EPIDEMIOLOGICAL ASSOCIATIONS OF TOXIN EXPOSURE AND NEURODEGERATIVE DISEASE NEED EXPERIMENTAL SUPPORT: TOXICITY DATA STRENGTHEN BMAA HYPOTHESIS

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Introduction

The neurotoxic amino acid ß-N-methyl-L-alanine (BMAA*)* is formed by cyanobacteria, but also by diatoms and dinoflagellates worldwide [1-4]. This water-soluble compound is biomagnified in terrestrial and aquatic food chains, presumably following incorporation as a "false" amino acid into protein or following an association to protein [1, 5-6]. BMAA should consequently be considered as a naturally occurring persistent environmental contaminant. Cyanobacterial blooming is promoted by eutrophication and may become an increasing future problem because of global warming. Human exposure to BMAA may occur through direct contact with water-borne cyanobacteria during recreational activities, via contaminated drinking water supplies or following food-chain transfer.

BMAA originally caught attention when proposed as a causative agent of the ALS-parkinsonism dementia complex (ALS/PCD), a neurodegenerative disease complex described among the indigenous people of Guam [7-9]. The rapid change in incidence and onset of ALS/PDC, suggested that environmental factors contribute to the disease, with dietary factors as one candidate. BMAA was identified in cycad tree (*Cycas micronesica)* roots and in the cycad tree seeds. Subsequent studies by Cox and coworkers demonstrated about two orders of magnitude higher concentration of BMAA in flying foxes eating these seeds, than in the seeds [1,8]. As both cycad tree seed flour, and flying foxes were part of the local, traditional diet at the time, the population of Guam was heavily exposed to BMAA. In an early attempt to reproduce the disease complex, experimental animals were exposed to BMAA for prolonged periods of time. Motor neuron toxicity could indeed be produced in cynomolgus monkeys, though at extremely high doses [10].

BMAA research was revitalized when BMAA was found to be formed by all major groups of cyanobacteria including cyanobacterial symbionts and free-living cyanobacteria [1-2]. Given the recently demonstrated bioaccumulation of BMAA in mussels, oysters, shellfish, and fish, there seems to be several hitherto unforeseen routes exposure to BMAA in humans [11]. Recent studies have suggested potential associations between consumption of local seafood or exposure to cyanobacteria via aerosolization, and an increased incidence of the neurodegenerative disease ALS [12-15].

As revealed by recent experimental studies, BMAA is a developmental rodent toxicant that can produce an array of effects in the adult brain following exposure during the neonatal period [16-19]. These alterations are preceded by site-specific uptakes of BMAA in the fetal and neonatal brain that are higher than those in the adult brain. In a recent study, adult rats exposed to BMAA neonatally displayed abundant intraneuronal fibril formation in the *hippocampus*, a hallmark of neurodegenerative disease [19].

This presentation aims to discuss relations between experimentally induced proteomic, degenerative and functional changes in the adult brain of rodents exposed to BMAA during the neonatal period, and epidemiological associations of exposure to cyanobacteria and neurodegenerative disease. Several changes in rodents are compatible with those observed clinically and *post mortem* in human

neurodegenerative disease. A comparative analysis of experimental and epidemiological data will help determine the potential role of BMAA and other neurotoxicants as risk factors for human neurodegenerative disease.

Materials and methods (experimental studies)

Neonatal rats were given single subcutaneous injections of BMAA on postnatal days (PND) 9-10 [16-19]. Animals were killed at time-points ranging from two weeks - 6 months after dosing. Selected brain regions were subjected to histochemical, histopathological or electron microscopic examination. Brain sections through *hippocampus* were used for proteomic MALDI-imaging mass spectrometry. In addition, neonatally exposed adult rats were subjected to behavioural analysis. To determine the disposition of BMAA during early life-stages, pregnant or neonatal mice injected with ^{14}C-BMAA were subjected to autoradiographic imaging studies. Excretion in milk was determined using lactating dams and suckling pups [20].

Results and discussion

The hypothesis that BMAA is involved in the etiology of human neurodegenerative disease gains support from studies at Guam, and more recent publications claiming potential associations between cyanobacterial exposure and ALS in other parts of the world [12-15]. Furthermore, BMAA has been reported to be present in brain tissue from Alzheimer´s disease patients [21]. The BMAA hypothesis remains controversial, however, and there is still a lack of validated human exposure data.

Migrants from Guam have developed ALS/PDC decades after they left the island [22]. This suggests that the etiological process had occurred during childhood or adolescence. Epidemiological studies have further reported that exposure to cycads during young adulthood, but not adulthood, is a risk factor for neurodegenerative disorder among inhabitants of Guam. BMAA has a low neurotoxic potency in adult rodents compared to young animals, and the access of BMAA to the adult rodent brain was found to be to be limited. In contrast, autoradiographic imaging revealed that radiolabelled BMAA is transferred across the blood-brain barrier in neonatal mice, with a distinct uptake in specific brain regions such as *hippocampus*, *striatum* and *cerebellum* [20]. Taken together the developing brain seems to be more vulnerable to BMAA than the adult brain.

The exposure period (PND 9-10) used in our experimental studies corresponds to the last trimester of pregnancy and the first few years of age in humans, and is characterized by rapid maturation of neuronal systems. Our finding that BMAA is efficiently transferred via milk to suckling pups, resulting in a high exposure to the neonatal brain, are therefore of particular interest for human risk assessment; they raise the possibility that exposure of breast-fed infants to BMAA could occur [20].

BMAA caused impairments in learning and memory function without any distinct morphological changes following low doses, whereas a high dose induced proteomic perturbations, as well as major histopathological changes [18]. Progressive neurodegenerative changes, astrogliosis, microglial activation and calcification appeared in the hippocampal CA1 region 3-6 months after neonatal exposure. As shown by immunohistochemistry, there was an increased staining for α-synuclein and ubiquitin in the area. Ultrastructural examination revealed intracellular deposition of of closely packed fibrils in neurons, axons and astrocytes in CA1 [19]. Laser capture microdissection and subsequent proteomic analysis of the affected site demonstrated enrichment of chaperones, cytoskeletal and intermediate filament proteins, and proteins involved in the antioxidant defense system. Several of the enriched proteins have previously been implicated in protein aggregation and fibril formation. Recent

studies have also demonstrated that BMAA may induce expression of TDP-43 and increase tau hyperphosphorylation in the rodent brain [23, 24].

MALDI imaging showed a dose-dependent decrease in expression of proteins involved in energy metabolism and intracellular signaling in the *hippocampus* six moths after neonatal exposure, suggesting a reduced neuronal signaling [18]. These changes are likely to contribute to cognitive impairments as *hippocampus* is essential for learning and memory. BMAA-treated animals had impaired spatial learning and memory suggesting that neonatal exposure preferentially affects neuronal systems that are important for spatial tasks in adult animals.

Our experimental design is based on the paradigm that exposure to toxicants during vulnerable early life-stages may result in effects that do not become fully manifest until adult age. The results raise the possibility that BMAA is a slow-acting toxin that can provoke or enhance neurodegeneration by continuous or repetitive low-dose exposure(s) in humans. The doses used in the neonatal animal studies are fairly high and do probably not mimic real-life exposures, which are low and prolonged over time. It is assumed, however, that protein misfolding or protein association caused by BMAA could be a critical mechanism for neurotoxicity. Although the kinetics of such incorporation is not yet known, only a minor fraction of a BMAA dose was found to be protein-bound one day after administration [25].

Conclusion

The risk posed by BMAA as a potential human neurotoxin merits further consideration, particularly if biomagnification of BMAA in food chains is confirmed and quantified. Biomarkers representing both human neurodegenerative disease and experimentally induced long-term neurodegeneration should be developed and validated. A coordinated toxicological and epidemiological risk assessment could include such biomarkers.

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