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Lack of behavioral and neuropathological effects of dietary β-methylamino-L-alanine (BMAA) in mice

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Abstract

β-Methylamino-L-alanine (BMAA) is an excitotoxin allegedly involved in ALS–parkinsonism–dementia complex (ALS–PDC), a neurological disorder found in Guam and its surrounding islands, in which motor neuron disease symptoms can present alone or can co-occur with parkinsonism and dementia. Although *in vitro* experiments have shown BMAA's neurotoxic properties, studies using adult animals and systemic administration which better model the case of environmentally-induced human neurodegenerative diseases have not supported the involvement of BMAA in these disorders. In order to better test the hypothesized role of BMAA in neurodegeneration, we fed adult mice BMAA at a dose (28 mg/kg body weight, daily for 30 days) that reproduces the natural levels and tested the animals with a battery of behavioural tests, the latter including the evaluation of motor coordination, motor neuron-mediated reflexes, locomotion, muscular strength and memory. We also assessed whether BMAA exposure triggers cell death in the central nervous system (CNS) of mice by examining neuronal numbers and glial response in the spinal cord and the brain. No motor, cognitive or neuropathological outcome resulted from this feeding paradigm. Our findings support neither the causal role of BMAA in neurodegeneration nor the specific involvement of this amino acid in ALS–PDC.

Keywords: ALS–PDC; β-Methylamino-L-alanine; Excitotoxicity; Neurodegeneration; Neurotoxin

1. Introduction

The etiology of amyotrophic lateral sclerosis (ALS), Parkinson's, and Alzheimer's diseases remains unknown. Although each of these diseases has early-onset familial forms, the vast majority of cases are sporadic, lending strong support to the notion that environmental factors, likely in the form of neurotoxins, could be largely causal. We have recently developed a mouse model of ALS–parkinsonism–dementia complex (ALS–PDC), a neurological disorder found in Guam and its surrounding islands, in which motor neuron disease symptoms or a form of parkinsonism associated with dementia usually occur as distinct disease entities. Some individuals, however, express both ALS and PDC. Neuropathologically, ALS–PDC is characterized by the presence of aggregated tau-containing neurofibrillary tangles and occasional Lewy bodies, while amyloid deposits are seldom present (Trojanowski et al., 2002). Based on extensive epidemiology, the most likely factor was thought to be the consumption of the seed of a cycad palm (*Cycas micronesica* K.D. Hill, formerly incorrectly referred to as *Cycas circinalis*) (see Kurland et al., 1994; Shaw and Wilson, 2003). Mice fed washed cycad flour show neurobehavioral deficits that resemble ALS–PDC features and correlate with losses of motor neurons in spinal cord, dopaminergic neurons in the substantia nigra as well as dopaminergic afferents in the striatum, and neurons in the hippocampus and cortex. In addition, signs of apoptosis and decreased tissue volumes have been seen in several central nervous system (CNS) areas, where decreased glutamate receptor and transporter expression and increased protein kinase C activity were also found (Shaw and Wilson, 2003; Schulz et al., 2003; Wilson et al., 2002, 2004). Overall, these studies showed that cycad toxins target motor and nigrostriatal dopaminergic neurons, while general signs of neuronal degeneration appear in other brain regions. One of the still unknown pieces of information is which cycad molecule(s) are responsible for the neurotoxic effects observed.

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β-Methylamino-L-alanine (BMAA) is an *in vitro* excitotoxin allegedly involved in ALS–PDC (Spencer et al., 1987b). The solubility of BMAA in water and its consequent absence in washed cycad flour have lead some researchers to propose an alternative explanation according to which BMAA is biomagnified by animal vectors, e.g. flying foxes, which were sometimes eaten as a delicacy and ceremonial food in traditional Guamian culture (Cox et al., 2003). BMAA has been shown to induce alterations in membrane conductance (Nedeljkov et al., 2005), increase intracellular calcium levels (Brownson et al., 2002) and lead to glutamate receptor-dependent neuronal death (Nunn et al., 1987; Pai et al., 1993) in *in vitro* systems. *In vivo* studies have also described neurotoxic effects when BMAA is administered to neonatal animals (Dawson et al., 1998) or delivered by intracerebroventricular injections (Rakonczay et al., 1991; Matsuoka et al., 1993; Chang et al., 1993; Bruijn et al., 1997). However, the studies using adult animals and systemic administration, which better model the case of environmentally-induced human neurodegenerative diseases, have not firmly supported the involvement of BMAA in these disorders. Perry et al. (1989) failed to find changes in brain neurotransmitter levels, neuronal numbers and behaviour of BMAA-fed mice, although these authors did not perform specific behavioural tests, but only general observations of mouse activity. On the other hand, neuropathological signs of brain damage have been described in rats (Seawright et al., 1990) and monkeys (Spencer et al., 1987a) exposed to very high doses of BMAA (300–2000 mg/kg body weight), that largely exceed the levels of this amino acid in the alleged environmental sources.

In order to better test the hypothesized role of BMAA as the toxin responsible for the Guamian ALS–PDC, we fed adult mice with BMAA at a dose that reproduces the proposed environmental levels and evaluated the animals by means of a battery of behavioural tests that has been previously proved to be sensitive to detect neurological deficits in cycad-fed mice (Wilson et al., 2002). In addition, we assessed whether BMAA exposure triggers cell death or inflammatory glial responses in the CNS of mice.

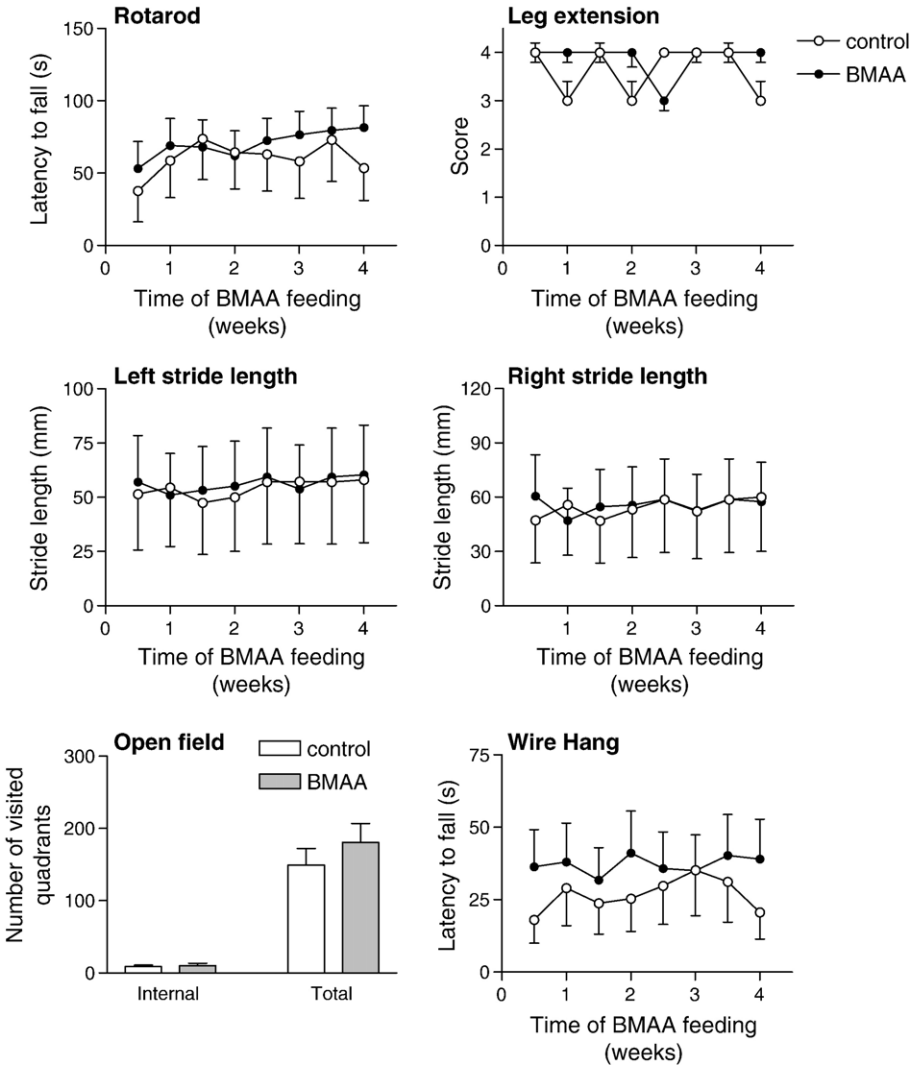

Fig. 1. Motor behavior of BMAA-fed and control mice. No significant performance differences were found between the two experimental groups in tests assessing motor coordination (rotarod), motor neuron-mediated reflexes (leg extension), locomotion (stride length and open field), or muscular strength (wire hang).

2. Methods

2.1. BMAA feeding

Six-month-old male CD-1 mice, weighing an average of 36± 2.7 g (mean±S.D.) at the start of the experiment, were randomly assigned to two experimental groups: mice fed with BMAA (Sigma, Cat. No. B-107) or with a control diet (n=7 for each group). The animals were housed one per cage in a room with a 12-h light/dark cycle at a temperature of 22 °C. The animals assigned to the BMAA-fed group received a mouse chow pellet (Purina® mouse chow) containing 1 mg BMAA (28 mg/kg BW) every day for 30 days. Control mice were given pellets of identical weight, but without BMAA. Pellets were given at 5:00 p.m. in the absence of standard food. On the next morning, the standard chow was supplied again *ad libitum*. Uneaten BMAA or control pellets were not found, which evidenced full consumption of the experimental diets.

The experimental protocol was approved by the Animal Care Committee of the University of British Columbia, in conformity with the Canadian Council on Animal Care guidelines.

2.2. Behavioral tests

To assess the impact of BMAA dietary exposure on motor and cognitive performance, mice were subjected to a previously described battery of behavioral tests (Wilson et al., 2002). All the tests were conducted between 1:00 p.m. and 4:00 p.m. twice a week, excepting for the radial arm water maze test, which was applied at the same time of the day but once a week and the open field test, which was conducted only once by the end of the feeding period.

In brief, the leg extension reflex test evaluated the presence (minimum score=0) or absence (maximum score=4) of clasping of the hind limbs when mice were suspended from the tail, as a measure of motor neuron functionality (Barneoud et al., 1999). In the rotarod test, the latency to fall from a rotating rod (speed: 24 rpm, maximum time: 2 min) was recorded and used as a measure of motor coordination (Crawley, 2000). The wire hang test, measured the latency to fall from an inverted grid and it is considered an index of muscular strength and motor neuron integrity (Paylor et al., 1999). The measurement of the distance between consecutive ipsilateral paw prints (stride length) was also used to evaluate gait and locomotion (de Medinaceli et al., 1982). The open field test provided a measure of activity and anxiety levels (Crawley, 2000).

As a memory test, we used the radial arm water maze (Clements et al., 2005), where the animals had to remember where an invisible escape platform was located in one of five arms of a submerged maze. Before the experimental feeding period started, the mice were pre-trained for 5 consecutive days on this task to ensure acquisition of the spatial map. During the feeding period, the animals were tested once a week. In both the pre-training and testing periods, mice were given 3 trials where they had 1 min to find the platform, 15 s to sit on the platform and 15 min of inter-trial resting time. The animals that did not find the platform in 1 min, were guided to it and received an escape latency score of 60 s. In addition to the escape latency, the number of entries to incorrect arms was also recorded.

2.3. Histochemical and immunohistochemical procedures

At the end of the BMAA feeding and behavioural testing period (30 days), mice from both groups were transcardially perfused with 4% paraformaldehyde under deep halothane narcosis. Their brains were dissected, fixed by immersion in 4% paraformaldehyde for 24 h, cryoprotected in 15% sucrose for another 24 h, embedded in OCT, frozen in dry-ice, and stored at −80 °C. Cryostat sections of 30 μm were immersed in Millonig's storage buffer and stored at 4 °C for analysis. With the aim of studying the effects of BMAA on neuronal viability and neuronal death, sections were mounted on glass slides and stained following a standard Nissl (thionine) protocol. For free-floating immunohistochemical procedures, other sections were washed three times in 0.1% phosphate buffered saline (PBS) and pre-incubated for 1 h at room temperature with a blocking solution containing 0.1% PBS with 0.3% Triton X-100 and 5% normal goat or rabbit serum. The sections were then incubated with anti-activated caspase 3 (Promega, 1:250, overnight at 4 °C), anti-choline acetyltransferase (ChAT, Chemicon, 1:50, 36 h at 4 °C), anti OX-6 (Serotec, 1:200, 1 h at room temperature), or anti-glial fibrillary acidic protein (GFAP, Calbiochem, 1:200, 3 h at room

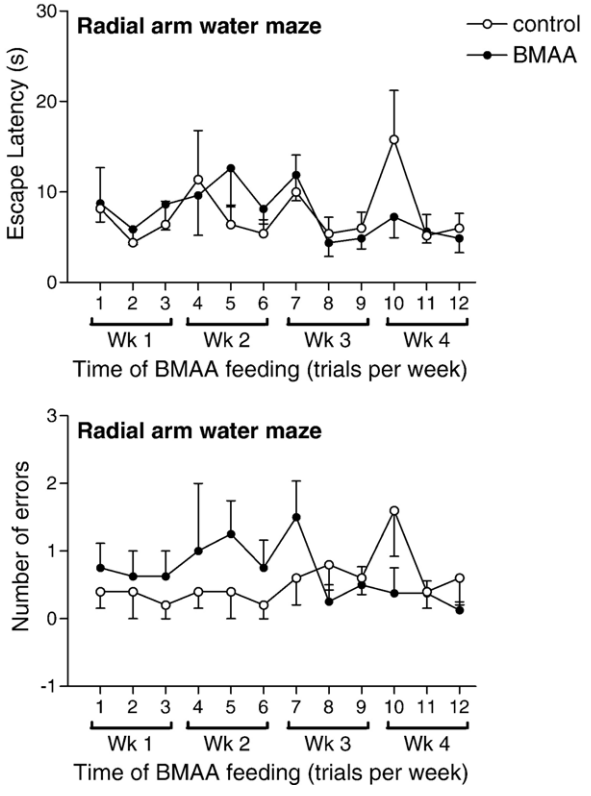

Fig. 2. Cognitive behavior of BMAA-fed and control mice. The dietary exposure to BMAA did not affect previously acquired information. BMAA and control animals spent similar time in finding the submerged escape platform (upper panel) and made comparable number of errors (lower panel). Although BMAA-fed mice showed an apparently higher number of errors (overall means: control=0.5; BMAA=0.8), this difference was not significant.

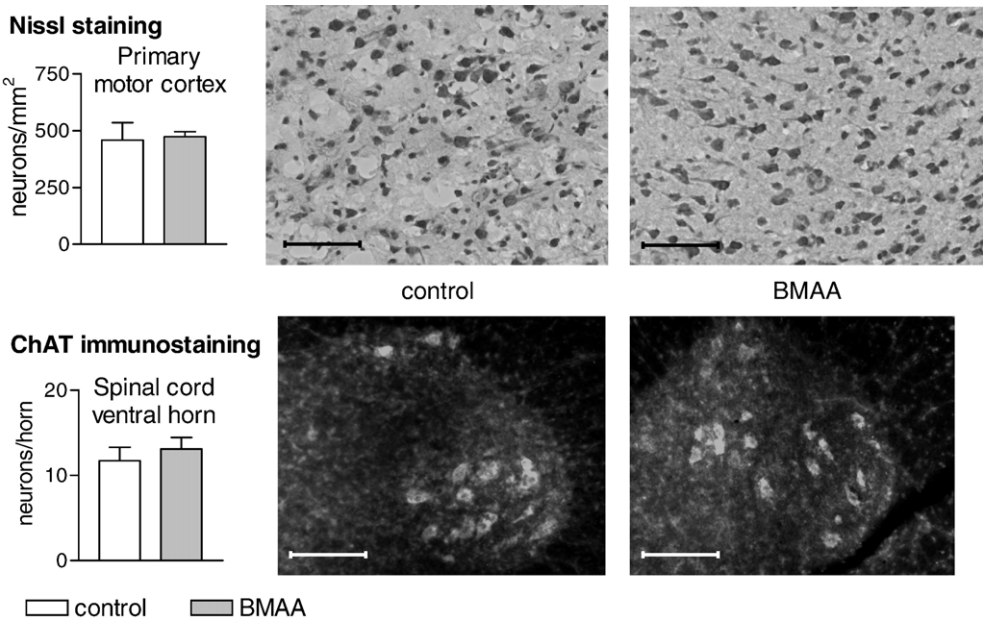

Fig. 3. Neuronal counts and representative microphotos of Nissl staining of the primary motor cortex (upper panel, scale bar=25 μm) and ChAT immunostaining of the spinal cord ventral horn (lower panel, scale bar=120 μm). The neuropathological analysis of mice exposed to BMAA showed no CNS damage as illustrated by the similar morphology and number of upper and lower motor neurons.

temperature). Afterwards, the sections were washed in 0.1% PBS and incubated for 1 h at room temperature with fluorescence secondary antibodies diluted in blocking solution, washed in PBS and mounted with Entellan or Vectashield mounting medium. Counts of Nissl-stained and ChAT immunoreactive neurons were performed with the AxioVision 3.1 imaging software on pictures taken with a Zeiss Axiovert microscope and a focal high performance CCD camera.

2.4. Statistics

The behavioural data time courses were analyzed by means of repeated measures ANOVA, with Experimental Group as independent factor, and Trials as the repeated factor. Neuronal numbers from the histological assessments were compared using Student's *t*-test (Statistica 2000 software). Values are shown as mean±S.E.M. for each group.

3. Results

The dietary exposure to BMAA did not induce any observable change in the motor performance of the treated mice as evidenced by the results from five different behavioural tests of motor function (Fig. 1). The repeated measures ANOVA confirmed this observation by showing that the independent factor (Experimental Group) was non-significant for all the tests ($F_{1,12}<0.8$; $P>0.3$). Although BMAA-fed animals appeared to perform better in the wire hang test, this difference did not reach statistical significance ($F_{1,12}=0.81$; $P=0.38$). The anxiety levels of BMAA-treated mice, which are inversely proportional to the number of entries to internal zones of an open field (Crawley, 2000), were comparable to the levels of control animals as well ($P=0.74$; Student's *t*-test). Likewise, BMAA did not cause significant changes in the performance of the animals in a memory task (Fig. 2), evaluated as the escape latency in a radial arm water maze ($F_{1,12}=0.01$; $P=0.93$), although a non-significant trend towards an increased number of errors by BMAA-fed animals was apparent, but non-significant ($F_{1,12}=0.29$; $P=0.60$).

The neuropathological analysis of brains and spinal cords from BMAA-fed animals did not provide any sign of neuronal damage or activation of death pathways. We did not observe immunoreactivity to markers of apoptosis (activated caspase 3), astrocytosis (GFAP) or microglial activation (OX-6) in the CNS of either control or BMAA-treated mice (not shown). The neuronal number in the primary motor cortex (as delineated by Paxinos and Franklin (2001)) of the animals fed with BMAA, assessed by the Nissl staining, was not different from that of control mice (Fig. 3, upper panel). Other brain regions such as striatum and hippocampus also displayed normal morphology and cell numbers (not shown). Since the lower motor neurons are one of the main neuronal populations targeted by ALS–PDC (Schmidt et al., 2001) and cycad toxicity (Wilson et al., 2004), we assessed the ventral horn neurons by both Nissl staining and immunoreactivity to ChAT, a marker of cholinergic alpha motor neurons. Both methods failed to show changes in the number of lower motor neurons in BMAA-fed animals (see ChAT immunostaining in the lower panel of Fig. 3).

In our previous experiments using the same battery of behavioural tests and histological protocols, mice fed washed cycad that contained no BMAA or MAM showed motor and cognitive deficits, as well as, decreased neuronal numbers and signs of apoptosis in the ventral spinal cord and other CNS areas (Wilson et al., 2002, 2005; Schulz et al., 2003).

4. Discussion

The average concentration of BMAA in dry cycad seeds is in the order of 1 mg/g, with significant differences among the

internal substructures of the seeds (Duncan et al., 1989). A large proportion of the BMAA contained in cycad seeds is washed out during the traditional Chamorro preparation of cycad flour. This has ruled out the possibility of BMAA as the toxin responsible for cycad neurotoxicity and ALS–PDC. The BMAA hypothesis has been revisited by the recent proposal that Guamian ALS–PDC is caused by the consumption of flying foxes, a species of bat that allegedly eat cycad seeds and accumulate BMAA to a level of roughly 4 mg/g in dry skin tissue (Cox et al., 2003; Banack et al., 2003). According to this measurement, a human of 70 kg who eats two 500 g bats (from bat weights reported by Lindhe-Norberg et al., 2000), will incorporate 28 mg of BMAA per kilogram of body weight, or less considering the water content in fresh bats. This is the BMAA dose that our mice received on a daily basis for 30 days in the present experiment. No motor, cognitive or neuropathological outcome resulted from this feeding paradigm, using behavioural and histological protocols that were clearly able to detect significant neurological deficits in mice fed washed cycad for the same period of time (Wilson et al., 2002; Schulz et al., 2003). These results coincide with the early observations by Perry et al. (1989), who used an even higher dose, but did not perform quantitative behavioural analysis. In addition, to confirm the preservation of neuronal numbers in BMAA-fed mice, the more detailed neuropathological examination in the present work showed that *in vivo* BMAA exposure does not trigger cell death-related events, such as caspase 3 activation, astrogliosis, or microglial responses. To the best of our knowledge, this is the first report on the effects of dietary BMAA on cognitive and motor performance and CNS integrity in adult animals with a dosing paradigm that resembles the proposed environmental exposure.

Two main factors might account for the present absence of BMAA effects. First, pharmacokinetics studies have shown that BMAA reaches potentially toxic levels in the CNS when administered at doses orders of magnitude higher than the environmentally available levels (Duncan et al., 1991). Second, BMAA is a very weak glutamate agonist that triggers increases in intracellular calcium levels and signs of cell damage at concentrations in the millimolar range, compared to the low-medium micromolar concentration range at which most excitotoxins are active (Pai et al., 1993; Brownson et al., 2002). Other factors, for example, possible inter-species differences in the level of impact, and specifically, differences in the pharmacokinetics and distribution of BMAA between rodents and primates, or the need for longer toxin exposure times to observe neurological deficits, might account for the absence of observed pathological outcomes in the present experiments.

Overall, the existing data from animal models do not support a causal role for BMAA in ALS–PDC or other neurodegenerative disorders. In addition, a report concerning BMAA accumulation in archival brain tissue from ALS–PDC and Alzheimer's disease victims (Murch et al., 2004) was not replicated by a recent study using fresh samples and a larger cohort (Montine et al., 2005). This outcome raises the question of which molecules in cycad flour are the causal agents for of ALS–PDC. Our recent experiments suggest that sterol glucosides, non-soluble compounds abundantly present in plants, especially in cycad seeds, might be one of the main neurotoxic components (Marler et al., 2005). The discovery of the molecules triggering cycad actions on the CNS and ALS–PDC has relevance not only for this particular disease, but it will also shade light into the biological basis of other environmentally induced brain diseases (Wilson and Shaw, 2006).

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