

# Mechanisms of L-Serine Neuroprotection in vitro Include ER Proteostasis Regulation

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**Abstract**  $\beta$ -N-methylamino-L-alanine (L-BMAA) is a neurotoxic non-protein amino acid produced by cyanobacteria. Recently, chronic dietary exposure to L-BMAA was shown to trigger neuropathology in nonhuman primates consistent with Guamanian ALS/PDC, a paralytic disease that afflicts Chamorro villagers who consume traditional food items contaminated with L-BMAA. However, the addition of the naturally occurring amino acid L-serine to the diet of the nonhuman primates resulted in a significant reduction in ALS/PDC neuropathology. L-serine is a dietary amino acid that plays a crucial role in central nervous system development, neuronal signaling, and synaptic plasticity and has been shown to impart neuroprotection from L-BMAA-induced neurotoxicity both in vitro and in vivo. We have previously shown that L-serine prevents the formation of autofluorescent aggregates and death by apoptosis in human cell lines and primary cells. These effects are likely imparted by L-serine blocking incorporation of L-BMAA into proteins hence preventing proteotoxic stress. However, there are likely other mechanisms for L-serine-mediated neuroprotection. Here, we explore the molecular mechanisms of L-serine neuroprotection using a human unfolded protein response real-time PCR array with genes from the ER stress and UPR pathways, and western blotting. We report that L-serine caused the differential expression of many of the same genes as L-BMAA, even though concentrations of L-serine in the culture medium were

ten times lower than that of L-BMAA. We propose that L-serine may be functioning as a small proteostasis regulator, in effect altering the cells to quickly respond to a possible oxidative insult, thus favoring a return to homeostasis.

**Keywords** Unfolded protein response · ER stress · Neuroprotection · L-serine · L-BMAA

## Abbreviations

AD	Alzheimer's disease
AMPA	$\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ALS	Amyotrophic lateral sclerosis
ALS/PDC	Amyotrophic lateral sclerosis/parkinsonism-dementia complex
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BCA	Bicinchoninic acid
L-BMAA	$\beta$ -N-methylamino-L-alanine
BiP	Binding immunoglobulin protein
CHOP	C/EBP homologous protein
CNS	Central nervous system
Ct	Cycle threshold
eIF2	Eukaryotic initiation factor 2
eIF2 $\alpha$	Eukaryotic initiation factor 2 subunit alpha
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
IRE1	Inositol-requiring enzyme 1
Grp78	78 kDa glucose regulating protein
Hsp70	Heat shock protein 70
NFTs	Neurofibrillary tangles
PD	Parkinson's disease
PDI	Protein disulfide isomerase

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mGluR5	Metabotropic glutamate receptor 5
NMDA	<i>N</i> -methyl-d-aspartate receptor
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
Tg	Thapsigargin
UPR	Unfolded protein response
UTC	Untreated control
XBP1	X-box binding protein-1

## Introduction

$\beta$ -N-methylamino-L-alanine (L-BMAA) is a neurotoxic non-protein amino acid synthesized by cyanobacteria that has been linked to Guamanian amyotrophic lateral sclerosis/Parkinsonism-dementia complex (ALS/PDC) (Cox et al. 2003) and Alzheimer's disease (AD) (Endres and Reinhardt 2013). It has also been suggested that chronic exposures to cyanobacterial blooms elsewhere in the world may be linked to increased risks of sporadic amyotrophic lateral sclerosis (ALS) (Bradley and Mash 2009; Bradley et al. 2013, Caller et al. 2015). Several mechanisms of BMAA-induced cell death have been reported, including interactions with the metabotropic glutamate receptor 5 (mGluR5), the *N*-methyl-d-aspartate (NMDA) receptor, kainate, the cystine/glutamate antiporter (system  $x_c^-$ ) and the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Rao et al. 2006; Lobner 2009; Liu et al. 2009; Arif et al. 2014). In vitro studies using human cell cultures have demonstrated that misincorporation of L-BMAA into nascent proteins leads to the formation of autofluorescent deposits in the cytosol and nucleus, increased activity of lysosomal proteolytic enzymes and the induction of endoplasmic reticulum (ER) stress and apoptosis (Okle et al. 2012; Dunlop et al. 2013; Shen et al. 2016).

L-serine is a naturally occurring amino acid that plays a crucial role in central nervous system (CNS) development, neuronal signaling, and synaptic plasticity [(Tabatabaie et al. 2010) and see also review in this issue (Metcalf et al. 2017)]. In vivo studies with nonhuman primates whose diet was supplemented with equal amounts of L-serine and L-BMAA over 140 days, showed significantly fewer neurofibrillary tangles (NFTs) than those dosed with L-BMAA alone (Cox et al. 2016); and control animals showed no NFT neuropathology. Although L-serine inhibits misincorporation of L-BMAA into proteins (Dunlop et al. 2013), the consistent ratio of protein-bound to total L-BMAA in brain tissues of animals in the different treatments suggested the likelihood of other mechanisms of L-serine neuroprotection (Cox et al. 2016).

Endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) is associated with neurodegenerative diseases but whether this process is a cause or a consequence of neurodegeneration has not been clarified (Doyle et al. 2011). The UPR is a protective mechanism that

responds directly and rapidly to the accumulation of misfolded and/or unfolded proteins in the lumen of the ER by activating a signaling cascade in the cytosol, mediated via transmembrane molecular sentinels in the ER membrane (Walter and Ron 2011). This response helps to clear unfolded proteins by increasing the size of the ER and the concentration of molecular chaperones within it and by decreasing the influx of newly made proteins into the ER (Walter and Ron 2011). Activation of the UPR results in both attenuation of translation of new proteins and transcriptional activation of stress response genes which serves to relieve ER stress and return cells to homeostasis (Walter and Ron 2011). However, sustained activation of the UPR can target the cell for death through apoptosis.

Little is known about the mechanisms by which L-serine imparts neuroprotection. A recent phase I human clinical trial of ALS patients taking L-serine supplements (30 g/day), demonstrated an 85 % reduction in disease progression as measured by the slope of ALSFRS-R scores (versus placebo) (Levine et al. 2016). But even at the lowest dose of L-serine (1 g/day), patients showed a 14 % reduction in the slope of ALSFRS-R scores, indicating that neuroprotection imparted by L-serine is dose-dependent. In addition to studies with non-human primates (Cox et al. 2016), this observed neuroprotective effect of L-serine is supported by studies in human cell cultures that show L-serine protects against the induction of apoptosis by preventing misincorporation of L-BMAA in the place of L-serine residues in proteins (Dunlop et al. 2013).

In an effort to better understand the mechanisms of L-serine neuroprotection, we utilized a real-time PCR array to examine the effect of L-BMAA and L-serine on the differential expression of genes, and western blotting to measure changes to key proteins from the ER stress and UPR pathways.

## Materials and Methods

**Reagents** L-BMAA (Cat. No. B107), L-serine (Cat. no. S4311), Tri Reagent® (Cat. no. T9424), 2-propanol (Cat. no. I9516), 1-bromo-3-chloropropane (Cat. no. B9763), and thapsigargin (Cat. no. T9033) were purchased from Sigma Aldrich and were of molecular biology grade where appropriate.

**Cell Culture** SH-SY5Y human neuroblastoma (ATCC® CRL-2266™) were brought out of cryo storage at passage doubling (PD) 11 and passaged to PD 23, by seeding in 25 cm<sup>2</sup> flasks, then culturing in 150 cm<sup>2</sup> flasks. Cell culture methods have been described previously (Dunlop et al. 2017, in this issue). Treatments were as follows: no treatment (UTC), L-serine 100  $\mu$ M (L100), L-BMAA 1000  $\mu$ M (B1000) for 17 h. For Grp78 western blots, a positive control of thapsigargin (Tg) was dissolved in DMSO to make a 1 mM

stock, then this was stored at  $-20\text{ }^{\circ}\text{C}$ . For a working solution, the stock was diluted in culture medium to  $1\text{ }\mu\text{M}$ , sterilized, and cells were incubated for 16 h. All incubations were conducted in duplicate wells (technical duplicates) and in triplicate independent incubations ( $n = 3$ ).

**RNA Isolation and cDNA Synthesis** RNA was isolated using Sigma TRI Reagent® according to the manufacturer's instructions as has been described previously (Dunlop et al. 2017, in this issue). RNA was reverse transcribed to cDNA using the RT<sup>2</sup> First Strand Kit provided as part of the QuantiTect Rev. Transcription Kit Array (QIAGEN, Cat. no. PAHS-089Z) which includes a genomic DNA removal step, as per the manufacturer's instructions. cDNA from the same RNA samples was synthesized in duplicate (technical replicates), then pooled, then the cDNA from three independent incubations pooled and loaded onto the array ( $n = 3$ ).

**Qiagen Array** The cDNA was loaded onto the real-time Human Unfolded Protein Response RT<sup>2</sup> profiler PCR Array (QIAGEN, Cat. no. PAHS-089Z) in combination with RT<sup>2</sup> SYBR® Green qPCR Mastermix (Cat. no. 330529) and qPCR run on an Applied Biosystems™ ViiA™ 7 Real-Time PCR System according to the manufacturer's instructions. Cycle threshold (Ct) values were exported to an Excel file to create a table of CT values, uploaded to the Qiagen data analysis web portal (<http://www.qiagen.com/geneglobe>), samples assigned to controls and test groups, and finally, Ct values were normalized based on an automatic selection from a full panel of reference genes. Fold-changes were calculated using the delta Ct method (Livak and Schmittgen 2001), in which delta Ct is calculated between gene of interest and an average of housekeeping genes, followed by delta-delta Ct calculations [ $\text{delta Ct (Test Group)} - \text{delta Ct (Control Group)}$ ]. Fold change is then calculated using  $2^{-(\text{delta delta Ct})}$  formula. All methods complied with minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al. 2009).

### Protein Extraction from the Phenol Layer

Proteins were extracted from the phenol layer of TRI® Reagent (Sigma Aldrich, St Louis, MO) according to the manufacturer's instructions with the following modifications: following drying of the protein pellet under a vacuum for 5–10 min in a vacuum concentrator (Savant Speed Vac® SC110), approximately  $100\text{ }\mu\text{L}$  1% SDS in water was added to each pellet, then the pellets were mashed with a pestle. The protein lysate was vortexed and heated at  $50\text{ }^{\circ}\text{C}$  for 40 min until it was dissolved (Likhite and Warawdekar 2011; Simões et al. 2013). Tubes were spun in a bench-top centrifuge

(10 min,  $14,000\times g$ ), then the protein-rich supernatant aliquoted into clean low-protein bind tubes and the protein concentration determined using Bicinchoninic Acid (BCA) Kit for Protein Determination (Cat. no. BCA1, Sigma Aldrich). Samples were stored at  $-20\text{ }^{\circ}\text{C}$  until required for western blotting.

### Western blots

$40\text{ }\mu\text{g}$  of protein was separated in the first dimension, then western blots run and quantitated as described previously (Dunlop et al. 2017, in this issue). Primary antibodies were diluted 1/200 in tris-buffered saline with 0.5% TWEEN 20 (Cat. no. P1379, Sigma Aldrich) (TBS-T), at pH 7.4. Primary antibodies were as follows: caspase-3 p17 antibody (B-4) mouse monoclonal (Cat. no. sc-271028, Santa Cruz Biotechnology), Grp78 polyclonal (H-129) rabbit (Cat. no. sc-13968, Santa Cruz Biotechnology), protein disulfide isomerase (PDI) (C-2) mouse monoclonal (Cat. no. sc-74551, Santa Cruz Biotechnology) and secondary antibodies (diluted 1/10,000), mouse IgGκ binding protein-HRP (m-IgGκ BP-HRP) (Cat. no. sc-516102, Santa Cruz Biotechnology) and anti-rabbit IgG whole molecule HRP (Cat. no. A6154, Sigma Aldrich).

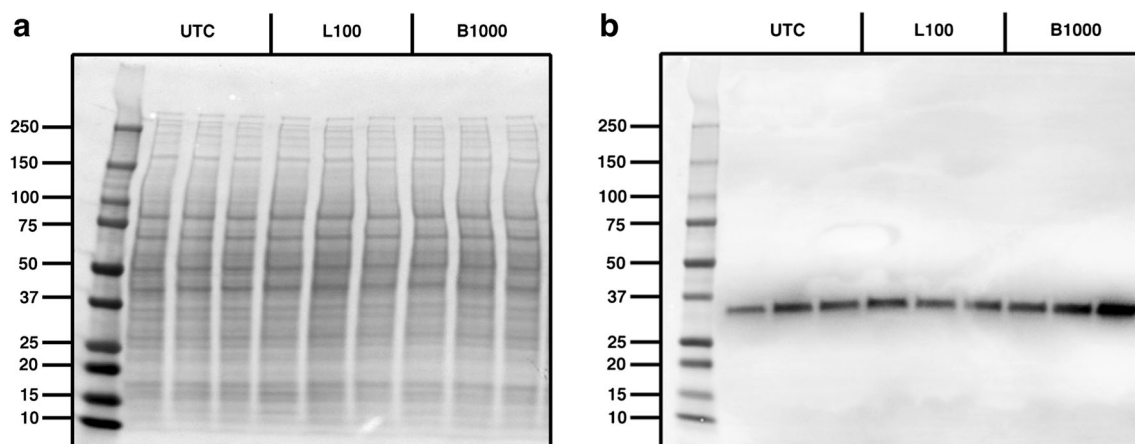
## Results

### Caspase-3 Activation

To ensure we were working at sub-toxic concentrations of L-serine and L-BMAA, we measured the activation of the proapoptotic enzyme, caspase-3, in cells that were untreated (UTC), treated with L-serine  $100\text{ }\mu\text{M}$  (L100) or L-BMAA  $1000\text{ }\mu\text{M}$  (B1000) for 17 h. We did not observe a band in the 17 kDa region of the membrane, indicating that procaspase-3 (32 kDa) was not being converted to active caspase-3 (17 kDa) under any of the conditions. These observations indicate that the cultures were not undergoing apoptosis and we were working with sub-toxic doses (Fig. 1b). A representative membrane stained with Ponceau S is shown in Fig. 1a, to demonstrate equal protein loading.

### Gene Array

A list of differentially regulated genes according to their L-serine/control or L-BMAA/control ratio is presented in Table 1. We report that the pattern of differential expression imparted by L-serine and L-BMAA, even though the concentration of L-serine was ten times lower, was very similar, as indicated by the heat maps generated from the human UPR array (see Fig. 2a, b).



**Fig. 1** Pro-caspase-3 (32 kDa) was not converted to active caspase-3 (17 kDa) in response to any conditions, indicating cells were not undergoing apoptosis and all treatments were sub-toxic. SH-SY5Y human neuroblastoma cells were treated as described in “Materials and Methods”. 40  $\mu$ g total protein was separated by SDS-PAGE then transferred overnight onto nitrocellulose and stained with Ponceau S to check

for transfer efficiency and equal protein loading (a). Membranes were probed overnight for pro-caspase-3 (a band at 32 kDa for pro-caspase-3 and 17 kDa for active caspase-3). The lack of a band at 17 kDa indicates these cells are not undergoing apoptosis (b). Note: Panel A is a representative Ponceau S stain to illustrate equal protein loading

**Genes That Encode Proteins Involved in Unfolded Protein Binding** Chaperone containing TCP1 subunit 4 and 7 (CCT4 and CCT7) code for proteins that are involved in molecular chaperoning and proper folding of newly synthesized proteins; in vitro, they are known to play a role in the folding of actin and tubulin.

Both L-serine (100  $\mu$ M) and L-BMAA (1000  $\mu$ M) increased the transcription of these two chaperones: CCT4, 5.05-fold and 4.72-fold, and CCT7, 3.93-fold and 3.74-fold, respectively. By contrast, heat shock 70 protein 1B (HSPA1B) which is involved in stabilizing pre-existing proteins against aggregation and mediating the folding of newly translated polypeptides in the cytosol as well as within organelles, was downregulated by L-serine (down by 2.17-fold) but not by L-BMAA. Binding immunoglobulin protein (BiP) associated protein (S11) encodes a resident ER N-linked glycoprotein that functions as a nucleotide exchange factor for another UPR protein to facilitate protein folding. SIL1 was downregulated by L-BMAA (by 1.85-fold) but not L-serine.

**Genes that Encode Proteins Involved in Heat Shock** The most consistent changes to gene expression shared by both L-serine and L-BMAA were observed in genes coding a family of proteins that play a role in protecting cells from apoptosis during ER stress, the highly conserved DNAJ/HSP40 family of proteins. Represented here by DNAJB9, DNAJC4, DNAJB2, and DNAJC3 (also known as P58IPK), these genes were upregulated to a very similar degree by both L-BMAA and L-serine (Table 1).

This DNAJ/HSP40 gene family, which share common substrates with HSP70s, encodes proteins involved in regulating molecular chaperone activity, as well as binding to exposed hydrophobic residues of unfolded and nascent polypeptides

(Qiu et al. 2006). Some DNAJs/HSP40s are able to bind proteins directly, yet others target HSP70 activity (Qiu et al. 2006).

For example, the DNAJB9 gene encodes an ER co-chaperone which binds to misfolded proteins as well as forming part of the ER-associated degradation (ERAD) complex, the latter critical for clearing misfolded proteins from the lumen during ER stress. The DNAJB2 gene encodes a protein that contributes to the translocation and ubiquitin-dependent proteasomal degradation of misfolded proteins from the ER (Shiber and Ravid 2014). The DNAJC3 gene encodes a co-chaperone protein that interacts with glucose binding protein 78 kDa (Grp78, also known as BiP) which is an ER-localized member of the HSP70 family, as well as a molecular sensor of ER stress. Grp78 tightly binds the three transmembrane ER stress molecular sensors, ATF6, PERK, and IRE1, rendering them inactive, but disassociates when misfolded proteins accumulate in the ER lumen. This dissociation enables the molecular sensors to initiate the UPR (Lee 2005).

In addition to its role as a co-chaperone, DNAJC3 also constitutes an important component of the negative feedback loop that blocks cellular signaling via phosphorylation of eukaryotic initiation factor 2 alpha (eIF2 $\alpha$ ). Eukaryotic initiation factor 2 (eIF2) phosphorylation occurs in response to ER stress, and signals the cell to downregulate global protein translation and switch to degradation of accumulated proteins. This functions to clear accumulating mis- and unfolded proteins from the ER lumen and return the cell to homeostasis. DNAJC3 is a direct inhibitor of a eIF2 $\alpha$  kinase, PERK, thus upregulation of DNAJC3 could be considered pro-apoptotic if the cell is undergoing ER stress.

That both L-serine and L-BMAA increase the transcription of genes from the J protein family to a similar degree suggests

**Table 1** Genes differentially regulated by L-serine 100 μM and L-BMAA 1000 μM. RNA was extracted from triplicate cultures of SH-SY5Y cells and transcribed to cDNA using the Qiagen QuantiTect Rev. Transcription Kit (Cat # 205311) then spotted onto Qiagen RT<sup>2</sup>-profiler PCR array and real-time run on the Applied Sciences ViiA™ 7 Real-Time PCR System in 96 well plates. Both L-serine and L-BMAA induced the differential expression of a wide range of genes that encode proteins involved in the unfolded protein response

Classification	Gene name	Abbreviation	Grid ref.	Role	Fold change		
					Upregulated		
					L-serine	L-BMAA	L-serine L-BMAA
Unfolded protein binding	Chaperonin containing TCPI1, subunit 4/7	CCT4	A09	Folding of newly translated polypeptide substrates	5.05	4.72	
	Heat shock 70 kDa protein 1B	CCT7	A10	Heat shock protein	3.93	3.74	
	BIP-associated protein	HSPA1B	C11	Protein translocation and folding in ER			- 2.17
	DnaJ heat shock protein family (Hsp40)	SIL1	F10	ER co-chaperones which are part of the ERAD complex	1.76	1.73	
Heat shock proteins and chaperones	DNAJ4	DNAJB9	B06	Involved in recognizing and degrading misfolded proteins	2.44	2.44	
	DNAJB2	DNAJB2	B05		1.71	1.85	
	DNAJC3	DNAJC3	B08		2.33	2.14	
	HSP70 protein	HSPA1L	C12	Hsp70-like gene	2.69	1.57	
Protein folding	ER protein 44	ERP44	C06	Oxidative protein folding in the ER	1.65	2.07	
	Calnexin	CANX	A08	Calcium regulation in the sarcoplasmic reticulum and ER	3.07	2.89	
	Calreticulin	CALR	A07		2.50	2.13	
	Eukaryotic translation initiation factor 2A	EIF2A	B12	Functions in the early steps of protein synthesis	1.55		
Apoptosis	Bcl2-associated X protein	BAX	A06	Activates apoptosis	1.94	1.73	
	Ataxin 3	ATXN3	A05	Deubiquitinating enzyme	2.62	2.42	
	CHOP	DDIT3	B02	Promotes apoptosis via ER stress		1.82	
	Heat shock protein family A (Hsp70) member 1B	HSPA1B	C11	HSP70 family member			- 2.17
Transcription factors	Protein phosphatase 1 regulatory subunit 15A	PPP1R15	E11	Reverses the shut-off of protein synthesis initiated by stress-inducible kinases and facilitates stress recovery			- 2.07
	Mitogen-activated protein kinase 10	MAPK10	D11	Member of the MAP kinase family	1.88	1.56	
	Activating transcription factor 6/4	ATF6B	A04	Activates target genes for the UPR during ER stress			- 1.61
		ATF6	A03		1.81	1.81	
		ATF4	A02		1.53	1.56	
	CCAAT/enhancer-binding protein beta	CEBPB	A11	Associates with DDIT3 (CHOP)			- 2.34
	ER to nucleus signaling 2	ERN2	C02	Signals to nucleus from ER			- 2.97
		SREBF1	F11	Transcription factor			2.67



Table 1 (continued)

Classification	Gene name	Abbreviation	Grid ref.	Role	Fold change			
					Upregulated		Downregulated	
					L-serine	L-BMAA	L-serine	L-BMAA
ER-associated protein folding, degradation, and ubiquitination	Sterol regulatory element-binding transcription factor	HTRA4	D07	Serine protease		2.12		– 1.68
	HtrA serine peptidase 4	EDEM1	B10	Protein processing in the ER	2.33	2.39		
	ER degradation enhancer	DERL2	B03	Degrades ER proteins	1.84	1.64		
	Degradation in ER-protein	HERPUD1ER	C10	Refolds ER proteins	1.83	1.62		
	Homocysteine inducible ER protein	ERP44	C06	Protein folding in the ER	1.65			
	Endoplasmic reticulum protein 44	UBE2G2	G04	Ubiquitination			– 1.68	
	Ubiquitin-conjugating enzyme E2G 2	VCP	G11	Forms a complex with HSC70			– 1.76	– 1.93
	Valosin-containing protein							

they share similar signaling pathways, at least with respect to ER stress and UPR.

Also under the classification of genes that encode heat shock protein activity, were genes encoding heat shock protein family A (Hsp70) member 1 like (HSPA1L) which in cooperation with other chaperones, stabilizes pre-existing proteins against aggregation and mediates the folding of newly translated polypeptides in the cytosol as well as within organelles. L-serine and L-BMAA increased the transcription of HSPA1L 2.69-fold and 1.57-fold respectively. Endoplasmic reticulum protein 44 (ERP44) which may have a role in the control of oxidative protein folding in the endoplasmic reticulum, was also induced 1.65-fold by L-serine and 2.07-fold by L-BMAA.

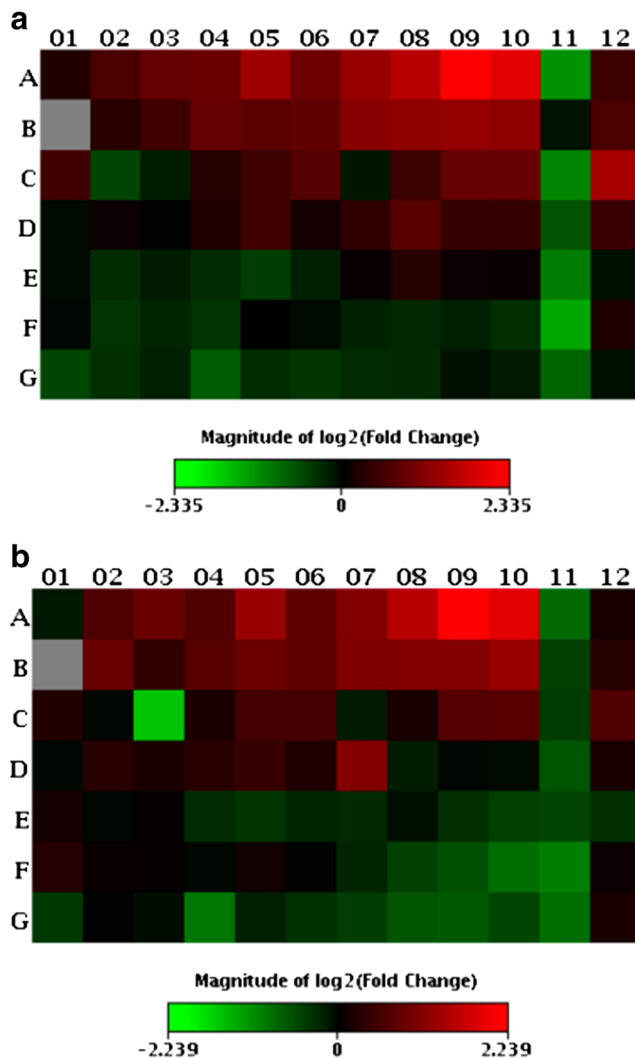
### Genes That Encode Proteins Involved in Protein Folding

Under the category of protein folding, L-serine and L-BMAA induced calnexin (CANX) and calreticulum (CALR) to similar degrees. CANX encodes a protein that is a calcium-binding, ER-associated protein that interacts transiently with newly synthesized N-linked glycoproteins, facilitating protein folding and assembly. CANX also seems to play a major role in the quality control apparatus of the ER by mediating the retention of incorrectly folded proteins. L-serine induced the transcription of CANX by 3.07-fold and L-BMAA by 2.89-fold.

CALR encodes a multifunctional protein that acts as a major calcium-binding (storage) protein in the lumen of the ER. Observations that it is also found in the nucleus suggest that it may have a role in the regulation of transcription. CALR promotes folding, oligomeric assembly, and quality control in the ER via the CALR/CANX cycle. L-serine induced the transcription of CALR by 2.50-fold and L-BMAA by 2.13-fold.

### Genes That Encode Transcription Factors

Several transcription factors key to the activation of the UPR during ER stress were also increased by both L-serine and L-BMAA. Activating transcription factor 6 (ATF6) is one of the three master regulators of ER stress, and under conditions of homeostasis, remains docked in the lumen of the ER, held inactive by Grp78. Once Grp78 senses unfolded or misfolded proteins, it detaches from ATF6, enabling it to translocate to the golgi where it dimerizes with X-box binding protein 1 (XBP1) and then travels to the nucleus to mediate the transcription of genes encoding ER chaperones. Activating transcription factor 6 beta (ATF6B) and ATF6 were both increased by L-serine (1.88-fold, 1.81-fold, respectively) and L-BMAA (1.81-fold, 1.81-fold, respectively). In light of evidence showing L-serine protects against L-BMAA mediated neurotoxicity (Dunlop et al. 2013; Cox et al. 2016), this observation is particularly interesting, especially given that ATF6 initiates the UPR during ER stress, thereby suggesting L-serine is activating the UPR.



**Fig. 2** Heat maps generated from the hybridization of cDNA synthesized from RNA extracted from SH-SY5Y cells from one of three treatments; no treatment (control), 100  $\mu$ M L-serine or 1000  $\mu$ M L-BMAA for 17 h. See “Materials and Methods” for details. Differential expression of genes by L-serine 100  $\mu$ M as a ratio of L-serine/UTC (control cultures) (a). Differential expression of genes by L-BMAA 1000  $\mu$ M as a ratio of L-BMAA/UTC (control cultures) (b). Green indicates downregulation whereas red indicates upregulation. Incubations were conducted in duplicate in six well plates, then repeated in triplicate ( $n=3$ ). L-serine and L-BMAA generated similar heat maps suggesting the two molecules mediate their effects by similar mechanisms

In support of this hypothesis is the observation that L-serine also induced the gene encoding activating transcription factor 4 (ATF4), another transcription factor in the UPR pathway that directly regulates the induction of CHOP in response to ER stress. CHOP is a sentinel of ER stress and rapidly induces apoptosis by regulating the transcription of ATF4 which promotes apoptosis by regulating the transcriptional induction of BBC3/PUMA—activators of outer mitochondrial membrane permeabilization, release of cytochrome c and apoptosis (Galehdar et al. 2010). ATF4 was induced by L-serine (1.53-fold) and L-BMAA (1.56-fold).

Among the transcription factors downregulated by L-serine and L-BMAA was a co-factor that associates with CHOP, CCAAT/enhancer binding protein beta (CEBPB) (2.34-fold down and 1.79-fold down, respectively), ERN2, a pro-apoptotic protein which induces translational repression through 28S ribosomal RNA cleavage in response to ER stress (2.97-fold down in response to L-BMAA), and MAPK10, a serine-threonine kinase responsible for regulating a wide variety of cellular functions (1.61-fold down in response to L-serine and 1.59-fold down in response to L-BMAA).

### Genes That Encode Proteins Involved in Folding, Degradation, and Ubiquitination

Both L-serine and L-BMAA increased the transcription of genes that code proteins involved in ER protein processing (EDEM1), refolding (HERPUD1ER) and degradation (DERL2). L-serine increased the transcription of ERP44 which codes a protein concerned with protein folding in the ER and L-BMAA induced HTRA4 which is a serine protease.

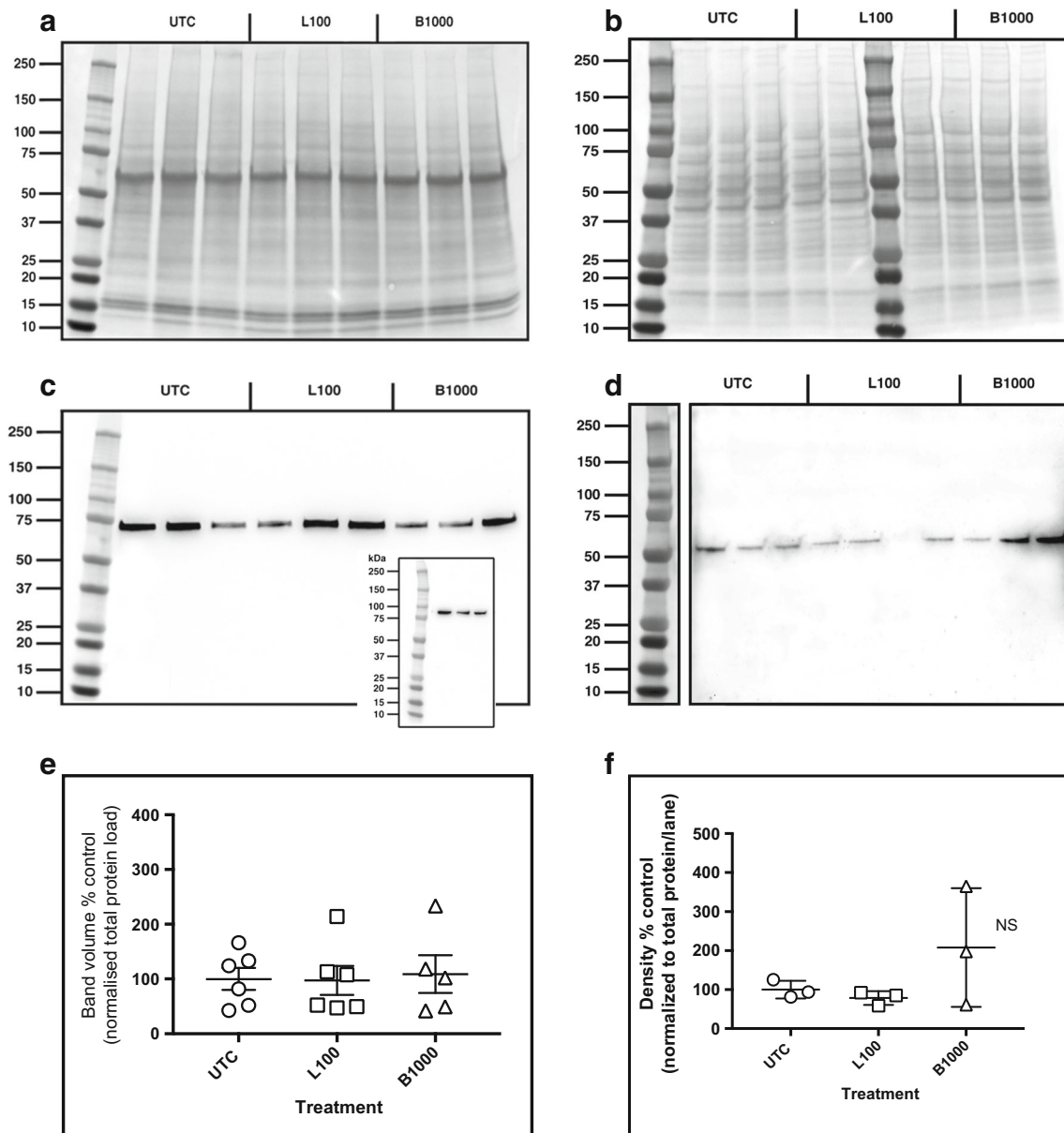
Downregulated by both L-serine and L-BMAA was valosin-containing protein (VCP) which encodes a protein that complexes with the inducible form of Hsp70, HSC70 and is concerned with ubiquitin-dependent protein degradation. L-serine also downregulated another gene that encodes a protein involved in ubiquitin conjugation, UBE2G2, and HSPA1B which is a member of the heat shock protein 70 family of proteins. In cooperation with other chaperones, Hsp70s assist in the correct folding of newly formed polypeptides (both in the cytosol and in organelles) as well as stabilize pre-existing proteins against aggregation.

**Genes That Encode Proteins Involved in Apoptosis** In the category of genes that encode proteins involved in apoptosis, L-BMAA induced the transcription factor C/EBP homologous protein (CHOP) but L-serine did not. Both however induced BAX which is a member of the BCL-2 family of proteins that contributes to programmed cell death or apoptosis via permeabilization of the mitochondrial membrane and release of cytochrome C (Brunelle and Letai 2009).

### Western Blots

Regulation of the UPR is not the exclusive domain of transcription, however, but also involves mRNA stabilization, translation, and post translational modification. Thus, we undertook western blots to examine changes to key proteins involved in the UPR.

Grp78 is not only a major ER chaperone with anti-apoptotic properties, but is considered a master regulator of ER stress, as evidenced by its ability to activate the UPR (Lee 2005). Although there were no observable change in the levels



**Fig. 3** Grp78 and protein disulfide isomerase (PDI) were unchanged in response to L-serine 100  $\mu$ M (L100) or L-BMAA 1000  $\mu$ M (B1000) versus control cells (UTC) after 17 h incubation. SH-SY5Y human neuroblastoma cells were treated, total protein extracted, SDS-PAGE run and western blotting undertaken as described in materials and methods. Ponceau S stains of nitrocellulose membranes to check for transfer efficiency and for use in protein normalization (a, b). The membrane was probed overnight for Grp78 (c) or PDI (d) then imaged using ECL. Inset: Grp78 positive control, 1  $\mu$ M thapsigargin, 16 h (c). Band densities were

acquired in BioRad ImageLab then normalized to total protein load/lane, converted to percentage of control and graphed in GraphPad Prism: Grp78 (e), PDI (f). One-way ANOVA with Tukey's multiple comparison test revealed no differences between any conditions. Incubations were conducted in duplicate wells (technical replicates) and in triplicate independent incubations ( $n=3$ ). Note: The molecular weight markers in (d) have been digitally spliced in from the corresponding Ponceau S stain showed in (b).

of Grp78 mRNA on the array, it is important to measure the change in Grp78 protein since the relationship between mRNA and protein is not always linear (Lee 2005). Western blots of Grp78 did not detect a difference in protein between any of the conditions examined (Fig. 3a, c, and e).

PDI is an important ER chaperone protein involved in the sequestration and refolding of misfolded proteins and

is rapidly induced when ER stress occurs. Western blots did not reveal any significant differences in the levels of PDI protein in any of the conditions examined (Fig. 3b, d, and f). This is consistent with previous observations from our own lab, where L-serine 100 or 500  $\mu$ M did not induce the translation of PDI, but 1000  $\mu$ M L-serine did (Dunlop et al. 2017, in this issue).



## Discussion

Data presented here shows that L-serine, at ten-times less the concentration of L-BMAA, differentially regulated many of the same genes and in some cases to the same degree as L-BMAA. This suggests that L-serine shares some of the same signaling pathways as L-BMAA.

This observation constitutes a new and novel mechanism that might contribute to L-serine mediated neuroprotection, as has been described in several models and by independent laboratories. For example, our lab reported a greater than 50% reduction in NFTs in five brain regions of non-human primates co-fed L-serine with L-BMAA including the temporal (dorsal and ventral), primary motor, entorhinal (posterior), and insula cortices (Cox et al. 2016). In human cell cultures, we reported the inhibition of L-BMAA protein misincorporation and subsequent abrogation of apoptosis when cells were co-incubated with L-serine (Dunlop et al. 2013). Levine and colleagues reported an 85% reduction in disease progression in ALS patients taking 30 g of L-serine per day, compared to controls (Levine et al. 2016).

These data presented here point to an as yet uncharacterized mechanism for L-serine neuroprotection which mimics some of the molecular pathways mediated by L-BMAA. This appears to be counter intuitive since it has been widely reported that L-BMAA is cytotoxic by a variety of mechanisms including activation of ER and oxidative stress, increased expression of CHOP, mitochondrial dysfunction and lactate dehydrogenase (LDH) release (Okle et al. 2012; Chiu et al. 2012; Dunlop et al. 2013; Chiu et al. 2013; Shen et al. 2016), whereas L-serine is reported as neuroprotective. Based on the data presented here, one of the mechanisms contributing to L-serine-mediated neuroprotection may involve activation of ER stress leading to induction of the UPR. In the context of the evidence so far supporting L-serine as a neuroprotective molecule, this could constitute a type of pre-emptive response, which could alert cells for a rapid response prior to being confronted with a neurotoxic insult.

Since ER stress and subsequent UPR does not rely solely on transcription for activation, we also measured changes to proteins by western blot. We report there were no significant changes in the levels of Grp78 or PDI under the conditions examined here. The observations for PDI are consistent with previous findings, also in SH-SY5Y cells, where L-BMAA 1000  $\mu$ M or L-serine 100  $\mu$ M did not increase translation of PDI after 17 h, but L-serine 1000  $\mu$ M did (Dunlop et al. 2017, in this issue). In addition to concentration dependence, timing is likely a critical factor here in detecting significant changes in UPR proteins. ER stress and UPR occurs rapidly in response to cell stress, thus it is likely changes will be observed much earlier than 17 h. We are currently exploring this hypothesis.

That L-serine differentially regulated many of the same genes as L-BMAA appears in direct contrast to its role as a neuroprotective molecule, however, activation of the UPR as a mechanism to adapt to ER stress and rescue cells from death has been described before. “Proteostasis regulators” (PRs) are a class of compounds that re-programme the ER by upregulating the UPR to favor the removal of accumulating and aggregating proteins. In vitro, tunicamycin or thapsigargin function as PRs; tunicamycin upregulates UPR proteins by inhibiting protein N-glycosylation and thapsigargin disrupts ER calcium homeostasis, leading to activation of the UPR (Osowski and Urano 2011).

Examples of pathologies where such a mechanism might prove to be useful in therapy include cystic fibrosis, various neurodegenerative diseases where protein deposition is a factor including ALS and AD, or lysosomal storage diseases such as Tay-Sachs (Wang et al. 2006; Mu et al. 2008; Chiang et al. 2012; Mu et al. 2008). Small molecule PRs that are currently in use experimentally and clinically include salubrinal, which inhibits the dephosphorylation of eIF2 $\alpha$  (Matsuoka and Komoike 2015), thus favoring the removal of mis- and unfolded proteins, celastrol, which induces the heat shock response, increases the expression of cytoplasmic chaperones, and activates the UPR (Westerheide et al. 2004), arimoclomol, which has shown promise in treating ALS (Benatar 2016), and 4-phenylbutyrate (4-PBA), which has been used to treat cystic fibrosis (CF) (for a review of PRs see Plate et al. 2016).

Given our observations presented here and in a complementary study in this issue, (Dunlop et al. 2017, in this issue), we propose L-serine may be a candidate for proteostasis regulation and this may contribute to the observed neuroprotection. Thus, by alerting cells to an impending insult via upregulation of heat shock genes and transcription factors, L-serine prepares the cell for a rapid response when confronted with a neurotoxic insult. These results provide interesting insights into the mechanisms of L-serine neuroprotection, and provide clues as to the reported efficacy of L-serine supplementation in human clinical trials of ALS patients.

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