MECHANISMS UNDERLYING

BETA-N-METHYLAMINO-L-ALANINE NEUROTOXICITY

IN DIFFERENT MODEL SYSTEMS

DISSERTATION

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"Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning."

Albert Einstein (1879-1955)

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Abbreviations

°C	degrees celsius
AD	Alzheimer's disease
ADP	adenosine diphosphate
AIF	apoptosis inducing factor
AL	antennal lobe
ALS	amyotrophic lateral sclerosis
ALS/PDC	amyotrophic lateral sclerosis/ Parkinsonism-dementia complex
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionate
ANOVA	analysis of variance
ATF4	activating transcriptional factor 4
ATP	adenosin triphosphate
BBB	blood-brain barrier
BGS	brain growth spurt
BMAA	β-N-methylamino-L-alanine
BSO	DL-buthionine-(S, R)-sulfoximine
Ca	calcium
cDNA	deoxyribonucleic acid
CHOP	C/EBP-homologous protein
CNS	central nervous system
DAB	2,4 diaminobutyric acid
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum- associated degradation
ERQC	endoplasmic reticulum quality control
EST	expressed sequence tag
GFP	green fluorescence protein
GSK	glycogen synthase kinase
h	hours
i.c.v.	intracerebroventricularly
i.p.	intraperitoneal
i.v.	intravenous
iGluR	ionotropic glutamate receptors
IRE-1	inositol-requiring protein-1
K	potassium
k	kilo (10^3)
LAT	large neutral amino acid transporter
LDH	lactate dehydrogenase
LMN	lower motor neurons
M	molar or moles per litre
m	milli (10^{-3})
MAD	median absolute deviation

MeDAP	methylated α, β-diaminopropionic acid
mg	milligram
mGluR	metabotropic glutamate receptors
min	minutes
MPP^+	1-methyl-4-phenylpyridinium
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na	sodium
NCBI	National Center for Biotechnology Information
NFT	neurofibrillary tangles
NMDA	N-methyl-D-aspartic acid
nNOS	nitric oxide synthase
OA	okadaic acid
PARP	poly(ADP-ribose) polymerase
PCR	Polymerase chain reaction
PD	Parkinson's disease
PER	proboscis extension reflex
pH	hydrogen ion concentration
PND	postnatal days
PP	protein phosphatase
RNA	messenger ribonucleic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcriptase
s.c.	solvent control
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SOD	superoxide dismutase
TCA	trichloroacetic acid
thaps	thapsigargin
UMN	upper motor neurons
UPR	unfolded protein response
UPS	ubiquitin-proteasome-system
U.S.	United States
v/v	volume per volume ratio
Δ	difference
λ	wavelength
µ	micro (10^{-6})

Publications and Presentations

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Zusammenfassung

Das erhöhte Auftreten von Amyotropher Lateralsklerose (ALS) in Kombination mit dem Parkinson-Demenz-Komplex (PDC) bei den Chamorros, den Ureinwohnern der amerikanischen Insel Guam sowie der benachbarten Inseln im Südwestpazifik, weckte in den 1960er Jahren die Aufmerksamkeit internationaler Forscherteams. In den folgenden Jahren zeichnete sich eine Korrelation zwischen dem Auftretenden von ALS/PDC und den traditionellen Essgewohnheiten (Mehl aus *Cycas* sp.) der Ureinwohner ab. Mit der Entdeckung der Substanz β-N-Methylamino-L-Alanin (L-BMAA), welche wahrscheinlich von Cyanobakterien selbst oder in deren Symbiose mit Palmfarnen (*Cycas* sp.) produziert wird, wurde der Fokus wissenschaftlicher Untersuchungen auf eine möglicherweise von L-BMAA induzierte Neurotoxizität als Verursacher der neurodegenerativen Erkrankung ALS/PDC gelenkt. Als toxischer Wirkmechanismus von L-BMAA wird häufig dessen Affinität zu Glutamatrezeptoren diskutiert, welche zu einer Exzitotoxizität führen könnte, die vergleichbar ist mit der durch den Neurotransmitter Glutamat induzierten Exzitotoxizität. In den letzten Jahren wurden Daten veröffentlicht, welche zeigen, dass ein spezifischer Transporter das Neurotoxin L-BMAA über die Blut-Hirnschranke von Ratten transportieren kann. Dies deutet darauf hin, dass L-BMAA auch intrazellulär vorkommen kann, wenn dieser Transporter in anderen Zellarten (z.B. Neuronen) exprimiert wird. Das intrazellulär vorkommende L-BMAA erhöht die Wahrscheinlichkeit, dass neben der Affinität von L-BMAA zu Rezeptoren weitere neurotoxische Mechanismen initiiert werden können, welche zu einer Neurodegeneration führen könnten.

Eine Biomagnifikation von L-BMAA wurde angenommen, um die hohen Dosen von L-BMAA, welche zum Erzielen eines toxischen Effekts in Tierversuchen benötigt wurden, erklären zu können. Diese Annahme wird von der Hypothese gestützt, dass L-BMAA in einer „proteingebundenen“ und einer „freien“ Form detektiert werden kann. Jedoch wurden bis dato noch keine experimentellen Daten publiziert, welche den Prozess der „Proteinbindung“ oder deren Auswirkungen auf neuronale Zellen erklären.

Basierend auf der Hypothese, dass L-BMAA ein langsam wirkendes Toxin ist, welches nicht (nur) durch eine Bindung an Rezeptoren, und somit exzitotoxisch, wirken kann, sondern auch intrazellulär vorkommt, wurden in dieser Doktorarbeit mögliche von L-BMAA induzierte toxische Mechanismen anhand eines *in vitro*- und eines *in vivo*-Modells untersucht. Die intrazelluläre Verfügbarkeit von L-BMAA kann hypothetisch zu einer Inkorporation von L-BMAA in Proteine oder zu einer starken Bindung von L-BMAA an Proteine führen. Sowohl die Inkorporation wie auch die Bindung hätten eine beeinträchtigte Proteinhomöostase zur Folge, was in einer progressiven Neurodegeneration sichtbar werden würde.

Die humane Neuroblastoma-Zelllinie SH-SY5Y wurde als *in vitro*-Modell verwendet um zu untersuchen, ob intrazelluläres L-BMAA die Proteinhomöostase beeinflussen kann. Nachdem SH-SY5Y Zellen mit L-BMAA exponiert wurden, konnte eine erhöhte Expression von CHOP sowie eine erhöhte Phosphorylierung von elf2α detektiert werden. Beide Proteine sind Markerproteine für den Endoplasmatischen Retikulum (ER)-Stress. Dies lässt den Rückschluss zu, dass erhöhte Mengen von Proteinen vorlagen, welche eine falsche oder keine Tertiärstruktur aufwiesen. Dieser Effekt wurde möglicherweise durch eine Inkorporation von L-BMAA in Proteine oder eine Anlagerung von L-BMAA an Proteine, welche an Faltungsprozessen im ER beteiligt sind, hervorgerufen und löste so die ER-Stress-Situation aus. Gleichzeitig führte intrazelluläres L-BMAA zu einer erhöhten Ubiquitinierung von Proteinen und zu gesteigerten proteasomalen (20S) sowie Caspase 12 Aktivitäten, wenn die SH-SY5Y-Zellen mit geringen L-BMAA-Konzentrationen exponiert wurden (≥1 mM). Hingegen wurde eine gesteigerte Menge an Sauerstoffradikalen (ROS) und oxidierten Proteinen nur beobachtet, wenn die Zellen mit hohen L-BMAA-Konzentrationen (≥1 mM) exponiert wurden. Diese Effekte konnten mit dem Radikalfänger Vitamin E minimiert beziehungsweise komplett abgefangen werden. Dies war jedoch nicht der Fall für die mit geringen L-BMAA-Konzentrationen induzierten Effekte der erhöhten Ubiquitinierung und der gesteigerten proteasomalen (20S) sowie Caspase 12 Aktivitäten. Somit bewirkten geringe intrazelluläre L-BMAA-Konzentrationen eine Fehlregulation der zellulären Proteinhomöostase und verursachten ER-Stress unabhängig von oxidativem Stress oder einer Exzitotoxizität.

Ein weiterer Effekt von L-BMAA war *in vitro* in SH-SY5Y-Zellen als Folge einer Anlagerung oder Bindung von L-BMAA an die Proteinphosphatase 2B (Calcineurin) zu beobachten. Hierbei induzierte L-BMAA eine gesteigerte Enzymaktivität von Calcineurin in dem neuronalen Modell SH-SY5Y sowie von isoliertem rekombinantem humanem Calcineurin. In SH-SY5Y-Zellen führte die gesteigerte Enzymaktivität zu einer erhöhten Dephosphorylierung (Serin 9) der Glykogen-Synthase Kinase 3β (GSK3β). Die so aktivierte GSK3β verursachte ihrerseits eine erhöhte Phosphorylierung des Tau-Proteins (Serin 202), welches für die Stabilisierung der miktotubulären Strukturen verantwortlich ist. Dissoziiertes und somit hyperphosphoryliertes Tau konnte in Patienten mit verschiedenen neurodegenerativen Krankheiten wie Alzheimer, ALS oder Parkinson detektiert werden und ist daher auch ein gebräuchlicher und anerkannter pathologischer Marker. Auch in den ALS/PDC-Patienten auf Guam wurde hyperphosphoryliertes Tau detektiert. Schlussfolgernd kann gesagt werden, dass das Toxin L-BMAA in dem humanen neuronalen Modell (SH-SY5Y) zu einer erhöhten Phosphorylierung des Tau-Proteins geführt hat, was auf eine erhöhte GSK3β- und Calcineurin-Aktivität zurückzuführen ist. Dieses Forschungsergebnis ist ein weiteres Beispiel dafür, dass eine Proteinphosphatase durch ein von Cyanobakterien produziertes Toxin oder Metabolit beeinträchtigt wird.

Obwohl bereits zahlreiche Tiermodelle verwendet wurden, um die Toxizität von L-BMAA und seinen möglichen neurotoxischen Wirkmechanismus zu erklären, waren nur sehr wenige der im Menschen zu beobachtenden Symptome im Tiermodell reproduzierbar. Ebenso wenig konnte der progressive Verlauf der Neurodegeneration in Tiermodellen durch L-BMAA induziert und somit beobachtet werden. Bis heute zeigt nur eine einzige Studie mit Cynomolgus-Affen, welchen hohe Dosen an L-BMAA verabreicht wurden, mit dem Menschen vergleichbare neurologische Auffälligkeiten. In der vorliegenden Arbeit wurde die Honigbiene (*Apis mellifera*) als Modellorganismus verwendet, um mögliche von L-BMAA initiierte molekulare Charakteristiken sowie neurologisch bedingte Verhaltensänderungen, welche bereits *in vitro* wie auch in menschlichen ALS-Patienten beobachtet werden konnten, zu reproduzieren. Durch eine neu entwickelte Methode war es möglich, die Entstehung von Sauerstoffradikalen, welche nach der Exposition mit L-BMAA im Gehirn der Bienen entstanden, zu beobachten. Ebenfalls konnten eine beeinträchtigte Ca^{2+}-Homöostase sowie eine veränderte neuronale Geruchsverarbeitung, welche auch in ALS/PDC-Patienten beschrieben wurden, in den mit L-BMAA exponierten Bienen detektiert werden. Experimentelle Ergebnisse, welche eine durch L-BMAA beeinträchtige Lernfähigkeit der Bienen zeigten, unterstützen zusätzlich die Hypothese, dass die Honigbienen geeignete Organismen sind, um Aspekte von ALS/PDC oder weiteren neurodegenerativen Erkrankungen zu untersuchen.

Zusammenfassend kann die Aussage getroffen werden, dass in dieser Dissertation gezeigt wurde, dass L-BMAA bereits in geringen Konzentrationen neurotoxische Kaskaden initiieren kann, wobei intrazellulär vorhandenes L-BMAA zur Aktivierung eines an den ER-Stress gekoppelten Apoptosemechanismus führt. Der ER-Stress wird hierbei wahrscheinlich durch die Inkorporation von L-BMAA in Proteine oder eine Anlagerung von L-BMAA an Proteine ausgelöst. Neben dem ER-Stress ist auch noch eine erhöhte Phosphorylierung des Proteins Tau durch die von L-BMAA beeinflusste Protein Phosphatase 2B (Calcineurin) ein eindeutiger Marker für das neurotoxische Potential von L-BMAA. Die beiden durch L-BMAA veränderten, progressive Neurodegeneration anzeigenden Biomarker ER-Stress sowie Hyperphosphorylierung tragen somit unabhängig von möglichen exzitotoxischen Effekten zur Erklärung des neurotoxischen Mechanismus bei, welcher durch einen einmaligen oder chronischen Kontakt mit L-BMAA hervorgerufen werden kann.

Diese Arbeit ist ein weiterer Schritt, um die Neurotoxizität von L-BMAA zu verstehen, und zeigt einen weiteren und bis dato unbekannten, Neurodegeneration initiierenden Mechanismus eines von Cyanobakterien produzierten Toxins auf. Desweiteren wird in dieser Arbeit mit der Honigbiene ein geeignetes Modell für die Erforschung von Toxin-induzierten neurotoxischen Effekten aufgezeigt.

Summary

In the 1960s international research teams became aware of a 50- to 100-fold increased incidence of symptoms of amyotrophic lateral sclerosis (ALS) in combination with Parkinsonism-dementia complex (PDC) among the native Chamorro population on the American island of Guam and neighboring islands in the South-West Pacific Ocean. In the following years a correlation of ALS/PDC occurrence and the native food consumption was hypothesized. The discovery of a new substance named β-N-methylamino-L-alanine (L-BMAA), produced by cyanobacteria or in their symbiosis with *Cycas* sp. seeds on Guam, set the focus of research on the neurotoxic potential of L-BMAA as a putative initiator of the neurodegenerative disease ALS/PDC. It has been suggested that the toxic mechanism of L-BMAA is initiated by a receptor-binding of L-BMAA which results in an overstimulation of the glutamate receptors and thus in an excitotoxicity comparable to glutamate-mediated excitotoxicity. As a specific transporter has been shown to mediate L-BMAA transport across the blood-brain barrier of rats, an intracellular availability of L-BMAA can be supposed if this transporter is also expressed in other cell types. Intracellular L-BMAA availability raises the possibility of further mechanisms involved in the neurotoxic acting of L-BMAA, thus possibly resulting in a progressive neurodegeneration.

Biomagnification of L-BMAA was postulated to explain the high dosages of L-BMAA needed in animal experiments to evoke the toxic effect. This was supported by the hypothesis that L-BMAA can become “protein associated” as it was detected in a protein-bound and a free form. However, no experimental findings were published elucidating the protein association of L-BMAA.

In this thesis, mechanisms underlying the L-BMAA-mediated neurotoxicity were investigated in *in vitro* as well as in *in vivo* models based on the hypothesis that L-BMAA acts as a slow toxin by a non-receptor-mediated mechanism. Intracellular availability of L-BMAA may result in a protein binding or incorporation, leading to an impaired protein homeostasis and, as a consequence, to progressive neurodegeneration.

Human neuroblastoma cells (SH-SY5Y) were used to examine *in vitro* if intracellular L-BMAA could impair the protein homeostasis of the neuronal model system. An increased expression of CHOP and an enhanced phosphorylation of elf2α, both endoplasmic reticulum (ER)-stress markers, hinted at an increased amount of unfolded proteins. The latter may be induced by incorporation into or attachment of L-BMAA to proteins and a subsequent altered tertiary structure of these proteins. Indeed, intracellular L-BMAA led to an increased protein ubiquitination, 20S proteasomal and caspase 12 activity following exposure to low L-BMAA concentrations (≥0.1 mM). However, increased amounts of reactive oxygen species (ROS) and oxidized proteins were only observable following high

L-BMAA concentrations (≥1 mM). These effects could be ameliorated by co-incubation with the ROS scavenger vitamin E, in contrast to the constant increase of protein ubiquitination, 20S proteasomal and caspase 12 activities which were not affected by vitamin E. Thus, low intracellular L-BMAA concentrations resulted in a dysregulation of the cellular protein homeostasis with ensuing ER-stress. The latter was independent from high concentration effects such as excitotoxicity and oxidative stress in the human SH-SY5Y cells.

A further effect of L-BMAA associated with a putative attachment or binding to the enzyme protein phosphatase 2B (calcineurin) was determined in SH-SY5Y cells. Thereby L-BMAA induced an increased activity of calcineurin in SH-SY5Y cells *in vitro* as well as of recombinant human calcineurin. The increased activity of calcineurin in SH-SY5Y cells led to a decreased phosphorylation of the glycogen synthase kinase 3β (GSK3β) at residue serine 9 and thus to its activation. This resulted in increased phosphorylation of the tau protein (serine 202), which promotes the assembly and stabilization of microtubular structures. In various neurodegenerative diseases like Alzheimer's or Parkinson's disease an increased amount of dissociated and thus hyperphosphorylated tau is a common pathological marker and was also detected in ALS and ALS/PDC patients on Guam. In summary, in the human neuronal cell model (SH-SY5Y), L-BMAA induced an increase of phosphorylated tau owing to the increased dephosphorylation of GSK3β and an increased activation of calcineurin. This finding demonstrates the neurotoxic potential of L-BMAA and is a further example for an impairment of a protein phosphatase by a cyanobacterial produced metabolite/ toxin.

Even though various animal models have been employed to investigate L-BMAA toxicity and its putative mechanism, the human neurodegenerative symptoms and the progressive onset were rarely reproducible in such models. To date only one study indicated symptoms comparable with human ALS/PDC in Cynomolgus monkeys following high L-BMAA dosage treatment. In this thesis, the bee (*Apis mellifera*, honeybee) was used as a model to reproduce L-BMAA initiated molecular characteristics as well as neurobehavioral symptoms which were observable in *in vitro* studies as well as in ALS/PDC patients. A newly established method enabled a real-time observation of the induction of reactive oxygen species (ROS) which were increased in the brain of the bees after L-BMAA exposure. An impaired calcium homeostasis and an impaired odor processing, both described for ALS as well as ALS/PDC patients, were also demonstrated in the bee model following L-BMAA-exposure. The reduced learning capability of L-BMAA treated bees adds to the hypothesis that the bee model is suitable for the research of at least some aspects of neurodegeneration in ALS or ALS/PDC.

In conclusion, the findings of this thesis clearly demonstrate that L-BMAA can initiate neurotoxic mechanisms even at low concentrations. Intracellular L-BMAA can cause the induction of an ER-stress-associated apoptotic pathway which is possibly due to an incorporation into or attachment of L-BMAA to proteins. The occurrence of

hyperphosphorylated tau, which resulted from the L-BMAA affected protein phosphatase 2B (calcineurin) and a consequent activation of GSK3β, is a distinct marker for the potential of L-BMAA to induce pathological neurodegenerative effects. Both of the hallmarks of neurodegeneration, ER-stress and hyperphosphorylated tau, can contribute to the elucidation of the toxic mechanism following low dose or chronic exposure with the "long-term acting", "slow toxin" L-BMAA irrespective of the already described acute excitotoxic effect of high dosage L-BMAA treatment.

This manuscript is a further step in the clarification of L-BMAA toxicity and adds a new neurotoxic mechanism to cyanobacterial produced toxins. It further describes a suitable *in vivo* animal model to study neurotoxic effects of potential neurotoxins.

Chapter 1: General Introduction

1.1 Exposure Scenarios to Beta-N-methylamino-L-alanine

The initial reason for the interest and following investigations of beta-N-methylamino-L-alanine (L-BMAA) were research reports of epidemiological studies concerning a 10- to 100-fold increased incidence of symptoms of amyotrophic lateral sclerosis (ALS) and Parkinsonism-dementia complex (PDC) in combination among the native Chamorro population on the American island of Guam and neighboring islands in the South-West Pacific Ocean (Arnold *et al.* 1953; Koerner 1952; Kurland and Mulder 1954; Mulder *et al.* 1954; Tillema and Wijnberg 1953). The geographically clustered occurrence of these neurodegenerative symptoms was discovered in the following years and various hypotheses concerning the cause of ALS/PDC were proven. However, an epidemiological study from 1967 to 1983 considering 899 Chamorro people who lived on Guam and Rota indicated that out of 23 variables, including medical and personal information, only one significant variable was given for the PDC symptoms – the traditional Chamorro food (Reed *et al.* 1987). Focusing on the traditional food two main hypotheses were discussed in the following years. On the one hand, it was hypothesized that deficiencies of calcium and magnesium in the Chamorro's food had led to a secondary hyperparathyroidism in the natives, which had resulted in an increased absorption of toxic metals and minerals (e.g. Al, Mn, Cd) and thus in an accumulation of these in the neurons. This accumulation as well as an accumulation of silicon with aluminum and calcium hypothetically could then have finally assisted the development of ALS/PDC symptoms (Gajdusek 1985; Garruto *et al.* 1989; Garruto *et al.* 1986). However, this hypothesis was undermined in the following years based on deficient correlations between a dietary intake of toxic metals and ALS/PDC (Durlach *et al.* 1997). Also the values of Ca, P and Mg as well as toxic metals (Al, Cd, Cu, Fe, Hg, Mn, Pb and Zn) were not different between ALS/PDC patients and control persons (Ahlskog *et al.* 1997; Kurland *et al.* 1994; Reed *et al.* 1987). Therefore, the second hypothesis became more probable, proposing that a toxic component in the Chamorro's food had affected the ALS/PDC incidence. As early as 1963, Whiting (1963) postulated the seeds of the local tree *Cycas circinalis*, which were processed by the native Chamorro population as flour, as a possible risk factor for the incidence of ALS/PDC on Guam. As it was known that *Cycas* produce hepatotoxic and carcinogenic chemical compositions such as cycasin (Nishida *et al.* 1955; Riggs 1956) these research teams looked for a toxic amino acid, comparable to the lathyrism-inducing α-amino-β-oxalylaminopropionic acid found in *Lathyrus* sp. (Murti *et al.* 1964). Vega and Bell (1967) found this toxic non-proteinogenic amino acid BMAA and demonstrated its toxicity in chicken and rats (Vega *et al.* 1968). As

BMAA was found in the seeds of *Cycas* and as it was also found in the flour used by the natives (Banack and Cox 2003a; Duncan *et al.* 1989; Duncan *et al.* 1991; Kisby *et al.* 1992a; Murch *et al.* 2004a) this putative route of exposure for BMAA, resulting in a neurodegeneration and thus in the symptoms of ALS/PDC, seemed reasonable (Spencer *et al.* 1987b). However, the concentrations of BMAA in the traditionally prepared flour of the Chamorro were comparably very low (about 30 mg/kg dry weight cycad seed flour) (Duncan *et al.* 1990) and a long latency period was described. This was contradictory to experimental findings pointing out that much higher BMAA concentrations would be needed for BMAA-mediated toxicity. Thus Cox *et al.* (2003) hypothesized, as described below, that a biomagnification of BMAA occurred on Guam. Thereby the cyanobacterially-produced BMAA is accumulated by symbiosis in *Cycas* (*Cycas micronesica*), whose seeds were eaten by local flying foxes (*Pteropus mariannus mariannus*) (Figure 1), which also form part of the traditional Chamorro cuisine and are enjoyed in their entirety as a delicacy (Monson *et al.* 2003). Following the hypothesis of Cox *et al.* (2003) the extinction of two flying fox populations is linked to the Chamorro's access to firearms in the course of the development of U.S. military bases on Guam. The reduction of the flying fox population thereby correlates with the increase of ALS/PDC cases on Guam (Cox and Sacks 2002).

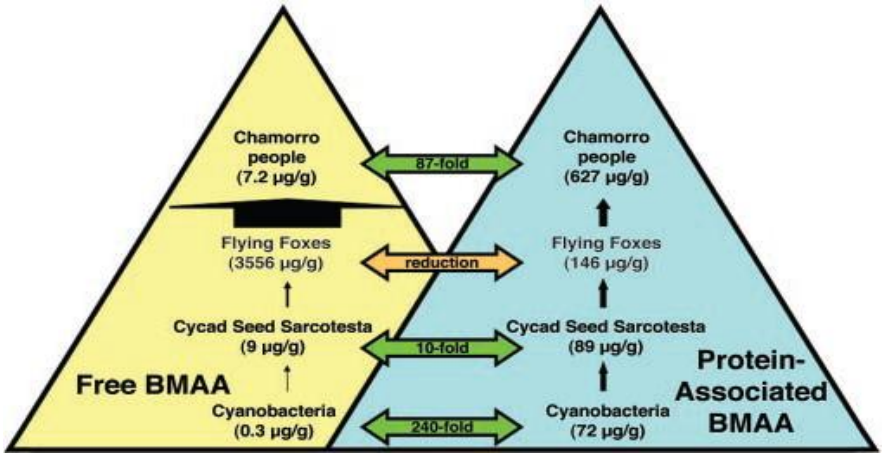

Figure 1: Scheme of the biomagnification hypothesis of BMAA. BMAA may become biomagnified in cyanobacteria, flying foxes and the native Chamorro people on Guam (yellow scheme) whereby BMAA may become associated to proteins resulting in an increased amount of BMAA in humans which raises its toxic potential (Murch *et al.* 2004a). Copyright (2004) National Academy of Sciences, U.S.A.

However, even if BMAA can become accumulated in *Cycas* as well as in flying foxes the increased amount of BMAA in the diet of the Chamorro could not explain the slow progression and the delayed outbreak of ALS/PDC symptoms. The fact that in native Chamorros who emigrated new cases of ALS/PDC occurred 1 to 34 years after leaving Guam indicates that there must be a long latency period between BMAA exposure and disease outbreak (Román 1996). Therefore Spencer *et al.* (1991) termed BMAA as a "slow toxin" indicating a delayed toxic mechanism. The putative delayed toxic mechanism was

theoretically explained by Murch *et al.* (2004a) hypothesizing that BMAA can exist in a free and in a protein bound form, in cyanobacteria and flying foxes as well as in humans. The protein bound BMAA is aggregated as an "endogenous neurotoxic reservoir" which can be metabolized and slowly released inducing its neurotoxicity during further processes in the cerebral tissues by different mechanisms, for example an incorporation or association of BMAA into/ with proteins resulting in a misfolding and functional loss of these proteins, or a putative binding to receptors or metal ions resulting in a receptor-mediated toxicity or a depletion of these ions. It has to be mentioned that the biomagnification and the "endogenous neurotoxic reservoir" hypothesis were constructed only on the basis of epidemiological data, a statement indicating that BMAA could possibly be a "slow toxin" and publications showing the appearance of different amounts of free and protein bound BMAA in cyanobacteria (Cox *et al.* 2003), flour (Murch *et al.* 2004a), flying foxes (Cox and Sacks 2002) and brain tissues from Chamorro patients with ALS/PDC (Cox *et al.* 2003).

1.1.1 Putative Exposure Scenarios outside of Guam

The high concentrations of BMAA (627 ±141 µg BMAA⁄ g brain tissue (mean ±SEM)) found in the brain tissue of Chamorro ALS/PDC patients was not locally restricted to Guam. In Canadian Alzheimer's disease (AD) patients also 95 ±32 µg BMAA⁄ g brain tissue (mean ±SEM) were found. Additionally, in the U.S. 134 ±12.8 µg BMAA⁄ g brain tissue (mean ±SEM) have been found in AD patients and 111 ±14.6 µg BMAA⁄ g brain tissue (mean ±SEM) in ALS patients (summarized by Pablo *et al.* (2009)). This indicates that on the North American continent, where the traditional food differs from that on Guam, further exposure alternatives must be given. Indeed, various epidemiological studies report a spatial ALS clustering whereby the neurotoxin BMAA might have impaired the human nervous system:

- In the year 1987, Spencer and colleagues described two cases on Papua Barat (former Irian Jaya) and Kii Peninsula (Japan) where the use of crude seeds and material of *Cycas* in traditional medicine and as poultices may have led to or promoted neurological disorders resulting in the appearance of ALS/PDC symptoms (Spencer *et al.* 1987c; Spencer *et al.* 1987d). However, it has to be mentioned that neither the content nor the concentration of BMAA or/and further *Cycas* toxins in the traditional medicine or poultices were researched.
- An epidemiologic case ascertainment study in the 1990´s described a significantly elevated risk of ALS for 2.5 million service personnel who served during the First Gulf War (1990-1991) in Iraq. A significantly elevated risk of suffering from ALS up to 10 years after the First Gulf War was reported not only for deployed active military, air force and army personnel, but also for deployed non-military personnel

in Iraq (Horner *et al.* 2003). Cox *et al.* (2009) determined BMAA and 2,4-diaminobutyric acid (DAB) in cyanobacterial crusts and mats found in the Gulf region and particularly of Qatar. They hypothesized that the dust disturbed during military activities contained cyanobacteria and their toxins. A potential exposure scenario for serving US military personnel stationed in Iraq was therefore the inhalation of aerosols containing BMAA (and other cyanotoxins) resulting in an uptake of BMAA and neurological disorders in the following decade (Cox *et al.* 2009).

- Caller *et al.* (2009) identified a spatial clustering of ALS in Enfield, a town in Grafton County, New Hampshire, located in the neighborhood of New Hampshire lake and Lake Mascoma. The incidence of ALS was 10 to 25 times higher than the rest of the U.S.A. As they detected the neurotoxin BMAA in one of the lakes (Lake Mascoma) they hypothesized that the direct contact by ingesting BMAA-contaminated lake water and/or inhaling its aerosols or the indirect contact by consuming fish in which BMAA had become accumulated may have led to the symptoms of ALS in this region.

Even though some reports of geographical clustering of ALS have been published, there is still a "substantial lack of adequate studies addressing the environmental factors that may trigger ALS" (Caller *et al.* 2012). Thus, the clustered ALS cases are hard to link to one or more specific environmental factors as a relatively low incidence rate complicates the detection of ALS cases, and additionally a long and a inconsistent period of latency as well as a high mortality rate lead to a delayed and a time-limited analysis of possible exposure factors and scenarios. However, based on improving detection methods and systems as well as an increased public interest and attentiveness toward potential environmental toxins more and more data indicating the occurrence of BMAA and potential contact scenarios for humans are becoming available.

For example, Pablo *et al.* (2009) recently published data demonstrating the availability of BMAA in brains of AD and ALS patients from the southeastern U.S. In parallel, analyses of different rivers and bays in south Florida by Brand *et al.* (2010) showed the occurrence of BMAA in shrimps, crabs and fishes. Whether a contact existed between the (dead) patients with accumulated BMAA in their brains and the regions of Florida, where BMAA occurs in potential aquatic food sources, cannot be confirmed, as it was not or just poorly documented.

In the Peruvian highlands the indigenous people use *Nostoc commune* as a food source as well as in medicine. Although Johnson *et al.* (2008) determined the occurrence of BMAA in various samples of *N. commune* from different locations, neurological disorders or abnormalities were not described within this population group. Whether the production of BMMA by *N. commune* is a relatively rare event and this is why the indigenous people show

no neurological impairments or whether the indigenous people are inherently non-sensitive to BMAA exposure needs further investigation. An accumulation of BMAA in the major food webs, which can possibly culminate in human consumption, seems to be increasingly realistic as it has been observed in the Baltic Sea, where cyanobacterial produced BMAA was shown to bioaccumulate in fish and shellfish via their consumption of zooplankton (Jonasson *et al.* 2010). Additional information on bioaccumulation and biomagnification in natural ecosystems were provided by Esterhuizen-Londt (2010), who determined the bioaccumulation of BMAA in model and naturally BMAA-exposed crop plants as well as the biomagnification of BMAA in animals (crocodile and fish) in a freshwater ecosystem where seasonal cyanobacterial blooms occur, and also by Metcalf *et al.* (2013), who determined BMAA in the feathers of the Lesser Flamingo, *Phoeniconaias minor*. Also reports about BMAA in high-price luxury food products such as shark fin soup (Mondo *et al.* 2012) and *Nostoc* soup (Roney *et al.* 2009) reflect that BMAA can occur in ecosystems and food outside of Guam whereby a chronic exposure may contribute to the development of neurodegenerative diseases and could therefore have important global health implications for the development of neurodegenerative diseases.

The above discussed putative exposure scenarios and findings on Guam and in the rest of the world indicate that the possible role of BMAA in the incidence of ALS/PDC is still neither completely understood nor proven. There is an ongoing dispute about the potential exposition scenarios on Guam (medicine, flour, flying foxes), the effective BMAA concentration the Chamorro had ingested or were indirectly exposed to, as well as the question if the symptoms of ALS/PDC are the results of a multifactorial exposure scenario (e.g., contact with BMAA and additional toxins such as cycasin from *Cycas* sp.). As in the last decade the number of reports concerning possible BMAA exposure scenarios for humans as well as putative BMAA-linked pathological findings increased, a closer look at BMAA and its possible role in human neurodegenerative diseases outside of Guam seems warranted.

1.2 Characteristics of Beta-N-methylamino-L-alanine

1.2.1 Structure of Beta-N-methylamino-L-alanine

Beta-N-methylamino-L-alanine (BMAA) was discovered and described for the first time by the researchers Vega and Bell (1967). They extracted an unknown substance from seeds of *Cycas circinalis* by high voltage electrophoresis, which they first described as γ-diaminobutyric acid (2,4-DAB) (Figure 2). However, further studies carried out by them showed that they had isolated BMAA and more specifically, the L-isomer of BMAA which shows high structural analogy to 2,4-DAB (Vega *et al.* 1968).

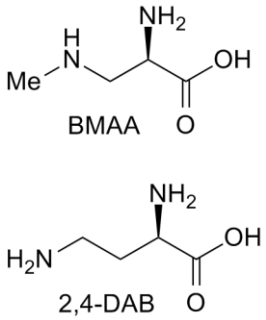

Figure 2: Chemical structures of BMAA and 2,4-DAB

The structure of BMAA displays a high degree of similarity to amino acids based on its configuration of a carboxyl group and a primary and secondary amine. BMAA is therefore mentioned in literature as an amino acid analogue (Abdulla and Campbell 1993) and is also known under the synonyms β-N-methyl-α, β-diaminopropionic acid, α-amino-β-methylamino propionic acid and methylated α, β-diaminopropionic acid (MeDAP).

Under certain physiological conditions (pH 7.4) one amino group can become protonated resulting in a neutral net charge of the molecule BMAA (Nunn and Ponnusamy 2009), which could possibly facilitate transport across membranes such as the blood-brain barrier (BBB) (Duncan *et al.* 1991).

1.2.2 Reactivity and Affinity of BMAA

Spencer *et al.* (1987b) postulated that a formation of metabolites and the neutral net charge characteristics of BMAA can result in an excitotoxic action (see chapter 1.3.1.1). Following this hypothesis Weiss and Choi (1988) published data indicating that BMAA needs physiological amounts of bicarbonate (10 mM) as cofactor for toxicity. The research of this "interaction" led Weiss and Choi (1988) to the conclusion that the reaction of BMAA and bicarbonate does not result in a complexation but rather in the appearance of a new compound – a carbamate. Indeed, the three-dimensional structure of carbamates show similarity to glutamate (Figure 3) (Weiss and Choi 1988; Weiss *et al.* 1989). An over-activation of glutamate receptors (e.g. NMDA) by these glutamate analogues could therefore possibly explain a receptor-mediated toxicity termed excitotoxicity. However, the total amount of two carbamates, α-N-carboxy-BMAA and β-N-carboxy-BMAA, was only 31% under physiological conditions in a 23 mM bicarbonate solution (Myers and Nelson 1990) and furthermore the carbamate reaction was reversible (Nunn and O'Brien 1989).

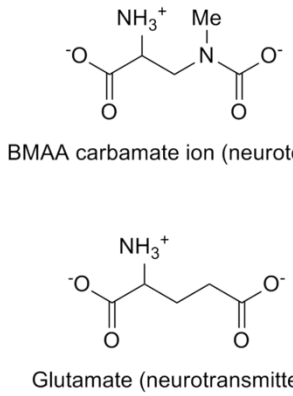

Figure 3: Structure of N-carboxy-BMAA (BMAA adduct) and the neurotransmitter L-glutamate.

In addition to the above mentioned carbamate building characteristic of BMAA with bicarbonate, a high affinity of BMAA for Cu^{2+}, Zn^{2+} and Ni^{2+} ions has also been reported (Nunn *et al.* 1989). The structure of the Cu^{2+}-BMAA complex was researched in the following years (Hursthouse *et al.* 1990) based on the assumption that the building of complexes as well as chelating of ions may have an impact on functionality and structure of proteins, for example by influencing the enzymatic activity via binding to ions in the active site or by binding to receptors and/or its cofactors (Esterhuizen-Londt *et al.* 2011; Nunn *et al.* 1989).

Even though BMAA shows characteristics of an amino acid and its structure is very similar to other unnatural β-amino acids, which have been demonstrated to become bound to t-RNA and have the potential to become incorporated (Hartman *et al.* 2007; Hartman *et al.* 2006), an incorporation of BMAA into proteins has not been demonstrated. Non-experimental based discussions therefore postulated that BMAA is a non-proteinogenic amino acid which can be found in a “free” and “protein bound” form (Murch *et al.* 2004a). The assumption of two “states” of BMAA is based on two extraction methods by which BMAA can be detected: The “free” form of BMAA is given following a trichloroacetic acid (TCA) precipitation and the ”protein bound” form of BMAA following acid hydrolysis of the TCA precipitated material. Both “states” of BMAA have been detected in *Cycas* sp., cyanobacteria as well as in animals and humans (Banack *et al.* 2006; Bradley and Mash 2009; Cervantes *et al.* 2012; Cox *et al.* 2003; Esterhuizen and Downing 2008; Murch *et al.* 2004b). The amount of the protein bound form of BMAA can be up to 120 to 240-fold higher than the free form (Ince and Codd 2005; Murch *et al.* 2004b).

1.2.3 Production and Producers of BMAA

BMAA was isolated for the first time from seeds of *Cycas circinalis* by Vega and Bell (1967). However, until this day no published data are available demonstrating a pathway by which

BMAA can be synthesized. The analysis of an expressed sequence tag (EST) library from cycad leaves by Brenner *et al.* (2003) hints to a theoretical synthesis of BMAA by a two-step biosynthetic pathway (Figure 4). Thereby the genes for two essential enzymes, a cysteine-synthase and methyl-transferase enzyme were identified in the library of *Cycas* sp.. These enzymes could theoretically transfer ammonia to β-carbon of a β-substituted alanine (cysteine, phosphoserine, o-acetylserine or cyanoalanine) or alternatively, catalyse the methylation in the second step for the BMAA synthesis pathway.

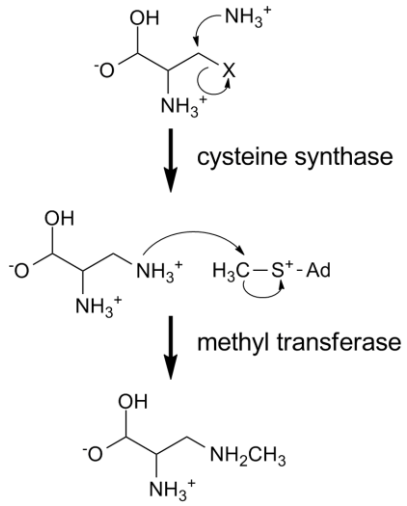

Figure 4: Proposed biosynthesis of BMAA, predicted on the basis of an analysis of an expressed sequence tag (EST) library. X= phosphoserine, cysteine, o-acetylserine or cyanoalanine; Ad-S-CH3=S-adenosylmethionine. (Adapted from Brenner *et al.* (2003)).

The biosynthesis of BMAA in cyanobacteria has still not been described. Only Araoz *et al.* (2010) postulated that the same biosynthesis pathway suggested by Brenner *et al.* (2003) is theoretically also possible in cyanobacteria as orthologous genes of the cysteine-synthase and methyl-transferase are present in the genome of cyanobacteria, too.

The existence of BMAA in the seeds of *Cycas circinalis* was described in the year 1967 (Vega and Bell 1967). Following analyses of the genus *Cycas* as well as of the BMAA content in leaf tissue, seeds, roots and flower showed variable concentrations of BMAA in different *Cycas* species, maturation states and especially in different parts (Banack and Cox 2003b; Cox *et al.* 2003; Dossaji and Bell 1973; Duncan *et al.* 1989; Kisby *et al.* 1992a; Murch *et al.* 2004a).

Focusing on coralloid roots of *C. micronesica* as these show a higher content of BMAA compared to roots with normal morphology, Cox *et al.* (2003) isolated and cultivated an axenic cyanobacterium of the strain *Nostoc* which synthesized BMAA independently of *C. micronesica* (0.3 µg g^{-1}). Hypothesizing that BMAA can be produced by cyanobacteria and the content of BMAA found in *Cycas* is just a result of a magnification of cyanobacterially-produced BMAA, based on a symbiosis of *Cycas* with cyanobacteria, further BMAA-

producing strains of cyanobacteria were discovered. Indeed Cox *et al.* (2005) found presence of BMAA in 27 out of 30 species or strains of free living cyanobacteria and Downing *et al.* (2011) described a *de novo* synthesis of BMAA in the axenic unicellular non-nitrogen fixing cyanobacterial culture, *Microcystis* PCC7806.

Contrary to the above mentioned findings indicating that BMAA can be produced by cyanobacteria and numerous reports indicating that BMAA was detected in different cyanobacterial species (Brand *et al.* 2010; Cervantes *et al.* 2012; Esterhuizen and Downing 2008) there are also some findings indicating that only some rare cyanobacteria can produce the substance BMAA. Based on these data and the detection methods used for BMAA there is still an ongoing scientific controversy about the occurrence of BMAA and if it is produced in cyanobacteria or in *Cycas* (Kruger *et al.* 2010; Marler *et al.* 2010; Snyder and Marler 2011).

1.2.4 Transport and Metabolism of BMAA

As BMAA can be found in diverse genera of *Cycas* and is located in different plant parts at different concentrations (Banack and Cox 2003b; Cox *et al.* 2003; Dossaji and Bell 1973; Duncan *et al.* 1989; Kisby *et al.* 1992a; Murch *et al.* 2004a) and following the hypothesis of BMAA as a product of a symbiosis of cyanobacteria and *Cycas*, a transport of BMAA within plants is obvious. Data from various publications showed that BMAA cannot only be found in plants and cyanobacteria but also in the brain region of humans as well as in animals (Banack and Cox 2003a; Murch *et al.* 2004b) which also necessitates a transport of BMAA within humans and animals.

For the risk assessment calculation of BMAA for humans, a determination of the bioavailable concentration of BMAA in the body of humans/ animals seemed necessary to enable the examination of the concentration of BMAA which could possibly be involved in causing neurodegenerative or other pathological symptoms. Experiments in Cynomolgus monkeys showed that nearly 79% of i.v. injected isotope-labeled BMAA were bioavailable in the body of the monkeys and maximum 20% were metabolized on first pass; 2.1% of total BMAA were excreted (Duncan *et al.* 1992). Additional data about the bioavailability of BMAA in cerebrospinal fluid were published by Kisby *et al.* (1988). They applied BMAA by oral dosing to Cynomolgus monkeys which resulted in a time-dependent increased appearance of BMAA in their cerebrospinal fluid. In the same publication they detected BMAA in the brain tissue of Sprague-Dawley rats following i.p. BMAA treatment for 14 days. The bioavailability of BMAA in rats was found to be similar as in monkeys (Duncan *et al.* 1991). The occurrence of BMAA in the brain regions of monkeys as well as rats leads to the hypothesis that BMAA can/ must be able to cross the BBB. However, only a low, homogeneous distribution of BMAA in the brains of adult C57BL mice following a single i.v.

BMAA injection was found (Karlsson *et al.* 2009a). Furthermore, the injection of BMAA into pregnant and neonatal mice revealed the accumulation of high BMAA amounts in fetal and neonatal brains, respectively (Karlsson *et al.* 2009b). Additionally, a transplacental transfer of BMAA was shown. The observation that BMAA in the mice brains was dependent on their developmental stage allows the presumption that a change in the BBB permits BMAA to cross. A changed transporter expression is conceivable as a developmental modulation of BBB transporters is known (Auguy-Valette *et al.* 1978; Vannucci and Simpson 2003). One of these transporters, whose expression level and functionality in the BBB as well as in the placenta is dependent of the developmental stage of the organism, is the large neutral amino acid transporter (LAT) (Boado *et al.* 2004; Ritchie and Taylor 2001). In fact Smith *et al.* (1992) demonstrated that the uptake of BMAA into the brain across the BBB is possible via the LAT1 in rat brains with an *in situ* brain perfusion technique. They also conjectured that the uptake of BMAA via the LAT1 might be sensitive to factors such as age, diet, metabolism and disease.

As well as the above mentioned LAT system, a cystine/ glutamate antiporter (Xc- system) was also discussed as a possible transporter for BMAA (Lobner *et al.* 2007). A direct toxic mechanism of BMAA was then assumed linked to the Xc- system. It was mentioned that the competition of BMAA with cystine at the Xc- system could lead to glutathione depletion and therefore to oxidative stress (Liu *et al.* 2009; Lobner *et al.* 2007).

1.2.5 *In vivo* Effects of BMAA

Soon after the discovery of the putative neurotoxin BMAA an animal model was used to characterize the (neuro-) toxicity and obtain knowledge about the putative mechanism of action of BMAA. Vega *et al.* (1968) administered BMAA (0.34–0.82 mg/g body weight) intraperitoneal (i.p.) to R-X-S chickens as well as young female Wistar rat pups (0.68-1.64 mg/g body weight) to identify this molecule as a putative neurotoxin from *Cycas* which, as they hypothesized, could be responsible for the raised incidence of the neurodegenerative disease ALS/PDC on Guam. Indeed both animal models showed an impaired coordination of the legs following treatment with the L-isomer of BMAA, whereas the D-isomer had no effect on the animals. The group around Vega and Bell also showed that i.p. applied DL-BMAA (2.52 and 3.34 mg/g body weight) in the mice model could lead to comparable impairments and uncoordinated movements as in rats and chickens (Polsky *et al.* 1972). However, a chronic subcutaneous (s.c.) administration of a lowered amount of DL-BMAA (0.28 mg/g body weight) resulted in no obvious observable neuronal dysfunctions. Therefore, the authors concluded that BMAA might not be the toxic component directly involved in the generation of ALS/PDC on Guam as the effects in their model organism were neither progressive nor chronic and all neurological impairments

followed immediately after the application of L-/ DL-BMAA (i.e. no long latency). Typical neuropathological findings were also not observable.

Some years later, when Ross and Spencer (1987) found behavioral impairments described as “whole body shaking” and “wobbling” in CD-1 mice in response to an intracerebroventricular (i.c.v.) administration of BMAA, the hypothetical correlation of BMAA ingestion and ASL/ PDC outbreak has attracted increasing attention of international scientists. This was especially based on their finding that a correlation of the neuro-behavioral impairments and the applied BMAA dose exists, as an increased BMAA concentration induced an extended duration of “whole body shaking” and wobbling, and, that the BMAA-induced impairments could be ameliorated by application of an N-methyl-D-aspartic acid (NMDA) receptor antagonist (AP7). Lindström *et al.* (1990) focused on this finding and demonstrated data from Sprague-Dawley rats which were administered BMAA intracisternally (400 µg/ 150-200 g body weight) or intracerebrally (10 µg/ 150-200 g body weight) into the susbstantia nigra. They concluded, based on the histology and monoamine levels, that subacute concentrations of BMAA can activate NMDA receptors and selectively affect noradrenergic neurons, whereas dopaminergic neurons were affected by nonselective mechanisms. The involvement of further subtypes of excitatory amino acid receptors (e.g., NMDA, EAA and AMPA) was suggested following chronic BMAA exposure of Sprague-Dawley rats (10 to 60 days; 500 µg/ 200-250 g body weight) with or without specific non-NMDA and NMA receptor antagonists (Matsuoka *et al.* 1993; Rakonczay *et al.* 1991), which also demonstrated physical impairments including body shaking and jerky movement.

These findings support the hypothesis of Seawright *et al.* (1990) that BMAA can act as an excitotoxin. In their experiments young rats showed functional disturbances in movement following a single high dose i.p. treatment with racemic BMAA (4 mg/g body weight). The neurophysical impairments became more emphasized in the following 48 hours. These progressive neurological disorders were also observable following a single L-isomer BMAA (0.9 mg/g body weight) or a chronic L-isomer BMAA treatment (0.5 mg/g body weight per day) over several days. The progressive development and the cumulative effects of BMAA treatment were not only observable in the behavioral dysfunctions of the rats but also in the degeneration of specific cerebellar neurons such as cerebellar stellate, basket, Purkinje and Golgi cells, whereas granule cells and the central nervous system (CNS) remained unaffected. They therefore concluded that neurons using GABA as neurotransmitter (GABAergic) can selectively be impaired by BMAA via an excitotoxic mechanism. This putative acute excitotoxic mechanism was also observable in Swiss Webster mice following 1-10 µmol i.c.v. BMAA racemate administered acutely. Motor-neuronal effects (ataxia, ptosis, rolling, unsteady gait, scratching, forelimb clonus, hyperlocomotion, myoclonic jerks, jumping, clonic muscle spasms, tonus, recovery, hypolocomotion) were manipulable by co-treatment with specific inhibitors (Smith and Meldrum 1990). The inhibitors for the

α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)/quisqualate subtype of glutamate receptors reduces thereby the activation more effectively than inhibitors for the NMDA or kainate subtypes which lead the authors to the conclusion that the latter receptor subtypes are not involved in the BMAA-mediated excitotoxicity.

Dawson *et al.* (1998) researched neurochemical and neurobehavioral effects of subcutaneously (s.c.) administered L-BMAA (100 and 500 mg/kg body weight) in postnatal injected Sprague–Dawley rats. They found treatment- as well as sex-dependent changes in the neurobehavior and in motor functions as well as in the neurochemistry of the spinal cord in the rats. However, they stated that the neurochemical differences in BMAA treated rats were not consistent with changes in ALS patients' tissues and therefore not related to a direct excitotoxic mechanism of BMAA but rather to subtle modification of postnatal neural development. This hypothesis of non-excitotoxic acting BMAA was supported by histopathological findings in mice presented by Buenz and Howe (2007) who indicated that BMAA (10 ml of 100 mM BMAA) can provoke *in vivo* injuries in hippocampal neurons and therefore has a neurotoxic potential *in vivo*. However, as the intracranially (intrastriatal) injected BMAA did not lead to a complete destruction of the hippocampal structure and the injuries of pyramidal neurons were just limited and sporadically impaired, they discussed that besides higher BMAA concentrations and longer incubation times the neurotoxin "BMAA predisposes cells to other types of damage" (Buenz and Howe 2007) which could lead to a more intensive and a more similar pathology of ALS/PDC. Indeed in the following years, Karlsson and colleagues published data indicating that BMAA, i.v. injected (0.91 µg⁄ kg) to C57BL⁄ 6J mice, has a high affinity to melanin and therefore accumulates in a time-dependent manner in melanin containing tissue like eyes, kidney, liver and pancreas (Karlsson *et al.* 2009a) in mice and neuromelanin-containing motor neurons in the trochlear chiasma under the optic tectum in frogs. However, only a low and homogeneous distribution of BMAA was found in the brain tissue of mice. In follow up experiments Karlsson and colleagues changed their model organism and administered BMAA (200 or 600 mg/kg) twice to neonatal Wistar rats during the brain growth spurt (BGS) (Karlsson *et al.* 2009b). The BGS is a central state during the CNS development as during the BGS various important neurodevelopmental changes occur (e.g., growth of axons and dendritic cells, establishment of neuronal networks, synaptogenesis) and a characteristic dramatic increase in brain weight is observable at postnatal days (PND) 4–9 in rats (Dobbing and Sands 1979; 1973). These applications during the BGS (PND 9 and 10) resulted in a BMAA uptake over the BBB (discussed above) and in acute behavioral deficits (impaired locomotor ability and hyper-activity) (Karlsson *et al.* 2009b). Treatment of Wistar pups during the BGS with 50 mg/kg and 200 mg/kg resulted in an impairment of learning and memory functions, however without any morphological abnormalities. Morphological abnormalities were only observable in the neonatal hippocampus following high-dose (600 mg/kg) treatment (Karlsson *et al.* 2011). Besides these short- and long-term behavioral effects Karlsson *et al.*

(2012) also determined histopathological changes (neuronal degeneration, neuronal cell loss, calcium deposits and astrogliosis) in the hippocampus of the adult rats six months after their neonatal exposure (twice during the BGS with 150 mg/kg body weight and 600 mg/kg body weight BMAA) and changes in various protein levels in this region. They observed a decrease of free ubiquitin and changes in the expression of S100β, histones, calcium, calmodulin-binding proteins and guanine nucleotide-binding proteins. Also α-synuclein was detected following high-dose BMAA treatment, whereas hyper-phosphorylated tau protein (AT8) was not observable. These changes of proteins and enzyme levels following BMAA treatment can be seen in *in vivo* and *in vitro* data published and reviewed by Kisby and Spencer (2011), which indicate that L-BMAA interferes with protein and RNA synthesis in different brain tissues of rats. This conclusion was made from experimental data demonstrating a reduced detectable amount of L-[1-^{14}C]-leucine in the motor cortex and the striatum and a reduced amount of [5-^{3}H]-uridine in the striatum and the cerebellum in BMAA pre-treated rats (daily i.p. BMAA injection (100 mg/kg body weight) for 14 days; finally 30 min exposure with labeled L-[1-^{14}C]-leucine (protein synthesis) or [5-^{3}H]-uridine (RNA synthesis)). Even though the reduction of L-[1-^{14}C]-leucine in the BMAA pretreated rats was not statistically significant, a distinct tendency was observable.

The observations and findings from rodent models, discussed above, suggest that BMAA can induce neurological changes and hence lead to a neurodegeneration similar to that observable in ASL/ PDC patients. However, some few observations in the mouse model revealed that BMAA applied by gavage (daily 28 mg/ kg body weight, for 30 days) (Cruz-Aguado *et al.* 2006) or by feeding pellets (daily minimum 200 mg/mg body weight) (Perry *et al.* 1989) is non-toxic as no neurobehavioral, physiological or neuropathological abnormalities were observable. This again demonstrates that the toxicity of BMAA depends on the model system.

For this reason, other models beside rodents were used to elucidate the putative toxicity of BMAA. Some few data from a primate model have been published (Kisby *et al.* 1988). These demonstrated the presence of BMAA in blood and cerebrospinal fluid in Cynomolgus monkeys (and rats) following oral administration over 12 months (100-350 mg/kg body weight), thus demonstrating its ability to cross the primates' BBB and become bioavailable to brain tissue. Earlier experiments with primates used cycad flour, whose BMAA content can only be estimated (Duncan *et al.* 1991). Additionally, the experimental design (replicates and endpoints) as well as the results were often not reproducible (Dastur 1964; Dastur and Palekar 1974) or the observed effects did not appear specifically in the treatment groups but also in the control groups (Garruto *et al.* 1989; Garruto *et al.* 1988). This made it nearly impossible to draw any conclusions on BMAA toxicity in primates from studies published before Spencer and colleagues demonstrated neurophysiological and neuropathological evidence that L-BMAA can induce motorneuronal dysfunctions in the

upper and lower motor neurons and the extrapyramidal system in Cynomolgus monkeys following a high-dose BMAA treatment (100-350 mg/kg body weight) by gavage for a duration of up to ten weeks (Spencer *et al.* 1987a; Spencer *et al.* 1987b). However, the main criticism in this study, besides the very high dosage of BMAA used, was that the observable effects in the Cynomolgus monkeys were acute and reversible and did not appear delayed and progressive, contrary to the symptoms of human neurodegeneration in ALS/PDC. Additionally, even though a transport of BMAA over the BBB was detectable, Duncan and colleagues concluded (Duncan *et al.* 1992; Duncan *et al.* 1991) from their own studies with monkeys and rats that the neurotoxin was poorly transported into the brains and became rapidly cleared from circulation which led to a temporary abandoning of the cycad/BMAA hypothesis and a cessation of primate experiments as reviewed by Karamyan and Speth (2008).

1.2.6 *In vitro* Effects of BMAA

As the meta-analysis of the data from *in vivo* studies in the model organisms (mice, rats and monkeys) makes it impossible to carve out a single toxic mechanism of the neurotoxin BMAA, researchers additionally focused on *in vitro* studies and methods to evaluate on the one hand the toxic potential of BMAA and on the other hand to identify the mechanism by which BMAA can induce neurodegeneration leading to the symptoms of ALS/PDC in humans.

In the first *in vitro* experiments, Ross *et al.* (1987) analyzed cellular pathological changes (postsynaptic vacuolation and shrunken cells) in cortical explants from mice. They demonstrated a reduction of histopathological changes and therefore a protection against BMAA (3.2 mM) induced neurotoxicity following co-treatment with the NMDA antagonist AP7 (0.1-1 mM). Experiments by Weiss *et al.* (1989) on NMDA channels using the patch clamp method demonstrated that the NMDA channel opening in primary cortical mouse neurons only occurs in the presence of BMAA (300 µM) dissolved in bicarbonate (10 mM). These findings correlated with their former findings of bicarbonate-dependent BMAA neurotoxicity (measured as LDH release) (Weiss and Choi 1988). As the opening of NMDA channels was only induced by high concentrations of BMAA, Rao *et al.* (2006) used primary embryonic spinal cords from mice to identify motor neurons as a selective target of BMAA (0.03 to 1 mM BMAA; LDH release) and demonstrated that BMAA can activate the AMPA/ kainate receptor with “far greater potency” (Rao *et al.* 2006) than NMDA channels. The activation of AMPA/ kainate receptor led to a rapid Ca^{2+} entry (1 mM BMAA) through Ca^{2+} permeable AMPA/kainate channels and consequently to ROS generation (1 and 3 mM BMAA), probably produced by mitochondria (Rao *et al.* 2006). The potential neurotoxicity of 0.05 to 1 mM BMAA was demonstrated in the motorneuronal hybrid cell line NSC-34

from mice, too (Buenz and Howe 2007). In addition to its acting at ionotropic receptors (NMDA and AMPA/kainate) Lobner *et al.* (2007) published data indicating that 3 mM BMAA can also induce toxicity through the activation of the metabotropic mGluR5 receptor, whose activation can also be found to be implicated in Parkinson's disease (PD) (Marino *et al.* 2003). An activation of both (ionotropic and metabotropic) receptors was also demonstrated in primary cultures of striatal neurons of mice (Manzoni *et al.* 1991). Even though in the primary mixed cortical cells from mice (Lobner *et al.* 2007) the toxicity was only significant following treatment with 1, 3 and 10 mM BMAA, they found that concentrations as low as 10-100 µM BMAA can potentiate the toxic effects of known toxic substances (NMDA, Fe^{2+}, BSO, MPP^{+} and amyloid β), as measured by LDH release. This finding and an observed oxidative stress (ROS development following 3 mM BMAA treatment), which was not reducible by the inhibition of NMDA or mGluR receptor activity led the authors to the conclusion that the BMAA-mediated toxicity may underlie multiple mechanisms. Thus the neurodegeneration may not only be due to ionotropic or metabotropic receptor activation but also to a hypothetical competition of BMAA with cystine at the cystine/ glutamate antiporter leading to a decreased cystine uptake and depletion of intracellular glutamate causing a depletion of glutathione and therefore a reduced radical scavenging capability in neuronal cells (Figure 5) (Lobner *et al.* 2007). In a follow-up study they hence demonstrated additional data concerning an inhibition of the cystine uptake by the system Xc^{-} (the cystine/ glutamate antiporter), increased glutamate release and a decreased cellular glutathione level following 3 mM BMAA treatment in the same *in vitro* model (Liu *et al.* 2009).

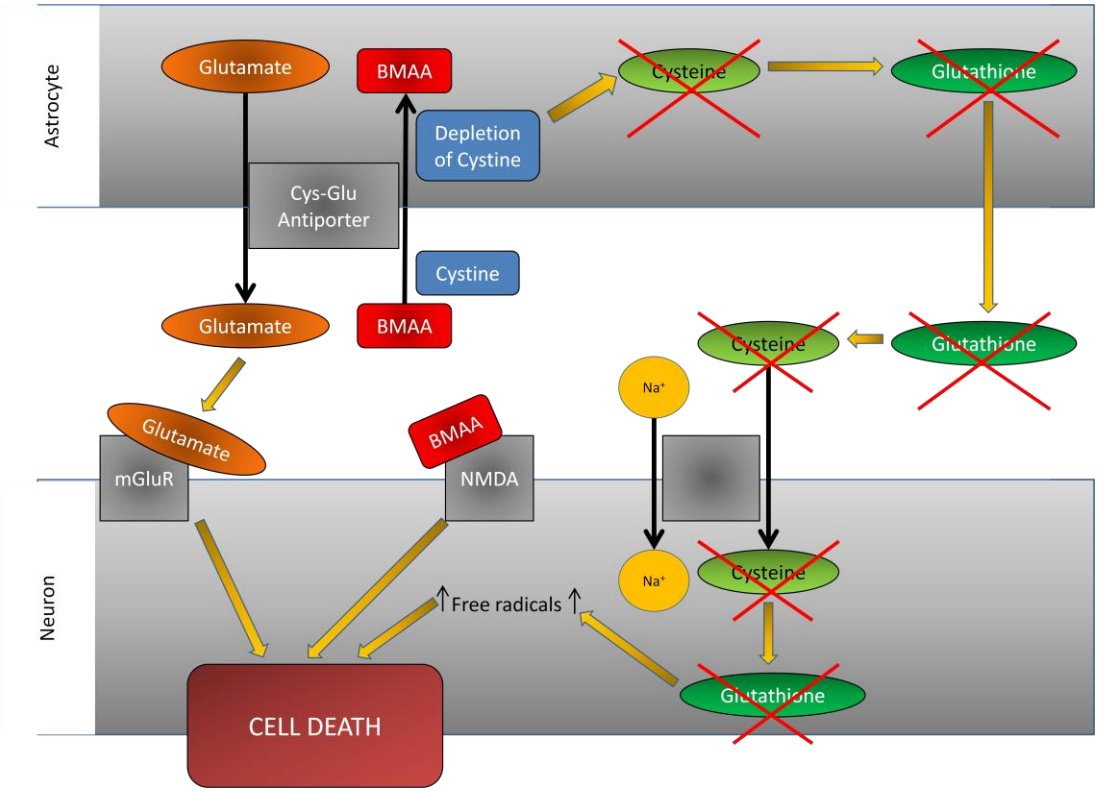

Figure 5: Putative mechanism for a BMAA-induced neurotoxicity via the activation of ionotropic and metabotropic receptor activation and/or the transport of BMAA over the cystine-glutamate antiporter resulting in a depletion of glutathione in astrocytes and neurons (Adapted from Lobner (2009)).

As well as *in vitro* data based on mice cells some experiments carried out in *in vitro* rat cells yielded comparable results to those of *in vitro* mice cells. As Richter and Mena (1989) demonstrated in primary cortical neurons of rats by binding assays using [^{3}H]-glutamate, bicarbonate ions are required for the interaction of BMAA (1 mM) with the glutamate receptor and therefore for a (non-determined) putative neurotoxicity *in vitro* in rat cells, which is analogous to the findings from *in vitro* experiments with mouse cells (Weiss and Choi 1988; Weiss *et al.* 1989). According to a putative ionotropic and metabotropic receptor activation of BMAA in mouse cells (Rao *et al.* 2006) experiments in rat brain slices indicated the activation of both receptor types by BMAA, too (Copani *et al.* 1991; Copani *et al.* 1990). These findings were determined by various uptake and binding assays. Additionally, an impaired Ca^{2+} homeostasis and the hydrolysis of polyphosphoinositides led the authors to suggest that BMAA can induce neuronal death in the rat cells. An impaired Ca^{2+} homeostasis was also found in whole brain cells from neonatal (<24h) rats whereby 5 mM BMAA induced an increase of the intracellular calcium levels if BMAA was applied in the presence of bicarbonate (Brownson *et al.* 2002), whereby the kind of cells affected was not described in detail. Cucchiaroni *et al.* (2010) focused on the specific effects of BMAA on the substantia nigra pars compacta and their dopaminergic cells, as an impairment of this

region and its neuronal loss is a described characteristic in sporadic Parkinson's disease (Beal 1998; Ince and Codd 2005). In these cells again a membrane-associated action of BMAA was demonstrated at the mGlu receptor with the activation of transient receptor potential channels resulting in cytosolic Ca^{2+} accumulation as well as a BMAA-induced cytochrome-c release and ROS production. A "potential synergistic effect" of BMAA and NMDA, defined as "larger Ca^{2+} accumulations" by co-treatment with both substances, was reported and thus the occurrence of "neuronal stress" and BMAA could theoretically cause dysfunctions and delayed injuries in the "metabolically compromised dopaminergic cells" (Cucchiaroni *et al.* 2010).

Besides the implications of BMAA in the brain tissue and in homogenates of livers and kidneys from rat, the occurrence of dimethylated BMAA (2,3-diaminopropionic acid) indicates that BMAA can be metabolized in these tissues by an N-demethylase (Nunn and Ponnusamy 2009). 2,3-diaminopropionic acid with the cofactor bicarbonate is described to be toxic to neurons (Weiss *et al.* 1989) and thus may be involved in progressive neurodegeneration. Significantly reduced concentrations of taurine and serine in slices of rat brain cortex following 10 mM BMAA treatment were interpreted by Nunn and Ponnusamy (2009) to possibly be the result of a BMAA-induced inhibition of the amino acids synthesis or a BMAA-affected export of those. In a final statement the authors declare that BMAA caused "a number of distinct biochemical changes" in their experiments and thus the neurotoxic mechanism of BMAA is "multifaceted" and likely "varies from species to species".

As further *in vitro* studies with primary nerve cells of leeches showed comparable results as the *in vitro* mice and rat data, for example a BMAA-induced depolarization by an increased membrane permeability, an activation of non-NMDA ionotropic glutamate receptors and an impaired Na^+ and K^+ homeostasis (Lopicic *et al.* 2009; Nedeljkov *et al.* 2005), Chiu *et al.* (2012) were the first to demonstrate effects of BMAA in a human *in vitro* model system with primary cells. They isolated primary neurons from human fetal brains and showed BMAA-mediated cytotoxicity (as determined by LDH release). This neurotoxicity could be prevented by co-treatment with MK801. This finding led them to their conclusion, even though control experiments were missing, that all subsequent effects were NMDA receptor-mediated. Thus the excitotoxicity led to the induction of Ca^{2+} influx, ROS development by stressed mitochondria, resulting DNA damage and the increased occurrence of neuronal nitric oxide synthase (nNOS) and caspase 3 cleavage, which may lead to activation of an apoptotic pathway. The authors themselves mentioned that the presented data were the same as those already described in mice, rat and leeches and therefore no new scientific insights were gained except the description of developing NOS. Thus they concluded that BMAA has a "similar effect on human neurons" as on those of rodents and leeches *in vitro* and *in vivo*. Although they assigned all observed effects to the excitotoxic acting of BMAA, Chiu and colleagues also agreed, based on ROS development

without bicarbonate, that BMAA could become toxic by an NMDA receptor-independent process, because it is not able in its native state to bind as a non-carbamate to glutamatergic receptors (Chiu *et al.* 2012). Contrary to the observations of Chiu *et al.* (2012) in primary human cells, data from one differentiated (NT-2) and two non-differentiated human neuronal cell lines (SH-SY5Y and SK-N-MC) demonstrated only a weak toxicity, which was comparable to the toxicity of β-alanine as determined by LDH release following 5 days BMAA treatment (Lee and McGeer 2012). The toxicity of BMAA was 300- to 400-fold lower than that of glutamic acid and a range of other excitotoxins (e.g. kainic acid, quisqualic acid, ibotenic acid, domoic acid, and quinolinic acid). The authors concluded from these data furthering combination with other published *in vitro* and *in vivo* studies, which showed that high concentrations of BMAA were needed to induce observable effects, that BMAA is a weak neurotoxin and further, that its action at glutamatergic receptors is weak (Lee and McGeer 2012).

Summarizing the available *in vitro* data it is clear that BMAA can act on different cell types from animals or humans with different intensity. The observable sensitivity toward BMAA is therefore highly variable. Nearly all hypotheses and therefore experiments focused on a putative receptor-mediated toxicity of BMAA with classical endpoints such as ROS development, LDH release with or without antagonists for specific metabotropic or ionotropic receptors, as well as Ca^{2+} influx into the cytosol. However, some data concerning a putative downstream apoptotic pathway, for example caspase 3 cleavage (Chiu *et al.* 2011) or cytochrome-c (Cucchiaroni *et al.* 2010) release from mitochondria, are available.

1.3 Putative Mechanism of Action for BMAA

1.3.1 Receptor-mediated Toxicity

Chiu *et al.* (2011) reviewed most of the published *in vivo* as well as *in vitro* data dealing with the receptor-mediated toxicity of BMAA. They concluded that BMAA is present *in vivo* as a carbamate (discussed above) which thus results on the one hand in an inhibition of the cystine/ glutamate antiporter (Figure 6 V) and on the other hand in a binding and activation of metabotropic (mGluR) and ionotropic (NMDA and AMPA) glutamate receptors (Figure 6 I). The inhibition of the antiporter system Xc^- leads to an intracellular depletion of glutathione as cystine is no longer available for its synthesis (Figure 6 VII). The depletion of glutathione can – theoretically – support the increased production of ROS in BMAA exposed neurons as glutathione is an important antioxidant which can rescue the cells from different kinds of ROS by a direct, non-enzymatic reaction with these radicals (Saez *et al.* 1990; Winterbourn and Metodiewa 1994). The activation of metabotropic and ionotropic

glutamate receptors (Figure 6 I) would lead to an impaired ion homeostasis (Figure 6 II) as the activation of the metabotropic G-protein-coupled glutamate receptor (mGluR) results in the activation of G-proteins (reviewed by Niswender and Conn (2010)) and a release of Ca^{2+} from the endoplasmic reticulum (ER) (reviewed by Bellone *et al.* (2008)) into the cytosol. The increased Ca^{2+} or activation of the mGluR can in turn open calcium-activated potassium channels, resulting in a decreased amount of intracellular potassium (reviewed by Conn and Pin (1997)). Activation of the ionotropic glutamate receptors (iGlurR) AMPA and NMDA, which are ligand-gated nonselective cation channels, allows the influx of Na^{+} and Ca^{2+} into and K^{+} efflux out of cells (reviewed by Traynelis *et al.* (2010)), again resulting in an impaired intracellular ion homeostasis (Figure 6 II) as well as an NMDA receptor-mediated release of noradrenalin (Figure 6 III). This release of noradrenalin was observed in mice (Lindström *et al.* 1990) and rat experiments. Chiu *et al.* (2011) additionally mentioned, when referring to the electrophysiological effects of BMAA on the Retzius nerve cells of leech (Nedeljkov *et al.* 2005), that cells can become depolarized by an impaired ion homeostasis resulting from glutamate receptor activation. The authors stated that neuronal cell death can be induced by BMAA in response to the uncontrolled increase of the intracellular Ca^{2+} levels. These are mediated by disrupted mitochondrial function (Figure 6 VII and VIII) indicated by increased ROS levels (Chiu *et al.* 2012; Cucchiaroni *et al.* 2010; Lobner *et al.* 2007; Rao *et al.* 2006; Santucci *et al.* 2009) and a cytochrome c release into the cytoplasm. The above mentioned authors suppose that the main toxic mechanism of BMAA is therefore its excitotoxic action, leading to an apoptotic cell death.

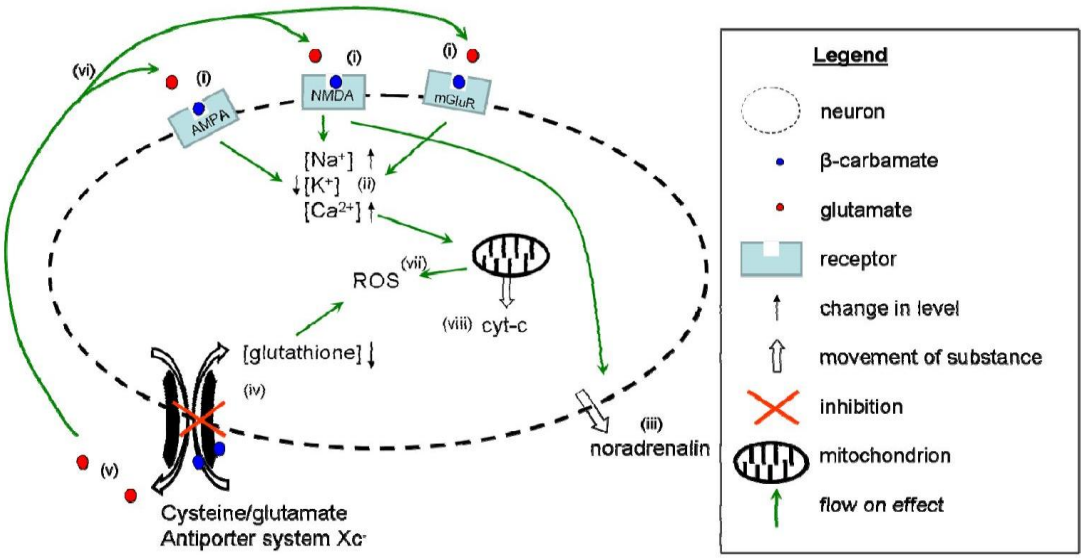

Figure 6: Hypothetical model for the neurotoxic action of BMAA on neurons, summing up various *in vivo* and *in vitro* data by Chiu et al. (2011). Thereby BMAA acts by binding to glutamate receptors or inhibition of the cystine/ glutamate antiporter (Xc- system).

1.3.1.1 Excitotoxicity

The excitotoxic mechanism results in cell death caused by a hyperactivation of glutamate receptors by agonists. In the mammalian CNS the excitatory neurotransmitter glutamate is the major excitatory neurotransmitter. An overactivation of the glutamatergic neurons by a prolonged exposure to the excitatory amino acid glutamate (or analog) can result in injuries, degenerations and ultimately neuronal death. An overstimulation of glutamate and glutamatergic pathways could contribute to the development of various neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, as well as Huntington's disease (HD) (reviewed by Dong *et al.* (2009)).

The activation of excitatory effects can be induced by three major types of ionotropic glutamate receptors and some metabotropic receptors. The ionotropic glutamate receptors (iGluR), which are activated by NMDA, AMPA and kainic acid (KA), are ligand-gated ion channels and permeable to various cations (reviewed by Danysz and Parsons (2003)). Their over-activation causes an excessive influx of cations into the neuronal cell. NMDA as well as AMPA receptors are Ca^{2+}-favoring glutamate-gated ion channels. Overstimulation of these receptors causes an increase of intracellular Ca^{2+} resulting in impaired catabolic enzyme activities and characteristic excitotoxic downstream effects like mitochondrial membrane depolarization, caspase activation, production of toxic oxygen and nitrogen free radicals. These can ultimately induce apoptosis or necrosis. In contrast, the activation of the metabotropic glutamate receptors (mGluR) is coupled to intracellular G-proteins and therefore mediates a slower synaptic response than iGluR. Various subgroups of mGluR induce different pathways. The mGluR1 and mGluR5 subunit subtypes are linked to the inositol trisphosphate (IP3)/Ca^{2+} signal transduction pathway. This pathway affects the activation of protein kinase and the stimulation of a Ca^{2+} release from neuronal stores such as the ER, leading to a delayed cell death (reviewed by Michaelis (1998)).

A further store of cellular Ca^{2+} and simultaneously a regulator of Ca^{2+} homeostasis are the mitochondria (White and Reynolds 1995). The electron transport chain in mitochondria generates a proton electrochemical gradient by which Ca^{2+} can become sequestered into the mitochondrial matrix leading to the depolarization of the mitochondrial potential (reviewed by Gunter and Gunter (1994)). An uncontrolled influx of Ca^{2+} into mitochondria decreases the electrochemical gradient and finally leads to reduced ATP synthesis (Wang *et al.* 1994). To counteract the reduced ATP synthesis rate, ATP-dependent Ca^{2+} pumps become initiated (Schinder *et al.* 1996). These three mechanisms – the accumulation of intramitochondrial Ca^{2+}, the ensuing reduced ATP synthesis, and the increased ATP usage by the Ca^{2+} pumps – were discussed by Schinder *et al.* (1996) as the main causes for excitotoxic-induced cell death. Additionally, the Ca^{2+} overload can evoke a rise in ROS by the aberrant mitochondrial electron chain function (reviewed by Peng and Jou (2010)), resulting in the production of oxygen metabolites such as H_2O_2, which can also induce an

increase of intracellular Ca^{2+} levels (Herson *et al.* 1999). The reactive oxygen metabolites can lead to an oxidation of proteins (Andreyev *et al.* 2005) and an opening of the mitochondrial permeability transition pores (Gunter and Pfeiffer 1990; Zoratti and Szabo 1995) resulting in cytochrome c release and a swelling of mitochondria and the collapse of the mitochondrial membrane potential (Budd and Nicholls 1996). In addition to the above mentioned pathological findings, the generation of nitric oxide (NO) with an activation of poly (ADP-ribose) polymerase (PARP), the release of mitochondrial apoptosis inducing factor (AIF) (Hong *et al.* 2004), Smac/DIABLO (Shibata *et al.* 2002) and pro-caspases can be induced by the Ca^{2+} overloading of mitochondria (reviewed by Green and John C. Reed (1998)).

1.3.2 Incorporation or Binding of BMAA

As well as the receptor-mediated toxicity of BMAA a binding or incorporation of BMAA into proteins has also been discussed. Murch *et al.* (2004a) recommended that a possible pathway for a delayed toxicity of BMAA can be based on the incorporation of the "non-protein amino acid" BMAA into proteins. This would affect on the one hand the tertiary folding structure of proteins, leading to an impaired biological activity of neuronal proteins, and on the other hand it could lead to the truncation of proteins during the translation mechanism or to a collapse of proteins after their release from the ribosomes (Murch *et al.* 2004a). However, such an incorporation or impaired translation had never been observed until then, with the exception of an insertion of an (R)-2-Alkyl-2-amino-3-(methylamino)-propionic acid residue into an artificial peptide by Seebach *et al.* (1994). The hypothesis of Murch *et al.* (2004a) were advanced by Field *et al.* (2011) suggesting that the BMAA-affected peptide bonds or post-translational modifications can lead to abnormalities in the protein collagen. As changes in collagen were observed in "assumed control Chamorros" who showed no clear symptoms of ALS, they hypothesized that either a large dose of BMAA or a prolonged exposure with low BMAA concentrations would be needed to induce ALS symptoms and that the exposure to smaller doses could result in the collagen changes (Field *et al.* 2011) as observed in the putative control group of the Chamorro. First experimental evidence that BMAA can affect translational processes was provided in cells of various brain regions of rats which were administered BMAA i.p. for 14 days. Less incorporated radioactive labeled leucine was detectable in the motor cortex and the striatum in long-term BMAA exposed rats than in control animals which might result from an impaired protein synthesis (Kisby and Spencer 2011). The first general proposal of impaired protein synthesis by Kisby and Spencer (2011) was supported by results from Karlsson *et al.* (2012) who demonstrated an alteration in the expression of S100β, calcium- and calmodulin-binding proteins and histone and guanine nucleotide-binding proteins, which could possibly be based on various impaired pathways following BMAA treatment

but also on translational and pre-translational disturbances. These alterations were accompanied by severe lesions including neuronal degeneration, cell loss, calcium deposits, and astrogliosis in the hippocampus indicating a neurodegenerative process already in progress. The reduction of around 60% of free ubiquitin levels in the same regions of the rat hippocampus led the authors to the conclusion that highly polyubiquitinated proteins are present in this region (Karlsson *et al.* 2012). This finding and the resulting conclusion permits the implication that BMAA treatment induces a polyubiquitination of proteins as they are deficient and malfunctional because of their misfolding or aggregation. These deficient proteins will then, after their tagging by a covalent attachment of multiple ubiquitin molecules, finally become degraded by ubiquitin-mediated proteolysis in the proteasome (reviewed by Ciechanover (1998)).

1.3.3 Genotoxicity of BMAA

Epidemiological studies showed no evidence for the presence of a genetic cause of the ALS/PDC disease on Guam or neighboring islands (Chen *et al.* 1996; Garruto and Yase 1986; Kurland and Mulder 1954). Nevertheless, various putative genetically caused alterations of modified proteins were investigated: The alteration of melastatin alleles (Hermosura *et al.* 2005) which regulate the homeostasis of intracellular Ca^{2+}, Mg^{2+}, and trace metal ion (Monteilh-Zoller *et al.* 2003; Nadler *et al.* 2001; Runnels *et al.* 2001; Schmitz *et al.* 2003) and thus could impair the homeostasis of ions leading to neurodegeneration; The genetic modification of apolipoprotein E, which is closely related to the formation of neurofibrillary tangles (NFT) composed of phosphorylated forms of the tau protein and its modification, is therefore a central genetic risk factor for neurodegenerative disease like AD (Ohm *et al.* 1999; Tesseur *et al.* 2000). Finally, the genetic modification of tau protein itself (Poorkaj *et al.* 2001) whose mutation and resulting aberrancy clearly plays a role in the pathogenesis of a variety of neurodegenerative brain diseases, even though the mechanisms by which tau mutations cause neurodegeneration vary and are partially unclear (reviewed by Wolfe (2009)). A direct link between a putative genotoxicity and the neurotoxin BMAA was found by Kisby *et al.* (1992b). In rodent brain tissue they demonstrated that BMAA can be enzymatically metabolized into formaldehyde which is known to be a potent genotoxin (reviewed by Speit and Schmid (2006)). The same authors demonstrated that BMAA is able to interfere with RNA synthesis in the striatum and cerebellum of BMAA-exposed rats (100 mg/kg L-BMAA, daily for 14 days) (Kisby and Spencer 2011) as radioactive labeled (incorporated) nucleoside uridine was reduced compared to the vehicle control in brain tissue.

1.3.4 Further proposed „Multiple Mechanisms“ for BMAA Toxicity

When Murch *et al.* (2004a) hypothesized that BMAA can become incorporated into proteins they added further suggestions to explain a BMAA-mediated toxicity. Besides the chelating characteristics of BMAA complexes for ions (Zn, Cu, Ca, and Al) a protein-association of BMAA resulting in its dimerization has also been suggested which could possibly lead to a binding of metal ions and thus could “alter the ionic balance in neuronal cells, generate free radicals, or even catalyze deleterious chemical processes” (Murch *et al.* 2004a). Additionally, the capture and release of metal ions by BMAA complexes could interfere with NMDA or AMPA receptors. Comparable to the hypothetical release of ions from BMAA complexes a hypothetical delayed release of BMAA from associated proteins was suggested to explain the “slow toxin” characteristics of BMAA postulated by Spencer *et al.* (1991). The hypothesis of a flux from protein-associated BMAA, and thus the “endogenous neurotoxic reservoir pool”, to free BMAA, which could be responsible for the induction of neurodegeneration, was picked up by Field *et al.* (2011) who suggested in their “hypothetical paper” that collagen might act as a reservoir of BMAA as 25% of protein in the body is collagen and can be recycled.

The explanations for BMAA toxicity discussed above reveal that besides the receptor-mediated toxicity of BMAA further mechanisms could be involved in the slow and progressive action of the neurotoxin, although little or no experimental data are available and some putative toxic mechanisms were just hypothetically described. Nevertheless, the findings and hypotheses summed up above indicate a tendency in the last ten years towards the theory, that the neurotoxicity of BMAA might be based on multiple mechanisms and consequently can also result in “different types of neurological diseases that have been associated with BMAA consumption” (Lobner *et al.* 2007). These multiple mechanisms, however, as Chiu *et al.* (2011) correctly pointed out, need yet to be defined as well as the “different types of neurological diseases”.

1.4 Amyotrophic Lateral Sclerosis/ Parkinsonism-Dementia Complex

Arnold *et al.* (1953) were the first to describe an increase of symptoms of the neurodegenerative disease amyotrophic lateral sclerosis (ALS) on Guam. Ensuring studies confirmed and amended their findings (Kurland and Mulder 1954; Mulder *et al.* 1954; Tillema and Wijnberg 1953). However, besides the symptoms of ALS described by Arnold *et al.* (1953), the study of Mulder *et al.* (1954) additionally revealed the occurrence of symptoms similar to PD, sometimes accompanied by dementia (Parkinsonism-dementia

complex (PDC)). Hirano *et al.* (1966) suggested that the symptoms of both neurodegenerative diseases, ALS and PDC, are clinical variants of a single disease entity. This was in contrast to their early conclusions where, based on clinical and pathological data, they postulated, that both symptoms were related to distinct diseases (Hirano *et al.* 1961). A reevaluation of clinical and neuropathological findings led Elizan *et al.* (1966) to the conclusion that ALS and PDC patients from Guam often suffer from features of both diseases concurrently. This correlation of ALS/PDC was affirmed by an epidemiologic survey conducted by the same coworkers (Reed *et al.* 1966).

1.4.1 Symptoms of ALS/PDC in the human Population on Guam

The neurodegenerative disease ALS/PDC is also known under the term “Lytico Bodig” as the native Chamorro population described the disease, whereby “lytico” or “lytigo” paraphrases the progressive paralysis in the onset, alike to symptoms of ALS. “Bodig” resembles the characteristics of PD with and without associated dementia.

The symptoms of ALS on Guam were quite similar to those observed in “classic” ALS (Kurland and Mulder 1954; Rodgers-Johnson *et al.* 1986): it is a paralytic disease which is characterized by a progressive loss of both upper motor neurons (UMN) and lower motor neurons (LMN). The progressive degeneration and loss of UMN, which are located in the motor cortex of the frontal lobe, results in hyperreflexia, an extensor plantar response, and an increased muscle tone. The UMN can also influence the LMN as they are connected by corticobulbar and corticospinal neurons in the brain stem and the spinal cord. The loss of LMN induces weakness, muscle wasting (atrophy) and cramps, hyporeflexia, and fasciculation as LMN innervate striated muscles (summarized by Kinsley and Siddique (1993)). In both ALS forms (classic and Guam variant) the progressive paralysis leads to the death of the patients, usually by the compromise of the respiratory muscles. The average disease duration in classic ALS is approximately three years, significant variations are possible. The ALS patients on Guam generally survived for approximately 2 to 4 years following initial diagnosis. However, occasional cases where a more rapid progression of the illness or a prolonged survival of the patients occurred were reported (Reed *et al.* 1975; Rodgers-Johnson *et al.* 1986; Uebayashi *et al.* 1980).

The Parkinson’s disease aspect occurred on Guam with classical marked features like tremor, rigidity as well as bradykenesia (Hirano *et al.* 1961). Common for the PDC patients on Guam was also a plastic “lead-pipe” rigidity, dysarthria and dysphagia as a result of extrapyramidal and cortico-bulbar dysfunctions (Chen and Chase 1985). Progressive axial and distal rigidities as well as a generalized motor slowness and profound difficulty in initiating volitional movements were also described for developing ALS/PDC on Guam. However, besides the above mentioned symptoms, the festination and the propulsion,

which are observable in classic PD, are lacking in the PDC patients of Guam. As implied by the name Parkinsonism-dementia complex (PDC) the above mentioned symptoms were sometimes paired with dementia, reminiscent to the cognitive decline in AD. Characteristic for the dementia in PDC is the progressive degeneration of nearly all intellectual faculties and recent memory deficits. The personality and behavioral changes were accompanied by an impaired orientation in time and place (reviewed by Murakami (1999)).

During the final phase of the ALS/PDC disease the patients on Guam were bedridden and various symptoms comparable to ALS, PD and AD occurred in parallel leading to the death of the patients as summarized in Table 1.

Table 1: Comparison of behavioral changes occurring in the major neurodegenerative diseases and ALS/PDC (adapted from Schulz *et al.* (2005)). X= feature is present; 0= feature is not present in all cases; - = feature is not observable in any case.

	ALS/PDC	ALS	PD	AD
Cognitive				
memory deficits	x	0	0	x
disorientation with regard to time, place and person	x	-	0	x
personality changes	x	0	0	x
olfactory deficits	x	x	x	x
Motor				
muscle weakness, atrophy	x	x	x	-
tremor	x	-	x	-
bradykinesia	x	-	x	-
postural instability	x	-	x	-
gait disturbance	x	-	x	-

1.4.2 Pathological Markers for ALS/PDC in the human Population on Guam

Few pathological as well neuropathological markers have been researched concerning their involvement in the increase of ALS/PDC symptoms on Guam. The most prominent pathological hallmark in ALS/PDC is the widespread occurrence of neurofibrillary tangles (NFT) (McGeer and Steele 2011) which were observed for the first time in ALS/PDC patients post mortem by Hirano *et al.* (1961). Since then, various scientists have confirmed these findings and broadened the pathological knowledge of NFT in Guam ALS/PDC by demonstrating that the ultrastructure as well as biochemical properties are quite similar to

those of AD and numerous other neurodegenerative diseases (Buee *et al.* 2000; Lee *et al.* 2001). Numerous NFT were found throughout the hippocampus, the entorhinal cortex, the neocortex, and the brain stem (Buee *et al.* 2000; Hirano *et al.* 1968; Hirano *et al.* 1966; Mawal-Dewan *et al.* 1996; Miklossy *et al.* 2008). The NFT in ALS/PDC are characterized by bundles of paired helical filaments (PHF) composed of aggregated hyperphosphorylated tau (Buee-Scherrer *et al.* 1995; Bussiere *et al.* 1999). The single tau gene on the long arm of chromosome 17 can undergo alternative mRNA splicing as well as post-translational modifications including phosphorylation (reviewed by Buee *et al.* (2000)). Consequences of the alternative mRNA splicing are six tau proteins which are normally expressed in the adult human CNS (Goedert *et al.* 1989a; Goedert *et al.* 1989b). All six isoforms of tau are expressed in ALS/PDC which is again similar to AD (Buee-Scherrer *et al.* 1995). As the phosphorylation of these tau proteins by kinases and phosphatases regulates the different roles of tau-like microtubule assembling, cell sorting and neuro-developmental steps, the abnormal phosphorylation of tau is the most prominent pathological feature for the neurodegenerative disease ALS/PDC (McGeer and Steele 2011). Tau is able to aggregate and form PHF (Buee *et al.* 2000), which possibly can impact the course of neurodegeneration. Additionally, no mutations in tau itself have been identified in Guam ALS/PDC patients so far (Sundar *et al.* 2007) which emphasizes the impact of an impaired phosphorylation onto the forming of NFT and PHF.

As ALS/PDC also exhibits characteristic symptoms of sporadic ALS as well as PD and AD disease the involvement of proteins which are pathologically relevant for these diseases were investigated. Indeed, the protein α-synuclein, which was initially identified as a precursor in the non-Aβ component of AD (Iwai *et al.* 1995) and observable as constituent of Lewy bodies and Lewy neurites (further pathological markers in AD patients) (Spillantini *et al.* 1997), was detected post mortem in various brain regions such as the cerebellum and the amygdala in ALS/PDC patients (Forman *et al.* 2002; Miklossy *et al.* 2008; Sebeo *et al.* 2004; Yamazaki *et al.* 2000).

Also the TAR DNA-binding protein 43 (TDP-43) was reported in the spinal cord and brain tissue of Guam ALS/PDC patients (Maekawa *et al.* 2009; Miklossy *et al.* 2008). TDP-43 is a highly conserved ribonucleoprotein affecting for example the regulation of gene transcription, mRNA splicing, and also RNA stability (Buratti and Baralle 2001; Strong *et al.* 2007). Even though its physiological functions need further research and the mechanistic role in neurodegeneration remains speculative a translocation from TDP-43 to the cytoplasm and the following forming of ubiquitinated aggregates, also known as TDP-43 proteinopathy, has been described for various neurodegenerative diseases including amyotrophic lateral sclerosis (Sreedharan *et al.* 2008), frontotemporal lobe degeneration (Benajiba *et al.* 2009) as well as PD and AD (Chanson *et al.* 2010; Lippa *et al.* 2009; Markopoulou *et al.* 2008).

In comparison to the above mentioned biomarkers the presence of amyloid plaques, which can be formed by an accumulation of extracellular Aβ fibrils, was inconsistently found in Guam ALS/PDC patients (Gentleman *et al.* 1991). However, the rare Aβ peptides detected in Guam ALS/PDC patients were tangle-associated amyloid deposits (McGeer *et al.* 1997) and their immunological profile showed similar characteristics to Aβ peptides found in amyloid plaques of AD and pathological aging (Schmidt *et al.* 1998).

Besides the research of possible biomarker proteins and their involvement in the ALS/PDC pathology on Guam also the gene pool of the Chamorro populations were investigated. However, until recently no evidence of a genetic cause for the ALS/PDC on Guam had been found (reviewed by McGeer and Steele (2011)). Chen *et al.* (1996) analyzed the apolipoprotein E as polymorphism is hypothesized to lead to the formation of NFT in AD (Saunders *et al.* 1993) and the CYP2D6 gene as its mutation is linked to a slower metabolism of toxins (and xenobiotics) (reviewed by Zanger *et al.* (2004)). They found no overrepresented genes or mutations in ALS/PDC patients' genes compared to healthy natives. Natives from Kii Peninsula (Japan) who were diagnosed with ALS/PDC symptoms were examined concerning mutations analysis in 19 genes. In the genes related to ALS or frontotemporal lobar degeneration (SOD2, SOD3, ALS2/alsin, SMN1, PGRN, ANG, VEGF, VCP, VAPB, DCTN1, CHMP2B, TARDBP, TDP-43), to tauopathy (GSK3β) and to Parkinson's disease (α- synuclein, LRRK2, parkin, DJ-1, PINK1, ATP13A2) no causative mutations were found in any exons nor in exon–intron boundaries (Tomiyama *et al.* 2008).

Only Hermosura *et al.* (2008) found a putative involvement of a genetic factor in the onset of ALS/PDC. They concluded that the occurrence of a heterozygous variant of the melastatin allele TRPM7 (T4821) in some Guam ALS/PDC patients led to an increased susceptibility of the patients as TRPM7 codes a calcium-permeable cation channel and "its disruption may, under certain conditions, contribute to disease states" of ALS/PDC (Hermosura *et al.* 2008). The "certain conditions" were defined previously by the authors as the low level of calcium and magnesium in Guam rivers and the high levels of bioavailable transition metals such as manganese (Hermosura *et al.* 2005).

The above summarized biomarkers indicate that in addition to the genetic markers a broad arsenal of biomarkers is relevant for the diagnosis of ALS/PDC. Analogous to the combined symptoms of the ALS, AD and PD in ALS/PDC patients (Table 1) also the biomarkers discovered for ALS/PDC to date are a composition of specific ALS, AD and PD biomarkers.

1.4.3 ER-Stress as an Aspect in Neurodegenerative Diseases

A common characteristic sign of many neurodegenerative diseases such as AD, PD and ALS is the aggregation, accumulation and deposit of misfolded proteins. As the aggregation of

proteins can affect various cell signaling systems and neuronal connectivity, there is overwhelming evidence for their involvement in cell death pathways leading to neurodegeneration (reviewed by Lindholm *et al.* (2006)).

To prevent misfolding and possible subsequent aggregation of proteins under physiological conditions, proteins run through various quality control mechanisms in different compartments in the (neuronal) cell. One of these mechanisms is the protein quality control in the endoplasmic reticulum (ER) called unfolded protein response (UPR). The impaired quality control mechanism and the resulting incompetence to restore the protein homeostasis in the cells are indicated by the presence of aggregates of misfolded proteins in many neurodegenerative diseases (Hoozemans and Scheper 2012). To prevent and counteract an accumulation of misfolded proteins, which would result in an ER-stress situation and an imbalance of the protein homeostasis, cells possess three main stress sensors which are evolved into a complex network of signaling events that target multiple cellular responses (Table 1).

First, the protein synthesis rate and the translocation of proteins into the ER can be reduced by the involvement of inositol-requiring protein-1 (IRE-1) which is able to sense the protein-folding status in the ER and to stimulate cytoplasmic effector proteins that interact with the transcriptional or translational apparatus (reviewed by Schröder and Kaufman (2005) and by Bernales *et al.* (2006)). This is controlled by a not completely understood mechanism whereby IRE-1 oligomerizes following a dissociation of the ER chaperone and the UPR regulator protein BiP (also known as glucose-regulated protein (GRP78)) from the lumenal domains. The trans-autophosphorylation of the IRE-1 kinase domain activates an effector function resulting in the endonucleolytic cleavage of X-box binding protein-1 (XBP-1) mRNA (reviewed by Ron and Walter (2007)). This IRE1-dependent splicing event results in the translation of XBP1, the general activator of UPR target genes (Yoshida *et al.* 2001) (Figure 7A). Also the precursor (unspliced) XBP1 mRNA can be translated, but this protein is labile and represses UPR target genes (Yoshida *et al.* 2006). The UPR target genes, which become transcribed by the binding of XBP1, are proteins that promote ER-associated degradation (ERAD) of misfolded proteins, protein quality control and phospholipid synthesis (Yoshida *et al.* 2003). Additionally, activation of IRE1 by its phosphorylation can also recruit the tumor necrosis factor receptor-associated factor-2 (TRAF2) which is linked to the Jun N-terminal kinase (JNK) (Urano *et al.* 2000) and to the caspase 12 activation both of which can initiate cell death (Yoneda *et al.* 2001). The caspase 12 mediated pathway is independent of death receptors and mitochondria (reviewed by Szegezdi *et al.* (2003)), although caspase 12 is subject of controversy as several mutations leading to an inactivation were described for the human caspase 12 gene (discussed by Fribley *et al.* (2009))

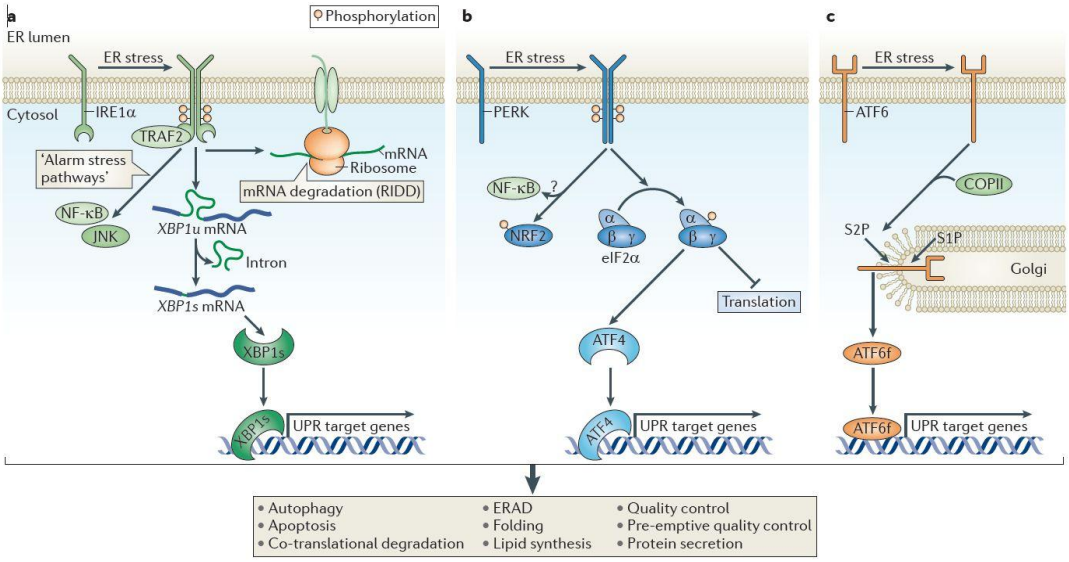

Figure 7: Scheme of the three main mechanisms by which the unfolded protein response (UPR) is regulated (Hetz 2012). A: IRE1α dimerization induces processing of unspliced X-box-binding protein 1 (XBP1u) mRNA to the active spliced transcription factor XBP1 (XBP1s). XBP1s controls the transcription of genes responsible for protein folding, ER-associated degradation (ERAD), protein quality control and phospholipid synthesis. B: Phosphorylated und thus activated PERK induces phosphorylation of eukaryotic translation initiator factor 2α (eIF2α) resulting in the translation of ATF4. ATF4 controls transcription of genes involved in autophagy, apoptosis, amino acid metabolism and antioxidant responses. C: ER-stress induces transport of ATF6 from the ER to the Golgi apparatus by an interaction with the coat protein II (COPII) complex. Processing of ATF6 on the Golgi apparatus by site 1 protease (S1P) and S2P leads to the release of cytosolic domain fragment (ATF6f) which controls genes encoding ERAD components and also XBP1. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, (Hetz 2012), copyright (2012).

The second way to handle an increased amount of unfolded proteins is regulated by the activation of the transcription factor 6 (ATF6) which represents a group of ER-stress transducers that encode basic Leu zipper (bZIP) transcription factors, including ATF6α, ATF6β, cyclic AMP-response element-binding protein (CREB3)/ LUMAN, old astrocyte specifically-induced substance (OASIS)/ CREB3L1, BBF2 human homologue on chromosome 7 (BBF2H7)/ CREB3L2, cyclic AMP-responsive element-binding protein hepatocyte (CREBH)/ CREB3L3 and CREB4/ CREB3L4 (reviewed by Asada *et al.* (2011)). ATF6 can become translocated to the Golgi if ER-stress occurs. There, ATF6 is cleaved first by site-1 proteases (S1P) at its ER luminal domain and in a second step by site-2 protease (S2P) at the intra-membrane region followed by the release of the cytosolic fragment (ATF6f) that controls genes encoding ERAD components and XBP-1 (Haze *et al.* 1999; Lee *et al.* 2002; Yamamoto *et al.* 2007) (Figure 7C).

The third of handling an ER-stress situation is the activation of the protein kinase RNA-like ER kinase (PERK) (Figure 7B). PERK, similarly to IRE-1 consists of an ER-localized type I transmembrane protein with a lumenal stress-sensing domain. The dissociation of BiP from

the lumenal domains leads to the activation and thus to an oligomerization of PERK and an activation of cytoplasmic protein kinase domain resulting in the trans-autophosphorylation. The trans-autophosphorylation initiates the phosphorylation of the α-subunit of eukaryotic translation initiation factor-2 (eIF2α) at Ser51 and maybe also the phosphorylation of the erythroid 2-related factor 2 (NRF2) which is involved in the redox metabolism. The phosphorylation of eIF2α leads to the translation of mRNA of the activating transcriptional factor 4 (ATF4) which controls a broad range of genes involved in pro-survival genes related to redox balance, amino acid metabolism, autophagy, protein folding, and also at a chronic stage to apoptosis (reviewed by Ameri and Harris (2008) and by Schröder and Kaufman (2005)).

If a UPR situation remains unresolved and therefore ER-stress exists over a longer time, possibly also linked to a chronic stress situation or irreversible ER damages, an apoptotic pathway can become initiated directly by the UPR (reviewed by Hetz (2012)). The mechanism behind the switch from early adaptive responses of the UPR to the initiation of an apoptotic pathway as well as the pathway itself is not completely understood. However, it seems verified that the core mitochondrial apoptotic pathway is under the control of the B cell lymphoma 2 (BCL-2) protein family. These proteins control caspase activation as well as the transcription initiation of further pro-apoptotic players like BCL-2-interacting mediator of cell death (BIM), p53 upregulated modulator of apoptosis (PUMA)/ BBC3, and C/EBP-homologous protein (CHOP)/ GADD153. A detailed overview of a putative model concerning cell fate decisions under ER-stress was given by Hetz (2012) in his review.

In addition to the induction of an apoptotic pathway it was also reported that chronic ER-stress and therefore a prolonged UPR can possibly affect the activity of tau kinases or phosphatases which could affect the phosphorylation of tau proteins (Figure 8). Indeed, Kim *et al.* (2005) as well as Song *et al.* (2002a) demonstrated the ER-stress dependent activation of glycogen synthase kinase 3β (GSK3β) which is a major tau kinase (reviewed by Hanger and Noble (2011)). Additionally, an increased presence of the UPR marker phosphor-PERK was observed *in vivo* in AD and frontotemporal dementias (FTDs) patients with distinct tauopathies (Hoozemans *et al.* 2009; Nijholt *et al.* 2012) which led to the suggestion that if the phosphorylation and thus the activation of GSK3β can be regulated by the UPR kinase PERK, as shown by Baltzis *et al.* (2007), the chronic ER-stress situation could possibly directly contribute to the phosphorylation of tau and thus to the development of NFT and a neurodegeneration (Hoozemans and Scheper 2012).

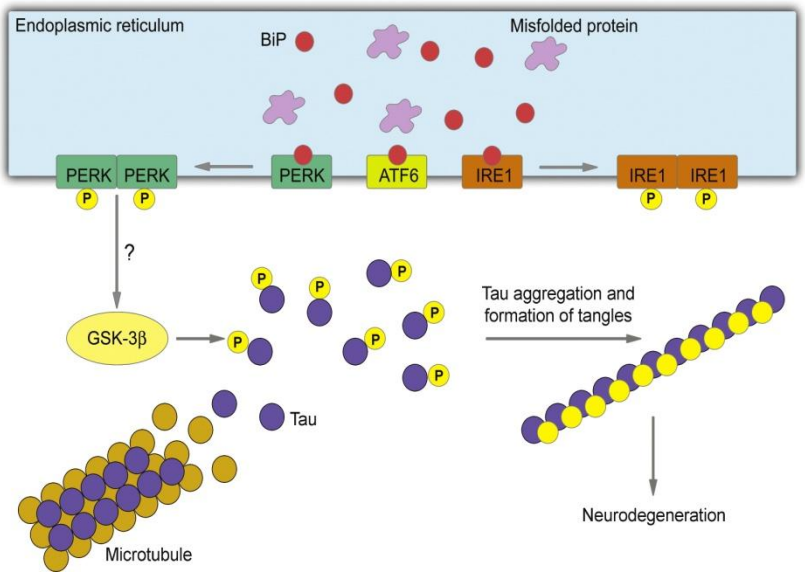

Figure 8: Model for a UPR-mediated activation of GSK3β with a resulting hyperphosphorylation of tau protein (Hoozemans and Scheper 2012). Reprinted from Publication Hoozemans and Scheper (2012), Copyright (2012), with permission from Elsevier.

1.4.3.1 Degradation of uncommon Proteins by the ERAD

The UPR manages the up- and down-regulation of genes involved in the expression of chaperones and other folding factors in the eukaryotic cells to accomplish an overload of misfolded proteins in the ER. Consequently, also genes, which are involved in ER-associated degradation (ERAD) and thus in the ubiquitin/proteasomal degradation to promote the degradation of irreversibly misfolded, non-functional and/or aggregated proteins, are regulated by the UPR (Casagrande *et al.* 2000; Friedlander *et al.* 2000; Ng *et al.* 2000; Travers *et al.* 2000). Thereby, proteins which fail the ER quality control (ERQC) as their folding was delayed or an illegitimate conformation arose are either subjected to a further folding cycle to achieve a functional folded state or are subjected to the ERAD (reviewed by Ellgaard and Helenius (2003)). Misfolded proteins with lesions in their cytoplasmic, intramembrane or ER-luminal regions can be recognized by chaperons like the 70 kDa heat-shock protein (Hsp70)-family members (calnexin and calreticulin) as well as protein disulphide isomerases and targeted to the retrotranslocon for their dislocation from the ER to the cytoplasm and/or to the E3 ligases where they become polyubiquitinated (Figure 9). The polyubiquitinated misfolded protein substrate is displaced in its entirety into the cytoplasm where 19S cap receptors in the 26S proteasome or proteasome-associated proteins recognize it and initiate the de-ubiquitination by removing the polyubiquitin tag, whereby ubiquitin is recycled. The de-ubiquitination “misfolded” substrate becomes degraded in the 20S catalytic core of the proteasome into 2–30 amino acid peptides (reviewed by Vembar and Brodsky (2008) and by Guerriero and Brodsky (2012)). These

peptide fragments can be further processed by other cellular proteases (Kisselev *et al.* 1999).

If, however, the concentration of misfolded and/or potentially toxic protein aggregates increases or the ERAD is directly impaired, resulting in a compromised ERAD efficiency, further compensatory pathways like the above discussed apoptotic pathway or a complementary autophagy-mediated destruction can be induced (Kruse *et al.* 2006).

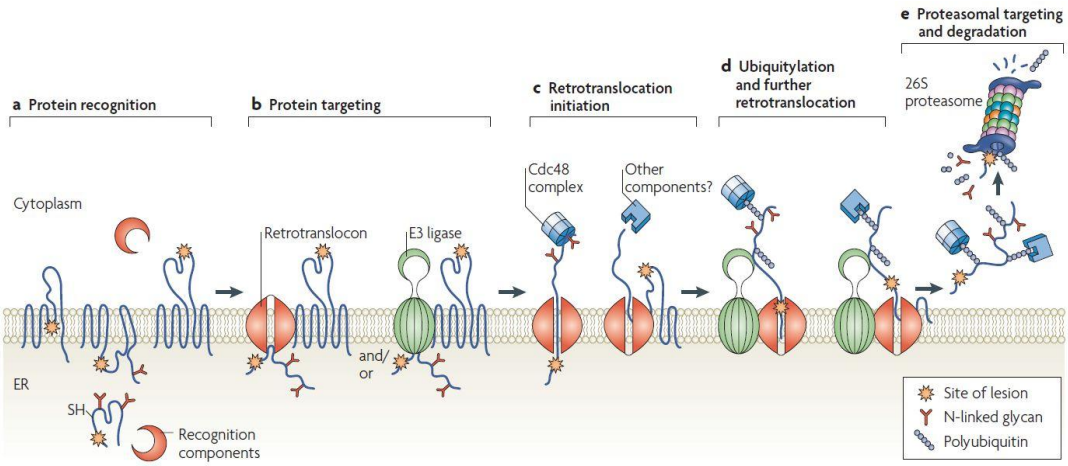

Figure 9: Overview of the ER-associated degradation (ERAD) (Vembar and Brodsky 2008). Unfolded or misfolded proteins in the ER lumen, cytosol and intramembrane will be recognized and targeted to a retrotranslocon or the E3 ligase. Following ubiquitination and further retrotranslocation proteins undergo proteolysis in the proteasome. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, (Vembar and Brodsky 2008), copyright (2008).

The described mechanism of UPR and ERAD involve a complex network of signaling events that target multiple cellular responses. Therefore, it is not conclusive that impairments in these systems can lead to enduring damage. Indeed, there is emerging evidence that ER-stress situation and thus UPR as well as ERAD pathways are critical for the development and/or pathology of various human diseases (reviewed by Zhao and Ackerman (2006)) including neurodegenerative diseases like AD, PD, ALS, HD and prion-related disorders (reviewed by Doyle *et al.* (2011)). Yet it has to be mentioned that the proposed specific mechanisms, which could be involved in the process of neurodegeneration (reviewed by Matus *et al.* (2011)), are the subject of current research and scientific discussions.

1.4.3.2 Impact of UPR and ERAD on ROS Production

As the ER lumen is an oxidizing environment, an oxidizing protein folding can take place there. When nascent proteins enter the ER, disulfide bonds are formed in the oxidizing environment providing the correct maturation and functionality before proteins leave the

ER. The forming of disulfide bonds is catalyzed by protein disulphide isomerase (PDI) which accepts electrons from thiol residues in the polypeptide chain substrate resulting in its oxidation. The reduction process of the PDI is accomplished by the family of ER oxidoreductases. The oxidoreductase can transfer their additional electron to molecular oxygen for recycling which results in increased ROS (reviewed by Chakravarthi *et al.* (2006) and by Ferreiro *et al.* (2012)). Additionally, PDI can become reduced by glutathione. Hence, an oxidation of glutathione can occur in two ways: As a consequence of the PDI reduction and as a consequence of its scavenging properties toward ROS. This shift from reduced glutathione to oxidized glutathione is known as glutathione depletion. The depletion of glutathione in the ER consequently lowers the ability of glutathione to scavenge from further raising ROS (reviewed by Malhotra and Kaufman (2007a)).

ROS can also become generated in mitochondria as a consequence of an increased cytosolic Ca^{2+} level caused by prolonged UPR or the accumulation of unfolded proteins in the ER. Different molecular mechanisms concerning the effect of Ca^{2+} on the mitochondrial ROS emission (reviewed by Adam-Vizi and Starkov (2010)) are controversially discussed as experimental observations cannot yet explain completely the cause-effect relationship between mitochondrial Ca^{2+} overload and the production of ROS (Adam-Vizi and Starkov 2010).

Even though it has been postulated that nearly 25% of ROS development in cells results from oxidative protein folding (Tu and Weissman 2004), the pathway of ROS generation following ER-stress is not completely understood and thus needs further research.

Chapter 2: Working Hypotheses

2.1 Part I

Previous research focused mainly on the excitotoxic and therefore receptor-mediated effects of BMAA to explain a neurodegeneration following BMAA exposure. Thereby the development of ROS and an impaired Ca^{2+} homeostasis were often used as affirmation of the excitotoxic characteristic of BMAA *in vitro*. However, these changes can be linked to mechanisms other than excitotoxicity. As mentioned above, an increased level of ROS as well as an impaired Ca^{2+} homeostasis are also observable during an ER-stress situation (reviewed by Malhotra and Kaufman (2007a)). Additionally, altered glutamate receptor levels and consequently their properties, as observed by Chang *et al.* (1993) in rats following BMAA treatment, are not exclusively associated to an over-activation of glutamate receptors. They are also linked to a repeated stress situation (Yuen *et al.* 2012) like ER-stress, especially since in neurons the UPR pathway is also involved in the trafficking of glutamate receptors (Shim *et al.* 2004). Additionally, the excitotoxic hypothesis of BMAA is mainly based on its carbamate forming in the presence of bicarbonate and as the resulting three-dimensional structure could be similar to glutamate. However, under physiological conditions (in a 23 mM bicarbonate solution) only 31% of BMAA formed carbamate (Myers and Nelson 1990) in a reversible reaction (Nunn and O'Brien 1989). Thereby, the toxic potential of at least 69% of BMAA has been disregarded which could possibly complement the already described acute, high-dose toxic findings following short-term BMAA exposure respectively mechanisms and pathways which are impaired following long-term, low-dose L-BMAA exposure. Such a mechanism was discussed hypothetically by Murch *et al.* (2004a), who suggested a slow release of BMAA after it has become incorporated or attached to proteins and thus biomagnification. Some few experiments support the hypothesis that BMAA is able to become attached to or incorporated into proteins (Kisby and Spencer 2011).

The first objective of this thesis was to investigate the hypothesis that L-BMAA can enter the human neuroblastoma cells SH-SY5Y *in vitro*. The intracellular non-cytotoxic and non-excitotoxic L-BMAA becomes attached to or incorporated into proteins which initiate various cell responses as specified in the following sub-hypotheses.

1. In consequence of the incorporation or attachment of proteins, taking place at/ in the ER, the neuronal cells will initiate the UPR and the ERAD to handle increasing amounts of misfolded or unfolded proteins and thus the ER-stress situation.

2. Non-cytotoxic and non-excitotoxic L-BMAA concentrations can initiate prolonged UPR und thus an ER-stress situation which can initiate apoptotic pathways.
3. Increased ROS following L-BMAA exposure is not the initiator for the UPR/ ER-stress situation and thus for the ERAD or initiated apoptotic factors but rather a subsequent or independent mechanism.

2.2 Part II

The reason for the symptoms of ALS/PDC are still not completely understood and no definitive diagnostic markers are given for ALS/PDC which suggests the possibility that ALS/PDC can also occur outside of Guam (McGeer and Steele 2011). Indeed, there are few reports of Filipino migrants and American military personnel from Guam showing similarities in clinical and pathological markers to the native Chamorros of Guam (Chen *et al.* 1982; Forno and O'Flanagan 1973). Different biomarkers have been used for the detection and pathological description of ALS/PDC (reviewed by McGeer and Steele (2011)), however, the focus was set mainly on the expression of genes and proteins and not on their functionality or on their cofactors. For example: no association of ALS/PDC to a gene variant or mutation was found in the sequenced tau gene (Sundar *et al.* 2007). ALS/PDC is described, however, as the most aggressive form of all tauopathies (McGeer and Steele 2011) and hyperphosphorylated tau and resulting NFTs and PHF were reported (Hirano *et al.* 1961; Mawal-Dewan *et al.* 1996; McGeer *et al.* 1997). Nevertheless, only a small set of data is available concerning enzymes involved in the phosphorylation and de-phosphorylation of tau although an impairment of tau related phosphatases by other cyanobacterial toxins (micocystins) is known (Feurstein *et al.* 2011). Kihira *et al.* (2009) concluded from their immunohistochemical data from Guam and Kii ALS/PDC patients that the glycogen synthase kinase-3beta (GSK3β) might have a function in the progression of NFT and PHF forming.

Focusing on cyanobacterial produced BMAA as a possible cause for ALS/PDC, the detection of BMAA in brain tissues of Chamorro ALS/PDC patients, Canadian AD patients, and in American ALS and AD patients post mortem (Banack *et al.* 2007; Murch *et al.* 2004b; Pablo *et al.* 2009) as well as the tau aggregation and neurofibrillary tangle formation, typical for ALS/PDC (Miklossy *et al.* 2008; Winton *et al.* 2006), indicate two main aspects: On the one hand, that an uptake of BMAA outside of Guam is possible and on the other hand, that there could be a link between BMAA exposure and the occurrence of tauopathies in ALS/PDC patients.

The second objective of this thesis was to investigate the hypothesis that L-BMAA can induce a hyperphosphorylation of the tau protein in the human *in vitro* model SH-SY5Y. A

putative mechanism by which L-BMAA can induce a hyperphosphorylation is specified in the following sub-hypothesis:

1. The activity of protein phosphatase 2B (calcineurin) can become impaired by L-BMAA.
2. The activity of glycogen synthase kinase-3beta (GSK3β) can become up-regulated by L-BMAA-affected calcineurin and thus modulates the increased phosphorylation of the tau protein.

2.3 Part III

Various animal models were used to research and characterize the ALS/PDC disease on Guam. Thereby, BMAA causes neurotoxic abnormalities like functional disturbances and neurodegenerative changes comparable to motor dysfunctions in most *in vivo* models. However, the behavioral and the neurodegenerative characteristics of ALS/PDC observable in living humans and in *post mortem* brain tissue, respectively, were not reproducible in the animal models (reviewed by Karamyan and Speth (2008)). This could be linked to the fact that neurodegenerative disorders have up to now only been described in humans but not in other species, e.g. rodents (Shaw 2001; Sillevis Smitt and de Jong 1989). Further, as correctly pointed out by Karamyan and Speth (2008) and Shaw and Wilson (2003), the lack of a definitive hypothesis for the toxic mechanism of BMAA and divergent estimations concerning the effective exposure dose in the native Chamorro on Guam as well as the long latency period between the uptake and the expression of the symptoms complicate and complex the development of an animal model.

Apart from the above mentioned criticisms and difficulties in finding an adequate model, Spencer *et al.* (1987b) were able to reproduce pathological findings in Cynomolgus monkeys which were similar to ALS/PDC symptoms in humans. This led Karamyan and Speth (2008) to the suggestion “to use great apes to mimic ALS/PDC” as they feature just few “functional differences in organization of neuronal structures” when compared to humans.

The third objective of this thesis was to investigate the hypothesis that L-BMAA can cause (neuro-)toxic effects in a non- human model organism, *Apis mellifera* (honeybee), which are also observable in human ALS/PDC patients as well as in cellular *in vitro* model systems. *Apis mellifera* was a particularly promising model since many insects are well-established model organisms for genetic, molecular, behavioral and neuronal analysis. *Apis mellifera* was chosen as the bees show cognitive abilities although their nervous system is relatively simple. This model can be used to understand the impact of L-BMAA on the organization of

neuronal structures and thus the molecular and physiological effects of the neurotoxin L-BMAA which will be investigated in the following sub-hypothesis:

1. Preclinical symptoms of ALS/PDC in humans, for example cognitive abilities and olfactory processing, can become impaired in the model system *Apis mellifera* following L-BMAA treatment.
2. Low concentrations (µM) of the neurotoxin L-BMAA can affect typical markers of toxicity, which are also associated to neurodegenerative diseases, like the generation of ROS and the Ca^{2+} homeostasis in the living model system *Apis mellifera*.
3. *Apis mellifera* could spread the neurotoxin L-BMAA within its colony.

Chapter 3: Manuscript I

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

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Running Title: L-BMAA induced dysregulated protein homeostasis.

3.1 Abstract

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 non-excitotoxic 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 (≥0.1 mM, 48 hours) 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 (≥1 mM, 48 hours) increased reactive oxygen species and protein oxidization, which were partially ameliorated by co-incubation with vitamin E. L-BMAA-mediated cytotoxicity was observable 48 hours following ≥2 mM 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.

3.2 Introduction

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.* 1987b), 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 i.v. injection (Duncan *et al.* 1992; Karlsson *et al.* 2009b). 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.* 2004a; 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 non-proteinogenic 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 non-excitotoxic 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 non- excitotoxic 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 (≥0.1 mM for 48 h corresponding to ≥11.8 mg/l) leads to neuronal protein misfolding and ER-stress, and thus could be a contributing factor in the onset and progression of motorneuron disorders as observed in the case of ALS/PDC in Guam.

3.3 Materials and Methods

3.3.1 Chemicals and Reagents

All chemicals, unless otherwise stated, were of the highest analytical grade commercially available. L-BMAA (Sigma-Aldrich) was dissolved in 10 mM NaHCO3. Nominal concentrations were used in the experiments.

3.3.2 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 used in all experiments. They were cultured in plates for three 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 (Supplemental Figure 13). Passages 4- 9 of SH-SY5Y cells and passages 3- 6 of HEK-293 cells were used for all experiments.

3.3.3 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´).

3.3.4 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 9 µM ^{14}C-L-BMAA for different time periods as indicated, washed 4x with modified PBS (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4),

lysed with 0.5 M NaOH, acidified with 2 M HCl, to avoid quenching by luminescence, and transferred to the scintillation cocktail (Quicksafe A, Zinsser Analytic, Frankfurt, Germany) for scintillation counting (5min).

3.3.5 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.

3.3.6 Fluorophotometric Quantitation of Oxidative Stress

SH-SY5Y cells were pre-treated for 45 minutes with the fluorescent ROS indicator dye 2‘,7‘-dichlorofluorescein-diacetate (Sigma-Aldrich). After washing (3x) 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).

3.3.7 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 (17500 g, 20min, 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 2 M Tris base/30% glycerol (Shacter 2000). Oxidized proteins were quantified via slot blot analysis using a rabbit anti-DNP antibody (#D9656, Sigma-Aldrich).

3.3.8 Superoxide Dismutase Activity

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

3.3.9 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).

3.3.10 Immunodetection of Proteins

Following treatment of SH-SY5Y with L-BMAA for 48 hours cells were lysed with ice-cold extraction buffer (10 mM triethanolamine [Tris] base, 140 mM NaCl, 5 mM EDTA, and 0.1% [vol/vol] Triton X-100; pH 7.5) and centrifuged (17500 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).

3.3.11 L-BMAA Incorporation into Proteins

Radioactive labeled [1-14C]-L-BMAA (1.81 mM; 2,035GBq/mmol) as well as ^{14}C-L-alanine (617 µM; 5,994 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 (20min) using 0.2 volumes 100% trichloroacetic acid (TCA). After centrifugation (5min, 10000 g) pellets were washed 3x 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)

3.3.12 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 post-test 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 $p<0.0001$ (****).

3.4 Results

3.4.1 Uptake of L-BMAA via LAT1

Expression of the large neutral amino acid transporter 1 (LAT1) was demonstrated at the mRNA and protein level and thus are potentially capable of transporting L-BMAA (Figure 10A and B). 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- 40min of ^{14}C-L-BMAA exposure (Figure 10C). ^{14}C-L-alanine exposure resulted in a comparable uptake pattern. Co-incubation of ^{14}C-L-BMAA with the Lat1 substrate L-leucine (10 mM) or the Lat1 inhibitor 2-aminobicyclo-2,2,1-heptane-2-carboxylicacid (BCH, 22 mM) for 17 min resulted in a significantly ($p<0.05$) reduced uptake of ^{14}C-L-BMAA (Figure 10D). 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.* 1992; Duncan *et al.* 1991; 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 bi-directional L-BMAA transporters in SH-SY5Y cells.

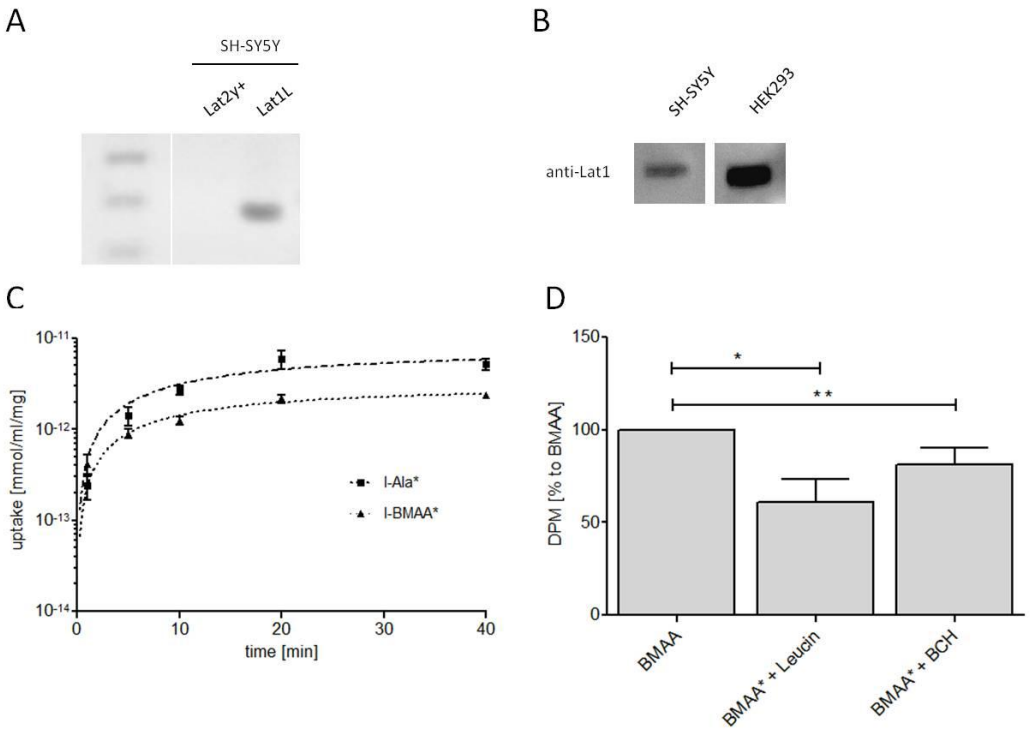

Figure 10: **A:** LAT1 expression at the mRNA (**B**) and protein level; the LAT1 protein expression in HEK293 cells served as expression control. **C**: Uptake of ^{14}C-L-BMAA (9.05 µM) and ^{14}C-L-alanine (1.54 µM) (N = 5). **D**: Reduced uptake of ^{14}C-L-BMAA (9.05 µM) co-treated with L-leucine (10 mM) and BCH (22 mM) for 17 min (N = 5). Values (mean ±SEM) are expressed as percentage of L-BMAA uptake; statistics: one-sample t-test.

3.4.2 L-BMAA Cytotoxicity

A significant reduction in cell viability was observed at 2 mM L-BMAA following 48 h and at concentrations ≥ 1 mM L-BMAA after 96 h of exposure (Figure 11A-C). Consequently, effects observed at concentrations < 2mM L-BMAA in 48 h exposure settings, see below, are non-cytotoxic 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 (Supplemental Figure 14). 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 caspase3/7 activity, possibly a regulatory element for the apoptotic chromatincondensation (Supplemental Figure 15), was observed at L-BMAA concentrations ≥2 mM only (Figure 11D). The latter effects were ameliorated by addition of the ROS scavenger vitamin E (220 µM).

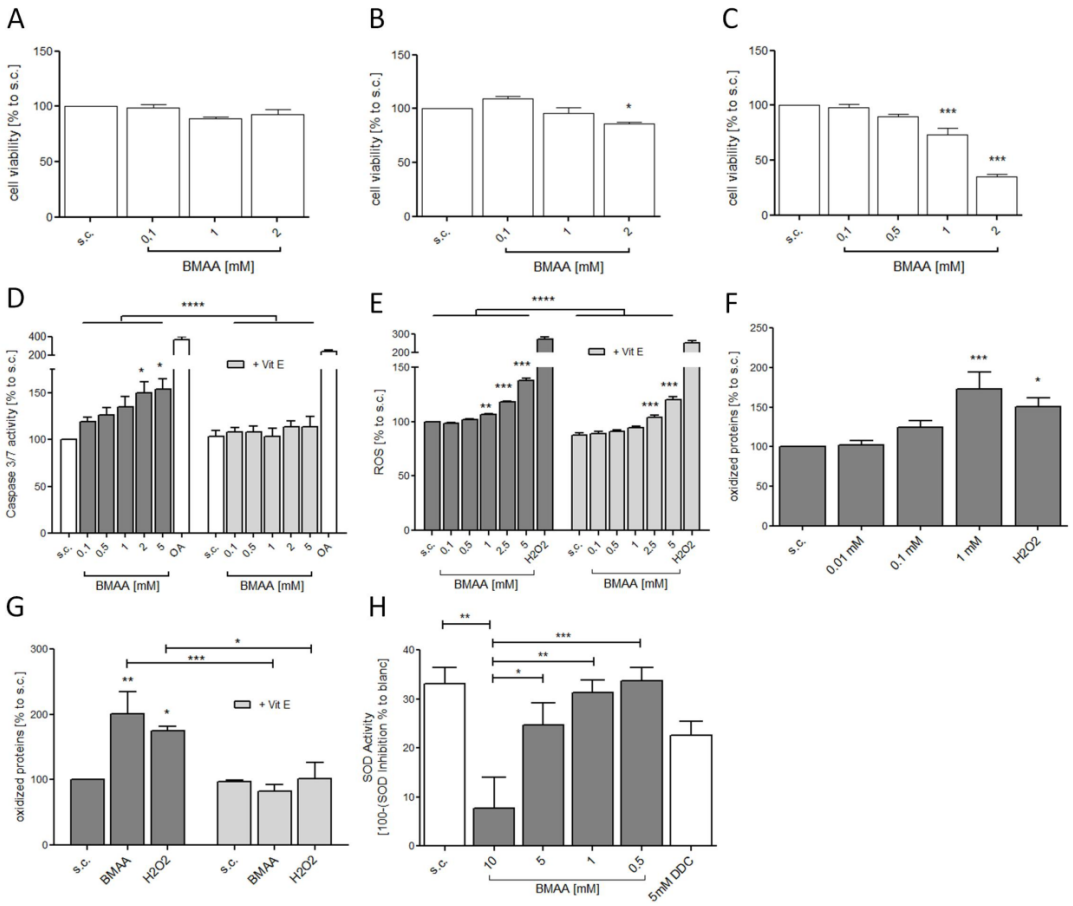

Figure 11: Cell viability and ROS-linked effects of L-BMAA in SH-SY5Y cells. **A-C**: Cell viability of SH-SY5Y cells following 24 h (**A**), 48 h (**B**) and 96 h (**C**) 0.1 mM to 2 mM L-BMAA treatment (N = 3). Low cytotoxicity of 2mM L-BMAA was observed primarily in SH-SY5Y cells after 48 h (**B**). 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- 5 mM L-BMAA or 30 nM 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- 5 mM L-BMAA or co-treated with vitamin E (220 µM) for 45 min (N = 7). **F**: Relative levels of oxidized proteins in SH-SY5Y cells, treated with 0.01, 0.1 and 1 mM L-BMAA (N = 9) and (**G**) co-treated with 1 mM L-BMAA and vitamin E (220 µM) for 48 h (N = 5,). **H**: Inhibition of the human erythrocyte SOD-1 *in vitro*; solvent control (s.c.), 0.5- 10 mM L-BMAA (N = 5).

3.4.3 L-BMAA Treatment increased ROS and Oxidized Proteins

Treatment of SH-SY5Y cells with 0.1 to 5 mM L-BMAA for 45 min resulted in a significant increase of ROS (Figure 11E). 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 1 mM L-BMAA (Figure 11F). Co-exposure with vitamin E significantly reduced the observed L-BMAA- or H_2O_2 - induced ROS and oxidized protein levels (Figure 11G). 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 (≥ 10 mM) had a significant inhibitory effect (Figure 11H).

3.4.4 ROS independent L-BMAA-induced ER-Stress and Protein Binding

Exposure of SH-SY5Y cells to <2mM concentrations of L-BMAA for 48 h (Figure 11B) resulted in a significantly increased level of ubiquitinated proteins and proteasomal activity (Figure 12A and C). Although co-incubation with vitamin E (220 µM) resulted in the expected reduction of ubiquitinated proteins (Figure 12B) 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 1 mM 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 (Figure 12D), 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 (Supplemental Figure 16B-C) supporting the finding that L-BMAA induces ER-stress in a cell autonomous manner.

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

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 (Figure 12F), this was not the case for L-BMAA after protein denaturing SDS gel-electrophoresis (Supplemental Figure 17). 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.

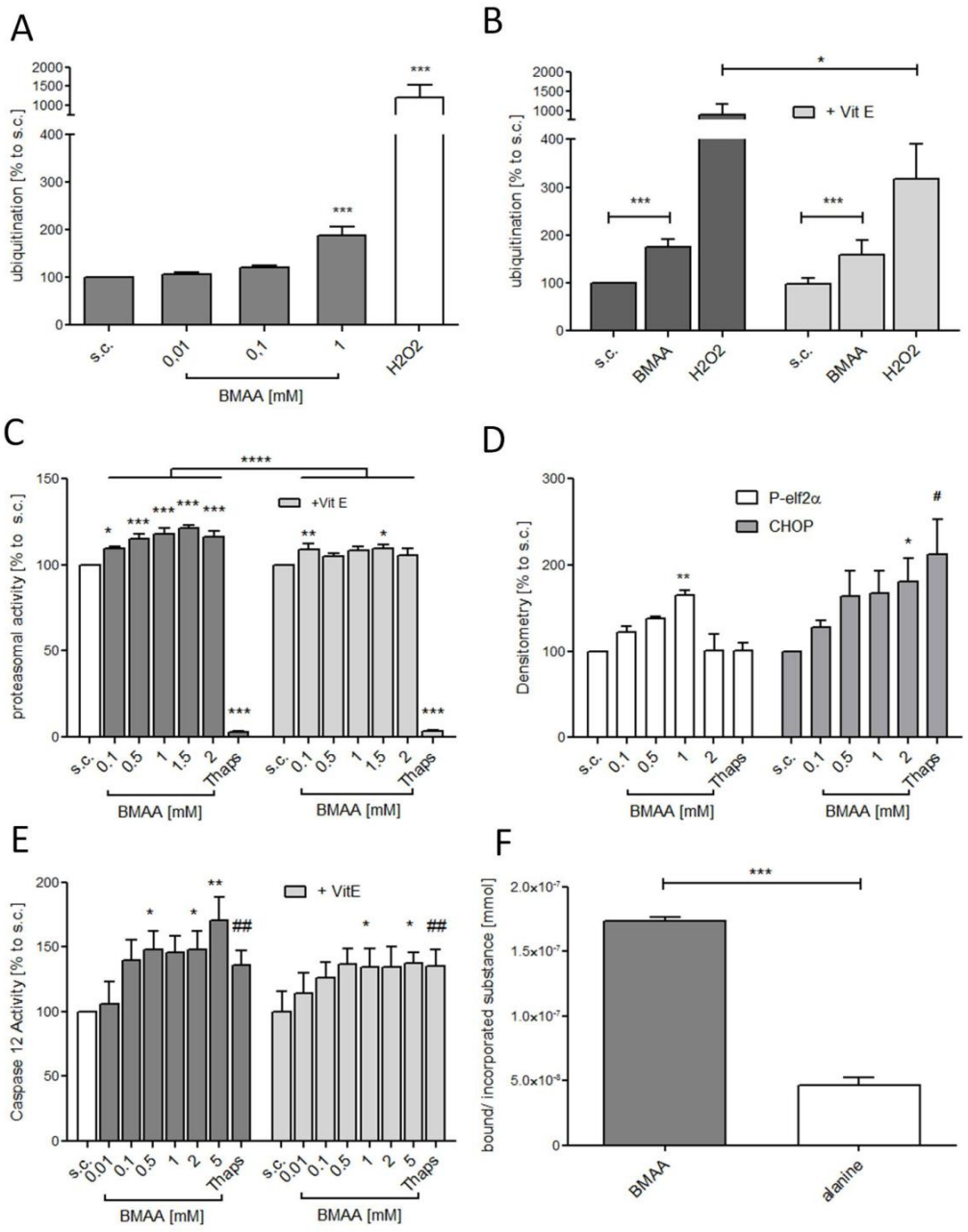

Figure 12: Impairment of protein processing and degradation in SH-SY5Y cells following L-BMAA exposure. **A**: Ubiquitinated proteins in SH-SY5Y cells treated with 0.01- 1 mM L-BMAA (N = 4) and (**B**) co-treated with 1 mM L-BMAA and vitamin E for 48 h (N = 7). Co-exposure of L-BMAA and vitamin E did not affect the ubiquitination status of the SH-SY5Y cells. **C**: Proteasomal activity in SH-SY5Y cells treated with 0.1- 2 mM L-BMAA (N = 10) and co- treated with vitamin E for 48 h (N = 6). L-BMAA induces an increased chemotrypsin cleavage activity of the proteasom; 0.8 nM 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 hours with 0,01- to 5 mM L-BMAA (-VitE, N = 7; +VitE, N = 5). There are no statistical differences between the treatments with or without vitamin E; 30 nM okadaic acid (OA) and 0.8 nM 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).

3.5 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 Chamorro's could have been exposed to via cycad flour are several orders of magnitude lower (Duncan *et al.* 1990; Kisby *et al.* 1988) than the daily dose 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 2003a). 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 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 i.v. injection (Duncan *et al.* 1992; Duncan *et al.* 1991; 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 (Figure 10A and B), whereby L-BMAA transport into SH-SY5Y cells (Figure 10C) can be partially inhibited with L-leucine and BCH (Figure 10D), 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 (0.1-1 mM) resulted in an increased ER-stress as suggested by the increased caspase 12 and 20S proteasomal activities (Figure 11 and Figure 12), enhanced phosphorylation of PERK, and increased expression of CHOP and BAG1. The latter results would corroborate the observations that intracellular BMAA influences 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 HEK293 and THP1 cells (Supplemental Figure 16), the finding that low concentrations of L-BMAA adversely effects 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 co-incubation with the ROS scavenger vitamin E (Figure 11E and G), 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 (Figure 12B, D and C), 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 co-incubation with vitamin E could suggests on one hand that caspase 3/7 activity, and thus the ensuing apoptosis (Supplemental Figure 15), is a result of the increased ROS, and on the other hand, that this occurs in SH-SY5Y cells only at L-BMAA concentrations ≥1 mM. 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 motorneurons 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 i.p. 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.* 2004a). It is noteworthy that the analysis of ^{14}C-L-BMAA incorporation in an *in vitro* protein synthesis assay (Figure 12E) 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.* (2009b) following i.v. 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.* 2004a; 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.

3.6 Acknowledgments

The authors gratefully acknowledge F. Pfister and M. Helmer for the technical assistance.

3.7 Funding

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3.8 Supplementary Data

3.8.1 Supplemental Material and Methods

3.8.1.1 Cytotoxicity

Cytotoxicity of L-BMAA was determined via MTT reduction in SH-SY5Y cells following treatment with glutamic acid, NMDA or CdCl for 24, 48 and 96 hours in 96 well plates in sextuplets.

3.8.1.2 Hoechst Staining

Cells were treated with 3 mM L-BMAA for 72 hours. To stain apoptotic chromatin condensation cells were washed with PBS, fixed with 4% paraformaldehyde and stained with 2.5 µM Hoechst (Sigma-Aldrich).

3.8.1.3 Western Blotting

Anti-phospho-PERK (Santa Cruz Biotechnology, California, U.S.A.); anti-phospho-elf2α, anti-PERK, anti-chop and anti-BAG1 (Cell Signaling Technology, Boston, MA, U.S.A.).

3.8.2 Supplemental Figures

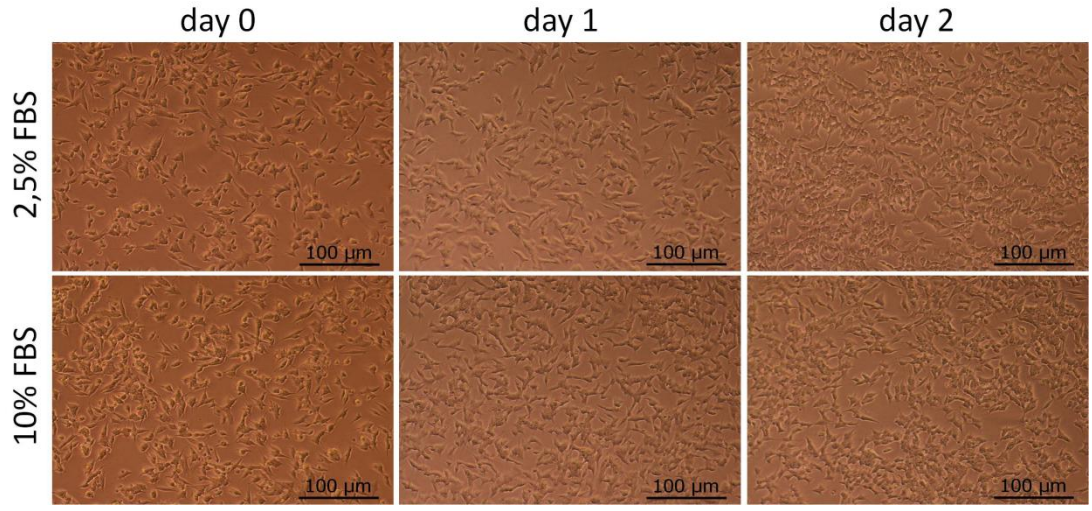

Figure 13: Morphology of SH-SY5Y following 10% FBS and 2.5% FBS treatment for 48 h.

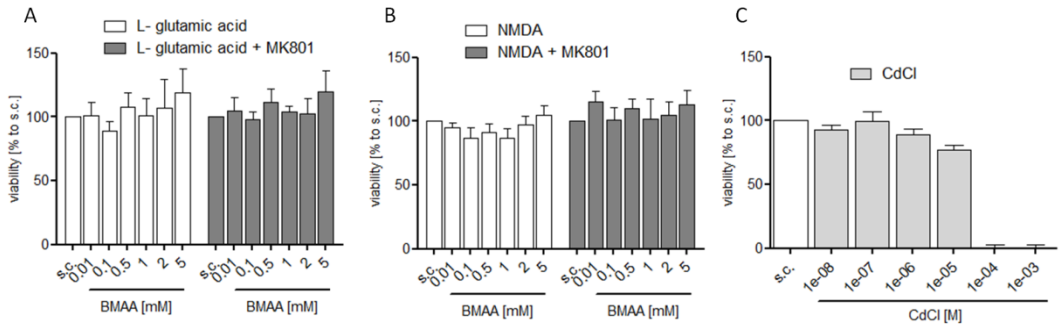

Figure 14: Cytotoxicity of glutamic acid, NMDA and the positive control CdCl

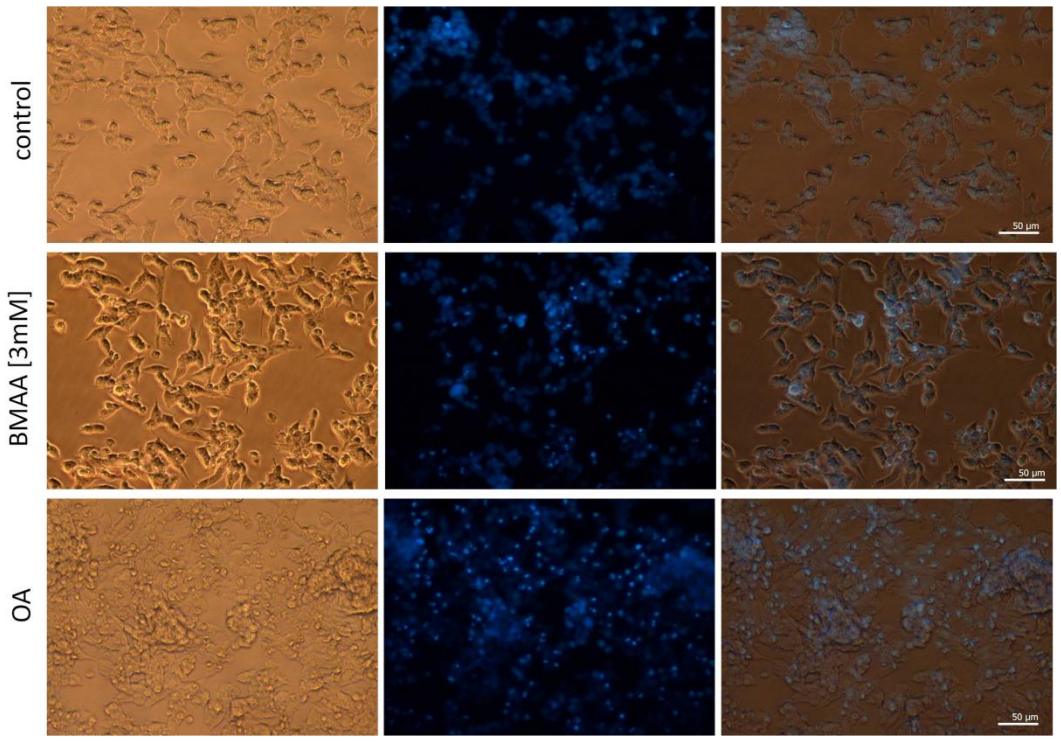

Figure 15: Apoptotic chromatin condensation in SH-SY5Y following 3mM L-BMAA treatment for 72 h.

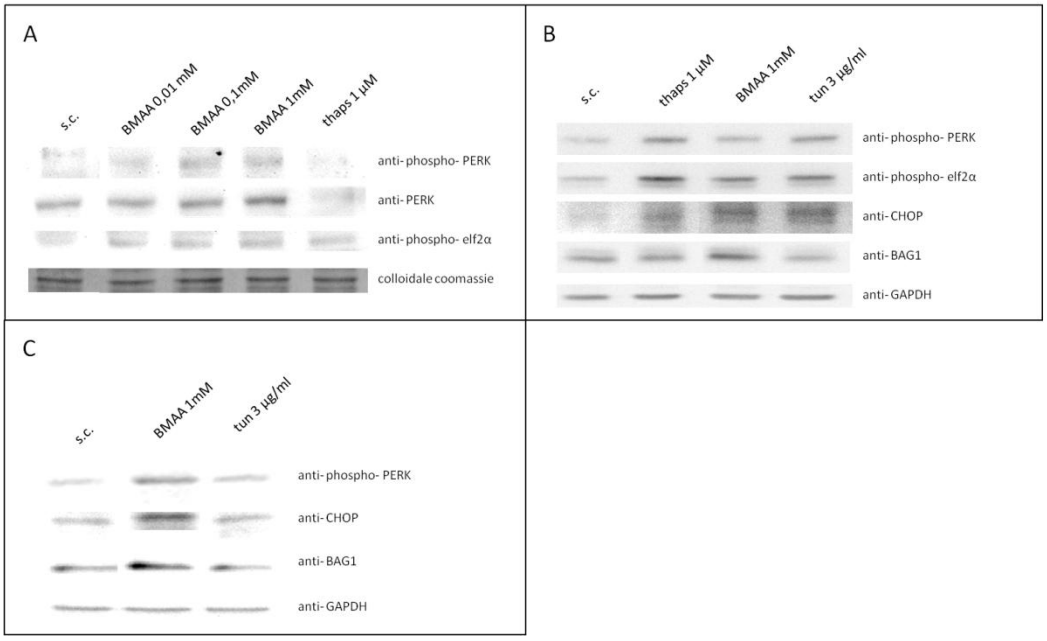

Figure 16: Unfolded protein response (UPR) and ER-stress marker proteins in human SH-SY5Y, THP1 and HEK-293 cells.

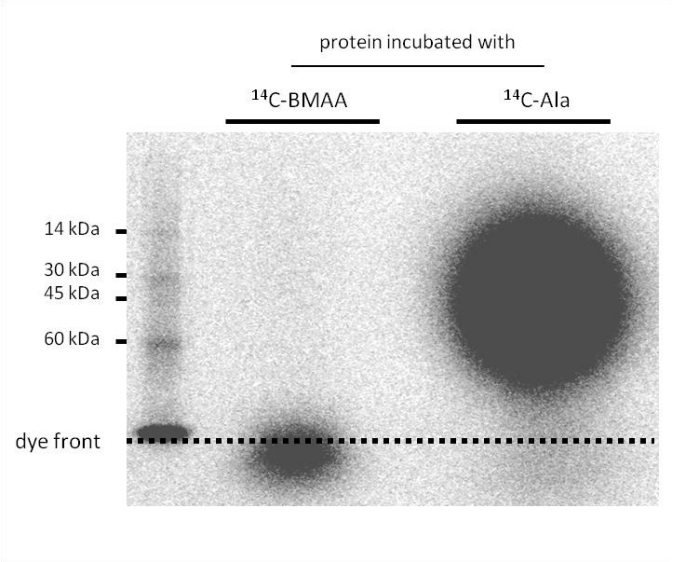

Figure 17: Detection of ^{14}C-L-BMAA and ^{14}C-L-alanine following SDS gelelectrophoresis.

Chapter 4: Manuscript II

L- BMAA-mediated Tau Hyperphosphorylation is associated with Calcineurin (PP2B)-dependent increased GSK3β (Ser9) Dephosphorylation

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4.1 Abstract

Exposure to the cyanobacterial neurotoxin beta-N-methylamino-L-alanine (L-BMAA) is discussed as one of the possible factors involved in the etiology of the neurodegenerative processes underlying the human amyotrophic lateral sclerosis/Parkinsonism-dementia complex (ALS/PDC). One of the hallmarks of these neurodegenerative processes is increased phosphorylation of the microtubule-stabilizing protein tau. Consequently, the human neuroblastoma cells SH-SY5Y were exposed to L-BMAA to elucidate whether L-BMAA treatment in vitro results in tau hyperphosphorylation and activation/deactivation of upstream critical enzymes, i.e. calcineurin (protein phosphatase 2B (PP2B)) and glycogen synthase kinase 3β (GSK3β). Positive controls employed for activation/deactivation of calcineurin and GSK3β were calmodulin, calcineurin inhibitor VIII, insulin and cyclosporine A, respectively. Exposure of SH-SY5Y to 2.5 and 4 mM L-BMAA for 45 min resulted in significantly increased tau hyperphosphorylation. Commensurate with this effect L-BMAA exposure resulted in increased calcineurin activity and calcineurin-dependent dephosphorylation of GSK3β at Ser9, thereby suggesting that a yet undefined interaction of

L-BMAA with calcineurin or upstream regulators of calcineurin results in abnormal activation of calcineurin. Whether this mechanism also occurs at low L-BMAA concentrations, i.e. at concentrations representative of the chronic exposure situations in humans, remains to be determined.

4.2 Introduction

An elevated incidence of amyotrophic lateral sclerosis/Parkinsonism-dementia complex (ALS/PDC) compared to the mainland of the USA was recorded in the native Chamorro population on the island of Guam in the 1960s (Spencer *et al.* 1987b). Subsequently, research focused, among other possible factors, on the neurotoxin β-N-methylamino-L-alanine (BMAA). BMAA is reportedly produced by cyanobacteria and assumed to be present at low concentrations in the traditional food staples consumed by the Chamorro (Cox *et al.* 2003), e.g. the flour made from cycad seeds.

The involvement of BMAA in the etiology of ALS/PDC has been and is heavily disputed (Steele and McGeer 2008), especially as the concentrations of BMAA required to induce NMDA receptor-mediated excitotoxicity (Liu *et al.* 2009; Rao *et al.* 2006) are extremely high and thus unlikely to occur under the realistic scenarios of long-term, chronic low-dose exposure (Lobner *et al.* 2007). Due to the amino acid structure a putative incorporation and/or protein attachment/aggregation of BMAA has been suggested (Banack *et al.* 2010; Field *et al.* 2011). Supporting the latter hypotheses, exposure of the human neuroblastoma cells SH-SY5Y to moderate concentrations of BMAA was shown to result in an impairment of protein homeostasis, increased protein oxidation and ER-stress followed by caspase 12 activation (Okle *et al.* 2013b). These effects occurred independent of reactive oxygen species (ROS) known to result from NMDA receptor-mediated excitotoxicity. Similarly, BMAA exposure induced, impaired RNA and protein synthesis were demonstrated in brain explants of mice and rats (Kisby and Spencer 2011). Moreover, neonatal treatment of six-month-old rats (postnatal days 9-10) with (460 mg/kg) BMAA resulted in an altered expression of various neuronal proteins, e.g. calcium- and calmodulin-binding proteins, and induced severe lesions in the adult hippocampus including neuronal degeneration, cell loss, calcium deposits and astrogliosis (Karlsson *et al.* 2012). Similarly, acute exposure of *Apis mellifera* (honeybees) to low mM concentrations of BMAA resulted in a dysregulated calcium homeostasis and increased levels of ROS in bee brains, while subchronic BMAA exposure led to impaired cognitive capacities (Okle *et al.* 2013a).

Increased tau hyperphosphorylation was reported in some ALS/PDC patients of Guam (Guiroy *et al.* 1993; Hirano *et al.* 1968; Hirano *et al.* 1961; Shankar *et al.* 1989) and is considered a general hallmark of neurodegenerative diseases, e.g. AD (Mirra *et al.* 1991). The accumulation of hyperphosphorylated tau within neurons destabilizes the microtubular

network, leading to an impaired axonal transport, and finally results in the formation of neurofibrillary tangles (NFTs) and neuronal death (Kuret *et al.* 2005; Spires-Jones *et al.* 2009). Dephosphorylation of tau is under the control of protein phosphatases (Gong *et al.* 1994a; Gong *et al.* 1994b; Yamamoto *et al.* 1988). An inhibition of protein phosphatases, e.g. by the marine biotoxin okadaic acid (OA), consequently leads to the accumulation of phosphorylated tau protein, which is why OA has been used to model tauopathy (tau hyperphosphorylation, NFTs) in many cellular systems *in vitro* and AD in rats *in vivo* (Zhang and Simpkins 2010). Among the compounds known to inhibit serine/threonine protein phosphatases (Ser/Thr-PPs), the cyanobacteria-produced microcystins (MCs) (Dietrich *et al.* 2008) have been demonstrated to produce tau hyperphosphorylation in murine neurons *in vitro* (Feurstein *et al.* 2010; Feurstein *et al.* 2011) as well as tau hyperphosphorylation and brain pathology associated with learning and memory impairment in rats *in vivo* (Li *et al.* 2012). The latter findings gained relevance due to the suggestion of an association between cyanobacterial blooms, possibly producing MCs or BMAA, in New Hampshire and development of ALS (Caller *et al.* 2009). However, the lack of obvious exposure to MCs in the ALS/PDC patients of Guam would *a priori* exclude MCs as a factor in the etiology of the tauopathy observed. On the other hand, based on structural considerations BMAA is unlikely to be able to directly inhibit Ser/Thr-PPs, thus suggesting that other pathways, e.g. an increased expression or half-life of tau phosphorlyation kinases, could be involved. Indeed, glycogen synthase kinase 3 (GSK3), specifically its isoform GSK3β, plays a major role in the onset of neurodegenerative diseases like AD and ALS. Increased activation of GSK3β leads to tau hyperphosphorylation and NFTs (Kaytor and Orr 2002). Moreover, increased GSK3β activity (Yang *et al.* 2008) was reported in the cortical regions of ALS patients (Yang *et al.* 2008), while the inhibition of GSK3β in an ALS mouse model led to reduced symptom onset and progression of neurodegeneration (Koh *et al.* 2007). The activity of GSK3β is regulated by the phosphorylation and/or dephosphorylation at various Ser/Thr residues by various kinases, e.g. PKB (Sutherland *et al.* 1993), PKA (Cross *et al.* 1995) and Wnt (Fukumoto *et al.* 2001). Deactivation of GSK3β kinase activity is achieved by phosphorylation of the N-terminal Ser9 residue (Forde and Dale 2007), whereas activation is achieved by protein phosphatase 2B (PP2B, calcineurin) via dephosphorylation of the Ser9 residue, as already demonstrated in SH-SY5Y cells (Kim *et al.* 2009). Consequently, increased or prolonged calcineurin activation would lead to an increased dephosphorylation of GSK3β at Ser9 and thus an increased GSK3β kinaseactivity and, as a consequence, would result in tau hyperphosphorylation (Figure 18). As calcineurin is phosphorylated by autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II (Hashimoto *et al.* 1988) and BMAA exposure of neonatal rats resulted in an over-expression of calcium- and calmodulin-binding proteins (Karlsson *et al.* 2012), the question was thus raised whether the non-proteinogenic BMAA could result in increased calcineurin activity thereby activating GSK3β and consequently resulting in tau hyperphosphorylation in SH-SY5Y cells. Indeed, an activation of GSK3β via calcineurin, subsequent tau phosphorylation

and caspase 3 cleavage was observed *in vitro* in SH-SY5Y cells following tunicamycin-induced ER-stress (Song *et al.* 2002b), while the latter was induced in SH-SY5Y cells following BMAA exposure (Okle *et al.* 2013b).

As expected, short exposure of SH-SY5Y to mM concentrations of BMAA resulted in a significantly increased calcineurin activity, increased dephosphorylation of GSK3β at Ser9 and a significant increase of phosphorylated tau protein. In contrast, the calcineurin inhibitor cyclosporine A (Cycl A) induced a significantly increased phosphorylation of GSK3β at the Ser9 residue compared to control or BMAA treatment. The results therefore suggest that BMAA-induced tau hyperphosphorlyation resulted from increased calcineurin and GSK3β activation. Whether BMAA also influences this pathway by an altered expression of Ca^{2+}/calmodulin-dependent protein kinase II expression remains to be established. However, these findings do suggest a role of BMAA in the pathways underlying the pathological events leading to neurodegenerative diseases.

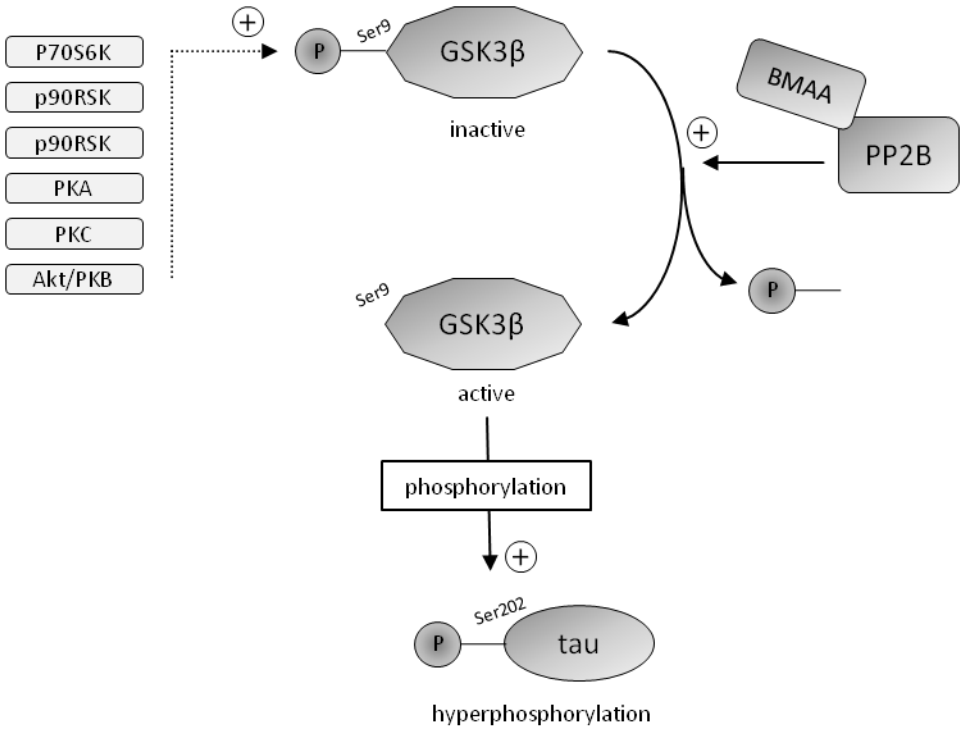

Figure 18: Increased calcineurin (PP2B) activity leads to a dephosphorylation of GSK3β at the residue Ser9 resulting in its activation. Hyperphosphorylated tau protein may become accumulated based on the raised GSK3β activity in the human neuroblastoma cells SH-SY5Y. Inhibitory phosphorylation of GSK3β at Ser9 can be accomplished by various kinases (reviewed by Cohen and Frame (2001)) which were not subject of this research.

4.3 Material and Methods

4.3.1 Chemicals and Reagents

All chemicals, unless otherwise stated, were of the highest analytical grade commercially available. L-BMAA (Sigma-Aldrich) was dissolved in 10 mM NaHCO3. Nominal concentrations were used in the experiments.

4.3.2 Cell System

The human neuroblastoma cell line SH-SY5Y (DSMZ, Braunschweig, Germany) was 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. SH-SY5Y cells were precultured in plates for 3 days (10% FBS, 5% CO_2, 37°C) before they were used in experiments.

4.3.3 Calcineurin Activity

The activity of calcineurin was measured using the calcineurin phosphatase assay kit in accordance to the manufacturer´s instructions (Enzo Life Science, Lörrach, Germany). Activity of 20 U/µl recombinant calcineurin was determined following 45 min L-BMAA exposure (37°). Calcineurin activity in SH-SY5Y cell extract was measured using 1 mg/ml extracted protein in the assay as the concentration of functional calcineurin in the extract was unknown. Proteins were extracted by lysing cultured SH-SY5Y cells in 2x calcineurin buffer for 5 min on ice using a syringe (21G).

4.3.4 Immunodetection of Proteins

Exposed SH-SY5Y cells were lysed in ice-cold extraction buffer (10 mM triethanolamine (Tris)-base, 140 mM NaCl, 5 mM EDTA, 0.1% (v/v) Triton X-100; pH 7.5) and centrifuged (17500 g, 20 min., 4°C) to obtain a cytosolic fraction following treatment with 2.5 mM L-BMAA or control substances for 30 min. Equal amounts of protein (determined by BCA assay (Thermo Fisher Scientific, Bonn, Germany) were loaded onto a 10% SDS gel and proteins were detected with specific primary antibodies: anti-phospho-Tau (#ABIN284769 (AT8), antikörper-online.de, Aachen, Germany), anti-Tau (#4019, Cell Signaling Technology,

Boston, MA, U.S.A.), anti-phospho-GSK3β (Ser9) (#5558P), anti-phospho-GSK3β (Thr390) (#3548S). Equal amounts of loaded protein were verified by a Colloidal Coomassie gel-staining. Densitometric quantification was done using Quantity One software (Bio-Rad, Munich, Germany). Statistical analyses were carried out as described in chapter 4.3.5.

4.3.5 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 post-test to compare values within an individual treatment group; F-test followed by a two-tailed t-test to compare the concentration between single treatment groups; one-sample t-test to compare treatment groups with solvent control. Results shown are the mean ±SEM and were considered statistically significant when $p<0.05$, with significance levels indicated as: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

4.4 Results

4.4.1 L-BMAA increased Tau Phosphorylation

A significant increase of the phosphorylated tau (Ser202) to total tau ratio was observable in SH-SY5Y cells following treatment with 2.5 mM L-BMAA for 30 min (Figure 19). The positive control OA provided for an increased phosphorylated tau (AT8) to total tau ratio as expected. As calcineurin activity is insensitive to OA inhibition (Carballo *et al.* 1999; Fruman *et al.* 1992), the observed OA-induced tau-phosphorylation was thus independent of calcineurin and GSK3β phosphorylation.

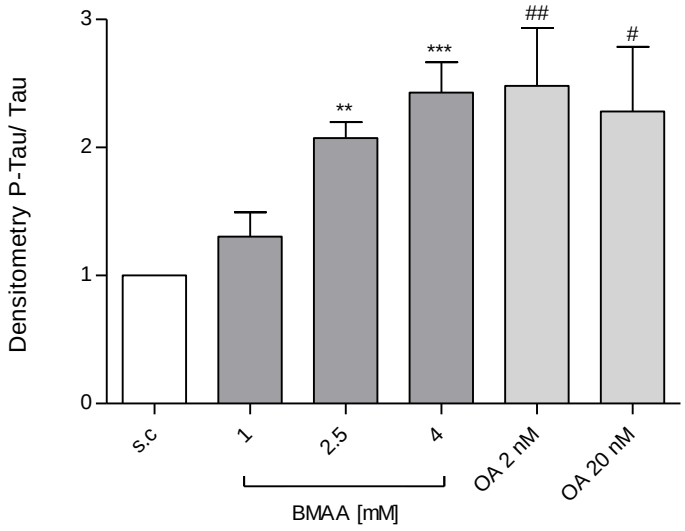

Figure 19: Increased phosphorylation (Ser202) of tau protein in SH-SY5Y cells following L-BMAA exposure. A significantly increased ratio of phosphorylated tau to total tau was observable in SH-SY5Y cells following 2.5 mM L-BMAA treatment for 30 min (N=4).

4.4.2 L-BMAA increased Calcineurin Activity

Exposure of 20 U/µl recombinant calcineurin with 0.08-8 mM L-BMAA increased calcineurin activity (Figure 20A). Although the data could suggest concentration-dependency, the associated variance within the individual concentration groups would rather suggest that concentrations of L-BMAA > 0.8 mM will increase calcineurin activity up to two-fold of control. As expected of negative and positive controls, the calcineurin inhibitor VIII (Millipore, Bedford, MA, U.S.A.) reduced calcineurin activity while correspondingly the calcineurin activator calmodulin increased calcineurin activity. Corresponding experiments with protein extract of SH-SY5Y cells showed comparable results (Figure 20B) to those obtained with the recombinant calcineurin (Figure 20A), albeit the increase in activity of calcineurin by L-BMAA was not as pronounced. However, the quantity of calcineurin within the SH-SY5Y protein extract was not determined, i.e. was not the same as for the recombinant incubations. Thus the direct comparison of SH-SY5Y protein extract with recombinant calcineurin is restricted to the qualitative effects of L-BMAA and the corresponding negative and positive controls.

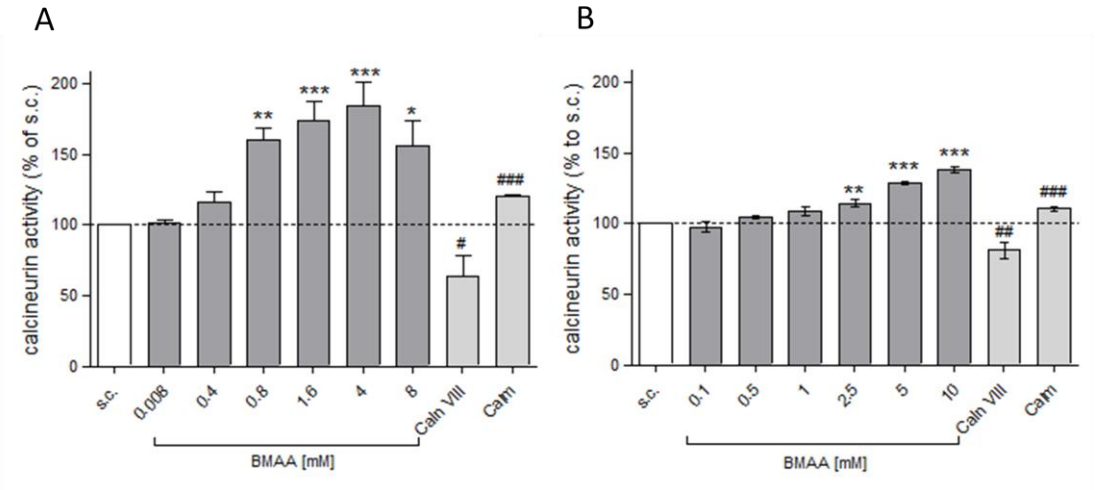

Figure 20: Calcineurin activity can be raised by L-BMAA treatment. **A**: 0.8 to 8 mM L-BMAA induced a significant increase of the activity of 20 U/µl recombinant human calcineurin (N=5). **B**: A significantly increased activity of calcineurin was observable in protein extract of SH-SY5Y following 2.5 mM L-BMAA treatment (N=3). The calcineurin inhibitor (CaIn VIII) induced a reduction of calcineurin activity, whereas additional calmodulin (Calm) induced an increase of the activity.

4.4.3 L-BMAA reduced GSK3β Phosphorylation at the Ser9 residue

Exposure of SH-SY5Y cells to the calcineurin inhibitor cyclosporin A (Cycl A) or insulin induced a significant increase of GSK3β phosphorylation at the Ser9 and Thr390 residues (Figure 21A and B) and thus an inactivation of GSK3β (Ma *et al.* 2012). In contrast, treatment with 5 mM L-BMAA resulted in a significant reduction of GSK3β phosphorylation at the Ser9 residue (Figure 21A). The combination treatment using L-BMAA and insulin or L-BMAA and Cycl A resulted in Ser9 phosphorylation comparable to control values, but significantly lower than values obtained with insulin and Cycl A alone. On the other hand, L-BMAA did not result in an increased or reduced phosphorylation at the Thr390 residue (Figure 21B), while the combination treatment with L-BMAA and insulin provided for phosphorylation levels at the THr390 residue comparable to those of the control, thus suggesting a lowering of the calcineurin activating capacity of insulin by L-BMAA. The phosphorylation levels at the Thr390 residue obtained with Cycl A or the L-BMAA and Cycl A combination treatment were comparable.

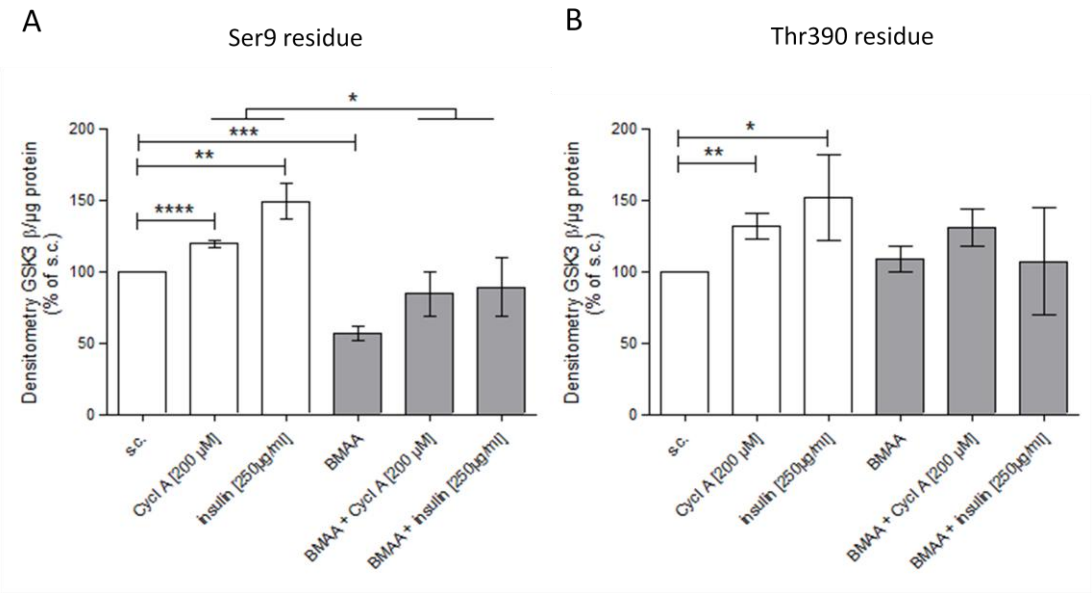

Figure 21: Impaired phosphorylation status of GSK3β following L-BMAA treatment. **A**: L-BMAA treatment induced a reduced phosphorylation of GSK3β at the Ser9 residue (N=4), whereas the phosphorylation status at the Thr390 residue (**B**) stayed unaffected (N=3). Cyclosporin A (Cycl A) and insulin provoked an increased phosphorylation at both residues, whereas co-treatment with L-BMAA only reduced the phosphorylation at the residue Ser9.

Treatment of SH-SY5Y cells with GSK3β inhibitor IX (Millipore, Bedford, MA, U.S.A.) or L-BMAA alone induced a decreased phosphorylation of GSK3β at the Ser9 residue. A more pronounced dephosphorylation at the Ser9 residue was achieved with the combination of GSK3β inhibitor IX and L-BMAA (Figure 22A). In contrast, treatment of SH-SY5Y cells with the GSK3β inhibitor IX resulted in a significantly increased phosphorylation of GSK3β at residue Thr390, while L-BMAA treatment had no effect at all, and the co-treatment of L-BMAA and GSK inhibitor reduced the phosphorylation significantly (Figure 22B). L-BMAA as well as GSK3β inhibitor IX treatment resulted in a marginally albeit significantly increased P-tau to total tau ratio (Figure 22C). Co-treatment with both L-BMAA and GSK3β inhibitor resulted in a 2.5-fold P-tau to total tau ratio. These findings indicate on the one hand that even if the phosphorylation of GSK3β at Ser9 residue is reduced by GSK3β inhibitor IX , it can be further reduced by co-treatment with L-BMAA. The same effect is observable if phosphorylation of GSK3β at Ser9 is increased by insulin/ Cycl A (Figure 21). Co-treatment with L-BMAA leads to a minored phosphorylation of Ser9 in SH-SY5Y in the same way. On the other hand, the stepwise reduced GSK3β phosphorylation at Ser9 residue and the stepwise increased phosphorylation of tau protein indicate the well-known correlation between GSK3β activity and its substrate tau.

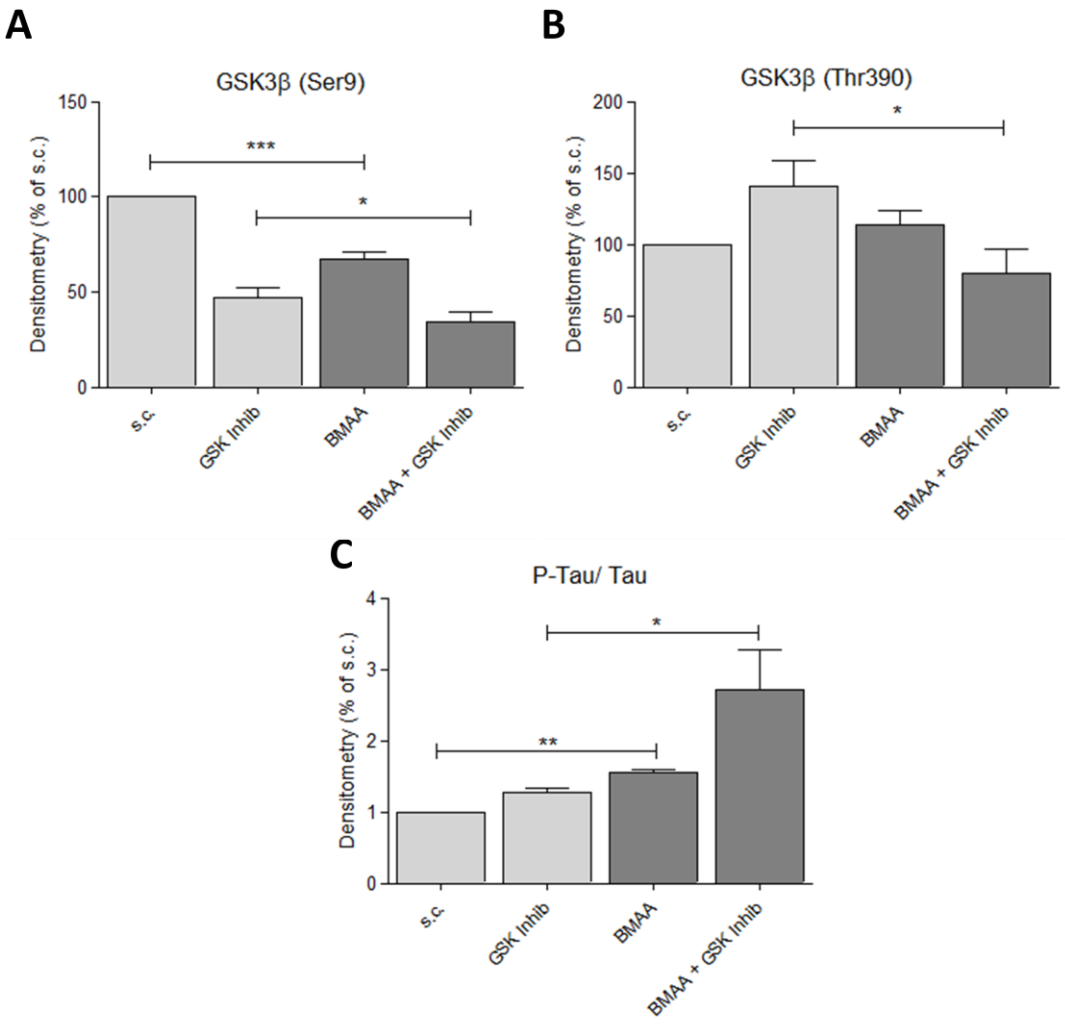

Figure 22: GSK3β inhibitor IX impaired phosphorylation of GSK3β and tau. GSK3β at residue Ser9 can become dephosphorylated following 10 µM GSK3β inhibitor IX (GSK Inhib) treatment as well as following 2.5 mM L-BMAA treatment (N=3) (**A**). Co-treatment with GSK Inhib and L-BMAA results in a further decreased GSK3β (Ser9) phosphorylation. Consequently, treatment with GSK Inhib or L-BMAA as well as co-treatment with GSK Inhib and L-BMAA resulted in an increased phosphorylation of tau protein (N=3) as the ratio of phosphorylated tau to total tau protein increased (**C**). L-BMAA had no effect on the phosphorylation status of GSK3β (Thr390) (**B**).

4.5 Discussion

Two main findings resulted from this research. First, L-BMAA can induce an increase of the phosphorylation of tau protein (Figure 19) in the human neuroblastoma cells SH-SY5Y. This can possibly lead to a pathological aggregation of tau protein in human brain and could thereby promote neurodegenerative processes. Second, the neurotoxin L-BMAA can affect the protein phosphatase 2B (calcineurin) resulting in its increased activity (Figure 20). To investigate a possible pathway which could explain the impaired phosphorylation status of

tau, the glycogen synthase kinase 3 β (GSK3β) was examined in the human neurocellular model SH-SY5Y. We chose the SH-SY5Y cells as a model system to eliminate a possible, already described activation of GSK3β by an NMDA receptor as NMDA receptors are inactive in SH-SY5Y cells (Jantas *et al.* 2008). Thus, L-BMAA cannot affect GSK3β by an activation of NMADA receptors in SH-SY5Y cells (Okle *et al.* 2013b).

The activity of GSK3β can be regulated by the phosphorylation and dephosphorylation at various serine or threonine residue sites. The phosphorylation of the serine 9 residue, located at the N-terminus of the protein, negatively regulates the kinase activity of GSK3β (Stambolic and Woodgett 1994) by an auto-inhibitory mechanism (Forde and Dale 2007). This is also the predominant regulator of GSK3 activity *in vivo* (Frame and Cohen 2001). A phosphorylation and therefore an inhibition of GSK3β at Ser9 can be achieved by various kinases and pathways, e.g. PKB (Sutherland *et al.* 1993), PKA (Cross *et al.* 1995) and Wnt signaling (Fukumoto *et al.* 2001). A possible dephosphorylating enzyme at the Ser9 residue leading to an activation of GSK3β is the protein phosphatase 2B (calcineurin) (Kim *et al.* 2009). This holoenzyme consists of two subunits, a catalytic A subunit (CnA) and a regulatory B subunit (CnB) (Aramburu *et al.* 2000), and its regulatory properties in physiological and pathological processes are controlled by two structurally similar but functionally different Ca^{2+}-binding proteins, calmodulin and calcineurin B (Klee *et al.* 1988). Dephosphorylation of GSK3β at the Ser9 residue by calcineurin led to a more efficient phosphorylation of the GSK3β substrate.

A well-known substrate for GSK3β *in vitro* (Hanger *et al.* 1992; Ishiguro *et al.* 1992) as well as *in vivo* (Lovestone *et al.* 1994) is the protein tau which can become phosphorylated by GSK3β at several residues (Wang *et al.* 1998). Consequently, a high GSK3β activity can result in an increased phosphorylation of tau. Increased tau phosphorylation (hyperphosphorylation) can destabilize the microtubule network as tau dissociates from mictotubular structures when it becomes phosphorylated. High amounts of phosphorylated tau could lead to impaired axonal transport and finally to the formation of neurofibrillary tangles (NFTs) and neuronal death (Kuret *et al.* 2005; Spires-Jones *et al.* 2009). These NFTs were observable in the ALS/PDC patients of Guam (Guiroy *et al.* 1993; Hirano *et al.* 1968; Hirano *et al.* 1961; Shankar *et al.* 1989) as well as in patients suffering from neurodegenerative diseases like AD (Mirra *et al.* 1991). Additionally a co-occurrence of abnormal hyper-phosphorylated tau and increased GSK3β levels were observable in AD (Leroy *et al.* 2007) and ALS patients (Koh *et al.* 2011). Therefore the activity of GSK3β and tau phosphorylation is assumed to play a major role in these neurodegenerative diseases.

As L-BMAA can induce an increased phosphorylation of tau protein (Figure 19) we investigated a putative upstream pathway which can possibly become activated or inhibited following L-BMAA treatment. As the main substrate of GSK3β is tau (Hanger *et al.* 1992; Ishiguro *et al.* 1993; Spittaels *et al.* 2000) and the enzyme can phosphorylate the tau

protein (Wang *et al.* 1998) and as additionally the inhibition of GSK3β can reduce the onset of symptoms and the disease progression in an ALS mouse model (Koh *et al.* 2007) we focused on this enzyme.

The increase of the calcineurin activity was a first hint that L-BMAA can affect the activity of protein phosphatases in SH-SY5Y cells (Figure 20). The functionality of the test system was verified as the calcineurin inhibitor VII as well as additionally added calmodulin affected the calcineurin activity in an expected manner in SH-SY5Y cells as well as of recombinant calcineurin.

A possible downstream effect of an increased calcineurin activity is a dephosphorylation of GSK3β at the Ser9 residue. Closer examination of the phosphorylation status of GSK3β (Figure 21A and Figure 22A) clearly indicates that the phosphorylation at the Ser9 residue decreases following L-BMAA treatment. In the same way increased phosphorylation by insulin or the calcineurin inhibitor Cycl A can be reduced following co-treatment with L-BMAA (Figure 21A). The increased phosphorylation of GSK3β following insulin treatment in SH-SY5Y cells was already described by Lesort *et al.* (1999).The dephosphorylation at Ser9 indicates the activation of GSK3β. In contrast, the phosphorylation status of GSK3β at the Thr390 residue remains unaffected by treatment or co-treatment with L-BMAA (Figure 21B). This hints towards an activation of GSK3β at the residue Ser9 by an L-BMAA-induced change of the calcineurin activity in the SH-SY5Y cells. The activation of GSK3β by a dephosphorylation at the residue Ser9 by calcineurin was already described by Kim *et al.* (2009) in the same cell line.

A plausible resulting phosphorylation of the tau protein (Sperber *et al.* 1995) in response to an increased GSK3β activity and an aggregation of the (hyper-)phosphorylated tau into tangle-like aggregates was investigated by Rankin *et al.* (2007). Indeed, L-BMAA treatment resulted in an increased dephosphorylation of GSK3β and an increased phosphorylation of tau protein (Figure 19). The manipulation of the GSK3β phosphorylation by GSK3β inhibitor treatment resulted in a decreased phosphorylation of GSK3β at residue Ser9 which consequently resulted in an increased phosphorylation of tau protein, putatively based on GSK3β activation. Contrary to expectation treatment of SH-SY5Y cells with 10 µM GSK3β inhibitor IX for 30 min induced a decreased phosphorylation of GSK3β at Ser9 residue and an increased phosphorylation of GSK3β at Thr390 residue and not vice versa. However, co-treatment of GSK3β inhibitor IX with L-BMAA resulted in a decreased phosphorylation of both GSK3β residues Ser9 and Thr390 and consequently in an increased phosphorylation of tau protein. This indicates once more that the L-BMAA-induced phosphorylation of tau protein is related to an upstream mechanism with GSK3β activation probably linked to an impaired and increased calcineurin activity. However, also chronic ER-stress, as observable in SH-SY5Y following BMAA treatment (Okle *et al.* 2013b), could directly contribute to the

phosphorylation of tau via GSK3 (Hoozemans and Scheper 2012)and would therefore be an interesting topic for further investigations.

The data presented above demonstrate a pathway which has to date not been considered, but one which may give an insight into the long-term, non-acute toxic acting of L-BMAA possibly causing the symptoms of ALS/PDC. The mechanism by which L-BMAA can influence the calcineurin activity in the human cell line SH-SY5Y as well as in experiments with recombinant calcineurin is not fully understood. Possibly, a mechanism comparable to the Ca^{2+}/calmodulin-binding to calcineurin, which induces conformational changes by displacing the auto-inhibitory domain and thus activating the phosphatase activity (Klee *et al.* 1998), is the source for the changed calcineurin activity following L-BMAA treatment. Indeed, a high affinity of L-BMAA for proteins - and therefore also enzymes – has already been discussed by various researchers (Banack *et al.* 2010; Field *et al.* 2011; Okle *et al.* 2013b). Furthermore, an altered expression of the regulatory Ca^{2+}/calmodulin-dependent protein kinase II or calcineurin-associated proteins (e.g. calmodulin or calmodulin-binding protein (BASP1/ NAP22A)) can influence the activity. An impairment of BASP1/NAP22A was already demonstrated in rat brains following BMAA exposure (Karlsson *et al.* 2012).

Even though the mechanism underlying increased calcineurin activity following L-BMAA treatment need further research, this is the first report of a (cyano)toxin that provokes a hyperphosphorylation of tau, not by an inhibitory mechanism of a protein phosphatase but by an activation of the protein phosphatase 2B (calcineurin). This results in an activation of the downstream pathway in the human neurocellular model system including impaired activity of GSK3β and tau hyperphosphorylation.

Chapter 5: Manuscript III

The Cyanobacterial Neurotoxin Beta-N-Methylamino-L-Alanine (L-BMAA) induces Neuronal and Behavioral Changes in Honeybees

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5.1 Abstract

The cyanobacterially produced neurotoxin beta-N-methylamino-L-alanine (BMAA) is thought to induce amyotrophic lateralsclerosis/Parkinsonism dementia complex (ALS/PDC)-like symptoms. However, its mechanism of action and its pathway of intoxication are yet unknown. In vivo animal models suitable for investigating the neurotoxic effect of BMAA with applicability to the human are scarce. Hence, we used the honeybee (Apis mellifera) since its nervous system is relatively simple, yet having cognitive capabilities. Bees fed with BMAA-spiked sugar water had an increased mortality rate and a reduced ability to learn odors in a classical conditioning paradigm. Using 14C-BMAA we demonstrated that BMAA is biologically available to the bee, is found in the head, thorax and abdomen with little to no excretion. BMAA is also transferred from one bee to the next via trophallaxis resulting in an

exposure of the whole beehive. BMAA bath application directly onto the brain leads to an altered Ca2+ homeostasis and to generation of reactive oxygen species. These behavioral and physiological observations suggest that BMAA may have effects on bee brains similar to those assumed to occur in humans. Therefore the bee could serve as a surrogate model system for investigating the neurological effects of BMAA.

5.2 Introduction

The cyanobacterially produced neurotoxin beta-N-methylamino-L-alanine (BMAA), originally discovered in cycads of the South Pacific, has been controversially discussed as a causative agent of the neurodegenerative disease amyotrophic lateral sclerosis/ Parkinsonism dementia complex (ALS/PDC). However, neither the occurrence of BMAA-producing cyanobacterial strains nor the mechanism by which BMAA possibly induces neurodegeneration nor the pathway of intoxication in humans is understood. The disease symptoms reported for ALS/PDC patients on Guam were similar to those observed in ALS, Alzheimer's (AD) as well as in Parkinson's disease (PD) patients, i.e. memory deficits, olfactory deficits, disorientation, personality changes, muscle weakness/atrophy, bradykinesia and gait disturbance. However, in contrast to AD and PD patients the symptoms reported for Guam ALS/PDC patients occurred either simultaneously or in a sequential development (reviewed by Schulz *et al.* (2005)).

Since it has been reported that also cyanobacteria produce BMAA and more BMAA-producing cyanobacterial strains are being discovered (Banack *et al.* 2007; Cervantes *et al.* 2012; Cox *et al.* 2005), a hypothetical link between the occurrence of these cyanobacteria, possible human exposure and an increased incidence of sporadic ALS cases is discussed (Caller *et al.* 2009; Cox *et al.* 2003; Gunnarsson *et al.* 1996). Indeed, high levels of BMAA were reported in brains of Canadian AD patients (Murch *et al.* 2004b) possibly having been exposed to cyanobacteria or dietary supplements thereof, thus supporting the hypothesis that chronic BMAA exposure is more ubiquitous than expected and that BMAA may play a role in the etiology of neurodegenerative diseases other than ALS/PDC. Supporting the latter, a recent study reports a two-fold higher BMAA concentration in the brains of AD and ALS patients of North America (Pablo *et al.* 2009). These finding raised speculations as to whether or not BMAA is involved in the etiology and/or progression of neurodegenerative diseases.

BMAA is a contaminant of the traditional food and medicine of the native Chamorro people (Steele and Guzman 1987), thus providing a basis for the association of BMAA exposure and the high incidence of ALS/PDC and thus neurodegenerative diseases (Caller *et al.* 2009; Cox *et al.* 2009). As BMAA was demonstrated to bioaccumulate along the aquatic major food webs (Jonasson *et al.* 2010), highest levels are found in the top predators (Jonasson *et al.*

2010) (Esterhuizen-Londt 2010). Thus it is not surprising that BMAA was also found in shark fins meant for human consumption (Mondo *et al.* 2012). Accordingly, low-level exposure of humans to BMAA could be much more prevalent than originally anticipated.

The mechanisms underlying BMAA-induced neurodegenerative effects are currently heavily disputed. One hypothesis suggests an excitotoxic effect via glutamate receptors (reviewed by Chiu *et al.* (2011)). Another, non-exclusive, hypothesis proposes an incorporation or binding of BMAA into/to proteins (Murch *et al.* 2004a), resulting in endoplasmatic reticulum (ER) stress and impaired protein homeostasis (Okle *et al.* 2013b). Indeed, the latter are considered the underlying key mechanisms causal to many human neurodegenerative diseases. Some *in vitro* experiments also suggest action via multiple mechanisms (Lobner *et al.* 2007; Murch *et al.* 2004a), including increased reactive oxygen species (ROS) and an impaired Ca^{2+} homeostasis (reviewed by Chiu *et al.* (2011)). Even though these effects indicate an acute toxicity of BMAA they are also described to initiate pathways which are common in progressive neurodegenerative processes (e.g. accumulation of oxidized proteins, impaired ion homeostasis, initiation of apoptotic pathways).

Unfortunately, most of the *in vivo* experiments in monkeys, rats, mice and chickens do not present the typical pathology of human ALS/PDC (Karamyan and Speth 2008), suggesting that the routine model organisms used in investigative toxicology are not ideal for elucidating the assumed BMAA-induced neurodegeneration in humans. Consequently, it was considered important to find relatively simple model organisms with relevance for humans and that allow detection of physiological and behavioral endpoints related to human neurodegenerative diseases e.g. zebrafish, *C. elegans, Drosophila*, or bees (Bilen and Bonini 2005; Gama Sosa *et al.* 2012). Insects appear particularly promising since many of them are well-established model organisms for genetic, molecular, behavioral and neuronal analysis. Honeybees (*Apis mellifera*) have become an important model species in neuroscience. We employed bees to investigate the neurotoxicity of BMAA since bees can be trained reliably to odors (conditioned stimulus, CS) and using sugar water as the corresponding control (unconditioned stimulus, US) in a classical conditioning paradigm (Giurfa *et al.* 2001; Kuwabara 1957; Matsumoto and Higa 1966).

Human ALS/PDC patients demonstrate both, learning and odor perception deficits, as discussed above. The latter two endpoints are readily testable via olfactory learning in bees. More importantly, the step from physiology to behavior is short in these insects: i.e. physiological techniques to measure odor-evoked activity patterns have been established (Galizia *et al.* 2000; Galizia and Vetter 2004), providing direct access to brain network effects induced by the neurotoxin treatment. Projection neurons (PNs) were chosen as PNs act as readout neurons of the primary olfactory center in the bee (the antennal lobe, comparable to the human olfactory bulb). Calcium imaging was employed as endpoint since

BMAA appears to impair calcium homeostasis *in vitro* (Brownson *et al.* 2002; Cucchiaroni *et al.* 2010; Rao *et al.* 2006). Consequently the bee experiments we present here demonstrate the acute toxicity of BMMA as shown by a dysregulated calcium homeostasis and increased levels of ROS. Moreover chronic BMAA treatment impairs cognitive capacities. Finally, due to trophallaxis, BMAA exposure can spread from individual bees to the whole population, thus suggesting that BMMA could be transferred into honey. In summary the data show effects of BMAA in bees similar to those expected to occur in humans subsequent to neurotoxin exposure, thereby also demonstrating the advantages of the bee as a model organism for mechanistic investigation of human neurotoxins.

5.3 Material and Methods

5.3.1 Animals

All experiments were conducted with summer bees of the European honeybee (*Apis mellifera*) between April and October 2011. Summer bees have an average life span of 41 days (Sakagami and Fukuda 1968).

5.3.2 Mortality Rate

Bees were caught at the entrance to their hives or feeders nearby and placed in meshed cages ("bugdorms", 30x30x30 cm, model: DP1000, MegaView Science Co., Ltd., Taichung 40762, Taiwan) (four bugdorms at a time, 150 bees each). Feeders within the bugdorms consisted of a petridish (15 cm diameter) filled with moist paper tissues to provide humidity in the surrounding area and a watch glass (7 cm diameter), fixed to the base with white wax, in which food was delivered with a syringe through the grids. Bugdorms were placed in a dim room (~22°C). For two days (pre-treatment) bees were fed sugar water (1.25 M) *ad libitum* to allow adjustment to the new situation. Subsequently they were fed with sugar water (1.25 M) *ad libitum* with either 9% of bee saline (in mM: 130 NaCl, 7 CaCl2, 6 KCl, 2 MgCl2, 160 saccharose, 25 glucose, 10 HEPES, pH 6.7, 500 mOsmol) or 9% bee saline with L-BMAA for a final L-BMAA concentration of 5 mM. To determine the rate of mortality, dead bees were counted and removed from the bugdorms daily.

5.3.3 Conditioning

Eight bees were randomly picked from each group of bugdorms on day 1 and 5 of the treatment. They were immobilized on ice and placed into plastic holders. 10 min before conditioning the ability to show a proboscis extension reflex (PER) was tested by touching the antennae with sugar water. Bees failing the PER test were discarded. All others were conditioned to the odor 1-hexanol (Sigma Aldrich, Munich, Germany) as described in Rath *et al.* (2011). They received 6 training trials with an inter-trial interval of 10 min. Each trial consisted of 20 sec airflow to habituate, 4 sec odor paired with sugar water (1.25 M) after 3 sec creating a 1 sec overlap of odor and reward. Sugar water was given for 3 sec. The sugar water was delivered to the bees' antennae with a needle on a syringe filled with sugar water. Once PER was triggered the needle was held to the proboscis to feed the bees for the remaining time. After pairing, bees were left in the airflow for 11 sec before removing them. 30 min after the last trial, short-term memory was tested by exposing the bee to the 1-hexanol-stimulus and monitoring the PER.

5.3.4 Odor Stimulation

For conditioning as well as Ca^{2+}-imaging the odor was applied as described by Rath *et al.* (2011). The odor was 1-hexanol (Sigma Aldrich, Munich, Germany) diluted 1:100 in mineral oil. 200 µl of the diluted odor was put on a cellulose strip (Sugi, REF 31003; Kettenbach GmbH KG, Eschenburg, Germany) and placed in a 3 ml syringe (Norm-Ject; Henke-Sass, Wolf GmbH, Tuttlingen, Germany). This syringe was placed in the custom-built computer-controlled six-channel olfactometer (Szyszka *et al.* 2011) and odor pulses of 1-hexanol were delivered throughout the experiment. The odor air stream was 300 ml/min controlled by a flowmeter (Analyt-MTC GmbH, Müllheim, Germany) and was injected into a continuous carrier air stream (1200 ml/min), which was directed to the bee via a glass tube (0.7 cm diameter, 1 cm in front of the bees' antennae). Thus, there was a total air stream of 1500 ml/min going to the bee's head, with an air speed of 0.65 m/s. Continuous air suction behind the bee cleared away residual odor.

5.3.5 Staining, Preparation and Ca^{2+} Imaging

Brains were prepared and imaged as described (Galizia and Vetter 2004; Szyszka *et al.* 2011). Bees were caught from the entrance of the hive, immobilized, and projection neurons were stained by applying a crystal of dye (Fura-2-Dextran, Sigma Aldrich, Munich, Germany) via glass electrodes between the calyces of the mushroom bodies, aiming at the ACT axonal tracts of antennal lobe projection neurons. After the dye had traveled to the

antennal lobes, the living bees were fixed, the head capsule opened, and the antennal lobes exposed. Imaging was done using a CCD camera (Imago QE; Till Photonics). Each measurement consisted of 232 double frames (340 nm, 380 nm) taken at a rate of 8 Hz. The odor was presented from frame 72 to 104 (4 sec). During the first recording at around frame 144 the amount of 50 µl of either saline solution or L-BMAA in saline solution (2.5 mM) were added to the brain to a final concentration of approx. 625 µM L-BMAA. Spontaneous activity was measured before and after each odor stimulus (frames 1-104, Figure 26). A measurement lasted for 28 sec. Measurements were taken before and 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, 60 min after applying either L-BMAA or saline solution.

5.3.6 ROS Detection in Bee Brains

Bees were caught and fixed and their brain was made accessible (Galizia and Vetter 2004). 100 µl of $DCFH_2$-DA (205.2 µM; Sigma Aldrich, Munich Germany) in saline solution was bath-applied to the brain (205.2 µM). The bee, sitting in a moist glass box, was put into the fridge (4°C) for at least 30min. Then the $DCFH_2$-DA was removed by rinsing the head capsule four times with 100 µl saline. The imaging set up was the same as for the Ca^{2+} imaging. Excitation light was 480 nm, dichroitic mirror was 495 nm and emission filter was 505 nm LP or 515 nm LP depending on the setup used. Each measurement lasted 1 min and consisted of 6 bouts of 3 sec length every 10 sec, every bout at 8Hz (24 frames each, 144 frames total). After the first measurement either 100 µl or 50 µl of L-BMAA in saline solution (final concentrations: 625 µM or 1.25 mM) or just saline solution or 300 µM 1-methyl-4-phenylpyridinium (MPP^+) (Sigma Aldrich, Munich, Germany) in saline solution were added to the brain (final concentrations: 150 µM or 300 µM). Measurements were taken at 0, 2, 6, 10, 20, 30 min subsequent to L-BMAA (or saline control) application.

5.3.7 Location of L-BMAA in the Bee's Body

Bees were caught, immobilized on ice and fixed in modified Eppendorf reaction tubes, still being able to show a PER for feeding. Then they were placed into a moist plastic container and fed with sugar water (1.25M) for 2 days for acclimatization. On day 3 they were given 15µl of sugar water (1.25M) with ^{14}C-BMAA (60µM) (BIOTREND, Cologne, Germany; 0.45mM). The following days they were fed with sugar water (1.25M) only again. Samples were taken 24h, 48h or 72h after treatment and handled as described above. Observations longer than 3 days were not possible with this experimental set-up as bees did not survive longer than day 3, most likely due to the stress of being fixed in a harness.

5.3.8 Intra-Species Transfer

Twenty bees were caught from the entrance of the hives and put in a box (13x6x5 cm) with 2 compartments (10 bees each) separated by a net and with a net as a lid. One compartment contained a plastic cup (Greiner Bio-One GmbH, Frickenhausen), glued to the base, to provide food. Wax and propolis were placed inside to override the smell of the glue. The first day the bees in the compartment with the cup (fed-group) were fed with 3 ml of sugar water (1.25 M). In the following days the fed-group was fed with 3 ml of ^{14}C-L-BMAA (60 µM) in sugar water. After 48 h the sugar water with ^{14}C-L-BMAA was eaten up by the fed-group. All bees were simultaneously shock frozen in liquid nitrogen and dissected into three parts (head, abdomen, thorax). Each part was placed into 600 µl tissue solubilizer Biolute-S (SERVA, Heidelberg, Germany) and lyzed (50°C; shaking, 48 h). 200 µl of the sample was added to 4 ml scintillation cocktail (Quicksafe A, Zinsser Analytic, Frankfurt, Germany). Radioactivity of acidified (600 µl 2M HCl) samples was determined in a LS6500 liquid scintillation counter (Beckmann, Munich, Germany).

5.3.9 Data Analysis and Statistics

Custom-written programs in IDL (RSI, Boulder, CO, U.S.A) were used for the analysis of the imaging data (Ca^{2+}, ROS measurements, Figure 25). Processing and analysis of the imaging data as to the signal strength and the spontaneous activity was carried out as described in Rath *et al.* (2011). Signals were calculated as F340/F380 and the baseline shifted to 0 by subtracting the average signal before the 1-hexanol stimulus (frame 4 to 69). For the analysis of the Ca^{2+}-level F340/F380 only was calculated and the traces were put at the different time points into relation to the time before adding the drug by dividing the value from a specific glomerulus by its value before the drug application. From these values the median absolute deviation (MAD) (Formula 1) was calculated. The MAD is a more “robust” parameter to analyze variability than squared error parameters such as standard deviation (Huber 2004).

The statistical analyses of mortality data (Figure 23A) was done in R 2.15.0 (R Development Core Team 2012) and carried out by oikostat (Ettiswil, Switzerland; www.oikostat.ch). A generalized linear mixed model was used with Poisson error distribution, log-link function and an offset to analyze the number of bees that died every 24 hours for each treatment group (mortality rate). The logarithm of the number of bees that were alive at the beginning of each 24 hour-interval (i.e. the number of bees “at risk”) was used as offset. The mortality rate was used as outcome variable. Treatment, the linear and quadratic term of time and the interactions between treatment and both (linear and quadratic) time variables were used as predictor variables. Interaction, Date x Treatment, was defined as

"cohort" and included as a random factor to account for repeated measures of the same group of bees. Date was included as an additional random factor to account for seasonal effects. An observation level random factor to account for the substantial overdispersion was also included. Stepwise unimportant interactions and quadratic terms were dropped as assessed by the BIC (Burnham and Anderson 2002) and the 95% credible intervals (CrI-95) of the parameter estimates.

Bayesian method was used to assess the significance and to quantify uncertainty of the parameter estimates and model predictions (Bolker *et al.* 2008). The function sim of the R-package arm (Gelman and Hill 2007) was used to simulate 2000 sets of random values from the joint posterior distribution of the model parameters assuming non-informative prior distributions. The 2.5% and 97.5% quantiles of these random values for each parameter were used as the lower and upper limits of the 95% credible intervals (CrI). From the model the posterior predictive distributions for the number of surviving bees in each cohort was simulated.

The statistical analysis for the acquisition curve of the behavioral data (Figure 23B) was done with a logistic regression model, which fits the data well (Figure 27), with repeated measurements with treatment and trial as fixed effects and the PER as response variable. For the in between trials effects and the retention test we used a Pearson's Chi-squared test with Yates' continuity correction.

5.4 Results

5.4.1 L-BMAA increases the Mortality Rate

The mortality rate of bees fed with L-BMAA (end concentration 5 mM) dissolved in sugar water (3 runs, **Δ**, Figure 23A) and those of bees fed with equal amounts of saline solution in sugar water (3 runs, **o**, Figure 23A) were compared and the results were tested using a generalized linear model with binomial distribution. The mortality rate was significantly higher in L-BMAA-treated bees (estimate: 0.70 and a CrI-95: 0.33-1.02). The mortality difference was statistically significant after 24 h. It increased over time in both groups (significant positive effect of time 0.62 and a CrI-95: 0.41-0.83, Table 2).

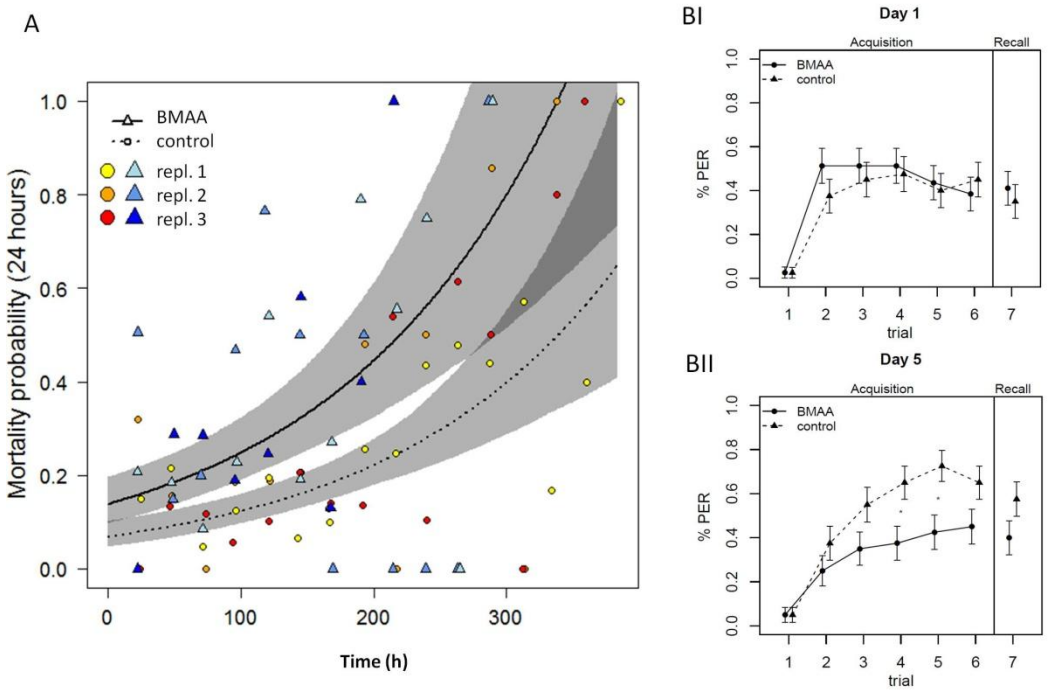

Figure 23: Behavioral effects. **A**: Mortality of bees over time (hours); **Δ**: exposed to 5 mM L-BMAA-supplemented food *ad libitum* (3 replicates, coded with different colors, n=525) and; **o**: control bees (3 replicates, coded with different colors, n=569). The model mortality function is given for the two cases with 95% credible interval (CrI-95) for the model prediction. Mortality in L-BMAA fed bees was significantly higher than in control bees. **B**: Learning curves and memory tests. **BI**: One day of feeding: no difference in learning curves or memory tests was evident between L-BMAA-exposed (n=40) and control (n=39) bees. **BII**: Five days of feeding: the acquisition curve and the memory recall test of L-BMAA-exposed bees (n=40) was consistently lower than of control bees (n=40) with a significant difference for the 4th and 5th training trial ($p<0.05$). Data show % of proboscis extension reflex (PER) as a fraction of the total number of bees per group; mean ±SEM.

5.4.2 Associative Learning and Short Term Memory is impaired by L-BMAA

To investigate the effect of L-BMAA on the bees' behavior, their short-term memory was tested in a classical conditioning paradigm. The bees were conditioned to an odor followed by a memory recall test after 30 min. Bees were fed for 5 days with L-BMAA. Both groups L-BMAA fed and sugar water fed (control) bees were compared. The control and the L-BMAA group of day 1 showed no difference in acquisition or memory recall (control: 36%; L-BMAA: 40%) (Figure 23B-I). In the L-BMAA and control groups the learning rate (~40%) was fairly low yet showed the typical learning curves: the proboscis extension reflex (PER) to the odor stimulus alone rose until saturation. On day 5, however, bees showed significant differences ($p<0.05$) in acquisition at the 4th and 5th trial (Figure 23B-II). In the L-BMAA group (n=40) considerably fewer bees learned to associate the odor with the reward

compared to the control group (n=40). The memory recall test, however, showed no significant differences between the control and the L-BMAA-exposed bees at day 5 although a trend of an impaired memory of L-BMAA-treated bees was observable (40% of the L-BMAA bees showed a PER, in contrast to 60% in the control group, p=0.07). A higher sample size might have shown the observed differences as statistically significant, albeit a difference of 0.2% PER between the control and the BMAA treatment at a background level of 0.55% PER represent a change of -137% over control.

5.4.3 Accumulation and Distribution of ^{14}C-L-BMAA in Bees

Bees were fed with 5 mM ^{14}C-L-BMAA sugar water (1.25 M) solution and the amount of ^{14}C-L-BMAA retained in head, thorax and abdomen 24 h, 48 h and 72 h after exposure was determined. The total amount of ^{14}C-L-BMAA remained constant over the 3 days tested (Figure 28) after application, suggesting that bees retain L-BMAA, and/or that L-BMAA was not excreted into the Malpigian tubuli. Generally L-BMAA accumulated more strongly in the abdomen than in the head or thorax (Figure 24A). The distribution of ^{14}C-L-BMAA in the head, thorax and abdomen relative to the dry weight was not significantly different after 24 h and 48 h (two-tailed t-test). The ^{14}C-L-BMAA in the head and thorax at 72 h was lower than at 24 h and 48 h, while the abdominal ^{14}C-L-BMAA remained at the same level as that observed at 24 h and 48 h.

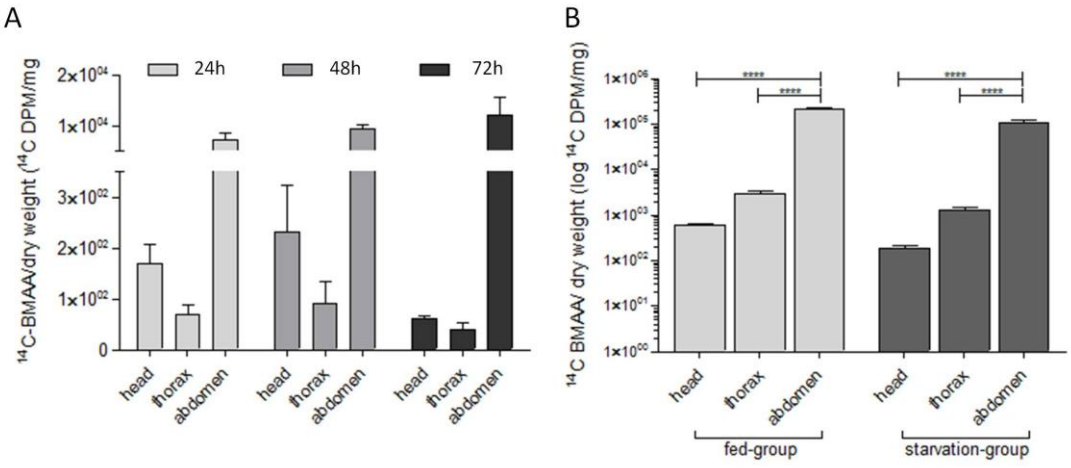

Figure 24: Distribution of ^{14}C-L-BMAA. **A**: ^{14}C-L-BMAA in the heads, thorax and abdomens 24 h (n=8), 48 h (n=6) and 72 h (n=4) after cessation of ^{14}C-L-BMAA exposure; mean ±SEM. **B**: Trophallaxis of L-BMAA. Non-fed bees (n=9) accumulated ^{14}C-L-BMAA after 48 h via trophallaxis from bees (n=11) with access to ^{14}C-L-BMAA sugar water. The highest ^{14}C-L-BMAA activities were found in the abdomen of both groups; mean ±SEM.

5.4.4 ROS Development

Optical imaging of ROS was used to determine whether direct application of L-BMAA onto the bee brain leads to an increased amount of reactive oxygen species (ROS). Exposure (30 min) to 1.25 mM L-BMAA significantly ($p<0.01$) raised ROS levels approximately 1.8-fold (Figure 25A) when compared to the value at the starting point (1 min). In comparison to L-BMAA, 300 µM of MPP^+ (positive control) raised ROS levels by 1.6-fold. This compound-induced, increased, ROS levels followed a concentration-response (Figure 29), while control ROS levels remained constantly low over the duration of the experiment (30 min).

5.4.5 L-BMAA leads to elevated Ca^{2+} Concentration in Brain Neurons

To test the theory that L-BMAA affects the functionality and activity of bee brains (Figure 30), bath-applied L-BMAA or saline solution was applied to the bee brains and intracellular calcium levels in the antennal lobes were recorded. There, projection neurons are constantly active even in the absence of odor stimuli. The resting calcium level is sufficiently high to determine a decrease of intracellular calcium subsequent to inhibitory input by at least two inhibitory networks (GABAergic and a PTX-insensitive and glomerulus-specific) resulting in an overlapping response profile of the glomeruli (Sachse and Galizia 2002). Simultaneous measurement of the relative change in total fluorescence in stained projection neurons, served as a record of changes in intracellular Ca^{2+}-concentration. Recordings were taken over a 60 min exposure period, whereby one recording was taken prior to compound application to the brains and then 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, 60 min after application. The intracellular calcium level over time was determined as F340/F380 relative to the first measurement (0 min). The MAD describes the degree of variability of the Ca^{2+} level within one group of bees at a given time point. A time-dependent increase in MAD was observed in the L-BMAA-treated bees (Figure 25B). The MAD in L-BMAA-exposed bees was significantly different from the MAD recorded for controls.

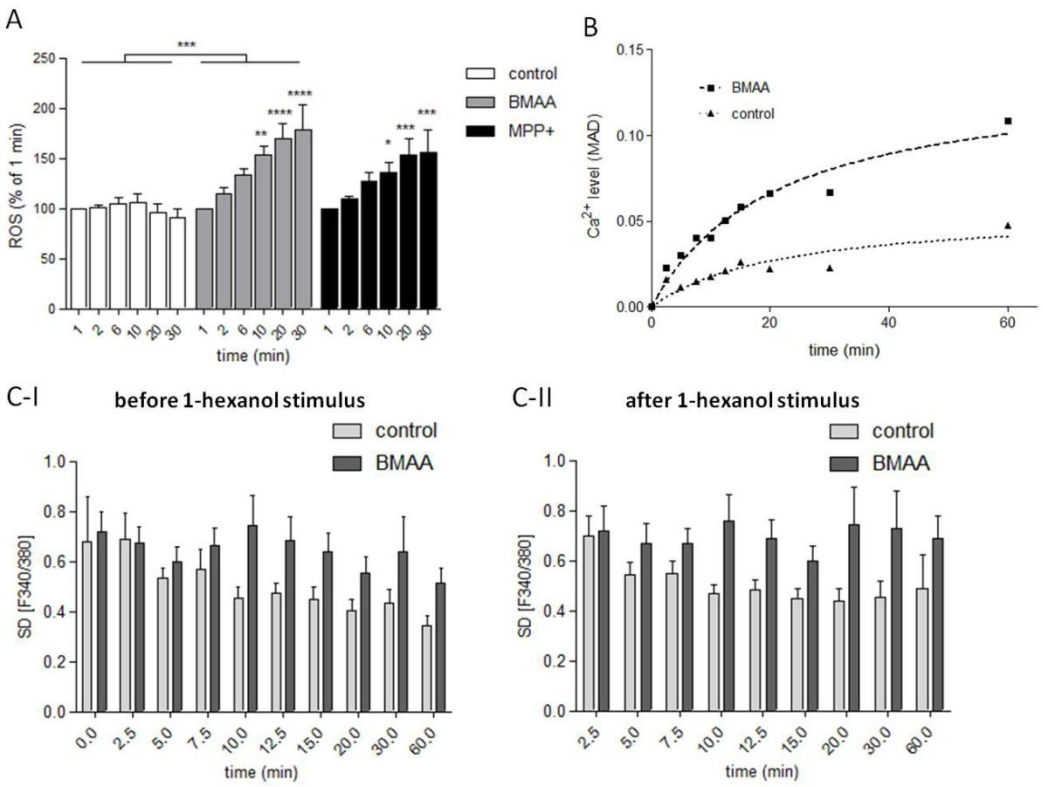

Figure 25: ROS level and Ca^{2+} homeostasis following L-BMAA exposure. **A**: ROS level: L-BMAA caused ROS development in the brain. 1.25 mM L-BMAA (n=5) induced an increase of 80%, MPP^{+} (positive control) with 300 µM 60%. Control levels remained constant; the effect is significant (P<0.001) (Two-way ANOVA with Bonferroni multiple comparisons) **B**: Ca^{2+} homeostasis in the bee brain. The median absolute deviation (MAD) of the variance in the Ca^{2+} level of L-BMAA bees (n=16) increased compared to the control bees (n=10) **C**: Increased spontaneous activity following a single L-BMAA or saline treatment over time (min). The spontaneous activity, defined as the standard deviation of the Ca^{2+} level was significantly higher (Two-way ANOVA) in acute L-BMAA (grey) exposed bee brains (n=16) than in the control bees (white) (n=10) before (P=0.0006) (**C-I**) and after (P≤0.0001) (**C-II**) the odor stimulus; mean ±SEM.

5.4.6 L-BMAA increases Spontaneous Brain Activity

Bee brain projection neurons are constantly active even in the absence of odor stimuli (1-hexanol), with irregular fluctuations of calcium concentration (Galan *et al.* 2006). With each odor-response measurement 8 sec of spontaneous activity before and 16 sec after the 1-hexanol-stimulus was recorded (example trace, Figure 31A). The spontaneous activity before (Figure 25C-I) and after (Figure 25C-I) the 1-hexanol-stimulus was greater in L-BMAA-exposed bees when compared to controls. In contrast L-BMAA exposure did not increase spontaneous activity in antennal lobe projection neurons during the 1-hexanol-stimulus (P=0.3315; Figure 31B).

5.4.7 Bees transfer L-BMAA via Trophallaxis

To test whether L-BMAA can be transferred between bees, two groups of bees from the same hive were placed in a single container separated by a net that would allow them to exchange food via trophallaxis. One group had unrestricted access to ^{14}C-L-BMAA-spiked sugar water (fed-group), while the other had no access to food (starvation-group). After 48h exposure, the highest ^{14}C-L-BMAA activity was found in the abdomen of the bees in both groups, with lower activities in the thorax and head. The latter proved that the starvation-group was fed with ^{14}C-L-BMAA by bees of the fed-group. The ^{14}C-L-BMAA activities in abdomen, thorax and head in the fed-group were generally higher than those recorded in the starvation-group (Figure 24B). As the bee abdomen contains the social stomach where bees store nectar for honey production and for trophallaxis, it is not surprising that the abdomen contained the highest activity of ^{14}C-L-BMAA.

5.5 Discussion

Since the public became aware of the neurotoxin BMAA in the 1960s, when there was an increased incidence of neurodegenerative symptoms of ALS/PDC on Guam, various putative BMAA intoxications and exposure scenarios have been reported for humans (Caller *et al.* 2009; Cox *et al.* 2009; Pablo *et al.* 2009; Spencer *et al.* 1987b). To reveal the biological and toxicological mechanisms as well as for better risk assessment, BMAA was tested in various animal models including monkeys, mouse, rat and *Drosophila* (Karamyan and Speth 2008). Several experimental paradigms were tested, including survival curves, behavioral and pathological observations, but the mechanism of action of BMAA remains elusive and the pathway of human intoxication is as unknown. The most important caveat in the existing classical toxicological assessment of BMAA is that very few behavioral and chronic exposure studies exist (Karlsson *et al.* 2009b; Spencer *et al.* 1987a; Zhou *et al.* 2010) that would allow extrapolation of the data for human risk assessment purposes. Indeed, BMAA acts as a “slow toxin”, as reviewed by Kisby and Spencer (2011), whereby the long-term exposure, metabolism and excretion appears to predominate the adverse reactions observed. In fact, prolonged BMAA exposure of bees (up to 300h) leads to a higher mortality probability (Figure 23A), suggestive of an accumulation of adverse events within the bees that finally lead to mortality. The latter findings are also corroborated by similar observations by Zhou *et al.* (2010) who reported reduced lifespan in *Drosophila* following dietary intake of BMAA. As we also found that learning capacity and odor processing were affected by chronic BMAA exposure (Figure 23B), the question was raised whether BMAA is taken into the brain of bees and thus elicits the adverse reactions and ultimately the increased mortality observed. In our experiments with ^{14}C-BMAA, we demonstrated that ^{14}C-BMAA (Figure 24A) remains present in the bees’ bodies for at least 3 days following a single BMAA feeding

bout. Moreover, the overall detectable activity of ^{14}C-BMAA did not decline significantly over time (Figure 28), albeit the observed redistribution of ^{14}C-BMAA between head, thorax and abdomen between 48 and 72h (Figure 24A) appeared to change. The latter data suggested that BMAA is bioavailable and redistributed amongst the bees compartments. That BMAA reaches the brain proper is supported by the behavioral data (Figure 23B) in conjunction with the functional data presented in Figure 25.

The observation that BMAA is distributed amongst bees via trophollaxis (Figure 24B) not only suggests that BMAA exposure can place a whole bee population at risk, but more importantly that given an environmentally relevant BMAA exposure is present (e.g. BMAA contaminated water from an algal bloom), BMAA could accumulate within bees with the concomitant potential of behavioral changes within the whole bee population. Moreover, although this still has to be conclusively demonstrated, BMAA could be transferred to honey and thus be available for human consumption.

The question, however, remains as to how BMAA induces the potential neurotoxicity *viz* learning impairment and finally mortality observed. Two major theories that are currently being discussed by various authors are "excitotoxicity" and the impairment of protein homeostasis due to "tight protein-binding" or a misincorporation into proteins (Banack *et al.* 2010; Field *et al.* 2011; Okle *et al.* 2013b), followed by ER-stress and inappropriate production of ROS. Indeed, impaired protein homeostasis with the concomitant downstream biochemical changes was observed at concentrations lower than those required for overt excitotoxicity in human neuronal cells (Banack *et al.* 2010; Field *et al.* 2011; Okle *et al.* 2013b). The latter suggests that these adverse effects may occur under a chronic accumulation scenario, whereas an acute high concentration BMAA exposure would result in excitotoxicity. Similar to our earlier findings, the application of 625µM or 1.25mM BMAA to the bee brain for 30min induced a significant and concentration dependent increase of ROS (40% or 80%, respectively) (Figure 25A, Figure 29) as well as an increased antennal lobe activity, as demonstrated by a higher Ca^{2+} influx (Figure 25B). While the former is testimony of ER-stress, the latter could result from BMAA excitotoxicity (Malhotra and Kaufman 2007b). An impaired Ca^{2+} homeostasis will affect most signaling processes in the olfactory area of the brain. Consequently, BMAA had an effect on spontaneous activity (Figure 25C). Interestingly, odor-responses were not affected in the antennal lobe (Figure 30). Therefore, additional experiments in higher brain areas (mushroom bodies) and for longer BMAA treatments are necessary in order to test whether sensory coding is affected, or whether BMAA is selectively influencing memory formation (Figure 23B).

Increased ROS was reported at high BMAA concentrations in mouse cortical cell cultures, in "whole brain cells" of neonatal rats (Brownson *et al.* 2002) as well as in human primary neuronal cells (Chiu *et al.* 2012). Moreover, treatment of human neuroblastoma cells with

high but non-excitotoxic concentrations of BMAA led to an early onset of ROS production resulting in the presence of oxidized proteins 48 h after the initial exposure (Okle *et al.* 2013b). We found BMAA-induced neurotoxicity in bees *in vivo* at concentrations lower and equal to those reported to produce adverse effects in human primary neuronal (Chiu *et al.* 2012) and neuroblastoma cells (Okle *et al.* 2013b). Thus our study presented here, is the first ever to demonstrate BMAA-induced ER-stress and neurotoxicity in an *in vivo* brain under real-time conditions. The absence of behavioral effects at 24 h of exposure despite the generation of ROS in the bee brains (Figure 24 and Figure 25) is not unexpected as demonstrable behavioral effects are assumed to occur only when appreciable levels of oxidized proteins have accumulated, thus effectively hampering normal brain function. Indeed, previous studies (Karlsson *et al.* 2012; Murch *et al.* 2004a; Okle *et al.* 2013b) suggest that BMAA binds to proteins thereby limiting protein functionality and consequently leading to protein dysfunction. This process is rather slow and thus testimony of inhibited protein homeostasis would be expected at a time-point when dysfunctional protein accumulation reaches a critical level. Above findings thus raise the question whether bees could serve as a model organism to study neurotoxic compounds in general and environmental exposure-associated neurodegenerative diseases e.g. ALS/PDC in particular. Indeed the often cited problem associated with contradictory findings on BMAA toxicity (reviewed by Chiu *et al.* (2011)) and atypical behavior in surrogate species (Cruz-Aguado *et al.* 2006; Karlsson *et al.* 2009a; Karlsson *et al.* 2009b; Spencer *et al.* 1987a; Zhou *et al.* 2010) underpins the need for an appropriate model for mechanistic investigations in a potential human risk assessment. As multiple mechanisms of action of BMAA are currently discussed (Chiu *et al.* 2011; Lobner *et al.* 2007; Murch *et al.* 2004a), yet a comprehensive picture explaining how BMAA would lead to neurodegeneration and ALS/PDC symptoms in humans is not yet available, bee studies could provide additional insight and endpoints critical for the hazard assessment of BMMA exposure in humans. Indeed, the bee *in vivo* system would allow understanding BMMA-linked impairment of neuronal integrity and thus inter- and intra-neuronal BMAA-induced changes including the characterization of the associated biochemical changes within the bees' brains.

The ubiquitous appearance of BMAA-producing cyanobacteria (Cox *et al.* 2005; Cox *et al.* 2009; Jonasson *et al.* 2010) and the rising number of reports associating neuro-degenerative symptoms, e.g. sporadic ALS, with potential chronic exposure to BMAA (Caller *et al.* 2009; Cox *et al.* 2009; Gunnarsson *et al.* 1996; Murch *et al.* 2004b; Pablo *et al.* 2009) emphasize the need for *in vivo* systems that allow mechanistic elucidation of potential neurological adverse effects of BMAA. With the bee system described here we have demonstrated the advantages of this *in vivo* animal model for better risk assessment of the cyanobacterial neurotoxin BMAA and its role in the etiology of neurodegenerative diseases in humans.

5.6 Conclusion

The ubiquitous appearance of L-BMAA-producing cyanobacteria (Cox *et al.* 2005; Cox *et al.* 2009; Jonasson *et al.* 2010) and the rising number of reports associating neuro-degenerative symptoms, e.g. sporadic ALS, with potential chronic exposure to L-BMAA (Caller *et al.* 2009; Cox *et al.* 2009; Gunnarsson *et al.* 1996; Murch *et al.* 2004b; Pablo *et al.* 2009), emphasize the need for *in vivo* systems that allow mechanistic elucidation of potential neurological adverse effects of L-BMAA. With the bee system described here we have demonstrated the advantages of this *in vivo* animal model for better risk assessment of the cyanobacterial neurotoxin L-BMAA and its role in the etiology of neurodegenerative diseases in humans.

5.7 Acknowledgements

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5.8 Supplementary Data

5.8.1 Supplemental Figures

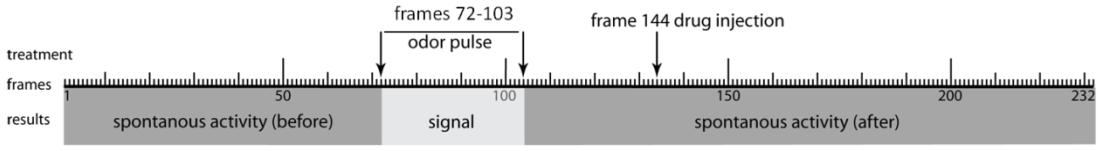

Figure 26: Time course of Ca^{2+} measurements. During frames 0 to 71 the spontaneous activity was recorded (before). From frames 72 to 103 an odor pulse was given and the signal was recorded (signal). From frames 104 to 232 again spontaneous activity is recorded (after). During the first measurement, at approximately frame 144, the compound (saline or L-BMAA) was added to the brain.

Formula 1: Median absolute deviation (MAD) is defined as:

$$MAD = median_i\left(\left|X_i - median_j\left(X_j\right)\right|\right)$$

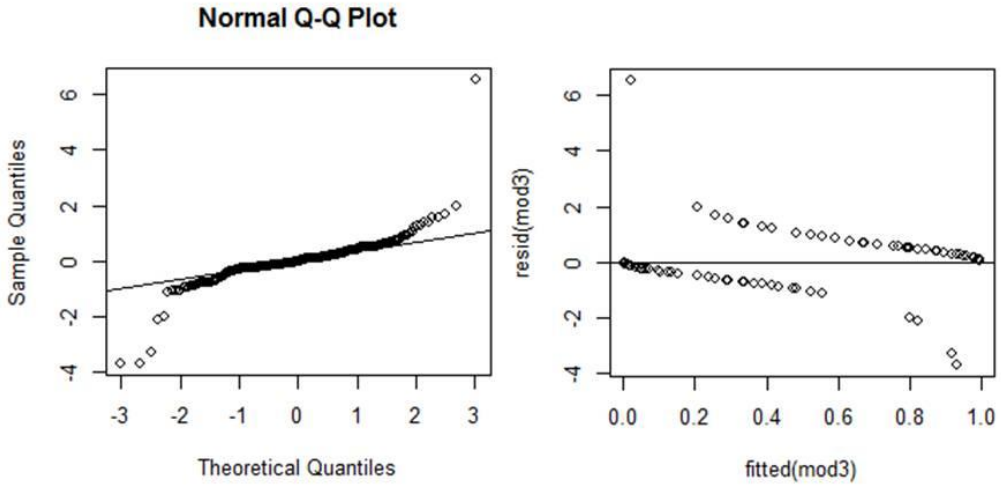

Figure 27: Plots for assessing how well the model fits the observed data.

Table 2: Parameter estimates of the final model for the mortality rate with deviance, likelihood, AIC and BIC.

AIC					
	BIC	logLik	deviance		
234.4	254.5	-109.2	218.4		
Random effects					
Groups	Name	Variance	Std.Dev.		
obsid	(Intercept)	3.9034e-01	6.2477e-01		
Cohort	(Intercept)	0.0000e+00	0.0000e+00		
Date	(Intercept)	1.9745e-21	4.4435e-11		
Number of obs: 78, groups: obsid, 78; cohort, 6; Datum, 3					
Fixed effects:					
	Estimate	Std. Error	z-value	Pr(>|z|)	
Fixed effects:	-1.5240	0.1129	-13.494	< 2e-16	***
Treatment L-BMAA	0.6967	0.1805	3.860	0.000113	***
Time.z	0.6304	0.1054	5.979	2.24e-09	***

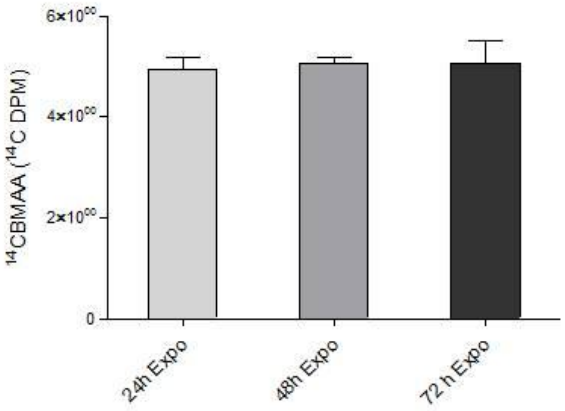

Figure 28: Accumulation of ^{14}C-L-BMAA. The total activity of ^{14}C-L-BMAA remains constant over 72 h; One-way ANOVA with Bonferroni multiple comparison Test; mean ±SEM.

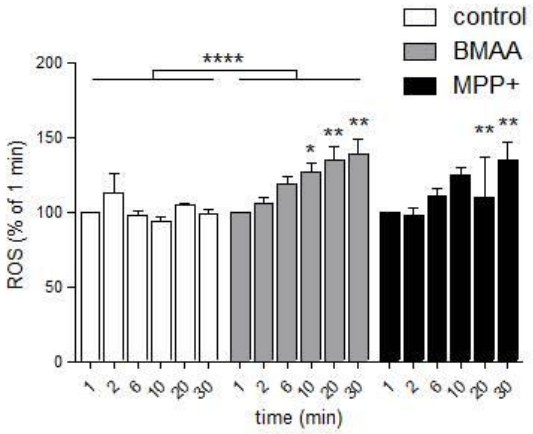

Figure 29: L-BMAA (625 µM) induced increase of ROS (40%) 30 min after application (n=6). (A), MPP$^+$ (positive control) with 150µM; Two-Way ANOVA, mean ±SEM.

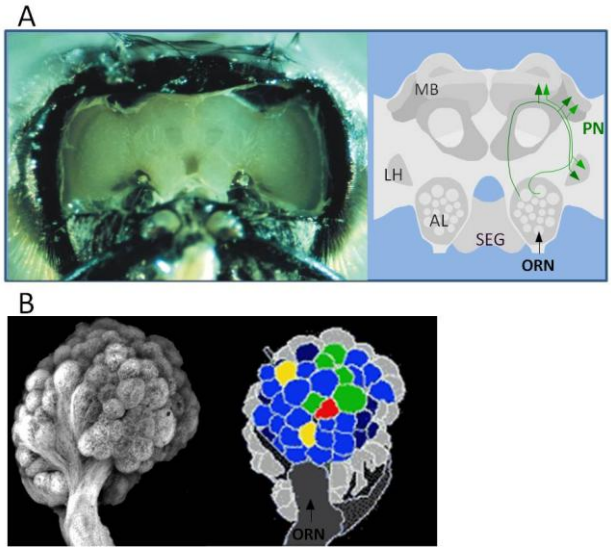

Figure 30: Bee central olfactory system. A: (left) view of the bee brain in the head capsule. A window was cut into the cuticle to expose the brain (yellowish). Note the antennae joints at the bottom. (right) schematic frontal view of the bee central olfactory system. Olfactory receptor neurons (ORN, black arrow) terminate in the antennal lobe (AL) glomeruli. Uniglomerular projection neurons (PN, green) transmit odor information from the AL to the mushroom body (MB) and to the lateral horn (LH). B: (left) confocal extended focus view of all receptor neurons entering the antennal lobe, (right) scematic view of an antennal lobe with glomeruli, and color coded odor response to the odor 1-hexanol (source: http://neuro.uni-konstanz.de).

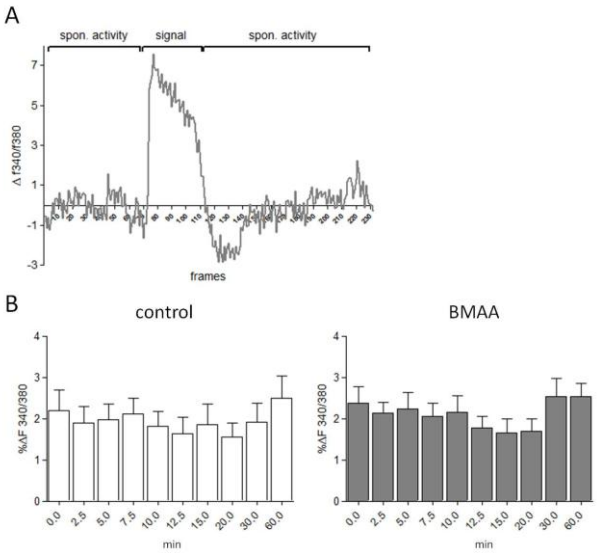

Figure 31: A: Example traces of Ca^{2+} recording with 8sec of spontaneous activity (frame 0-71), 4 sec of signal/odor response (frame 72-103) and another 16sec of spontaneous activity (frame 104-232), B: Signal strength in L-BMAA treated and control bees. The relative Ca^{2+} levels during a 4 sec odor stimulus over time (min) after treatment (application of saline or L-BMAA) was not significant (P= 0.3315; Two-Way ANOVA).

Chapter 6: Overall Discussion

6.1 BMAA – a putative Inducer of ALS/PDC on Guam and beyond?

Combined symptoms of the neurodegenerative diseases ALS and Parkinson's disease, sometimes combined with dementia, were described for the first time on the island Guam. Ever since its first occurrence on Guam, the definition of the diseases was complex as symptoms of the various neurodegenerative diseases occurred parallel and differed in intensity (Table 1) (Hirano *et al.* 1966; Kurland 1988). The combination of the sometimes sequentially developed features of ALS and Parkinson's disease was however the reason to summarize and define these symptoms as an individual disease – ALS/PDC. The definition of the disease was facilitated on Guam by the fact that the overall incidence of neurodegenerative disorders was 50- to 100-fold higher among the native Chamorro of Guam than elsewhere (Kurland and Mulder 1954) and that patients with neurodegenerative disorders on Guam were much younger than the average of patients with similar disorders elsewhere (Guam ALS/PDC: mean age 44 years (Kurland and Mulder 1954); ALS: 59 years; AD: 62 years; PD: 72 years (Eisen and Calne 1992)) as reviewed by Shaw and Wilson (2003). If a clustering of neurodegenerative diseases or the occurrence of neurodegenerative symptoms at a very young age was not given, the diagnosis of ALS/PDC would be quite difficult because the diagnosis of the diverse neurodegenerative diseases by themselves is complex as the symptoms can vary and disease pattern are often not sharply defined and thus do not distinctly border each other. It is therefore probable that if symptoms of different diseases co-occur in one disease, like in the case of ALS/PDC, sometimes false negative or false positive diagnoses can be concluded based on temporarily lacking symptoms and disorders or on the overlapping of symptoms, especially since neither definitive hypotheses nor finally proven causal factors are given for the age-related neurodegenerative diseases AD, PD, ALS and ALS/PDC (Shaw and Wilson 2003).

For the neurodegenerative disease ALS/PDC on Guam a putative causative factor has been discussed in literature, namely the ingestion of the cyanobacterially-produced neurotoxin BMAA which was probably concentrated in the traditional food or medicine of the native Chamorro people (Steele and Guzman 1987) or has become biomagnificated in flying foxes (Cox *et al.* 2003) which were also a food source of the Chamorro. However, the toxic potential of BMAA and its involvement in the induction and the progress of the neurodegenerative disease ALS/PDC are still controversially discussed (Cruz-Aguado *et al.* 2006; Ince and Codd 2005) as until now the symptoms of ALS/PDC have not been

completely reproducible in animal experiments. Only one Cynomolgus monkey study (Spencer *et al.* 1987b) reproduced neurodegenerative patterns following BMAA treatment, but the high BMAA doses used are still the subject of criticism (Duncan *et al.* 1990; Steele and McGeer 2008). However, as pointed out by Karamyan and Speth (2008), the lack of an adequate animal model is a general problem for the experimental investigation of chronic delayed neurotoxicity and progressive neurodegenerative diseases.

Closer examinations of look at epidemiological studies on neurodegenerative diseases and especially concerning symptoms of ALS or ALS/PDC, it is noticeable that the number of scientific reports dealing with the appearance of uncommon neurodegenerative diseases raises. Within the last decade, for instance, reports on a six-fold increased incidence of ALS in an Italian soccer team compared to the rest of Italy (Chio *et al.* 2008), a two-fold increased incidence of ALS symptoms in U.S. veteran soldiers deployed in various wars, for example, the Iraq War (Johnson *et al.* 2006), and a cluster of ALS patients in Enfield, New Hampshire, located in the neighborhood of New Hampshire Lake and Lake Mascoma (Caller *et al.* 2009), have been published. Some of the listed ALS clusters have already been discussed to be linked to an exposure to the cyanobacterial toxin BMAA, e.g. the soldiers who breathed cyanobacterial dust (Cox *et al.* 2009) and the citizens of Enfield by contact with water contaminated by algal blooms (Caller *et al.* 2009). However, this short summary also highlights, beside the increased occurrence of ALS, that cases of non-endemic ALS are often described when they occur in clusters. This raises the question whether there are also undescribed or unrecognized non-endemic ALS cases which were maybe caused by non-genetic predispositions as, for example, by an environmental factor like the neurotoxin BMAA. Indeed, the neurotoxin BMAA has also been found outside the island Guam in the last century, whereby the chance for a direct exposure for humans by ingestion was often conceivable as accumulated BMAA has been found in shrimps, crabs, fishes in ponds and rivers in the U.S. (Brand *et al.* 2010) as well as in fish and shellfish in the Baltic Sea (Jonasson *et al.* 2010), and thus in major food webs. Further, an exposure to BMAA outside of Guam seems to be a realistic scenario as BMAA was detected in brains of Canadian AD patients and American ALS and AD patients (Banack *et al.* 2007; Murch *et al.* 2004b; Pablo *et al.* 2009). Thus, an uptake of BMAA in humans seems possible and a correlation of the diagnosed neurodegenerative diseases (AD, ALS, PD) and the occurrence of BMAA in the Canadian and U.S. patients is thinkable, although not all symptoms of ALS/PDC observed in the patients on Guam were distinct in the Canadian and U.S. patients, which might have led to the classification AD, ALS and PD and not ALS/PDC. In summary, a risk of coming into contact with BMAA is given and this contact could possibly result in the development of neurodegenerative disorders.

To estimate the possible risk for humans it is important, on the one hand, to have knowledge and information concerning possible exposure scenarios and therefore effective

concentrations to which humans can be chronically or acutely exposed and on the other hand, to know the toxic mechanism of BMAA (discussed in the next paragraph).

The studies of Banack *et al.* (2007) and Cox *et al.* (2005) indicated that most cyanobacteria can produce the neurotoxin BMAA. Although this hypothesis needs further research and has to be assessed critically, it is relevant for a possible chronic or acute BMAA exposure as cyanobacteria are abundant worldwide in marine, brackish and freshwaters, including rivers, lakes, ponds and drinking water reservoirs, and are known to produce toxic metabolites there (summarized by Hoeger *et al.* (2005) and by O'Neil *et al.* (2012)). Thus, the risk for a direct contact with cyanobacteria and their toxin BMAA could occur worldwide and is not limited to Guam or nearby islands. Additionally, the observations of increasing mass occurrences of cyanobacteria may be based on global warming linked to the climate change (Paerl and Huisman 2009; Paerl and Huisman 2008; Paul 2008) and a worldwide increasing eutrophication of water bodies (O'Neil *et al.* 2012) supports the assumption that BMAA-producing cyanobacteria probably represent a growing risk for human health analog to the increased incidence with other cyanobacterial poisonings like microcystins, cylindrospermopsins, anatoxins and saxitoxins (Carmichael 2008).

6.2 Can ALS/PDC be caused by an Uptake of the cyanobacterial Toxin BMAA?

The direct contact of humans with the cyanobacterial toxin BMAA is conceivable worldwide- as discussed above. This raises two questions: which concentration of BMAA leads to neurotoxicity, and did chronic or acute exposure induce neurodegenerative disorders. Both questions are closely related to the toxic mechanisms of BMAA.

Up to now various mechanisms were discussed, partially with an experimental background and partially only theoretically. The receptor-mediated toxicity of BMAA, hence the binding to different glutamate receptors and a possible resulting excitotoxicity, can be interpreted as an acute toxic mechanism. Following a further hypothesis of Murch *et al.* (2004a), an accumulation of BMAA can occur if BMAA becomes incorporated into or attached to proteins hypothetically resulting in a delayed toxicity if protein-bound or incorporated BMAA is released with a delay and thus occurs as "free" BMAA. Then, the released "free" BMAA can hypothetically again act as an excitotoxin. A binding of BMAA to proteins was also postulated by Cox *et al.* (2003) to explain a biomagnification of the neurotoxin BMAA in the food chain of the ALS/PDC diseased Chamorro and thus toxicity by high BMAA concentrations observed in various experimental findings (reviewed by Chiu *et al.* (2011)). However, biomagnification as well as protein-binding theories are still controversially discussed as not enough experimental data are available supporting these theories.

The findings of this thesis can contribute to the elucidation of various disputable aspects and criticisms of the above mentioned hypothesis concerning the involvement of BMAA in the generation of ALS/PDC even though an explicit neurotoxic concentration for humans was not calculable based on the data of this thesis.

First of all, the occurrence of intracellular BMAA (Figure 10C and D) within a human neuronal cell system as well as the experiments with an *in vitro* translation system (Figure 12F) demonstrated that intracellular BMAA is able to accumulate in the cytosol and possesses a strong association to proteins. This association can also take place in humans as BMAA can cross the blood-brain barrier possibly facilitated by amino acid transporters like the large neutral amino acid transporter (Figure 10C-D) as already demonstrated in rats (Duncan *et al.* 1991). Indeed BMAA was detected in brains of human ALS/PDC patients *post mortem* (reviewed by Pablo *et al.* (2009)) which indicates that BMAA can become bioavailable in human brain tissue.

The intracellular BMAA raises the possibility of inducing a toxic mechanism in the cytoplasmic environment besides the already discussed excitotoxicity mediated by extracellular BMAA. As the unfolded protein response from the ER (Figure 12D) and even the ER-stress-induced apoptotic pathway can be initiated by BMAA (Figure 12E), it is probable that prolonged BMAA exposure raises the amount of misfolded or unfolded proteins as BMAA could become incorporated into proteins causing an abnormal tertiary folding structure of them. Further, an attachment or binding of BMAA to enzymes and proteins engaged in protein folding and, as a consequence, the affecting of them is also plausible. The increased proteasomal activity as well as the increased ubiquitination (Figure 12A-C) indicates that the *in vitro* cell system has become overloaded with misfolded, unfolded or aggregated proteins. The UPR as well as induced downstream mechanisms obviously cannot manage the emerging amounts of those proteins by initiated refolding processes in the ER and thus they are processed to become degraded. Whether further export and degradation processes like the autophagy-lysosome pathway, which also plays a major role in various neurodegenerative diseases (reviewed by Wong and Cuervo (2010)) like Parkinson's disease (Pan *et al.* 2008), can additionally become directly or indirectly initiated by intracellular BMAA needs further research. However, the raised ER-stress and the induction of the UPS as well as apoptotic mechanisms (Figure 12A-E) clearly indicate that BMAA can induce long-term neurotoxic effects following a single non-cytotoxic treatment as all of the above mentioned markers are common in various neurodegenerative diseases and the neurodegenerations occurring in these diseases are not mainly based on excitotoxic mechanisms. Additionally a non-excitotoxic acting of BMAA was supported by the fact that an acute excitotoxic effect of BMAA could be excluded as on the one hand no cytotoxicity was observable (Figure 11B), as confirmed by control experiments with NMDA and glutamic acid, both positive controls for NDMA and mGlu receptor-mediated excitotoxicity (Figure 14), and on the other hand as active NMDA

receptors are absent in the SH-SY5Y cells used (Jantas *et al.* 2008). Thus it can be assumed that already low, non-excitotoxic concentrations of BMAA could possibly cause neurodegeneration resulting in symptoms of ALS/PDC. Further it can be hypothesized that by the above discussed mechanisms already a single application of BMAA could affect the development of neurodegenerative lesions and that a chronic contact with low levels of BMAA by contaminated water or food could amplify these mechanisms. Therefore, the hypothesis of a biomagnification of BMAA on Guam (Cox *et al.* 2003) is not essential for explaining the incidence of ALS/PDC on Guam as also a chronic low concentration exposure to BMAA could induce neurodegeneration by an involvement of UPR, UPS and/or ER-stress.

The new finding of an induced ER-stress in response to BMAA exposure contributes to the elucidation of the neurotoxic mechanism/s behind the toxicity of BMAA. However, as ER-stress can also be initiated in non-neuronal cells (Figure 16), further research also has to focus on non-neuronal cells which could be affected and possibly impaired like, for example, neuronal-associated cells which are responsible for the neuronal integrity.

The observation of an increased phosphorylation of tau protein in SH-SY5Y cells (Figure 19) is a further indication that BMAA can act as a neurotoxin. The increased phosphorylation (hyperphosphorylation) of tau at specific sites can result in subsequent aggregation of the tau protein by forming NFT. NFT as well as hyperphosphorylation of tau are initial markers for neurodegeneration (Braak *et al.* 1994). Consequently the aggregation of tau and the formation of NFT can also be found in ALS/PDC patients and is a widespread prominent pathological hallmark for this neurodegenerative disease (McGeer and Steele 2011). The detection of the hyperphosphorylated tau proteins following BMAA treatment of SH-SY5Y cells thus satisfies the postulation of Steele and McGeer (2008) who proposed that factors which could be responsible for causing ALS/PDC, e.g. BMAA, should be able to induce and thus reproduce pathological markers, which are observable in ALS/PDC patients, in an experimental system. Thereby they explicitly mentioned the reproduction of tauopathy in experimental systems. Thus, the induction of a hyperphosphorylation of tau protein by BMAA supports the hypothesis of the highly neurotoxic potential of BMAA.

A phosphorylation of tau molecules can be catalyzed by the glycogen synthase kinase 3 (GSK3) (reviewed by Avila *et al.* (2012)) which is also abundant in the central nervous system (Frame and Cohen 2001). The activity of GSK3 is strongly associated to protein phosphatase 2B (calcineurin) which was affected following BMAA exposure (Figure 20). However, it is remarkable that the activity of calcineurin was not reduced but increased following contact with BMAA. The direct effect of the increased calcineurin activity, the activation of GSK3β by a dephosphorylation of the inhibitory phosphorylation at residue Ser9, was consequently also observable in the SH-SY5Y *in vitro* model (Figure 21). This activation of GSK3β resulting in an increased phosphorylation of tau protein (Figure 19) was manifested by experimental manipulations of calcineurin and GSK3β with specific inhibitors

and by co-treatment of them with BMAA (Figure 22). Thus the impairment of GSK3β by BMAA is a crucial factor for the BMAA-induced tau phosphorylation.
In addition to the phosphorylation of tau, the enzyme GSK3β also regulates the neural development by controlling key steps in neuro-developmental processes and consequently processes for microtubule dynamics as well as for proteasomal targeting and degradation (reviewed by Hur and Zhou (2010)). Consequently a change in the activity of GSK3β has been associated with many neurodegenerative and psychiatric diseases, such as AD, PD, schizophrenia, and autism spectrum disorders (reviewed by Lei *et al.* (2011) and by Lovestone *et al.* (2007)). Moreover, a close association of GSK3β to the ER was also reported. Thereby GSK3 plays a central role in the signaling of downstream effects in consequence of an ER-stress situation (Chen *et al.* 2004). In the pro-apoptotic signaling cascades for example, the expression of the death-inducing transcription factor C/EBP homologous protein (CHOP) can effectively become reduced by the inhibition of GSK3 (Meares *et al.* 2011). Hence, an over-activation of GSK3β, as it was observable in SH-SY5Y cells by the BMAA-induced increased calcineurin activation (Figure 20 and Figure 21), could possibly directly influence or induce apoptotic pathways. Thus, apoptosis in response to BMAA exposure could not only be induced by a protein misfolding and a prolonged UPR, which can result in ER-stress (as discussed above), but additionally also by a second pathway: an over-activation of GSK3. However, this hypothesis needs further data for validation.

The findings discussed above clearly indicate that BMAA can impair neurons as well as their development. However, as already mentioned by Chiu *et al.* (2011), the toxicity of BMAA can possibly be mediated by several mechanisms which need yet to be identified. Already the extracellular and intracellular BMAA raises the possibility of parallel occurring toxic mechanisms as discussed above. Whether these findings of a BMAA-induced tau hyperphosphorylation, protein oxidation, ER-stress and proteasomal degradation are autonomic toxic mechanisms or whether they can influence and regulate each other cannot be finally analyzed as general mechanisms and initial causes for an ER-stress situation, hypherphosphorylation of tau proteins and ROS generation themselves are not yet completely understood. Also it is still not completely understood whether and how ER-stress acts up- or downstream of a tau hyperphosphorylation and whether ER-stress can induce ROS or results from oxidative stress (Hoozemans and Scheper 2012; Malhotra and Kaufman 2007b).

Even though the presented experiments using the human neuroblastoma cell line SH-SY5Y explicitly demonstrate that BMAA can act as a neurotoxin, the lack of an adequate animal model (Karamyan and Speth 2008; Shaw and Wilson 2003) restricts these experimental *in vitro* findings as no toxic mechanism has been demonstrated *in vivo* for BMAA yet. Thus, further experiments to demonstrate that the observable toxic mechanisms *in vitro* can also lead to neuronal disorders and the disease pattern of ALS/PDC *in vivo* are needed. Also a

biomagnification, as described by Cox *et al.* (2003), is not necessary to explain the long-latency period of BMAA on Guam. The data of this thesis rather partially support the hypothesis of Murch *et al.* (2004a) as an incorporation into or an attachment of BMAA to proteins was indirectly demonstrated. A delayed release of protein-bound or protein-associated BMAA, as hypothesized by Murch et al. (2004a), was not investigated in this thesis and thus cannot be evaluated by the presented data. However, the prolonged UPR resulting in an ER-stress situation and the downstream initiation of an ER-stress-linked apoptotic pathway as well as the formation of hyperphosphorylated tau proteins and of oxidized proteins are distinctive in various neurodegenerative diseases like ALS, AD and PD. As the above described initiated pathways were affected following a single, non-acute toxic exposure with a modal BMAA concentration, a single exposure event or a chronic exposure with very low concentrations of BMAA could possibly be sufficient for the initiation of the slow and progressive process of neurodegeneration and the resulting neurological disorder and symptoms of ALS/PDC.

6.3 The Honeybee – not only a suitable Model for ALS/PDC?

The lack of a suitable animal model for the discovery of neuronal disorders and diseases is not only an issue for the reproduction of ALS/PDC symptoms following BMAA exposure (discussed above) but rather a general issue as neurotoxicity mostly appears delayed and chronic exposure scenarios as well as putative stressors and factors inducing a neurodegeneration are hard to isolate and only partially understood (Bradley and Mash 2009; Karamyan and Speth 2008). In the case of the lacking animal model for the ALS/PDC symptoms a further problem is that animal experiments carried out so far had no "definitive hypothesis for the mechanism(s) of neurotoxicity" of BMAA (Karamyan and Speth 2008). Additionally, most of the animal models showed no BMAA-induced irreversible progressive neurodegeneration and none of the BMAA-induced observed effects were clear reproductions of the behavioral and neurodegenerative alterations observed in human ALS/PDC patients (Bradley and Mash 2009; Karamyan and Speth 2008).

Typical features of ALS, PD, AD as well as ALS/PDC are, above others, an increase of ROS and resulting oxidative stress, an impaired Ca^{2+} homeostasis as well as deficits in cognitive behavior and the olfactory system (Ahlskog *et al.* 1998; Christen-Zaech *et al.* 2003; Tissingh *et al.* 2001). A determination of all features was practicable neither in rodent and monkey model nor in further models like *Drosophila*. Besides the already investigated animal models for the reproduction of ALS/PDC features, the bee model has never been proven as an adequate model for the neurodegenerative disease even though the bees' relatively simple nervous system allows manipulations and the analysis of the resulting consequences with regard to physiological and behavioral impairments which makes it an

important model species in neuroscience (Galizia *et al.* 2000; Galizia and Vetter 2004; Giurfa 2007; Matsumoto *et al.* 2012). Thus, in this thesis a new approach was developed in bees for the reproduction of marking features of ALS/PDC by measuring ROS generation and an impaired Ca^{2+} homeostasis directly in brains of living bees, following exposure to the neurodegenerative substance BMAA (Figure 25A-C). Beside the ROS development and an impaired Ca^{2+} homeostasis also an impaired olfactory processing (Figure 23B) and a deficit in cognitive capacities (Figure 23B) were demonstrated in bees following BMAA treatment. These findings clearly indicate, in addition to the increased mortality rate of BMAA-exposed bees (Figure 23A), that bees can become affected by BMAA. A focus of further research was set onto the trophallaxis of BMAA (Figure 24B) as bees are able to transport and spread the neurotoxin BMAA within a bee colony and maybe also to humans via honey since trophallaxis and honey production both take place in the honey stomach.

In summary, all the bio- and behavioral markers investigated in bees were affected by BMAA treatment. As the observed markers are common to various neurodegenerative diseases a closer look at these impairments and especially at the neuronal network and the neuronal integrity following BMAA exposure would be a promising approach to learn more about the toxic mechanism of BMAA. A genetic or pharmaceutical manipulation of defined endpoints (e.g. specific enzymes or cell structure proteins) in the bee and resulting consequences on the ROS generation, the Ca^{2+} homeostasis as well as on memory and behavior would promote a better understanding of toxic mechanisms leading to the impairment of the neuronal network and the neuronal integrity in bees. These findings could be used to design approaches to explain human neurodegenerative diseases like ALS/PDC as well as AD, PD or ALS concerning specific toxins, stressors or environmental conditions.

6.4 Overall Conclusion

Even though many questions concerning the molecule BMAA are still unanswered and need further hypothesis-oriented research, it can be concluded from the findings presented in this thesis that the molecule BMAA possesses toxic and, more specifically, neurotoxic characteristics. A putative excitotoxic action of BMAA by binding to receptors of neurons has already been supposed (reviewed by Chiu *et al.* (2011)). However, BMAA can also enter cells, most likely by means of transporters, as former studies demonstrated in rats (Smith *et al.* 1992) and as this thesis demonstrated in the human neuronal cell model (Figure 10C and D). Intracellular BMAA could be responsible for the induction of pathological processes by its attachment to or incorporation into proteins. This attachment or incorporation needs to be further elucidated in suitable models albeit initial supporting experimental findings were published by Kisby and Spencer (2011) and within this thesis (Figure 12F). An incorporation

of BMAA into proteins seems reasonable as the UPR and the resulting ER-stress-related apoptotic mechanism (Figure 12E) can be induced by BMAA in the human neuronal model system. The induced prolonged UPR, leading to an ER-stress situation, is on the one hand an indirect evidence for the incorporation into or the attachment of BMAA to proteins leading to the misfolding, and on the other hand, an approved marker for many neurodegenerative diseases like ALS, PD and AD. Indeed, various markers, which were also described for further neurodegenerative diseases (e.g. ALS, PD and AD), can be induced in the human neuronal model following BMAA exposure with non-acute, non-excitotoxic and non-cytotoxic concentrations. Besides an impairment of the protein phosphatase 2B (calcineurin) (Figure 20) and the resulting activation of GSK3β (Figure 21) which resulted in the hyperphosphorylation of tau proteins (Figure 19), an increased ubiquitination and proteasomal activity and a generation of ROS and oxidized proteins were demonstrated in the human neuronal model system following 48 hours BMAA exposure. Thereby BMAA initiated also the activation of an apoptotic pathway (Figure 11 D and Figure 12E). Concluding the observations of pathways which were initiated by BMAA, it can be reasoned that BMAA is a neurotoxin and also that the exposure with non-acute cytotoxic BMAA concentrations can lead to a neurodegeneration as general pathways and biomarkers which are involved in the pathology of known neurodegenerative diseases become initiated. Indeed, the occurrence of biomarkers and symptoms described for ALS, PD or AD were quite similar to those of the patients on Guam who may have been exposed to BMAA. Consequently this similarity and the combination of various ALS and PD symptoms of the native Chamorro led to the descriptive naming of the symptoms on Guam: ALS/PDC.

However, neither the mechanisms nor the biomarkers described in the neurodegenerative process of ALS, PD or AD have been completely understood and the mechanisms behind the neurotoxicity of BMAA are even less explored and proven. To discover pathological processes having regard to the progressive degeneration of neurons and the involvement of various factors, the use of animal models would be supporting. An advisable model could be the bee as the detection and analysis of pathological and neurobehavioral abnormalities described in human neurodegenerative processes were also observable in this model system. Thus, using the bee as a model organism for chronic, low-dose BMAA exposure scenarios could provide an insight into the progressive neurotoxic mechanisms of BMAA and the putative resulting impaired neuronal integrity.

Summarizing, the research of this thesis is a further step in the clarification of BMAA-mediated toxicity and proposes a new neurotoxic mechanism for a cyanobacterial produced toxin.

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Erklärung

Die vorliegende Arbeit wurde ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Weitere Personen, insbesondere Promotionsberater, waren an der inhaltlich materiellen Erstellung dieser nicht beteiligt. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Eigenabgrenzung / Kooperationen:

- Der MTT Assay mit CdCl (Kapitel 3 – Supplemental Material) wurde im Vertiefungskurs 2009 in der AG Humane und Umwelttoxikologie an der Universität Konstanz unter meiner Betreuung vorgenommen.
- Vorversuche zur Detektion und der Quantifizierung von ROS, von oxidierten Proteinen und der proteasomaler Aktivität (Kapitel 3) wurden im Vertiefungskurs 2010 sowie 2011 in der Arbeitsgruppe Humane und Umwelttoxikologie an der Universität Konstanz unter meiner Betreuung vorgenommen.
- Die in Kapitel 4 beschriebenen Experimente mit lebenden Bienen wurden in Kooperation mit Lisa Rath, Arbeitsgruppe Neurobiologie und Zoologie an der Universität Konstanz durchgeführt.

Alle weiteren Leistungen wurden, sofern nicht explizit angemerkt, von mir selbst durchgeführt.

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Oliver Okle

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