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The cyanobacterial neurotoxin beta-N-methylamino-L-alanine (BMAA) induces neuronal and behavioral changes in honeybees

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A B S T R A C T

The cyanobacterially produced neurotoxin beta N methylamino L alanine (BMAA) is thought to induce amyotrophic lateral sclerosis/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 ^{14}C BMAA we demonstrated that BMAA is biologically available to the bee, and 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 Ca^{2+} 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.

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).

Abbreviations: BMAA, β-N-methylamino-L-alanine; ROS, reactive oxygen species; PER, proboscis extension reflex; AL, antennal lobe.

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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. 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), the highest levels are found in the top predators (Esterhuizen Londt, 2010; Jonasson et al., 2010). Thus it is not surprising that BMAA was also found in shark

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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 endoplasmic reticulum (ER) stress and impaired protein homeostasis (Okle et al., 2013). 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 vitro* 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, *Caenorhabditis 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 they 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.

Material and methods

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

Mortality rate. Bees were caught from the entrance of their hives or feeders nearby and placed in meshed cages ("bugdorms", 30 × 30 × 30 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 with 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 m Osmol) or 9% bee saline with BMAA for a final BMAA concentration of 5 mM. To determine the rate of mortality, dead bees were counted and removed from the bugdorms every day.

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 s airflow to habituate, 4 s odor paired with sugar water (1.25 M) after 3 s creating a 1 s overlap of odor and reward. Sugar water was given for 3 s. The sugar water was delivered to the bees' antennae with a needle on a syringe filled with sugar water. Once a 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 s before removing them. 30 min after the last trial, the short term memory was tested by exposing the bee to the 1 hexanol stimulus and monitoring the PER.

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 stripe (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 in 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.

Staining, preparation and Ca^{2+} imaging. Brains were prepared and imaged as described (Galizia and Vetter, 2004; Szyszka et al., 2011). On the 1st day bees were caught from the entrance of the hive, immobilized, fixed in plexiglas holder, and projection neurons were stained by applying a dye (Fura 2 Dextran, Sigma Aldrich, Munich, Germany) via glass electrodes between the calyces of the mushroom bodies, aiming at the axonal tracts of the projection neurons going to the antennal lobes. Afterwards the head capsule was closed again. The next day the dye had traveled to the antennal lobes, the head capsule was reopened, 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 s). During the 1st recording at around frame 144 50 μl of either saline solution or BMAA in saline solution (2.5 mM) was added to the brain to a final concentration of approx.

625 μM BMAA. Spontaneous activity was measured before and after each odor stimulus (frames 1–104, Supplemental Fig. S1). A measurement lasted for 28 s. Measurements were taken before and 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, and 60 min after applying either BMAA or saline solution.

ROS detection in bee's brain. Bees were caught, fixed and their brain 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 30 min. Then the $DCFH_2$ DA was removed by rinsing the head capsule 4 times with 100 μl saline. The imaging setup was the same as for the Ca^{2+} imaging. The excitation light was 480 nm, the dichroitic mirror was 495 nm and the emission filter was 505 nm LP or 515 nm LP depending on the setup used. Each measurement lasted for 1 min and consisted of 6 bouts of 3 s length every 10 s, every bout at 8 Hz (24 frames each, 144 frames total). After the first measurement either 100 μl or 50 μl of 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, and 30 min subsequent to BMAA (or saline control) application.

Location of 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 (1.25 M) for 2 days for acclimatization. On day 3 they were given 15 μl of sugar water (1.25 M) with ^{14}C BMAA (60 μM) (BIOTREND, Cologne, Germany; 0.45 mM). The following days they were fed with sugar water (1.25 M) only again. Samples were taken 24 h, 48 h or 72 h after treatment and handled as described above. Observations longer than 3 days were not possible with this experimental setup as bees did not survive longer than day 3, most likely due to the stress of being fixed in a harness.

Intra species transfer. 20 bees were caught from the entrance of the hives and put in a box (13 × 6 × 5 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. On the 1st day the bees in the compartment with the cup were fed (fed group) with 3 ml of sugar water (1.25 M). In the following days the fed group was fed with 3 ml of ^{14}C BMAA (60 μM) in sugar water. After 48 h the sugar water with ^{14}C 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 2 M HCl) samples was determined in a LS6500 liquid scintillation counter (Beckmann, Munich, Germany).

Data analysis and statistics. For the analysis of the imaging data (Ca^{2+}, ROS measurements, Fig. 3) we used custom written programs in IDL (RSI, Boulder, CO, USA). For the processing and the analysis of the imaging data as to the signal strength and the spontaneous activity we proceeded 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 4–69). For the analysis of the Ca^{2+} level we calculated F340/F380 only and put the traces 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) (Supplemental Formula S2) was calculated. The MAD is a more "robust" parameter to analyze variability than squared error parameters such as standard deviation (Huber, 2004).

The statistical analysis of the mortality data (Fig. 1A) was done in R 2.15.0 (R Development Core Team, 2012) and carried out by oikostat (Ettiswil, Switzerland; www.oikostat.ch). We used a generalized linear mixed model with Poisson error distribution, log link function and an offset to analyze the number of bees that died every 24 h for each treatment group (mortality rate). 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 × 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. We further included an observation level random factor to account for the overdispersion that was substantial. 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.

The 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, we simulated the posterior predictive distributions for the number of surviving bees in each cohort.

The statistical analysis for the acquisition curve of the behavioral data (Fig. 1B) was done with a logistic regression model, which fits the data well (Supplemental Fig. S3), with repeated measurements with treatment and trial as fixed effects and the PER as response variable. For the in-between trial effects and the retention test we used a Pearson's Chi-squared test with Yates' continuity correction.

Results

BMAA increases the mortality rate

We compared the mortality rate of bees fed with BMAA (final concentration 5 mM) dissolved in sugar water (3 runs, Δ, Fig. 1A) with those of bees fed with equal amounts of saline solution in sugar water (3 runs, o, Fig. 1A), and tested the results using a generalized linear model with binomial distribution. The mortality rate is significantly higher in 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, Supplemental Table S4).

Associative learning and short term memory is impaired by BMAA

To investigate the effect of BMAA on the bees' behavior, we tested their short-term memory in a classical conditioning paradigm. We conditioned bees to an odor followed by a memory recall test after 30 min. We used bees fed for 5 days at which BMAA induced significant mortality differences, and bees fed for 1 day, where BMAA had no significant effect. In both groups BMAA fed and sugar water fed (control) bees were compared. The control and the BMAA group of day 1 showed no difference in acquisition or memory recall (control: 36%; BMAA: 40%) (Fig. 1BI). In the BMAA and control groups the learning rate (~40%) was fairly low yet with 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

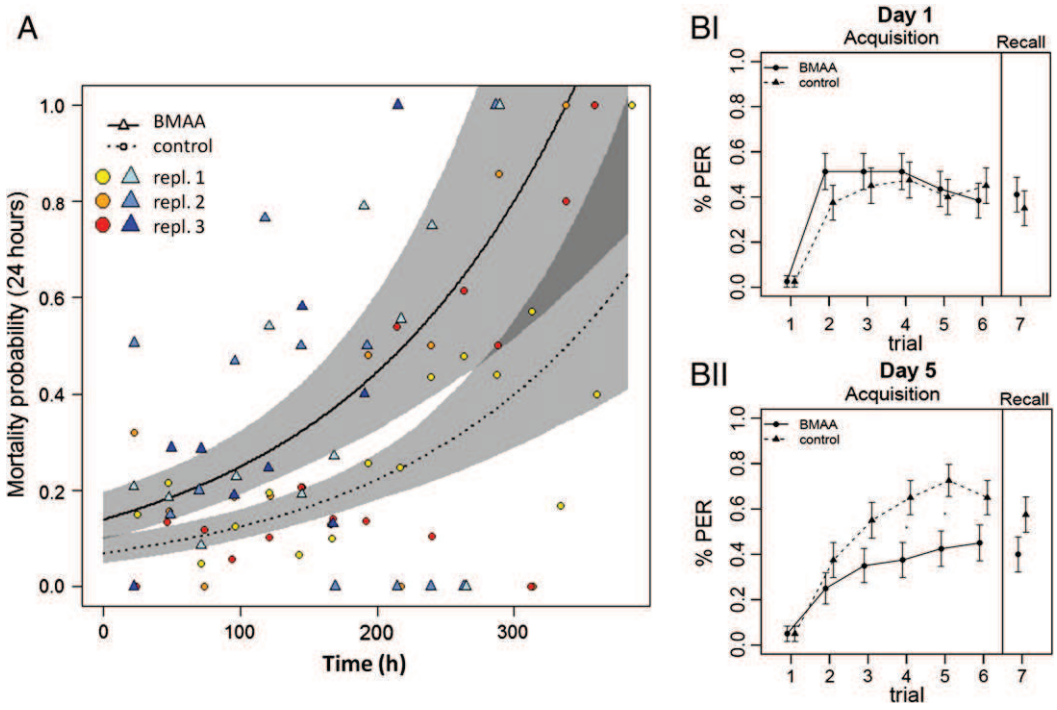

Fig. 1. Behavioral effects. A: Mortality of bees over time (hours); Δ: exposed to 5 mM BMAA supplemented food ad libitum (3 replicates, coded with different colors, n = 525) and; o: control bees (3 replicates, coded with different colors, n = 569). The model mortality function is given for the two cases with 95% credible interval (CrI-95) for the model prediction. Mortality in BMAA fed bees is significantly higher than in control bees. B: Learning curves and memory tests. BI: 1 day of feeding: no difference in learning curves and memory tests between BMAA (n = 40) and control (n = 39) bees. BII: 5 days of feeding: the acquisition curve and the memory recall test of BMAA bees (n = 40) was substantially 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) over the total amount of bees per group; mean ± SEM.

acquisition at the 4th and 5th trial (Fig. 1BII). In the 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, gave no significant differences between the control and the BMAA bees at day 5 although a trend was observable (40% of the 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.

Accumulation and distribution of ^{14}C BMAA in bees

We fed bees with 5 mM ^{14}C BMAA sugar water (1.25 M) solution and determined the amount of retained ^{14}C BMAA in head, thorax and abdomen 24 h, 48 h and 72 h after exposure. The total amount of ^{14}C BMAA remained constant over the 3 days tested (Supplemental Fig. S5) after application, suggesting that bees retain BMAA, and/or that BMAA is not excreted into the Malpighian tubule. Overall BMAA accumulated more strongly in the abdomen than in the head or thorax (Fig. 2A). The distribution of ^{14}C 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 BMAA in the head and thorax at 72 h was lower than at 24 and 48 h, while the abdominal ^{14}C BMAA remained at the same level as that observed at 24 and 48 h.

ROS development

We used optical imaging of ROS to determine whether application of BMAA onto the bee brain leads to an increased amount of reactive oxygen species (ROS). 30 min of exposure to 1.25 mM BMAA significantly ($p < 0.01$) raised ROS levels approximately 1.8 fold (Fig. 3A) when compared to the value at the starting point (1 min). In comparison to BMAA, 300 μM of MPP+ (positive control) raised ROS levels by 1.6 fold. This compound induced increased ROS levels followed a concentration response (Supplemental Fig. S6), while control ROS levels remained constantly low over the duration of the experiment (30 min).

BMAA leads to elevated Ca^{2+} concentration in brain neurons

As we assumed that BMAA affects the functionality and activity of bee brains (Supplemental Fig. S7), we bath applied BMAA or saline solution to the bee brains and recorded intracellular calcium levels in the antennal lobes. 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). The simultaneous measurement of the relative change in total fluorescence in stained projection neurons, served as a record of changes in the intracellular Ca^{2+} concentration. Recordings were taken over a 60 min exposure period, whereby one recording was taken prior to the compound application to the brains and then 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, and 60 min after application. The intracellular calcium level over time was determined as F340/F380 relative to the 1st 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 BMAA treated bees (Fig. 3B). The MAD in BMAA treated bees was significantly different from the MAD recorded for controls.

BMAA increases spontaneous brain activity

Bee brain projection neurons are constantly active also in the absence of odor stimuli (1 hexanol), with irregular fluctuations of calcium concentration (Galan et al., 2006). With each odor response

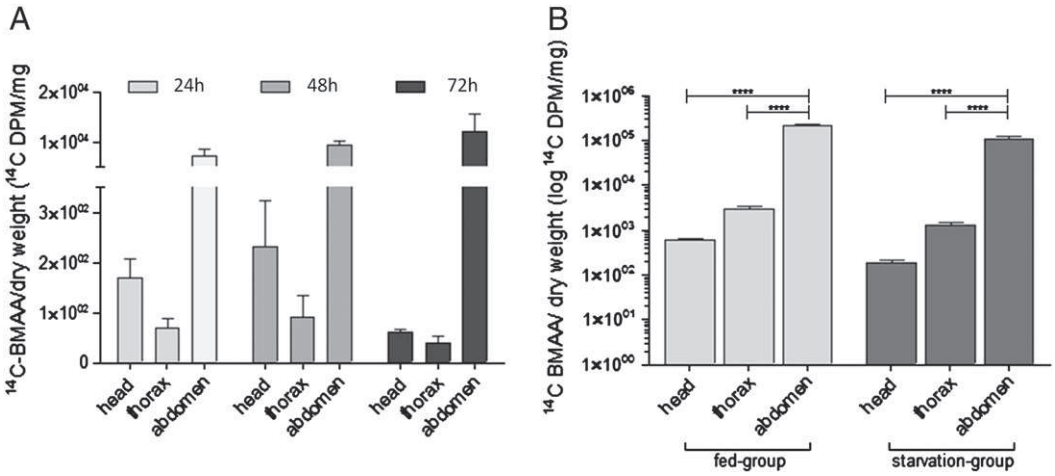

Fig. 2. Distribution of ^{14}C-BMAA. A: ^{14}C-BMAA in the heads, thorax and abdomens 24 h (n = 8), 48 h (n = 6) and 72 h (n = 4) after cessation of ^{14}C-BMAA exposure; mean ± SEM. B: Trophallaxis of BMAA. Non-fed bees (n = 9) accumulated ^{14}C-BMAA after 48 h via trophallaxis from bees (n = 11) with access to ^{14}C-BMAA sugar water. The highest ^{14}C-BMAA activities were found in the abdomen of both groups; mean ± SEM.

measurement we also recorded 8 s of spontaneous activity before and 16 s after the 1 hexanol stimulus (example trace, Supplemental Fig. S8A). The spontaneous activity before (Fig. 3C I) and after (Fig. 3C II) the 1 hexanol stimulus was greater in the BMAA bees when compared to the controls. In contrast BMAA exposure did not increase spontaneous activity in antennal lobe projection neurons during the 1 hexanol stimulus (p = 0.3315; Supplemental Fig. S8B).

Bees transfer BMAA via trophallaxis

To test whether BMAA can be transferred between bees we placed two groups of bees from the same hive 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 BMAA spiked sugar water (fed group), while the other had no access to food (starvation group).

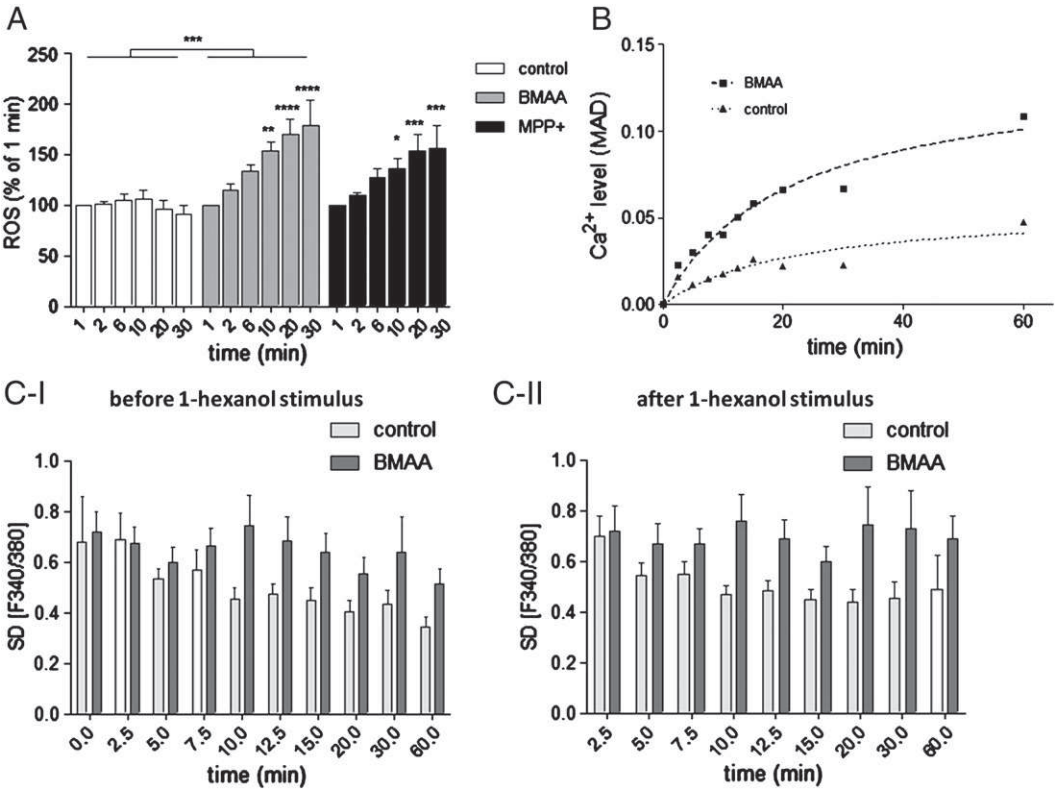

Fig. 3. ROS level and Ca^{2+} homeostasis following BMAA exposure. A: ROS level: BMAA caused ROS development in the brain. 1.25 mM BMAA (n = 5) induced an increase of 80%, MPP + (positive control) with 300 μM 60%. The control did not change; the effect is significant (P < 0.001) (two-way ANOVA with Bonferroni multiple comparisons) B: Ca^{2+} homeostasis in the bee brain. The median absolute deviation (MAD) of the variance in the Ca^{2+} level of BMAA bees (n = 16) increased compared to the control bees (n = 10) C: Increased spontaneous activity following a single BMAA or saline treatment over time (min). The spontaneous activity, defined as standard deviation of the Ca^{2+} level was significantly higher (two-way ANOVA) in acute BMAA (gray) 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.

After 48 h exposure, we found the largest ^{14}C BMAA activity in the abdomen of the bees in both groups, with lower activities in the thorax and head. The latter proved that starved bees were fed with ^{14}C BMAA by bees of the fed group. The ^{14}C BMAA activities in abdomen, thorax and head in the fed group were generally higher than those recorded in the starvation group (Fig. 2B). 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 BMAA.

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 appear to predominate the adverse reactions observed. Indeed, prolonged BMAA exposure of bees (up to 300 h) leads to a higher mortality probability (Fig. 1A), 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 (Fig. 1B), 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 (Fig. 2A) 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 (Supplemental Fig. S5), albeit the observed redistribution of ^{14}C BMAA between head, thorax and abdomen between 48 and 72 h (Fig. 2A) appeared to change. The latter data suggested that BMAA is bioavailable and redistributed amongst the bees compartments. That BMAA reaches the brain is supported by the behavioral data (Fig. 1B) in conjunction with the functional data presented in Fig. 3.

The observation that BMAA is distributed amongst bees via trophallaxis (Fig. 2B) suggests not only that BMAA exposure can place a whole bee population at risk, but also 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., 2013), 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., 2013). 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 625 μM or 1.25 mM BMAA to the bee brain for 30 min induced a significant and concentration dependent increase of ROS (40% or 80%, respectively) (Fig. 3A, Supplemental Fig. S6) as well as an increased antennal lobe activity, as demonstrated by a higher Ca^{2+} influx (Fig. 3B). While the former is a testimony of ER stress, the latter could result from BMAA excitotoxicity (Malhotra and Kaufman, 2007). 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 (Fig. 3C). Interestingly, odor responses were not affected in the antennal lobe (Supplemental Fig. S7). 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 (Fig. 1B).

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., 2013). 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., 2013). 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 (Figs. 2 and 3) 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., 2013) 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, 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, 2009; Jonasson et al., 2010) and the rising number of reports associating neurodegenerative 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.

Conflict of interest

None.

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