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L-BMAA Induced ER Stress and Enhanced Caspase 12 Cleavage in Human Neuroblastoma SH-SY5Y Cells at Low Nonexcitotoxic Concentrations

Oliver Okle,* Kerstin Stemmer,*,† Ulrich Deschl,‡ and Daniel R. Dietrich*,[1]

**Department of Human and Environmental Toxicology, University of Konstanz, 78457 Konstanz, Germany; †Institute for Diabetes and Obesity, Helmholtz Centre Munich, 85764 Neuherberg, Germany; and ‡Nonclinical Drug Safety Germany, Boehringer Ingelheim Pharma GmbH & Co. KG, 88397 Biberach an der Riß, Germany*

[1]To whom correspondence should be addressed at Department of Human and Environmental Toxicology, University of Konstanz, Jacob-Burckhardstrasse 25, 78457 Konstanz, Germany. Fax: +49 (0) 7531/88 3170. E-mail: daniel.dietrich@uni-konstanz.de.

The cyanobacterial β-N-methylamino-L-alanine (L-BMAA) is described as a low-potency excitotoxin, possibly a factor in the increased incidence of amyotrophic lateral sclerosis (ALS) and Parkinsonism-dementia complex (PDC) in Guam. The latter association is intensively disputed, as L-BMAA concentrations required for toxic effects exceed those assumed to occur via food. The question thus was raised whether L-BMAA leads to neurodegeneration at nonexcitotoxic conditions. Using human SH-SY5Y neuroblastoma cells, L-BMAA-transport, incorporation into proteins, and subsequent impairment of cellular protein homeostasis were investigated. Binding of L-BMAA to intracellular proteins, but no clear protein incorporation was detected in response to ^{14}C-L-BMAA exposures. Nevertheless, low L-BMAA concentrations (≥ 0.1mM, 48 h) increased protein ubiquitination, 20S proteasomal and caspase 12 activity, expression of the endoplasmic reticulum (ER) stress marker CHOP, and enhanced phosphorylation of elf2α in SH-SY5Y cells. In contrast, high L-BMAA concentrations (≥ 1mM, 48 h) increased reactive oxygen species and protein oxidization, which were partially ameliorated by coincubation with vitamin E. L-BMAA-mediated cytotoxicity was observable 48 h following ≥ 2mM L-BMAA treatment. Consequently, the data presented here suggest that low L-BMAA concentrations result in a dysregulation of the cellular protein homeostasis with ensuing ER stress that is independent from high-concentration effects such as excitotoxicity and oxidative stress. Thus, the latter could be a contributing factor in the onset and slow progression of ALS/PDC in Guam.

Key Words: **L-BMAA; neurotoxin; unfolded protein response; ALS/PDC; ER stress; ER-associated degradation.**

Amyotrophic lateral sclerosis (ALS), i.e., familial ALS and the sporadic ALS (sALS), is a progressive neurodegenerative disease whereby the etiology of sALS is mostly unknown, albeit among other factors the exposure to β-N-methylamino-L-alanine (L-BMAA), a cyanobacterial neurotoxin, is discussed.

Exposure to L-BMAA may have contributed to a geographically clustered increased incidence of ALS/Parkinsonism-dementia complex (PDC) in the native Chamorro population of Guam Island (Cox *et al*., 2003; Spencer *et al*., 1987), whether as the sole etiological factor or in conjunction with the co-occurring neurotoxic and carcinogenic cycasin demonstrated to be statistically associated with the historical incidence of ALS/PDC in Guam (Zhang *et al*., 1996). The hypothesis of L-BMAA-induced ALS/PDC appears to be supported by a recent report of increased L-BMAA concentrations in the brains of ALS- and PDC-affected patients of the United States compared with Huntington Disease patients or brains from patients that died from other causes other than neurodegenerative diseases (controls) (Pablo *et al*., 2009). However, only a few scientists have striven for a mechanistic explanation for the involvement of L-BMAA in neurodegeneration and ALS/PDC. Most of these publications focused on NMDA and mGluR5 receptor kinetics and therefore on the "excitotoxic mechanism" hypothesis of L-BMMA exposure (Kisby and Spencer, 2011). However, L-BMAA excitotoxicity occurs *in vivo* in rodents only at very high L-BMAA concentrations (> 100 mg/kg bw) (Duncan *et al*., 1991), i.e., far above the concentrations of L-BMAA the indigenous Chamorro could have been exposed to chronically (Duncan *et al*., 1990). Indeed, Duncan *et al*. (1991) calculated the worst-case daily exposure to L-BMAA in Chamorro at 0.36 mg/kg bw and the cumulative exposure at 10.8 mg/kg bw/month. The latter large discrepancies were also the grounds upon which L-BMAA was initially excluded as etiological agent of ALS/PDC. Moreover, overt excitotoxicity would suggest rapid development of neurodegenerative disorders, rather than the slow progression of ALS/PDC as observed following onset of the disease (Cucchiaroni *et al*., 2010). Due to the structural similarity of L-BMAA with alanine, the erroneous incorporation in cellular proteins during protein synthesis, thereby leading to protein misfolding, was hypothesized (Banack *et al*., 2010;

Field *et al*., 2011). Indeed, misfolded protein aggregation and deposition leading to apoptotic neuronal cell death is a hallmark of neurodegenerative disorders and prion diseases (Prusiner, 2012; Soto and Estrada, 2008). Moreover, current data strongly suggest that the ubiquitin proteasome system (UPS), and thus also the molecular processes of the unfolded protein response, and therefore endoplasmic reticulum (ER) stress (Schröder and Kaufman, 2005) play a central role in the clearance of abnormal, misfolded and oxidized, proteins (Bové *et al*., 2006; Goldberg, 2003). Failure of the UPS or protein structural changes, e.g., via reactive oxygen species (ROS) damage, can lead to the inability of neuronal cells to degrade ubiquitinated proteins (Sherman and Goldberg, 2001).

L-BMAA readily passes through the blood-brain barrier into the brain of cynomolgus monkeys and rats following iv injection (Duncan *et al*., 1992; Karlsson *et al*., 2009). Moreover, L-BMAA was reported to be either incorporated (Banack *et al*., 2010) or tightly associated with proteins (Banack *et al*., 2006; Murch *et al*., 2004; Pablo *et al*., 2009). Therefore, it is likely that neuronal uptake of L-BMAA could lead to misincorporation during protein synthesis, as shown for other nonproteinogenic amino acids (Hartman *et al*., 2007). The latter potentially results in protein misfolding and oxidation, thus leading to dysregulation of protein homeostasis with ensuing ER stress at L-BMAA concentrations lower than those known to elicit an excitotoxic response.

Human neuroblastoma cells (SH-SY5Y), lacking an active NMDA receptor (Jantas *et al*., 2008), were employed to investigate the nonexcitotoxic effects of low L-BMAA concentrations. The latter demonstrated that L-BMAA has an association with proteins, albeit covalent incorporation was not demonstrable, and that nonexcitotoxic or cytotoxic concentrations of L-BMAA led to increased protein oxidation and ubiquitination, 20S proteasomal and caspase 12 activity, expression of the ER stress marker CHOP, and enhanced phosphorylation of elf2α, thus suggesting that low L-BMAA concentrations (≥ 0.1mM for 48 h corresponding to ≥ 11.8 mg/l) lead to neuronal protein misfolding and ER stress and thus could be a contributing factor in the onset and progression of motor neuron disorders as observed in the case of ALS/PDC in Guam.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals, unless otherwise stated, were of the highest analytical grade commercially available. L-BMAA (Sigma-Aldrich) was dissolved in 10mM $NaHCO_3$. Nominal concentrations were used in the experiments.

Cell system. The human neuroblastoma cell line SH-SY5Y and human embryonic kidney (HEK) cell line HEK-293 cells (DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle medium (low glucose), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria) under standard condition (5% CO_2, 37°C) in flasks. Cyclic cells were used in all experiments. They were cultured in plates for 3 days under the same conditions above. On the third day, cells were starved by the reduction of FBS to 2.5%. The starvation did not result in a morphological change during the following 48 h treatment (Supplementary fig. 1). Passages 4–9 of SH-SY5Y cells and passages 3–6 of HEK-293 cells were used for all experiments.

Reverse transcription-PCR. Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) and reverse transcribed to cDNA using M-MuLV Reverse Transcriptase (New England BioLabs, Ipswich, MA). Subsequent PCR amplification was performed using OneTaq Polymerase (New England BioLabs) and an annealing temperature of 60°C for the large neutral amino acid transporter 1 (LAT1) primer pair LAT1L (5´-GAAGGCACCAAACTGGATGT-3´; 5´-GAAGTAGGCCAGGTTGGTCA-3´).

Radioactive assays. Uptake of radioactive ^{14}C-L-BMAA (^{14}C-methyl-L-BMAA; BIOTREND, Cologne, Germany) and ^{14}C-L-alanine (PerkinElmer LAS, Rodgau, Germany) was determined via liquid scintillation counting (LS 6500 liquid scintillation counter, Beckman, Munich, Germany). SH-SY5Y cells were treated with 9µM ^{14}C-L-BMAA for different time periods as indicated, washed 4× with modified PBS (136.9mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4; pH 7.4), lysed with 0.5M NaOH, acidified with 2M HCl, to avoid quenching by luminescence, and transferred to the scintillation cocktail (Quicksafe A, Zinsser Analytic, Frankfurt, Germany) for scintillation counting (5 min).

Cytotoxicity. Cytotoxicity of L-BMAA was determined measuring the reduction of thiazolyl blue tetrazolium bromide (MTT) in SH-SY5Y cells following L-BMAA treatment for 24, 48, and 96 h in 96-well plates in six technical replicates and in three independent experiments.

Fluorophotometric quantitation of oxidative stress. SH-SY5Y cells were pretreated for 45 min with the fluorescent ROS indicator dye 2´,7´-dichlorofluorescein diacetate (Sigma-Aldrich). After washing (3×) with modified PBS, SH-SY5Y cells were treated with various concentrations of L-BMAA. Fluorescence was recorded in the fluorescence detector (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland) after 45 min at 485 nm/528 nm (ex/em) (Zhao *et al*., 2007).

Slot blot analyses for oxidized proteins. Protein oxidation was equated to the number of carbonyl groups reacting with 2,4-dinitrophenyl hydrazine (DNPH, Sigma-Aldrich). After 48 h L-BMAA treatment, cells were lysed with ice-cold extraction buffer and centrifuged (17,500 × g, 20 min, 4°C). The cytoplasmic fraction was subsequently used for determination of carbonyl groups. Following an incubation with 1 volume 12% SDS and 2 volumes DNPH subsequently, the solution was neutralized with 1.5 volumes 2M Tris base/30% glycerol (Shacter, 2000). Oxidized proteins were quantified via slot blot analysis using a rabbit anti-DNP antibody (#D9656, Sigma-Aldrich).

Superoxide dismutase activity. Super oxide dismutase (SOD) activity was measured using 768 UI/l SOD from human erythrocytes, dissolved in modified PBS, and a spectrophotometric-based SOD Assay Kit (#19160-1KT-F, Sigma-Aldrich).

Caspase and proteasomal activity assays. Caspase 3/7 (Kit #G7790, Promega, Mannheim, Germany), Caspase 12 (Kit #K139-25 BioVision, CA), and 20S proteasomal activities (Kit #ABD-13456 AAT Bioquest, CA) were determined in technical duplicates using a 96-well-plate assay. Caspase 3/7 activity was determined measuring fluorescence at $\lambda_{em/ex}$ 485/527; caspase 12 activity at $\lambda_{em/ex}$ 400/505 and 20S proteasomal activity was determined after cleavage of LLVY-R110 at $\lambda_{em/ex}$ 498/520 (Infinite M200).

Immunodetection of proteins. Following treatment of SH-SY5Y with L-BMAA for 48 h, cells were lysed with ice-cold extraction buffer (10mM triethanolamine [Tris]-base, 140mM NaCl, 5mM EDTA, and 0.1% [vol/vol] Triton X-100; pH 7.5) and centrifuged (17,500 × g, 20 min, 4°C) to obtain a cytosolic fraction. Equal amounts of protein were loaded onto an SDS gel, and proteins were detected with specific antibodies such as anti-ubiquitin (#3933, Cell Signaling Technology, Boston, MA), anti-CHOP (#2895), anti-phospho-elf2α (#3398).

L-BMAA incorporation into proteins. Radioactive-labeled [1-14C]-L-BMAA (1.81mM; 2035 GBq/mmol) as well as ^{14}C-L-alanine (617µM; 5994 GBq/mmol) and an *in vitro* protein expression assay (Kit #88858, Pierce/Thermo Scientific, Rockford, IL) were employed to detect incorporation of L-BMAA during protein synthesis. Two micrograms of pCFE-GFP mRNA and amino acid mixtures without leucine were used in the human translation system. Radioactive-labeled proteins were precipitated on ice (20 min) using 0.2 volumes 100% trichloroacetic acid (TCA). After centrifugation (5 min, 10,000 × g) pellets were washed 3× with ice-cold acetone. Protein pellets were dissolved in tissue solubilizer (Biolute-S, SERVA, Heidelberg, Germany), transferred to scintillation cocktail (Quicksafe A, Zinsser Analytic), and acidified with HCl (2M) to avoid quenching by luminescence; radioactivity was determined for 5 min in the LS 6500 liquid scintillation counter (Beckman). Before and after precipitation, the proteins were loaded to a reducing 10% SDS gel visualized by autoradiography in FLA-9000 (Fujifilm, Düsseldorf, Germany).

Statistical analyses. Statistics were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Specific statistical tests were chosen according to the data type generated: one-way ANOVA with Dunnett's posttest to compare values within an individual treatment group; two-way ANOVA with Bonferroni multiple comparisons test for comparison of concentration effects between treatment groups; *F*-test followed by a two-tailed *t*-test to compare the concentration between single-treatment groups. Results shown are mean ± SEM and were considered statistically significant when $p < 0.05$, with significance levels indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

RESULTS

Uptake of L-BMAA via *LAT1*

Expression of LAT1 was demonstrated at the mRNA and protein level and thus are potentially capable of transporting L-BMAA (Figs. 1A and 1B). Exposure of SH-SY5Y cells to 9µM ^{14}C-L-BMAA resulted in a time-dependent increase of radioactive L-BMAA, with an uptake equilibrium at ~20–40 min of ^{14}C-L-BMAA exposure (Fig. 1C). ^{14}C-L-alanine exposure resulted in a comparable uptake pattern. Coincubation of ^{14}C-L-BMAA with the Lat1 substrate L-leucine (10mM) or the Lat1 inhibitor 2-aminobicyclo-2,2,1-heptane-2-carboxylic acid (BCH, 22mM) for 17 min resulted in a significantly ($p < 0.05$) reduced uptake of ^{14}C-L-BMAA (Fig. 1D). L-BMAA uptake in SH-SY5Y cells is thus considered to occur via the Lat1, as suggested earlier by an *in vivo* as well as *in vitro* study in rodents and monkeys (Duncan *et al*., 1991, 1992; Kisby *et al*., 1988; Smith *et al*., 1992). However, the finding that L-BMAA uptake could be reduced by 40 or 20% only by the respective Lat1 inhibitors suggests the presence of other mono- or bidirectional L-BMAA transporters in SH-SY5Y cells.

L-BMAA Cytotoxicity

A significant reduction in cell viability was observed at 2mM L-BMAA following 48 h and at concentrations ≥ 1mM L-BMAA after 96 h of exposure (Figs. 2A–C). Consequently, effects observed at concentrations < 2mM L-BMAA in 48 h exposure settings, see below, are noncytotoxic and thus can be considered as not having an acute cytotoxic origin. Excitotoxicity of L-BMAA in SH-SY5Y cells could also be excluded as control experiments with NMDA and glutamic acid, both positive controls for NDMA- and mGlu receptor-mediated excitotoxicity, respectively, remained negative (Supplementary fig. 2). The latter is also at least partially supported by earlier reports on the absence of an active NMDA receptor in SH-SY5Y cells (Jantas *et al*., 2008).

A significantly increased caspase 3/7 activity, possibly a regulatory element for the apoptotic chromatin condensation (Supplementary fig. 3), was observed at L-BMAA concentrations ≥ 2mM only (Fig. 2D). The latter effects were ameliorated by addition of the ROS scavenger vitamin E (220µM).

L-BMAA Treatment Increased ROS and Oxidized Proteins

Treatment of SH-SY5Y cells with 0.1–5mM L-BMAA for 45 min resulted in a significant increase of ROS (Fig. 2E). Commensurate with the demonstrated increased ROS following L-BMAA treatment, a significantly increased level of oxidized proteins can be detected following 48 h exposure to 1mM L-BMAA (Fig. 2F). Coexposure with vitamin E significantly reduced the observed L-BMAA- or H_2O_2-induced ROS and oxidized protein levels (Fig. 2G). A direct inhibitory effect of L-BMAA on the cellular antioxidant system, e.g., SOD1 activity, appears unwarranted as only very high L-BMAA concentrations (≥ 10mM) had a significant inhibitory effect (Fig. 2H).

ROS-Independent L-BMAA-Induced ER Stress and Protein Binding

Exposure of SH-SY5Y cells to < 2mM concentrations of L-BMAA for 48 h (Fig. 2B) resulted in a significantly increased level of ubiquitinated proteins and proteasomal activity (Figs. 3A and 3C). Although coincubation with vitamin E (220µM) resulted in the expected reduction of ubiquitinated proteins (Fig. 3B) in the positive control H_2O_2, this ameliorative effect was not observed in the L-BMAA treated cells, thus suggesting that L-BMAA-induced effects on the ubiquitin/proteasome system are not ROS-related. Indeed, treatment of SH-SY5Y cells with 1mM L-BMAA for 48 h resulted in an increased phosphorylation of the ER stress marker (de Haro *et al*., 1996) elf2α translation inhibitor and an increased CHOP expression (Fig. 3D), a protein involved in ER stress-mediated apoptosis (Wang *et al*., 1996). These findings suggest that micromolar to millimolar concentrations of L-BMAA induce ER stress in SH-SY5Y cells. Moreover, L-BMAA treatment increased phosphorylation of PERK and expression of CHOP and BAG1 in human HEK-293 cells and THP1 cells (Supplementary figs. 4B and 4C), supporting the finding that L-BMAA induces ER stress in a cell autonomous manner.

Additional to the L-BMAA concentration-dependent increase of CHOP expression, a significantly increased caspase 12 activity (Fig. 3E) was observed at L-BMAA concentrations ≥ 0.5mM, comparable to that observed for the ER stress-positive control, thapsigargin. This increased caspase 12 activity, however, was not L-BMAA concentration-dependent.

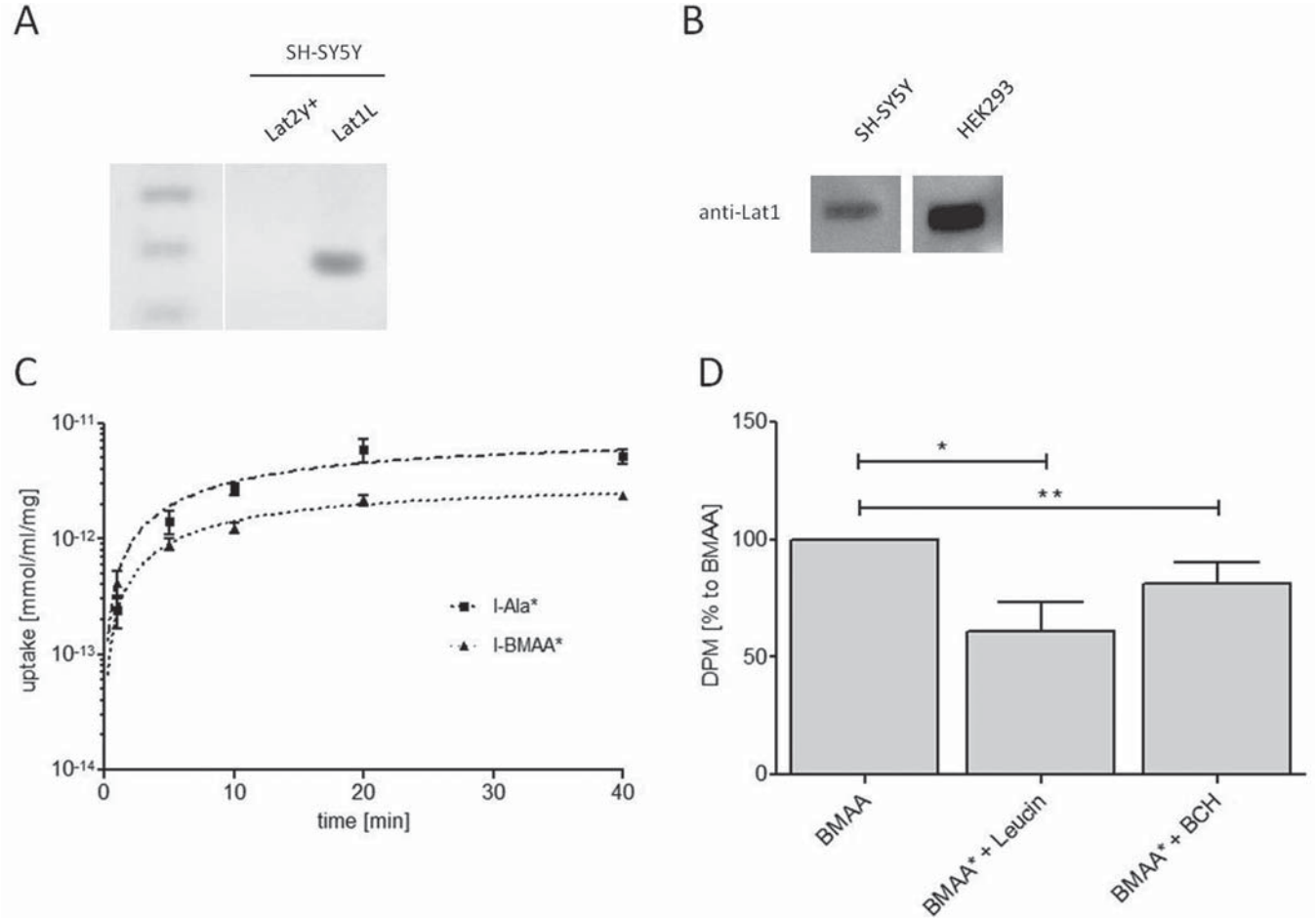

FIG. 1. (A) LAT1 expression at the mRNA and (B) protein level; the LAT1 protein expression in HEK-293 cells served as expression control. (C) Uptake of ^{14}C-L-BMAA (9.05µM) and ^{14}C-L-alanine (1.54µM) ($N = 5$). (D) Reduced uptake of ^{14}C-L-BMAA (9.05µM) cotreated with L-leucine (10mM) and BCH (22mM) for 17 min ($N = 5$). Values (mean ± SEM) are expressed as percentage of L-BMAA uptake; statistics: one-sample *t*-test.

L-BMAA-induced ER stress may have resulted from its incorporation during protein synthesis. Although ^{14}C-L-BMAA and ^{14}C-L-alanine were detectable following a TCA-mediated protein precipitation (Fig. 3F), this was not the case for L-BMAA after protein denaturing SDS gel electrophoresis (Supplementary fig. 5). The latter findings may suggest that the observed radioactivity in the precipitated protein following L-BMAA incubation most likely resulted from a strong association of L-BMAA with newly synthesized proteins and/or that covalently incorporated L-BMAA was too low to be detected with the method chosen.

DISCUSSION

In the continuing dispute of the potential causal involvement of L-BMAA in the etiology of ALS/PDC in the Chamorro population of Guam, the most critical fact arguing against a role of L-BMAA is that the daily doses to which Chamorros could have been exposed to via cycad flour are several orders of magnitude lower (Duncan *et al*., 1990; Kisby *et al*., 1988) than those used in animal models (Chiu *et al*., 2011) that displayed ALS- or PDC-like symptoms. Additionally, there is little evidence that fruit bats were a major dietary component of the Chamorro (Duncan and Marini, 2006) irrespective of their degree of L-BMAA contamination (Banack and Cox, 2003). However, the latter point entirely hinges upon the assumption that the mechanism underlying L-BMAA-induced ALS/PDC is based on the excitotoxic characteristics of L-BMAA (Holtcamp, 2012). Indeed, the concentrations of L-BMAA found to be excitotoxic to neuronal cells and motorneurons are so high (predominantly > 2mM; Chiu *et al*., 2011) that huge oral doses of L-BMAA would have to be ingested to arrive at neurotoxic concentrations (Duncan *et al*., 1991).

Yet as Kisby and Spencer (2011) and later Holtcamp (2012) correctly pointed out, L-BMAA may indeed have additional mechanisms by which it may interact with normal homeostasis and function of neuronal cells and thus possibly provide for continuous irreparable neuronal damage. The latter could act either by itself or in conjunction with cycasin to then provide

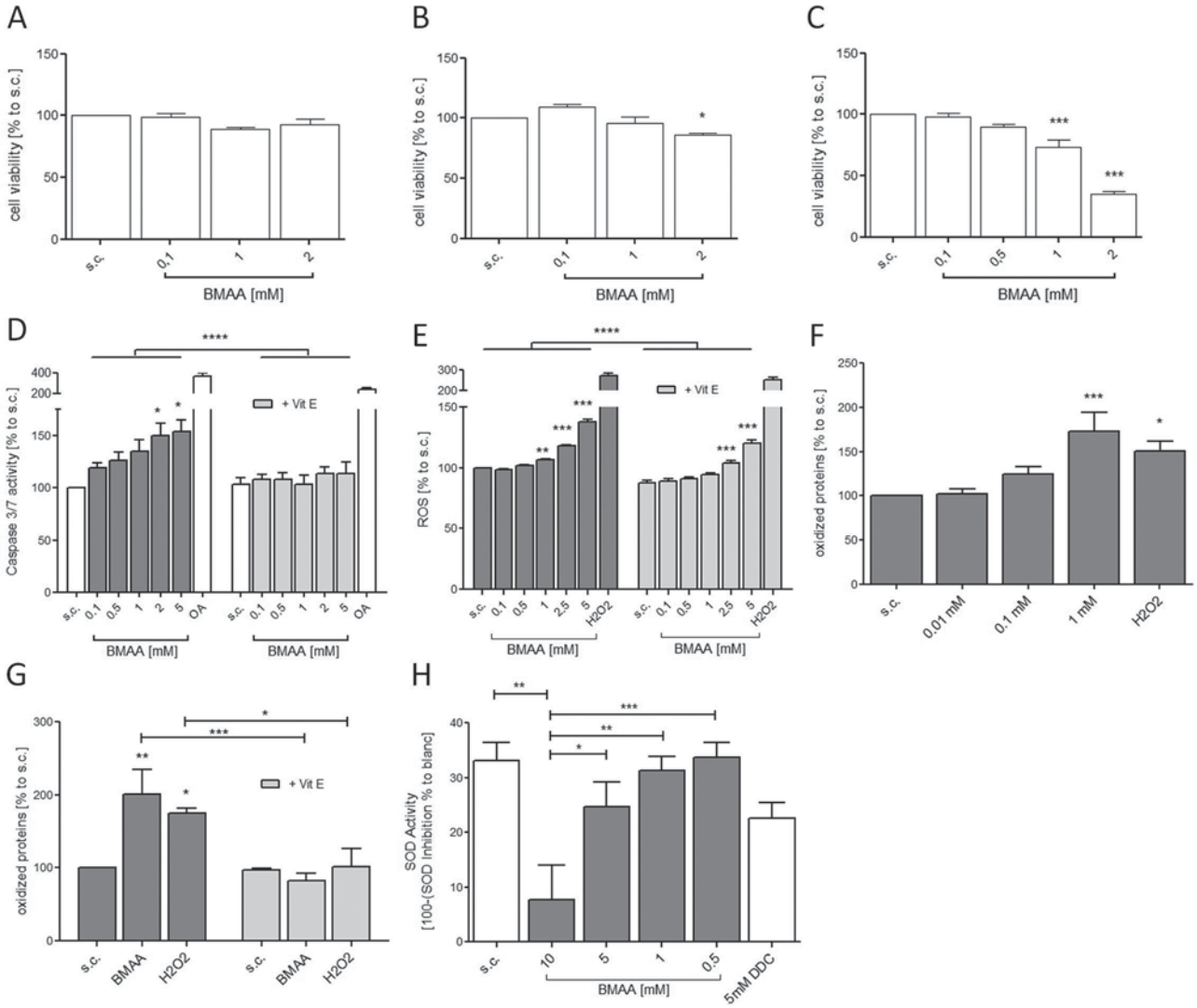

FIG. 2. Cell viability and ROS-linked effects of L-BMAA in SH-SY5Y cells. (A–C) Cell viability of SH-SY5Y cells following 24 h (A), 48 h (B), and 96 h (C) 0.1–2mM L-BMAA treatment ($N = 3$). Low cytotoxicity of 2mM L-BMAA was observed primarily in SH-SY5Y cells after 48 h (B). Following exposure for 96 h, a significant cytotoxicity was also observable for 1mM L-BMAA treatment (C). (D) Caspase 3/7 activity of SH-SY5Y cells treated for 48 h to 0.1–5mM L-BMAA or 30nM okadaic acid (OA), the positive control (−Vit E, $N = 10$; +Vit E, $N = 6$). (E) ROS in SH-SY5Y cells following exposure to 0.1–5mM L-BMAA or cotreated with vitamin E (220µM) for 45 min ($N = 7$). (F) Relative levels of oxidized proteins in SH-SY5Y cells treated with 0.01, 0.1, and 1mM L-BMAA ($N = 9$) and (G) cotreated with 1mM L-BMAA and vitamin E (220µM) for 48 h ($N = 5$). (H) Inhibition of the human erythrocyte SOD-1 *in vitro*; solvent control (s.c.), 0.5–10mM L-BMAA ($N = 5$).

for cumulate neurodegeneration that then leads to the observed symptoms of ALS/PDC. Prerequisite for neuronal effects of L-BMAA in humans is its capability to cross the blood-brain barrier and thus become biologically available. L-BMAA was demonstrated to cross the blood-brain barrier in rodents and monkeys following iv injection (Duncan *et al.*, 1991, 1992; Kisby *et al.*, 1988; Smith *et al.*, 1992). The latter could occur either by facilitated diffusion or transport by a specific transporter, e.g., LAT1 among others (Boado *et al.*, 1999). Indeed, L-BMAA blood-brain barrier transport was also demonstrated in rats (Smith *et al.*, 1992), which was competitively inhibited with the specific LAT1-substrate L-leucine and BCH, an inhibitor of LAT1. Similarly, LAT1 is functionally expressed in the undifferentiated human neuroblastoma SH-SY5Y cell line (Figs. 1A and 1B), whereby L-BMAA transport into SH-SY5Y cells (Fig. 1C) can be partially inhibited with L-leucine and BCH (Fig. 1D), suggesting that L-BMAA can enter SH-SY5Y cells also via other transporters or also by diffusion.

Treatment of SH-SY5Y cells with low L-BMAA concentrations (0.1–1mM) resulted in an increased ER stress as suggested by the increased caspase 12 and 20S proteasomal activities (Figs. 2 and 3), enhanced phosphorylation of PERK, and increased expression of CHOP and BAG1. The latter results would corroborate the observations that intracellular BMAA influences

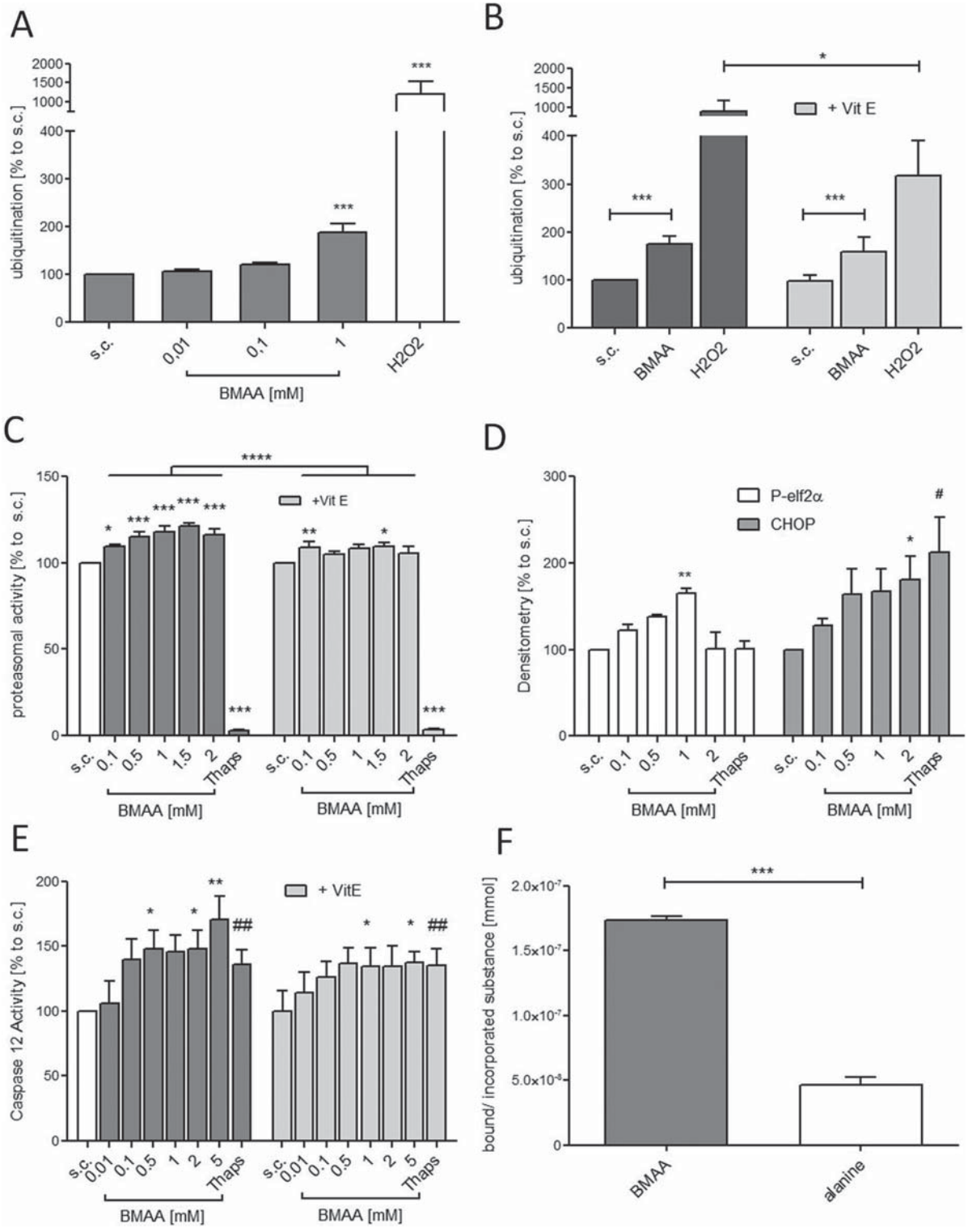

FIG. 3. Impairment of protein processing and degradation in SH-SY5Y cells following L-BMAA exposure. (A) Ubiquitinated proteins in SH-SY5Y cells treated with 0.01–1mM L-BMAA ($N = 4$) and (B) cotreated with 1mM L-BMAA and vitamin E for 48 h ($N = 7$). Coexposure of L-BMAA and vitamin E did not affect the ubiquitination status of the SH-SY5Y cells. (C) Proteasomal activity in SH-SY5Y cells treated with 0.1–2mM L-BMAA ($N = 10$) and cotreated with vitamin E for 48 h ($N = 6$). L-BMAA induces an increased chemotrypsin cleavage activity of the proteasome; 0.8nM thapsigargine (thaps) was used as proteasomal inhibition controls. (D) Increased CHOP expression and phosphorylation of elf2α ($N = 3$) in SH-SY5Y cells following L-BMAA exposure. (E) Caspase 12 activity of SH-SY5Y cells treated for 48 h with 10µM to 5mM L-BMAA (−Vit E, $N = 7$; +Vit E, $N = 5$). There are no statistical differences between the treatments with or without vitamin E; 30nM OA and 0.8nM thapsigargine (thaps) were used as control; one sample *t*-test to compare the thapsigargin control treatment with the solvent control, $p < 0.01$ (##). (F) Amount of radioactive "bound" ^{14}C-L-BMAA and ^{14}C-L-alanine in the human expression system after TCA precipitation ($N = 3$).

protein and mRNA synthesis in various brain regions of adult rats as reported by Kisby and Spencer (2011). Moreover, a modified protein homeostasis of various proteins was also reported in brain areas of rats possessing (neuro-) behavioral disabilities (Karlsson *et al*., 2012) upon exposure to L-BMAA, thus insinuating that L-BMAA adversely effects cellular protein homeostasis. As similar effects as observed for SH-SY5Y cells were also observed in human HEK-293 and THP1 cells (Supplementary fig. 4), the finding that low concentrations of L-BMAA adversely effect cellular protein homeostasis via ER stress, ROS, protein oxidation, and ubiquitination appears to be a general phenomenon in human cells. It is important to note that L-BMAA-induced ROS and ROS-associated protein oxidation were at least partially rescued via coincubation with the ROS scavenger vitamin E (Figs. 2E and 2G), as also observed previously by Liu *et al.* (2009) in cortical cell cultures of mice. This, however, was not the case for the L-BMAA-induced ubiquitination and increased caspase 12 and 20S proteasomal activity (Figs. 3B–D), thus suggesting that these are two entirely separate mechanisms by which L-BMAA affects protein homeostasis. The fact that, at least in SH-SY5Y cells, L-BMAA-increased caspase 3/7 activity was reduced following coincubation with vitamin E could suggest, on one hand, that caspase 3/7 activity, and thus the ensuing apoptosis (Supplementary fig. 3), is a result of the increased ROS, and on the other hand, that this occurs in SH-SY5Y cells only at L-BMAA concentrations ≥ 1mM. Consequently, lower (μM) L-BMAA concentrations could affect protein homeostasis without inducing immediate cell demise, whereas higher (mM) L-BMAA concentrations will induce ROS-mediated cell apoptosis, as also corroborated by previous *in vitro* investigations (Cucchiaroni *et al*., 2010). Above data thus suggest that L-BMAA-induced excitotoxicity most likely is not the predominant mechanism underlying the putative involvement of L-BMAA in ALS/PDC, as was also suggested in the recent review by Kisby and Spencer (2011). On the contrary, chronic exposure to low concentrations of L-BMAA and ensuing continuous dysregulated protein homeostasis may lead to a slow but continuous loss of motor neurons and neuronal cells and thus to the observed neurodegenerative effects reported. Indeed, similar mechanisms, i.e., involving increased caspase 12 activity and ER stress were reported to be at the core of neurotoxicity mediated by dysregulated protein homeostasis (Hetz *et al*., 2003; Rao *et al*., 2004). In addition, L-BMAA may be metabolized in the brain to a genotoxic metabolite, as reported for adult rats ip administered with 100 mg L-BMAA/kg bw (Kisby and Spencer, 2011). Thus, the lack of adverse effects of low concentrations of L-BMAA to organs other than the brain in humans, monkeys, and rodents acutely and chronically exposed to low concentrations of L-BMAA thus may lie in the difference of the regenerating potential of the respective organs and cells in dealing with the genotoxic formaldehyde and misfolded proteins generated.

The question thus remains as to how L-BMAA induces increased caspase 12 and 20S proteasomal activity. The latter may result from misincorporation of L-BMAA into proteins during protein synthesis (Field *et al*., 2011; Murch *et al*., 2004). It is noteworthy that the analysis of ^{14}C-L-BMAA incorporation in an *in vitro* protein synthesis assay (Fig. 3E) resulted in a "binding" of L-BMAA with proteins synthesized but did not provide clear evidence for incorporation of L-BMAA into proteins, albeit the latter may have failed due to restrictions of method sensitivity. Irrespective of the latter, some evidence demonstrating L-BMAA retention in the brain of L-BMAA exposed mice was provided by Karlsson *et al*. (2009) following iv injection of ^{3}H-L-BMAA (7.3 μg/kg) to 10-day-old mice, supporting the aforementioned "protein binding" of L-BMAA. Whether or not this "protein binding" of L-BMAA is of covalent nature, as suggested by various scientists (Banack *et al*., 2006; Murch *et al*., 2004; Pablo *et al*., 2009) to be the case for the Guam fruit bats, algae, and human brains, still needs to be indisputably proven.

In conclusion, the data presented here suggest an "interaction" of L-BMAA with intracellular proteins at low non-excitotoxic L-BMAA concentrations, resulting in dysregulated protein homeostasis and ER stress, and thus most likely in dysfunctional cells. Whether or not this "interaction" of L-BMAA requires covalent incorporation of L-BMAA into proteins remains to be determined. However, the current data do suggest that L-BMAA uptake into neuronal cells and the ensuing impaired intraneuronal protein homeostasis and also the formation of formaldehyde following exposure to low concentrations of L-BMAA, as suggested earlier by Kisby and Spencer (2011), could be a contributing factor in the scenario of chronic L-BMAA exposure, onset, and slow progression of neurodegenerative diseases, e.g., ALS/PDC in Guam.

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