, a number of controversies exist regarding L-BMAA, one of which is the possibility that when assaying for L-BMAA, isomers of L-BMAA are detected instead. This is of particular importance since there is debate concerning the presence of L-BMAA under various conditions. For example, L-BMAA is not always detected in the brains of neurodegenerative disease patients (Montine et al., 2005) or even in cyanobacteria (Rosen and Hellenas, 2008). One explanation proposed for the discrepancies in the results is that the detection of isomers of L-BMAA may confound some studies (Jiang et al., 2012). The fact that isomers of L-BMAA exist in nature, also leads to another possibility, that is, it is possible that these isomers are also involved in toxicity. BMAA has at least four isomers that are known to occur: L-BMAA and its mirror image D-BMAA, 2,4-diaminobutyric acid (DAB), and N-(2-aminoethyl)glycine (AEG). There are numerous studies showing the presence of L-BMAA, DAB, and AEG in biological samples (reviewed by: Lance et al., 2018) and they each appear to be produced by cyanobacteria,

diatoms, and dinoflagellates (Chatziefthimiou et al., 2018). There is only one study regarding the presence of D-BMAA in biological systems. Metcalf et al., (2017) did not find the presence of D-BMAA in cyanobacteria. However, they did find that when vervets, or mice, were treated with L-BMAA, it was only D-BMAA that was detected in the animal's central nervous system.

Since isomers of L-BMAA exist in nature, it is possible that they are important not only for complicating the analysis of L-BMAA levels, but also as potential neurotoxins themselves. We set out to determine the toxicity of each of the BMAA isomers in primary cortical cultures.

2. Material and methods

2.1. Cortical cell cultures

Mixed cortical cell cultures containing neuronal and glial cells were prepared from fetal (15–16 day gestation) mice as previously described (Lobner, 2000). Briefly, dissociated cortical cells were plated on 24 well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Astrocyte-enriched glial cultures were prepared as described for mixed cultures except they were from cortical tissue taken from post-natal day 1–3 mice. Cultures were maintained in humidified 5% CO_2 incubators at 37 °C. Mice were handled in accordance with a protocol approved by our institutional animal care and use committee and in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2. Induction of neuronal death

Experiments were performed on mixed cortical cultures 13–15 days in vitro (DIV). Toxicity was induced by exposure to the toxic agents for 24 h in media as described for plating except without serum. Experiments were performed without serum to prevent interference from the many, and not completely characterized, compounds found in serum. However, this does add the potential confound of the media being different for the experiments from that in which the cells were grown. In experiments involving receptor antagonists or free radical scavenger compounds they were added at the same time as the toxins.

2.3. LDH release

Cell death was assessed in mixed neuronal and glial cultures by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 h after the beginning of the insult. Blank LDH levels were subtracted from insult LDH values, and results normalized to 100% neuronal death caused by exposure to 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 (Lobner, 2000).

2.4. 14C-cystine uptake

For ^{14}C-cystine uptake, astrocyte-enriched glial cultures were washed into HEPES buffered saline solution and immediately exposed to ^{14}C-cystine (0.025 μCi/mL) for 20 min with or without the toxins present. Following ^{14}C-cystine exposure, all cultures were washed with HEPES buffered saline solution and dissolved in 250 μL warm sodium dodecyl sulfate (0.5%). A 200 μL aliquot was removed and added

to scintillation fluid for counting. Values were normalized to ^{14}C-cystine uptake in untreated controls on the same experimental plate.

2.5. Statistical analysis

Differences between test groups were examined for statistical significance by means of one-way ANOVA followed by the Bonferroni correction post-hoc test, with $p < 0.05$ being considered significant.

3. Results

It has been known since the 1980's that L-BMAA is neurotoxic in cortical cell culture, but that the induction of neuronal death by L-BMAA required high concentrations (Weiss et al., 1989). We repeated the studies using L-BMAA in mixed neuronal and glial cortical cultures and found similar results, with no significant toxicity occurring until an L-BMAA concentration of about 1 mM (Fig. 1A). Also, as we have found previously, D-BMAA (Metcalf et al., 2017), is a slightly more potent neurotoxin than L-BMAA (Fig. 1B). We now tested the toxicity of DAB and AEG under the same conditions in cortical cultures. We found that DAB was also a more potent neurotoxin than L-BMAA (Fig. 1C), and that AEG was the most potent neurotoxin (Fig. 1D). The EC50 for the induction of neuronal death for each compound was as follows: L-BMAA 1463 µM; D-BMAA 445 µM; DAB 365 µM; AEG 18 µM.

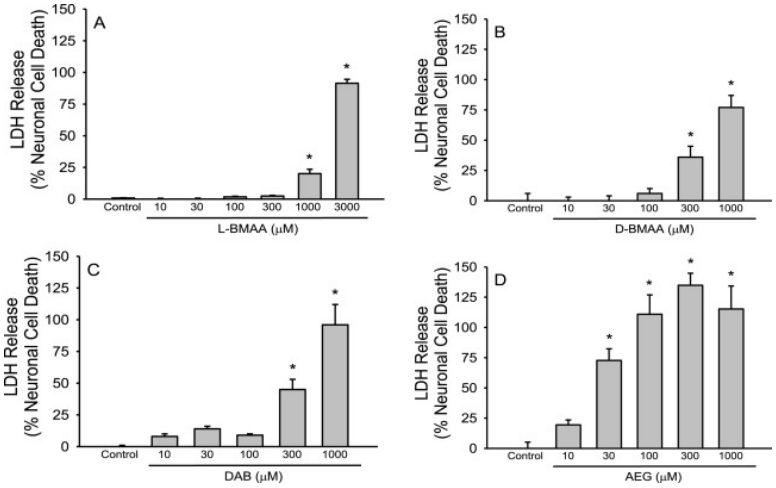

Fig. 1. Toxicity of varying concentrations of L-BMAA (A), D-BMAA (B), DAB (C), and AEG (D) following 24 h exposure of mixed cortical cultures to the toxins. Bars show % neuronal death (mean ± s.e.m, n = 8–16) quantified by measuring LDH release. * indicates significantly different from control.

We have previously determined the mechanism of L and D-BMAA toxicity in cortical cultures, with L-BMAA toxicity involving induction of free radicals as well as activation of NMDA and mGluR5 type glutamate receptors (Lobner et al., 2007), while D-BMAA toxicity was mediated by activation of AMPA type glutamate receptors (Metcalf et al., 2017). We now examined the mechanism of DAB and AEG toxicity. DAB toxicity was mediated by activation of NMDA receptors as toxicity was attenuated by the NMDA receptor antagonist memantine (Fig. 2A), while AEG toxicity was mediated by the induction of free radicals and the activation of mGluR5 receptors as its death was partially attenuated by the free radical scavenger Trolox and the mGluR5 receptor antagonist 6-methyl-2-[phenylethynyl]-pyridine (MPEP) (Fig. 2B). The AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo [f]quinoxaline (NBQX) had no effect on the toxicity of either compound (Fig. 2).

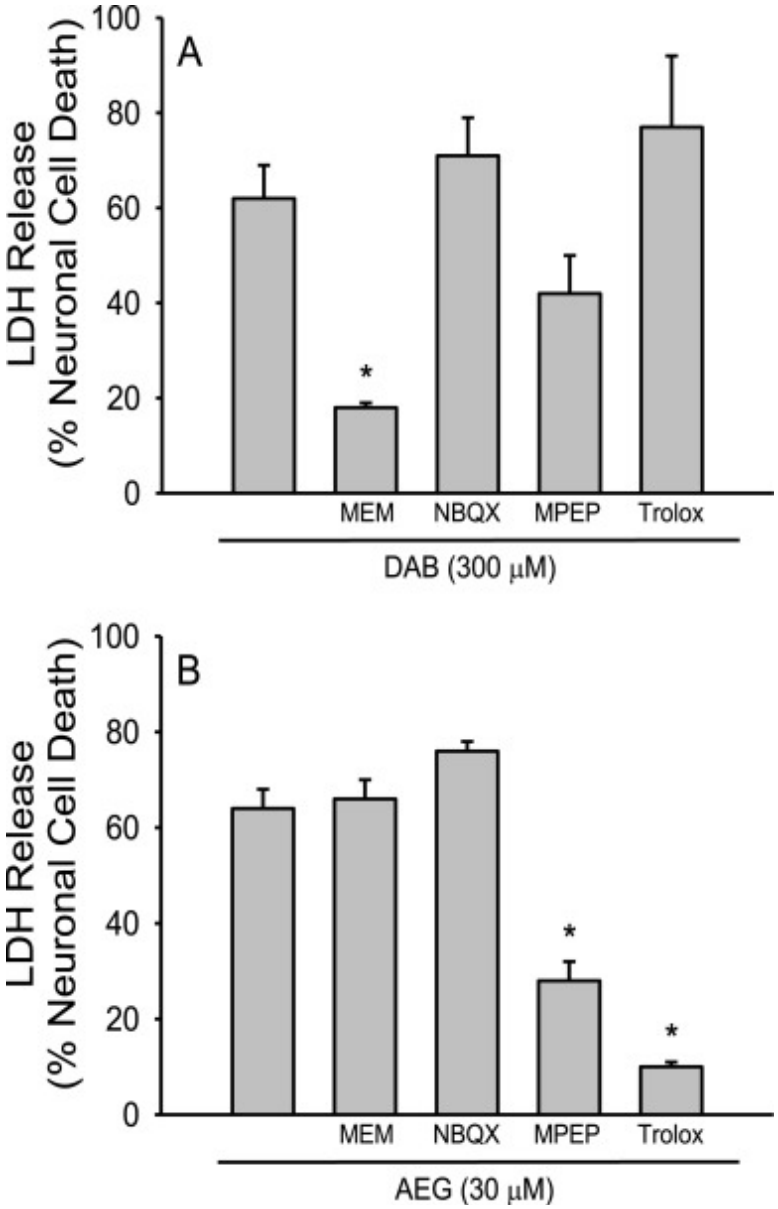

Fig. 2. Toxicity of DAB is mediated by NMDA receptors (A), and AEG by mGluR5 receptors and oxidative stress (B). Cell death assayed 24 h after exposure of mixed cortical cultures to the toxins with or without protective agents present. Bars show % neuronal death (mean ± s.e.m, n = 16) quantified by measuring LDH release. * indicates significantly different from control. MEM: 10 μM memantine, NBQX: 10 μM NBQX, MPEP: 50 μM MPEP, Trolox: 100 μM Trolox.

One possibility for induction of oxidative stress by L-BMAA and AEG is that they inhibit cystine uptake. Cystine is a precursor for production of the endogenous free radical scavenger, glutathione. In previous studies we have shown that about 80% of cystine uptake in astrocyte-enriched glial cultures is through system x_c- (Liu et al., 2012). Testing the effects of BMAA isomers on 14C-cystine uptake in astrocyte enriched glia cultures we found that L-BMAA at concentrations starting at 300 μM inhibited cystine uptake (3 A), while D-BMAA actually caused an increase in cystine uptake (Fig. 3B). DAB significantly decreased cystine uptake, but only at the high concentration of 1 mM (Fig. 3C). AEG decreased cystine uptake beginning already at a concentration of 10 μM, although the effect disappeared at the 1 mM concentration (Fig. 3D).

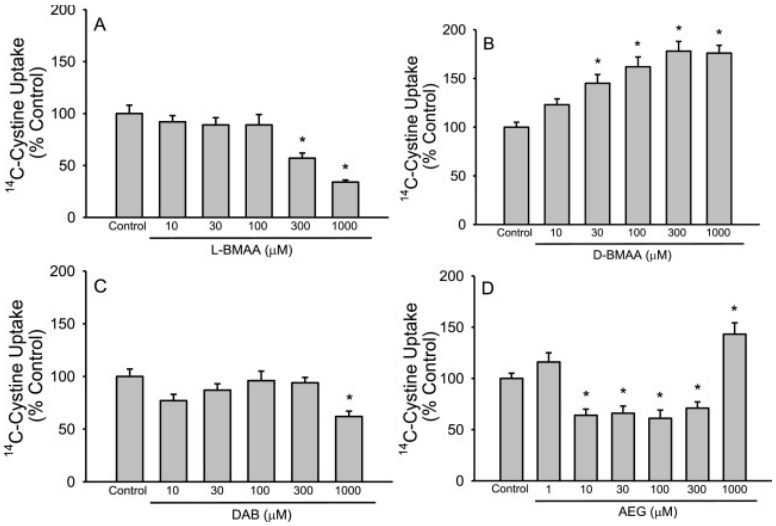

Fig. 3. ^{14}C-cystine uptake in astrocyte-enriched glial cultures in the presence of varying concentrations of L-BMAA (A), D-BMAA (B), DAB (C), and AEG (D). ^{14}C-cystine uptake was measured for 20 min with or without the toxins present. Results are expressed as mean ± s.e.m (n = 12) after normalizing to untreated control uptake. * indicates significantly different from control.

4. Discussion

The most surprising results obtained were that L-BMAA was a less potent toxin than its isomers and that AEG was by far the most potent neurotoxin. The importance of these findings is dependent on whether humans are likely to be exposed to these isomers. There are numerous studies involving the measurement of L-BMAA, and its isomers DAB and AEG, in the environment (reviewed by: Lance et al., 2018). Each of these isomers have been shown to accumulate in organisms that are consumed by humans, such as, mussels (Reveillon et al., 2014, 2015), oysters (Réveillon et al., 2014), ansd shark fins (Mondo et al., 2014). It is a harder to determine which of the compounds occur at the highest level in nature, as not all isomers are measured in each study, and results vary widely, likely dependent of the assay method. However, it is of interest that in shark fins, AEG levels were about 10 times higher than L-BMAA or DAB (Mondo et al., 2014). There is only one study regarding the presence of D-BMAA. Metcalf et al., (2017) did not find D-BMAA in cyanobacteria. However, they did find that when vervets or mice were treated with L-BMAA, there was only D-BMAA found in the animal's central nervous system, suggesting a conversion from L-BMAA to D-BMAA. Therefore, exposure of humans to each of these compounds is possible.

The current study was performed in cell culture and therefore does not directly address the question of toxicity due to in vivo exposure. L-BMAA toxicity has been studied in animal models, with variable results. Oral intake of L-BMAA by rodents has not shown to have any effects (Perry et al., 1989; Cruz-Aguado et al., 2006). Although oral consumption of L-BMAA induces features of neurodegenerative diseases in vervets (Banack and Cox, 2018) and there are numerous studies showing neurotoxicity caused by injections of L-BMAA in rodents (Caller et al., 2018). There is some evidence that DAB can have neurological effects in animals. Intraperitoneal injection of DAB in rats can cause convulsions (Chen et al., 1972) and enhance catalepsy induced by a GABA uptake inhibitor (Williams and Davies, 1979). There are no studies of D-BMAA or AEG toxicity in an animal model.

Correlating the concentrations of L-BMAA isomers tested in these studies to the concentrations found in the most relevant condition, that is, in the interstitial fluid surrounding neurons and astrocytes in the central nervous system is very difficult. Certain relevant facts are known about L-BMAA. It has been shown that approximately 80% of oral L-BMAA is absorbed (Duncan et al., 1991) and it is transported into the brain where it accumulates in a number of regions, including the hippocampus (Karlsson et al., 2009). The amount of L-BMAA found in the brains of Alzheimer's disease patients from North America was about one-fifth that found in ALS/PDC patients from Guam (111 ± 14 μg/g vs. 627 ± 141 μg/g). However, much of this L-BMAA is in a form described as protein associated and how this correlates with free L-BMAA in the interstitial fluid in the brain is unknown. Much less is known about the absorption, transport into the brain, and brain accumulation of the other BMAA isomers. These questions will need to be answered to the determine the importance of the current studies.

We have shown previously that toxicity of L-BMAA in cortical culture is mediated by action at NMDA and mGluR5 receptors, as well has induction of free radicals (Lobner et al., 2007), while D-BMAA induces neuronal death specifically through action at AMPA receptors (Metcalf et al., 2017). The current study indicates that DAB causes neurotoxicity by acting at NMDA receptors, while AEG acts both at mGLuR5 receptors and induces oxidative stress. Therefore, the common feature of L-BMAA and AEG toxicity is the induction of oxidative stress. The results are generally consistent with inhibition of cystine uptake through system x_c-playing a role in the oxidative stress mediated toxicity of L-BMAA and AEG. System x_c-is a sodium-independent amino acid transporter found primarily on astrocytes, which takes up one molecule of cystine into the cell in exchange for one molecule of glutamate (Bannai and Kitamura, 1980; Bannai, 1986). The cystine that is imported is quickly reduced to cysteine and used to synthesize glutathione (GSH) (Bannai and Kitamura, 1980). GSH is the main endogenous antioxidant in the brain and protects against oxidative stress; it can be released by astrocytes and used to protect neurons (Shih et al., 2006). Both of the compounds inhibit cystine uptake, but AEG is much more potent at both this action and at inducing neuronal death. In fact, the concentrations of L-BMAA and AEG to induce toxicity and their ability to inhibit cystine uptake are generally consistent. The compounds undoubtedly have actions other than effects on system x_c-. For example, L-BMAA can also act downstream in the glutathione pathway by inhibiting glutathione peroxidase and glutathione reductase (Esterhuizen-Londt et al., 2011). It is unknown whether AEG also has this action and it has in general been studied much less than L-BMAA. Also, the fact that AEG no longer inhibits cystine uptake at a concentration of 1 mM, yet is highly toxic at that concentration, indicates that it can induce toxicity through other mechanisms at high concentrations.

The reason for the difference in the effects of L-BMAA and D-BMAA, particularly on cystine uptake, where L-BMAA causes a decrease, while D-BMAA causes an increase, is not known. One possibility for the difference is that the function of L-BMAA is known to be altered by its interaction with bicarbonate to form a carbamate adduct. All of the current experiments were performed in the presence of bicarbonate. It is not known whether D-BMAA has a similar interaction with bicarbonate, but if it doesn't have this interaction, this could potentially account for its very different actions than L-BMAA.

A previous cell culture study failed to show the high potency of AEG toxicity that we observed (Main and Rodgers, 2018). However, that study was performed in a neuroblastoma cell line (SH-SY5Y cells) which likely has different sensitivity to various types of injury. For example, they observed that L-BMAA

toxicity was mediated by endoplasmic reticulum stress and disturbances in protein synthesis, folding, and turnover. The type of toxicity observed in the current study involved activation of glutamate receptors and effects on system x_c^-likely play a greater role in primary neurons than in cell lines. The fact that oxidative stress and glutamate receptor mediated toxicity occur during neurodegenerative diseases (Fan et al., 2017) suggests that the toxicity observed by BMAA isomers such as AEG in these studies may be relevant to disease conditions.

5. Conclusion

All four BMAA isomers are toxic, with L-BMAA surprisingly being the least potent toxin and AEG being the most potent. To date, emphasis has been placed on studying the potential role of L-BMAA in neurodegenerative diseases, however the potential exists that its isomers could contribute to these diseases. The production, distribution, and role in disease of BMAA isomers should be further studied.

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CRediT authorship contribution statement

Thomas Schneider: Writing - original draft, Methodology, Software, Investigation. **Catherine Simpson:** Investigation, Data curation. **Prachi Desai:** Investigation, Data curation. **Madeleine Tucker:** Supervision, Conceptualization, Validation, Writing - review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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