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Review Article

Western Pacific ALS-PDC: Evidence implicating cycad genotoxins

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ABSTRACT

Amyotrophic Lateral Sclerosis and Parkinsonism-Dementia Complex (ALS-PDC) is a disappearing neurodegenerative disorder of apparent environmental origin formerly hyperendemic among Chamorros of Guam-USA, Japanese residents of the Kii Peninsula, Honshu Island, Japan and Auyu-Jakai linguistic groups of Papua-Indonesia on the island of New Guinea. The most plausible etiology is exposure to genotoxins in seed of neurotoxic cycad plants formerly used for food and/or medicine. Primary suspicion falls on methylazoxymethanol (MAM), the aglycone of cycasin and on the non-protein amino acid β-*N*-methylamino-L-alanine, both of which are metabolized to formaldehyde. Human and animal studies suggest: (a) exposures occurred early in life and sometimes during late fetal brain development, (b) clinical expression of neurodegenerative disease appeared years or decades later, and (c) pathological changes in various tissues indicate the disease was not confined to the CNS. Experimental evidence points to toxic molecular mechanisms involving DNA damage, epigenetic changes, transcriptional mutagenesis, neuronal cell-cycle reactivation and perturbation of the ubiquitin-proteasome system that led to polyproteinopathy and culminated in neuronal degeneration. Lessons learned from research on ALS-PDC include: (a) familial disease may reflect common toxic exposures across generations, (b) primary disease prevention follows cessation of exposure to culpable environmental triggers; and (c) disease latency provides a prolonged period during which to intervene therapeutically. Exposure to genotoxic chemicals ("slow toxins") in the early stages of life should be considered in the search for the etiology of ALS-PDC-related neurodegenerative disorders, including sporadic forms of ALS, progressive supranuclear palsy and Alzheimer's disease.

1. Introduction

Western Pacific Amyotrophic Lateral Sclerosis and Parkinsonism-Dementia Complex (ALS-PDC) is a progressive neurodegenerative disease with multiple clinical phenotypes and of familial or sporadic origin that has been highly prevalent in the island communities of the southern Marianas (Guam and Rota), Honshu, Japan (Kii Peninsula), and New Guinea (Papua, Indonesia). The history of ALS-PDC in Kii-Japan and Guam can been traced back several hundred years [1–3] although the disease on Guam was earlier thought to have followed the period (1668–1898) of Spanish occupation [4]. In New Guinea, clinical ALS and atypical parkinsonism with dementia (P-D) existed prior to the foreign introduction of domestic animals and manufactured products [5]. Following the 1939–1945 Second World War (WWII), the extraordinarily high prevalence of ALS, and later of P-D, declined in Guam and Kii-Japan, and the disease predictably will soon have disappeared from

Abbreviations: AD, Alzheimer's disease; ALS, Amyotrophic Lateral Sclerosis; ALS-PDC, Amyotrophic Lateral Sclerosis and Parkinsonism-Dementia Complex; L-BMAA, β-*N*-methylamino-L-alanine; BSS-BSSG, β-sitosterol and glucoside; CAT, catalase; cKO, conditional knock-out; C9orf72, chromosome 9 open-reading frame 72; DM, diabetes mellitus; DMH, dimethylhydrazine; E17, embryonic day 17; FTLD, frontotemporal lobar degeneration; GABA, γ-aminobutyric acid; GADD, growth arrest and DNA-damage-inducible protein; GD, gestational day; GPX, glutathione peroxidase; *Grin2b*, glutamate receptor, ionotropic, *N*-methyl-D-aspartate 2B gene; GSK-3β, glycogen synthase kinase 3β; HDAC, Histone deacetylase; hNPC, human neuroprogenitor cell; HPLC, high performance liquid chromatography; IGF-1, Insulin-like growth factor 1; KO, gene knock-out; L-BOAA, β-*N*-oxalylamino-L-alanine; LC-MS-MS, Liquid chromatography-tandem mass spectrometry; LRPE, Linear Retinal Pigment Epitheliopathy; MAM, methylazoxymethanol; MAMac, methylazoxymethanol acetate; *MAPT*, *microtubule-associated protein tau*; MGMT, O^6-methylguanine DNA methyltransferase; NF-κB, Nuclear factor kappa B; NFT, neurofibrillary tangle; NSC, neural stem cells; O^6-mG, O^6-methylguanine; 8-oxoG, 8-oxoguanine; P-D, Parkinsonism-dementia; PND, postnatal day; PFC, prefrontal cortex; PSP, progressive supranuclear palsy; SOD, superoxide dismutase; Sp1, specificity protein 1; SRF, serum response factor; STZ, streptozotocin; TDP-43, TAR-DNA binding protein-43; WWII, Second World War.

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all three geographic isolates. The disappearance of a hyperendemic disease among diverse ethnic genotypes suggests the operation of one or more exogenous factors to which the affected populations were formerly and commonly exposed. The identity of the culpable environmental agent(s), the principal subject addressed here, has been pursued for over 70 years by clinical and experimental neuroscientists. This review draws on diverse sources of information relevant to ALS-PDC to identify the probable etiology of this perplexing disease and its relevance to related neurodegenerative disorders. The Supplement contains neglected aspects of ALS-PDC, including clinically silent developmental cerebellar and retinal abnormalities that, in some cases, preceded, anticipated and accompanied the adult onset of clinically apparent neurodegenerative disease. Attendant dermatological, olfactory and other abnormalities suggest a systemic rather than a purely neurologic disorder, analogous to the motor and non-motor features of Parkinson's disease [6]. The Supplement also details the results of recent molecular studies of relevance to the pathogenesis of ALS-PDC-related human neurodegenerative diseases.

2. Western Pacific ALS-PDC

2.1. Neurology

While there is general agreement that Western Pacific ALS-PDC is a single nosological entity [7], the epidemiological history in Guam and Kii-Japan post-WWII shows that the ALS phenotype peaked and then declined, only to be followed some years later by a similar pattern for the P-D form [8–11]. Most of the information comes from 70 years of research on Guam. In general, clinical onset of ALS occurred at an earlier age than that of P-D, and onset age advanced as disease incidence declined [12,13]. On clinical grounds, ALS (known as *lytico* by Chamorros on Guam) was similar to ALS elsewhere, while the extrapyramidal phenotype conformed to atypical parkinsonism with cognitive decline (P-D, *bodig*), sometimes in combination with signs of motor neuron disease. A dementia (D) form clinically equivalent to Alzheimer's disease (AD) was also later recognized on Guam [14,15]. All three phenotypes have occurred spontaneously in individuals or as a familial disorder, with examples of ALS in younger siblings (age 20 onward), P-D in those older (age 34 onward), and D in the oldest [16]. Many Guam cases had electromyographic evidence of peripheral neuropathy and multisystem autonomic involvement similar to but less severe than in multiple system atrophy [17,18]. By 2000, mean ages at onset were 55 years for ALS ($n = 10$), 68 years for PDC ($n = 90$) and 74 years for Guam dementia ($n = 83$) [14]. First-degree relatives of patients with ALS or PDC had a significantly higher risk for disease relative to the Guamanian population, whereas relatives of disease-free controls had a significantly lower risk [14]. Motor neuron disease in susceptible Chamorro sibships was up to 28 times greater than the lifetime risk for the general population [19].

2.2. Cellular neuropathology

While the neuropathology of ALS-PDC in Papua-Indonesia is unstudied, the brains of Guam and Kii-Japan cases show tau- and α-synuclein-dominated polyproteinopathies that variably include cellular inclusions positive for ubiquitin, Aβ proteins and TAR-DNA binding protein-43 (TDP-43) [20–23]. Aggregated paired helical filaments form neurofibrillary tangles (NFTs) of aberrant hyperphosphorylated tau very similar to those seen in AD [24]. Unlike AD and progressive supranuclear palsy (PSP), α-synuclein also accumulates in the brains of Guamanians with ALS-PDC [25]. NFTs characterize not only all three Guam phenotypes but also the brains of Chamorro people who were considered healthy prior to accidental death [22,26–33]. Discovery of the etiology and pathogenesis of Western Pacific ALS-PDC therefore promises to illuminate understanding of related sporadic neurodegenerative disorders.

2.3. Molecular neuropathology

The Guam ALS-PDC brain, like that of other tauopathies (AD, PSP, Frontotemporal Dementia linked to chromosome 17, Corticobasal Degeneration, Pick disease, Niemann Pick disease type C), shows markers of cell-cycle reactivation in neurons with tau pathology destined for degeneration [34–36]. Hyperphosphorylated retinoblastoma protein (pRb), a cell-cycle G_1-to-S phase checkpoint protein, was also elevated in Guam ALS-PDC neurons with and without NFTs [36]. Evidence of cell-cycle perturbation early in development, in the form of hyperploid (i.e. bi- and tri-nuclear) and misaligned neurons, occurs in the cerebellum of both Guam and Kii-ALS-PDC brains [37,38]. Moreover, in Kii ALS-PDC brains, there is reduced expression of growth-arrest and DNA-damage/binding genes (e.g., GADD-45 and GADD-153) [39]. GADD-45 proteins have been associated with numerous cellular mechanisms including cell-cycle control, DNA-damage sensing and repair, genotoxic stress, neoplasia, and molecular epigenetics [40]. Identification of predominant oxidative and nitrative DNA damage in the brains of Kii-Japan ALS-PDC [41] is consistent with early genotoxic stress. Collectively, these findings suggest that cell-cycle changes contribute to the underlying pathogenic mechanisms in ALS-PDC and other tauopathies.

The molecular pathogenesis of Guam and Kii ALS-PDC has also been linked to an abnormality in the ubiquitin–proteasome pathway (UPP) [42–44], the principal pathway for protein degradation in mammals. UPP is central to the regulation of most cellular processes including cell cycle and division, DNA transcription and repair, differentiation and development, the morphogenesis of neural networks and stress responses, among other key functions [45]. Ubiquitin-B^{+1} (UBB^{+1}), a frameshift mutant ubiquitin and dose-dependent UPP inhibitor found in AD brain [46,47] was also recently reported in Guam and Kii ALS-PDC neurons and astrocytes, and in association with NFT-like structures [42–44].

2.4. Epidemiology

Dramatic falls in the very high incidence of ALS occurred in association with the post-WWII recovery in Guam and Japan. In Japan's Kii peninsula, the 5-year average incidence rate for ALS was almost 110/100,000 in 1950 and < 20/1000,000 in 1990 [10] while, on Guam, the incidence of ALS in 1962 was 87/100,000 and 5/100,000 in 1985 [12]. In 1989, the annual age-adjusted incidence of Guam ALS and PDC was 7/100,000 and 22/100,000, respectively [48]. During the post-WWII period, westernization and modernization progressively replaced traditional sources of food and medicine in Guam, Japan and, later, in Papua-Indonesia.

Evidence consistent with a primary environmental etiology of ALS-PDC, and of prolonged latent periods between exposure to the disease trigger(s) and the clinical onset of phenotypes, emerge from the experience of migrants to and from populations hyperendemic for ALS-PDC (Fig. 1). Guam Chamorros ($n = 18$) who lived on-island from birth to age 18–63 years developed ALS 1–34 years after migration to the U.S. mainland, and a brother and sister aged 16 and 15 years developed motor neuron disease in California 7 and 4 years, respectively, after they had left Guam [49,50] Adolescence was estimated to be the critical exposure period for the acquisition of ALS and P-D risk prior to migration from Guam [65]. Four additional persons developed ALS within 1 to 14 years of their return to Guam after a long-term residence in the continental United States [50]. Filipino migrants to Guam developed ALS 1–29 years or P-D 13–26 years after arrival on the island [59,60]. Two Caucasians and two Filipinos who immigrated to Guam at the ages of 32, 39, 43 and 29 years (in 1955, 1971, 1969 and 1970) developed signs of PDC at the age of 63, 60, 52 and 57 years, respectively (mean duration from immigration to disease onset of 21–28 years) [55]. These data demonstrate that continuous exposure to the Guamanian environment (specifically, the Chamorro culture) increased the risk of

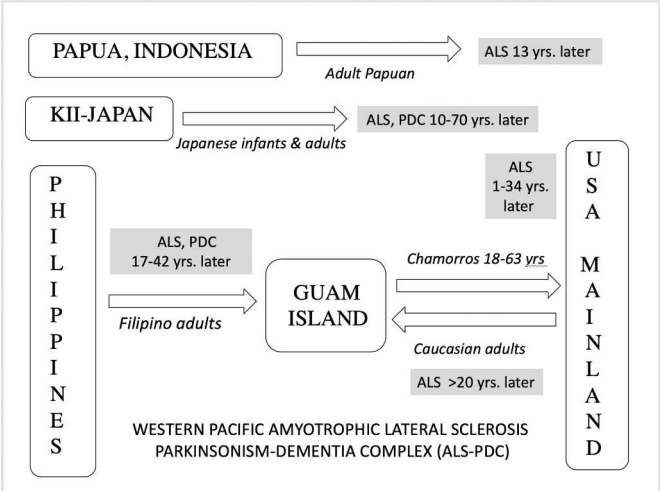

Fig. 1. Summary of ALS and P-D among migrants to or from populations hyperendemic for ALS-PDC. *Guam to US Mainland*: Through 1980, ALS developed in 21 Chamorro migrants (18/21 to California) of mean age 29.4 years (range, 18–63) after periods of absence from Guam of 1 to 34 years (mean 13.6 years); 4/21 developed ALS 1–14 years after returning to Guam. Mean ages at onset and death of the 21 subjects were 44.8 years (range, 19–66) and 48.8 years (range, 29–66), respectively [50]. Migrants of Chamorros born, educated and permanently resident on the U.S. mainland are unstudied but are not known to have developed ALS or PDC. *USA Mainland to Guam.* Of >10,000 stateside male construction workers who worked on Guam for one year or more from 1945 to 1954, there was no excess ALS through 1970 [51]; there was no later follow-up of these persons. However, some non-Chamorro U.S. soldiers stationed on Guam in 1944–45 for 1–2 months developed ALS 40 years later while living in New York City and three Gulf War/Era veterans with prior service on Guam developed ALS in the late 1990s [52–54]. Two Caucasians who migrated to Guam between 1955 and 1971 developed and died from autopsy-proven ALS >20 years later at age > 60 years [55]. The last certain case of *lytico-bodig* was ALS in a Caucasian who came to Guam in 1956 and who is the subject of a report by D. Perl [J.C. Steele et al., personal communication, 2019]. *Philippines to Guam:* Filipinos from different provinces who migrated to Guam post-WWII included three males who arrived in 1947, became friends, lived together in central Guam (Sinajana) and were employed as construction workers. Two of the three married Chamorro sisters whose family members had ALS. One developed ALS, the second PDC and the third PDC (all autopsy-proven) 17, 24 and 42 years respectively after arrival in Guam [56]. A possible excess of ALS among Filipino residents of Guam was evident by 1975 [57,58]. By 1980, ALS occurred in 9 Filipino migrants to Guam 1 to 29 years after their arrival and PDC in 2 migrants 13 and 26 years after arrival [59,60]. *Migration from high-risk areas of Kii-Japan: Kii-Kozagawa*: Four subjects aged 15–66 years (mean, 22) developed ALS up to 1–4 decades after migration [61,62]. *Kii-Hohara:* One subject developed ALS-PDC 73 years after migration from the Kii Peninsula at the age of three years [63]. *Migration from high-risk are of Papua, Indonesia (western New Guinea island):* A 35-year-old woman developed ALS 13 years after leaving the ALS-PDC epicenter in Osso village on the northern river Ia, and ALS affected another subject 14 years after treatment of a large wound with the neurotoxic pulp of cycad seed [64].

developing ALS-PDC [60]. Exposure may have been short, as illustrated by a Filipino who developed ALS only 36 months after moving to Guam [65]. Some non-Chamorro U.S. soldiers stationed on Guam in 1944–45 for only 1–2 months also developed ALS almost 4 decades later [66], and a subset of Gulf War/Era veterans who developed ALS in the late 1990s was born or saw military service on Guam, where their diet off-base included traditional Chamorro food served at frequent village fiestas [54].

Migration studies of Japanese in Kii Peninsula have revealed latency periods up to seven decades between exposure to the high-risk environment and clinical onset of neurodegenerative disease [63], with evidence of acquired risk at a very young age [61]. Four subjects, who moved from the high-risk area at ages 4, 5, 24, and 55 years developed clinical disease at 45, 63, 65, and 79 years-old, respectively. On the island of New Guinea, ALS developed in one native subject 13 years after migration from the epicenter of ALS-PDC in Papua, Indonesia.

Based on the epidemiology of Western Pacific ALS-PDC, it is logical to conclude that: (a) the culpable environmental agent(s) is present in, and likely common to, all three geographic foci of this neurodegenerative disease, (b) human contact with the agent(s) progressively reduced or ceased years or decades before the decline and eventual disappearance of cases, (c) exposures to the trigger factor(s) sometimes occurred early in life, while clinical brain disease emerged years or decades later, and (d) the causative agent(s) is capable of inducing both sporadic and familial disease, either because of common exposure or susceptibility from an heritable trait.

2.5. Genetic vs. Environmental trigger

The familial occurrence of hyperendemic neurodegenerative disease on Guam is well documented [67] and, in the early years of research investigation, this association was interpreted as indicative of a dominantly inherited genetic disorder, albeit with differing degrees of penetrance [49,57,68–70]. With evidence of declining disease incidence, both on Guam and in Kii-Japan, undefined gene-environment interactions were proposed, some advancing a primary genetic role [68,69,71], while others favored the reverse or an equal contribution [57,58,72–77]. In 1982, Gajdusek and Salazar [5] concluded a genetic etiology for ALS-PDC in New Guinea was unlikely, and later studies (2005–2012) of the affected population (Auyu and Jaqai linguistic groups) demonstrated declining prevalence of neurodegenerative disease [11,64] as had occurred on Guam and in Kii-Japan.

The burden of diverse genetic perturbations among Chamorros living on Guam or nearby islands has been reported to be surprisingly high. While approximately 80% (51 of 64) of Guam P-D patients had no pathogenic mutations, the balance (~20%) included 3 with homozygous *PTEN-induced putative kinase 1* (*PINK1.L347P*) mutations linked to parkinsonism, 2 heterozygous dynactin (*DCTN1 p. T54I*) mutations, 1 *fused in sarcoma* (*FUS p.P431L*), and 6 *alsin* (*ALS2*) mutations. Clinically unaffected controls ($n = 30$) were twice as likely than patients to have mutations in *leucine-rich repeat kinase 2* (*LRRK2*), *charged multivesicular body protein 2b* (*CHMP2B*) and *PINK 1* [78]. No mutations were found in *progranulin* unlike the many found in inherited forms of frontotemporal dementia [79].

With regard to Kii-Japan ALS-PDC, familial disease (vertical and horizontal) was present in approximately three-quarters of patients in the northern focus (Hohara) but genetic screening for mutations associated with inherited neurodegenerative disorders proved negative [2,80,81]. An extensive mutation analysis of three patients from two families with pathologically confirmed ALS-PDC found no mutation in 19 genes, including 12 ALS-FTLD-related genes, 6 parkinsonism-related genes, and *glycogen synthase kinase-3β* (*GSK3β*), the gene coding for tau kinase implicated in inherited tauopathies such as AD. Additionally, gene dosage was normal for *MAPT*, *α-synuclein*, *GSK3β*, *parkin* and *TDP-43*, which codes for TAR-DNA-binding protein 43 [82,83]. A few ALS patients in Kii-Japan had mutations in *C9orf72* or *optineurin* [84]. Other studies yielded negative results, missense mutations, single nucleotide polymorphisms, or hexanucleotide repeat expansions in a small minority of Guam and/or Japanese patients, but without overlap or commonality between the two [76,85–97]. No genetic studies have been performed on ALS-PDC cases in Papua-Indonesia.

Research efforts to identify a common genetic explanation for ALS-PDC in Kii and Guam continue but none has been found to date (*vide infra*). With the virtual disappearance of ALS-PDC on Guam, and similar trends in Kii-Japan and Papua-Indonesia, it seems probable that the cause of this disease is primarily if not exclusively exogenous [54]. Since the peak birth years for acquisition of ALS-PDC on Guam were between 1910 and 1929, with risk for disease acquisition disappearing for those born after the mid 1950s (J.C. Steele, personal communication), significant

population contact with the culpable agent(s) must have declined rapidly after the end of WWII, when disease incidence was at its peak. A similar conclusion can be reached for Kii ALS, the rates for which fell steadily from its post-War II peak incidence [10].

3. Environmental etiologic hypotheses

3.1. Infection

A positive family history of ALS on Guam (and the northern focus of Kii-ALS) potentially suggested the operation of a vertically transmitted infection [55]. However, there are no known infectious agents that selectively acted on the three Western Pacific populations with hyperendemic neurodegenerative disease. Van Nuis [98] suggested an avian distribution of a neurotropic virus. A link with poliomyelitis on Guam was ruled out [99]. In the 1940s, Guam experienced outbreaks of Japanese B encephalitis and mumps encephalitis [74,100] but P-D patients did not recall a preceding infection [26,27,56]. There is overlap between the neurofibrillary pathology of ALS-PDC and post-encephalitic parkinsonism [101], but ALS and synucleinopathy are absent in the latter [102]. The possibility of a kuru-like slow virus (prion) was ruled out when, in contrast to kuru, experimental transmission of ALS-PDC to primates failed [103]. Furthermore, TDP-43 inclusions, a prominent feature of Guam and Kii ALS-PDC, are absent from prion diseases [104]. TDP-43 and other intracytoplasmic protein deposits found in ALS-PDC and other neurodegenerative disorders have occasionally been described as 'prion-like', but this does not infer they were caused by an infection. In the New Guinea (Papua, Indonesia) ALS-PDC focus, the distribution of affected and nonaffected villages indicated that a communicable infectious etiology was unlikely [5]. In sum, evidence to support an infectious etiology in Western Pacific ALS-PDC is lacking. Discussed below is the suggestion that a parasitic infection links P-D with the concurrent retinal pathology reported in some individuals in Guam and Kii-Japan [55].

3.2. Minerals

The contribution of the local geological environment to the genesis of ALS-PDC has been of interest to several investigators. Considerable effort was invested in the possibility of atypical elemental exposure related to geochemical patterns, whether in Kii-Japan, Papua-Indonesia or Guam [5,105–111]. Water and plants used for food were reported to have a low content of calcium (Ca), magnesium (Mg) and zinc (Zn), and a correspondingly high content of manganese (Mn), iron (Fe), silicon (Si) and aluminum (Al). Chronic dietary deficiency since birth of Ca, Mg and Zn was proposed to induce a parathyroid-mediated increase in the gastrointestinal absorption of divalent cations that accelerated oxidant-mediated neuronal degeneration in the Guam Chamorro population. However, the normal serum parathyroid hormone status and alkaline phosphatase levels of Guamanians with and without neurodegenerative disease provided no support for this hypothesis [112].

Excess bioavailable Al was found post-mortem in the brains of Guam and Kii-Japan ALS-PDC cases [108,113], and intraneuronal accumulation of Al with or without Ca and Si was present in Guam CNS tissues [114,115]. Since Guam soil content of elutable Al was 42-fold higher than that of soils of ALS-PDC-free Jamaica or Palau, whereas dietary intake of Al and Ca was comparable, excess Al was proposed but not shown to enter Guamanians via inhalation [116,117]. Another study on Guam found no relationship between motor neuron disease and content of Ca, Mg or Al in soil and water, but there was a significant correlation with the concentration of iron (Fe) in water samples [19,65]. Al^{3+} modulates tau phosphorylation, and Fe^{3+} as well as Al^{3+} enhance both the formation of mixed oligomers and recruitment of α-synuclein in pre-formed tau oligomers [118,119]. Brain Fe levels were found in a small number of Guam ALS-PDC cases to be higher relative to controls [113] but other studies yielded conflicting results [120–122]. High levels of Fe and Mn were reported in the top soils and vegetation proximate to the former epicenter of ALS-PDC on Guam, and elevated levels of Mn in soil, food and cattle hair were found in regions of high-incidence human neurodegenerative disease in Kii-Japan [106,123]. Juvenile cynomolgus monkeys placed for 41–46 months on a low-calcium diet ($n = 2$), with or without supplemental Al and Mn (n = 2), exhibited mild Ca and Al deposition and degenerative changes in motor neurons of the spinal cord, brain stem, substantia nigra and cerebrum in excess of those seen in a single control monkey, but all animals remained clinically healthy without apparent behavioral deficits or neurological signs [124].

Taken in concert, these observations suggest some association between ALS-PDC and certain minerals in soil, drinking water, and vegetation [125]. However, this association is unlikely to be causal because rates of ALS-PDC declined in Papua-Indonesia in the presence of an unchanged water supply and a forest-based diet on which the indigenous population had long depended [64]. In sum, therefore, while differential mineral intake may be a risk factor, Steele and Williams [126] concluded the mineral hypothesis does not explain the etiology of ALS-PDC.

3.3. Autoimmune disease

A potential role for autoimmune mechanisms in motor neuron degeneration was proposed in sporadic ALS [127,128] and in Guam ALS-PDC, the latter linked to aluminum [129]. No consistent profile of autoimmunity was found in extensive serologic testing of subjects with or without Guam ALS-PDC [130]. Differences in serum Ig levels in ALS and P-D patients were attributed to repeated infections and abnormal immunoregulation accompanying immunodeficiency during the course of CNS disease, rather than to a specific antiviral or autoimmune response [131]. In sum, therefore, evidence to support an autoimmune mechanism in ALS-PDC is lacking, as in ALS [132].

3.4. Cycad toxicity

Reduction in the incidence of ALS-PDC in all three geographic foci of the disease has been associated with declining traditional use of a neurotoxic plant for both food and medicine, specifically the seed of female gymnosperms of the genus *Cycas*. Seed of these Fe- and Mn-dependent cycad plants formerly served as a traditional source of food for Guamanians, an oral tonic and folk medicine in Kii-Japan, and a topical medicine for the treatment of open wounds in Papua-Indonesia, all of which have been linked to the subsequent development of ALS-PDC [62,133–139], albeit in single well-documented cases in Kii and New Guinea. Ungulates grazing on cycad leaves develop a poorly defined neuromuscular disease accompanied by the loss of horns and hooves that are considered relevant to the neurological and dermatological changes in ALS-PDC, as discussed in the Supplement. *Cycas* spp. contain azoxyglycosides (notably cycasin and neocycasins) and non-protein amino acids (notably β-*N*-methylamino-L-alanine, L-BMAA), both of which have genotoxic and neurotoxic properties (*vide infra*).

Several human groups have used cycad seed or sago with differing degrees of detoxication as an emergency food source. The Aborigines of northern Australia traditionally crushed the seed, sun-dried the toxic pulp, loaded the pulp into a dilly bag and then placed the receptacle in running water for several days to effect complete detoxication [140,141]. Other less effective methods of detoxication may account for instances of motorsystem disease in this population [142]. Effective detoxication that renders cycad seed/sago harmless is achieved by prolonged fermentation and repeated washing, as practiced in the Nansei islands of southern Japan [143]. By contrast, some Chamorros of Guam consumed food products prepared from flour derived from the gametophyte of water-soaked and incompletely detoxified seed. Additionally, the fresh green seed cover or sarcotesta (0.2% cycasin content) was used to relieve thirst and the dried sarcotesta (0.35% cycasin) served as a confection [134]. Use of cycad seed for food on Guam was important during times of food shortage, especially following a hurricane or during times of conflict such

as WWII [74]. After the end of WWII, with Guam under U.S. Navy governance, reliance on cycad seed as a source of food and medicine declined progressively with the importation of U.S. products and the acculturation of Guamanians to a modern American lifestyle. The traditional Chamorro diet also included *fanihi*, a cycad-eating fruit bat (*Pteropus mariannus mariannus*), a once-common but now threatened species on Guam. The relevance of the fruit bat to the traditional diet has been variably described as "highly salient" to a delicacy, with less than once monthly consumption [144,145]. These animals were proposed to eat cycad seed, bioconcentrate L-BMAA and thereby intoxicate the Chamorro consumer [146,147], but an independent study using a powerful analytical technique that measured underivatized L-BMAA found no trace of the neurotoxin in comparable fruit bat specimens [148].

Studies have also shown that preference for traditional Chamorro food was significantly associated with an increased risk of P-D on Guam [58]. Furthermore, adjusted odds ratios and confidence intervals for picking, processing, and eating cycad seed in young adulthood were consistently elevated and significant for dementia (n=166), mild cognitive impairment (n=50), and P-D (n=21) on Guam [145]. Not linked to ALS-PDC was ingestion of the fruit bat (*syn*. flying fox) or use of cycad seed pulp as a topical medicine; ingestion of cycasin-containing fresh or dried sarcotesta was not addressed. Prasad and Kurland [4] noted that adults may be unable to recall receiving cycad-derived medicine that, as children, was administered by male/female indigenous healers (*surahanos/surahanas*) "who used cycad in poultices and as tonics". They also raised the possibility of a link between ALS-PDC and inhalation of cycad pollen during seed collection.

Guam Chamorro folklore associated the practice of handling and consuming cycad materials to *lytico*, many cases of which were diagnosed as ALS [133,134,149]. Chamorro children were said to have fallen acutely ill after eating cycad food products, and a few died if preparation was poor because detoxication was incomplete [150]. Some who ate poorly prepared cycad seed products developed sudden onset of nausea and vomiting after 1–2 days, followed by liver enlargement, convulsions, loss of consciousness, and death or recovery [151,152]. Analysis of cycad flour prepared Chamorro-style demonstrated the presence of cycasin and ten-fold-equivalent lower concentrations of L-BMAA [153]. Importantly, the concentration of cycasin, but not of L-BMAA, was significantly correlated with average annual age-adjusted incidence rates for ALS and P-D among Guamanian males and females [19,65]. Cycasin induces a cycad-like motorsystem disease in ruminants [154,155], and the potential role of its DNA-damaging aglycone methylazoxymethanol (MAM) in Guam ALS-PDC has been recognized earlier [156,157]. Comparison of the neurological and other features of ALS-PDC with those induced by MAM acetate (MAMac) in laboratory animals is described in the Supplement.

4. Cycad components and neurological disease

4.1. Cycasin and Methylazoxymethanol

The principal *Cycas* seed toxin cycasin (up to 2.5% *w*/w) and a number of other azoxyglycosides [158,159] are metabolized to the potent genotoxin MAM by β-glucosidase, an enzyme present in plant tissue and in the microbiome, skin and other tissues of animals and humans [160–162]. MAM is a potent radiomimetic, genotoxin, mutagen, teratogen, mitogen, hepatotoxin, pancreotoxin and carcinogen, as well as a developmental neurotoxin that can induce cellular injury both by oxidative stress and the alkylation and oxidation of guanine [163–166]. MAM alters gene expression in SY5Y human neuroblastoma cells and, in the presence of DNA damage and reduced DNA repair, enhances glutamate-modulated expression of tau mRNA in rat primary neurons [167,168].

Cycasin, a glucoside of MAM, can enter cells via glucose transporters [169,170] whereupon the sugar moiety is cleaved enzymatically by intracellular β-glucosidase [153]. Although β-glucosidase activity in intestinal bacteria is known to be responsible for liberating MAM in the gut of animals fed cycasin, its activity is reportedly 500-fold higher in the brains of rodents and presumably in humans [157]. The metabolism of MAM yields the highly reactive methyldiazonium ion that can methylate DNA, RNA, protein and other molecules, such as the amino acids glutamate and aspartate. An important unresolved question is whether MAM can systemically generate L-BMAA or an endogenous L-BMAA-like compound from tissue amino acids. Incubation of primate serum with MAMac yielded an aspartate derivative (Asp) and a L-BMAA-like compound (both uncharacterized) that increased in a concentration-dependent manner. As with L-BMAA alone, each of the two novel compounds generated by MAMac induced excitotoxic post-synaptic swelling in murine cortex cultures, and the aspartate derivative (Asp-D) also caused MAMac-like changes in neuronal chromatin. The acute neuropathological changes induced by Asp-D suggest that, in addition to MAM, both neuronal receptors and intracellular components (notably nucleic acids), are potential targets of cycasin derivatives [171].

Guam and Kii ALS-PDC brains show evidence of developmental perturbation comparable to that induced by MAMac in rodents. The response of the immature rodent brain to a single systemic treatment is strictly dependent on the stage of development and, secondarily, on the administered dose [172]. In rats, a single dose of MAMac given on or before gestational day 15 (GD 15) leads to microcephaly with abnormal cytoarchitecture of the cerebral cortex, striatum and hippocampus [173–175], including nodular heterotopia associated with increased susceptibility to convulsive agents [176,177]. Azizi and colleagues [165] reported that MAMac reduced the activity of cortical antioxidant enzymes (CAT, SOD, GPX) and increased nitric oxide levels in 1-month-old mice following injection on GD15, such that oxidative/nitrative stress appears to be a latent event in the MAMac rodent model of microcephaly. Steullet and colleagues [166] showed that DNA damage (i.e, 8-oxoguanine) was increased 164% in the prefrontal cortex of 2–3 month-old rats following an injection of MAMac on GD 17. Notably, predominant oxidative and nitrative DNA damage was shown to be frequently co-localized with tau in the brains of Kii-Japan subjects with ALS-PDC [41]. Rodents treated with 1,2-dimethylhydrazine or diazomethane, both of which produce the common metabolite MAM, induced both alkylation (O^6-methylguanine) DNA damage in target tissues [178,179] and indirectly damage brain DNA (8-oxoguanine) via an oxidative stress-mediated mechanism [166,180].

MAMac administered to rodents at or shortly after birth produces lifelong cerebellar dysplasia [172,181–184], which is directly comparable to sub-clinical structural abnormalities of the cerebellum found in a number of Kii and Guam ALS-PDC cases [37,38,185]. A mild bilateral cerebellar syndrome was also reported in some members of a motor neuron disease cluster of Australian Aborigines in Arnhem Land who consumed washed cycad seed [142]. Injection of newborn rats with MAMac reduced the weight of the cerebellum by 62%, the olfactory bulb by 65%, and the hippocampus by 18% [186]. The cerebellar pathology and ataxia in MAMac-treated neonatal mice was more pronounced in DNA-repair-deficient animals, and they were reversed in animals that overexpress DNA repair [187]. These studies indicate that DNA damage plays an important role in the neurodevelopmental changes induced by MAM.

MAMac also induces retinal dysplasia in laboratory animals when administered at specific stages of embryonic development. The neuroblastic layer folds to produce rosettes that are tubular in cross-section and which persist as retinal tracts in adult life [188,189]. These structures appear to correspond to the linear or vermiform tracts reported in Guam and Kii ALS-PDC [190–194] that were previously described in the former as "Linear Retinal Pigmentary Epitheliopathy" [195]. Retinal and cerebellar dysplasia can occur simultaneously in animals treated perinatally with MAM [196,197] but this concurrence has not been explored in ALS-PDC. Since the retina of the rat at birth is equivalent in developmental stage to the human retina at 4–5 months of gestation [198], this implies that human retinal dysplasia could result from fetal

cycasin/MAM exposure as early as the second trimester of embryonic development, while cerebellar dysplasia might be induced by later exposure.

The Supplement to this paper details the association between the experimental effects of MAMac on the developing mammalian brain and the cerebellar, retinal, olfactory, mental, skin and other abnormalities that have been variably documented to precede and accompany Guam and Kii ALS-PDC and related motorsystem disorders. While cause-effect proof is unavailable, clinical and experimental observations that support various linkages between MAM and ALS-PDC have been known for the last half-century but integrated here for the first time. In sum, the above-noted studies document anatomical and functional evidence in ALS-PDC that is consistent with prenatal, postnatal and adult exposure to the toxic actions of cycasin/MAM.

5. Other cycad chemicals

As with all plants, cycads such as *C. micronesica* (Guam) and *C. revoluta* (Japan) are chemical factories that synthesize and harbor multiple substances, including non-protein amino acids with neurotoxic potential, notably L-BMAA and β-*N*-oxalylamino-L-alanine (L-BOAA). L-BMAA has been heavily investigated in relation to ALS-PDC, while L-BOAA is a potent glutamate excitotoxin best known for its presence in Grass pea (*Lathyrus sativus*), prolonged consumption of which induces lathyrism, a non-progressive upper motor neuron disorder of humans and animals [199]. Gymnosperms like cycads, as well as modern plants that are globally used for food, also contain β-sitosterol and its corresponding glucoside, both of which have also been proposed to have neurotoxic potential relevant to the etiology of Guam ALS-PDC.

5.1. BSS-BSSG

β-Sitosterol (BSS, 24-ethylcholesterol) is the major phytosterol in higher plants along with its β-D-glucoside (BSSG) [200]; they are abundant in fruits, beans, nuts, seeds and plant-based beverages. While BSS and BSSG possess anti-inflammatory, antipyretic, antineoplastic, radioprotective and immune-modulating properties, they are generally considered to be non-toxic in rodents [201–203], except in mice with inactivated liver X receptor beta which itself leads to the pathological accumulation of sterols and lipids and motor neuron disease [204,205]. Several experimental studies have shown BSS affords significant protection against the deleterious effects of methylating and other agents that induce tumors [206–208]. The therapeutic properties of phytosterols have stimulated a number of controlled clinical trials that demonstrate BSS-BSSG reduces subtle immunosuppression associated with physical stress and promotes urinary flow in prostatic hyperplasia [209–212]. After 6 months of treatment with BSS, a follow-up study at 18 months for symptomatic prostatic hyperplasia found the phytosterol maintained efficacy and produced no major side effects [213].

Phytosterols interfere with multiple cell-signaling pathways, including cell cycle, apoptosis, proliferation, survival, invasion, angiogenesis, metastasis and inflammation [214] that may be useful for the treatment of cancers of the prostate, lung, breast and colon. Two studies have shown that BSS and BSSG induce apoptosis in cancer cells that is associated with unspecified DNA damage without strand breaks [215–217], but plant phytosterols including BSS lacked genotoxic properties in several bacterial, mammalian, and *in vitro* assays [218]. BSS-BSSG inhibited DNA polymerase-β lyase activity that is required to repair apurinic/apyrimidinic sites in the genome [219]. The therapeutic effects of BSS and BSSG might be due to their ability to inhibit repair of oxidative DNA damage by the base-excision repair pathway in cancer cells [220]. BSSG also promotes IGF-1-associated neural stem cell proliferation [221] and stimulates insulin secretion in isolated rat islets [222].

Canadian investigators reported CNS changes attributed to phytosterols in CD-1 mice fed pellets prepared from water-washed cycad flour samples [223]. These mice were considered to have developed an ALS-like syndrome, with loss of motor neurons and an eventual reduction of dopaminergic innervation of the striatum. Young adult Sprague Dawley rats fed washed cycad flour for up to 22 weeks developed locomotor changes, altered dopamine metabolism in dorsal striatum and, post-treatment, α-synuclein aggregates in substantia nigra *pars compacta*; these changes were also attributed to cycad BSS-BSSG [224]. In addition to these motorsystem disorders, cycad seed/flour-fed rats also exhibited extended and abnormal sleep periods early in the course of treatment, with a reduced number of hypothalamic cells positive for orexin, a neuropeptide that regulates arousal, wakefulness and appetite [225]. The Canadian investigators subsequently reported that 6–7-month-old, male CD-1 mice fed BSSG developed olfactory, motor and cognitive dysfunction associated with increased indices of apoptosis and intracellular α-synuclein aggregates appearing first in the olfactory bulb, then the striatum, the substantia nigra and, finally, hippocampal and cortical regions [223,226,227]. However, these results could not be replicated by three cooperating Canadian laboratories that also included the original research group [228]. Male CD and SD rats treated orally from 4 to 5 months of age with pellets with or without 3 mg BSSG failed to develop neurological deficits after 10–11 months. No group differences were observed with respect to gain of body weight, behavioral measures (including open-field and olfactory discrimination tests), motor function, micropositron emission tomography imaging of labeled dopamine terminals, and markers of neuroinflammation in the striatum and substantia nigra *pars compacta*. These negative results, coupled with the uneventful and widespread human therapeutic use of BSSG, call into question the reported neurotoxic properties of BSS-BSSG and their proposed relevance to the etiology of ALS-PDC.

While the induction of a neurological disorder in rodents fed washed cycad flour lends strong support for the hypothesis that cycad chemicals have primary etiologic importance in ALS-PDC, causal attribution to BSS-BSSG is difficult since these phytosterols are components of all plants consumed by humans, and their widespread therapeutic use is without reported adverse effect on the human CNS. According to the Canadian research group, the composition of the washed cycad flour that induced motorsystem disease in rodents "failed to show appreciable amounts of the cycad toxins, cycasin, MAM, or BMAA" by HPLC-MS analysis [223,224,229]. However, the extraction method that was used likely failed to remove all toxic azoxyglycosides present in the gametophyte of cycad seed formerly used by Chamorros to prepare flour. In support, a Guam cycad flour sample [230] that was extracted five times with 70% alcohol contained a low concentrations of cycasin (4.34 mg/g flour) and a substantially higher amount of an unknown β-glucoside that were both susceptible to β-glucoside activity [231]. The analysis of flour samples for L-BMAA, MAM and cycasin content also requires different HPLC conditions [232,233], but only one was reported by the Canadian research group. Washed cycad flour must be subjected to additional analytical procedures (e.g., chromotropic acid, addition of β-glucosidase) to establish absence of azoxyglycosides, especially since some may be 'bound' in cycad plant pulp [231]. For example, the cycad species (*Cycas revoluta*) associated with Kii-Japan ALS-PDC contains neocycasin A (β-laminaribioside of MAM) and neocycasin B (β-gentiobioside of MAM) plus a xylose derivative of macrozamin, which can undergo conversion to cycasin [234–237].

5.2. L-BMAA

β-*N*-Methylamino-L-alanine (L-BMAA) is a non-protein neuroexcitatory amino acid so named by Spencer in 1986 [136] but first isolated in the 1960s by Bell and colleagues from the seed of cycads associated with Guam ALS-PDC [238]. L-BMAA has been the subject of intense research and hundreds of papers which, through 2017, have been critically reviewed [239]. While the amino acid is produced by cyanobacteria, dinoflagellates and diatoms, the proposed endophytic cyanobacterial origin of L-BMAA in cycads [147] has been challenged

[240]. The concentration of L-BMAA in the female gametophyte tissue of the Japanese *C. revoluta* (0.32 mg/g dry weight) is lower than that reported (1 g/g dry weight) in seed of *C. micronesica* collected on Guam. L-BMAA was present in small amounts in flour prepared Chamorro-style from the washed gametophyte of *C. micronesica* [153,241] but, in marked contrast to residual cycasin, its concentration did not correlate with the historical incidence of ALS-PDC [19,65]. Nevertheless, prolonged treatment of adult macaques (*Macaca fascicularis*) with large oral doses of L-BMAA (100-315 mg/kg/day for 2-12 weeks) — which is highly bioavailable in primates [242] — induced an L-dopa-responsive motorsystem disease associated with degenerative or chromatolytic changes in cortical and spinal motor neurons plus brain amylaceous swellings containing structures resembling paired helical filaments and Hirano bodies [137,243]. Moreover, prolonged oral dosing of vervet monkeys (*Chlorocebus sabaeus*) with L-BMAA (210 mg/kg/d for 140 days) produced subclinical brain neurofibrillary tangles and amyloid plaques [244–246]. Neonatal rodents treated with large systemic doses of L-BMAA developed hippocampal damage and impaired learning as adults [247,248]. Especially in post-natal day 3 (PND3) rat pups, a large single subcutaneous dose (400 mg/kg) of L-BMAA induced behavioral deficits and ALS-PDC-relevant neuropathology when the animals were studied at 6 months of age [249]. Taken in concert, controlled animal studies, albeit many lacking the use of a negative control compound such as L-BOAA, demonstrate that large single doses or prolonged lower dosages of L-BMAA can reproduce features of ALS/PDC in rodents and primates, respectively. Since L-BMAA undergoes transplacental transfer and is secreted in maternal milk [250–252], the high susceptibility of the developing rat brain [253,254], albeit to large doses of L-BMAA, is of considerable interest in relationship to the etiology of ALS-PDC.

Free and protein-associated L-BMAA was reported in the brains of Guam ALS-PDC and Canadians with AD [255], but these observations were not confirmed in an independent study of Guam Chamorro P-D and AD brains [256,257]. L-BMAA was also not detectible by LC-MS-MS in the brains of Kii-Japanese subjects with ALS, ALS-PDC or ALS with dementia [258]. Elsewhere, links have been sought between clusters of sporadic ALS and exposure to cyanobacterial L-BMAA in water bodies and/or shellfish. One study identified an ALS cluster surrounding the Thau lagoon in France, the most important area of shellfish production and consumption along the Mediterranean coast, where mussels and oysters contained L-BMAA, especially during summer months [259]. A potential ALS cluster was also described among persons living near a water body with a history of L-BMAA-positive cyanobacterial algal blooms (Lake Mascoma) in New Hampshire, USA [260–262]. Other studies in New England linked poor lake water quality and phycocyanin concentration with increased odds of belonging to an ALS cluster [263,264] with positive association with water sports, particularly water skiing [265]. None of these studies has provided evidence of cause-effect relationships between L-BMAA exposure and ALS. Also not considered in the North American studies is the possible food use of ALS-associated False Morels [266] that harbor MAM-like compounds (hydrazines) and, in New England, these foodstuffs tend to grow on sandy soils near pine trees around lakes [267].

The neurotoxic mechanisms by which L-BMAA induces experimental neurodegenerative disease are not fully understood. While this methylated amino acid is a mixed ionotropic-metabotropic glutamate receptor agonist that induces oxidative stress [268–272], it is difficult to relate these excitotoxic properties to chronic progressive neurodegeneration. Some have suggested L-BMAA is misincorporated into neuroproteins (including synthetic β-amyloid) resulting in protein misfolding [273,274] but cell culture, rodent and primate studies have failed to detect L-BMAA in neuroprotein(s) [275–279]. The controversial proposal that L-serine is misincorporated into CNS protein [239] stimulated study of the effects of L-serine supplementation of L-BMAA-treated vervets and, based on a reported reduction of treatment-induced spinal cord pathology [246], a phase-1 trial of L-serine in patients with ALS proceeded with apparent absence of adverse drug effects [280].

Others have reported the genotoxic potential of L-BMAA to mussel and mouse cells [281–283] and, after nitrosation, to human cells [284]. Nitrosation of L-BMAA (*N*-BMAA) might occur *in vivo* from the reaction with nitrite to produce an alkylating agent with similar DNA-damaging properties to those of MAM. The latter mechanism might explain how L-BMAA induced cell-cycle dysregulation and heritable changes in embryonic rat striatal neurons [285]. While millimolar concentrations of L-BMAA induced apoptosis, much lower concentrations (50–100 μM) reduced the differentiation of rat striatal neural stem cells (NSCs) into astrocytes, oligodendrocytes and neurons, and altered the morphology of neurons [285]. In addition, L-BMAA did not alter the cell cycle of corresponding post-mitotic striatal neurons, but it did reduce the percentage of NSCs in $G_{0/1}$ phase and increased the percentage of those in $G_{2/M}$ phase. The effect of BMAA on the NSC cell cycle was heritable (D1 and D2 daughter cells) indicating that L-BMAA can induce permanent cell-cycle changes. Such changes in daughter NSCs did not reduce their viability, an observation consistent with results from previous *in vitro* and *in vivo* studies when neurons are forced into cell-cycle reentry [286,287]. At comparable concentrations (16–128 μM), L-BMAA induced genomic instability in human peripheral blood cells without production of DNA strand breaks or oxidative stress [283]. In contrast, higher concentrations of L-BMAA (>500 μM) induced both oxidative stress and DNA strand breaks in mixed cultures of human neurons and glia [288] or murine neural stem cells [282], suggesting that lower concentrations of the methylated amino acid disrupt the cell cycle and induce genotoxicity by a different mechanism, possibly by production of a nitrosylated derivative [284]. These observations are consistent with the notion that L-BMAA induces cell-cycle changes in neurons comparable to those observed in the brains of Guam PDC patients and related tauopathies. Such changes could be related to the genotoxic effects of L-BMAA [282–284], especially following early-life exposure to low concentrations of the cycad toxin. These same properties are shared by the principal cycad genotoxin MAM [289,290]. Additional studies are required to sort out the relative importance of L-BMAA- and MAM-induced cell-cycle changes to those in ALS-PDC [see Supplement].

6. Mechanisms of cycad toxicity

6.1. Genomic instability

While the roles of L-BMAA, MAM, or their metabolites are individually, together, or with other factors, plausibly responsible for triggering ALS-PDC, it appears highly probable that this occurs primarily through the induction of genomic instability. There is wide acceptance that MAM is a potent genotoxin that induces alkyl and oxidative DNA lesions (O^6-mG, N7-mG, 8-oxoG) in many murine organs, including the brain [166,180,290–292]. MAM induces oxidative DNA damage most likely via hydroxy radicals that form during autooxidation in the presence of metals such as iron [293] or by inhibiting antioxidant enzymes [165]. While both occur in cancer and neurodegenerative diseases, interest focuses on O^6-mG and 8-oxoG DNA lesions because they are pro-mutagenic for cycling cells and appear to be pro-cytotoxic in non-cycling cells, notably neurons [294,295]. Human and murine O^6-mG lesions are subject to direct repair by O^6-mG methyltransferase (MGMT) [296]. Failure to repair O^6-mG lesions in cells undergoing division increases risk of mispairing with thymine during DNA replication, resulting in GC➔AT transitions and frameshift mutations in bacteria [297]. MGMT enzyme activity is especially required during cell division but, in post-mitotic nerve cells, activity appears to be very low such that neurons should be highly susceptible to MAM. Given the low capacity of brain vs. liver tissue to repair O^6-mG lesions [298–300], repeated exposure to alkylating agents such as MAM would result in mounting DNA damage and genomic instability [157,290]. This may explain why proteins involved in DNA repair, such as TDP-43, accumulate over time. Even cells with mitotic potential, such as renal cells, accumulate MAM-induced pro-mutagenic/carcinogenic O^6-mG lesions because transcriptional activation of *Mgmt* or any other DNA-

repair enzymes fails to occur [301], perhaps because the MAM metabolite formaldehyde epigenetically silences *Mgmt* expression [302,303]. Oxidative DNA damage has also been observed in the liver and colon of rodents following administration of the MAM precursors 1,2 dimethylhydrazine (DMH) and azoxymethane [180]. 8-oxoG levels were elevated dose-dependently in the colon of young rats (~1 month old) 24 h after administration of a single subcutaneous injection of DMH (25 m/kg, 50 mg/kg, 100 mg/kg) [180]. 8-oxoG levels were also elevated in the colon of adult mice after twice weekly intraperitoneal injections of azoxymethane (25 mg/kg) [292]. These studies demonstrate that MAM indirectly induces oxidative DNA damage in non-neural tissues through a transition metal-catalyzed mechanism [293] or by reducing antioxidant enzymes [165]. Such mechanisms may explain the elevated levels of 8-oxoG detected in the prefrontal cortex of the MAMac animal model of schizophrenia [166].

6.2. Cell-cycle changes

Although neurons undergo terminal differentiation by withdrawing from the cell cycle to remain quiescent in the G_0 phase [304], aberrant cell-cycle activation of post-mitotic neurons is a key molecular mechanism in AD and other human neurodegenerative disorders and animal models thereof [[304–308], Supplement]. Remarkably, cell-cycle events can be maintained [307] *in vivo* in affected neurons for weeks to years before apoptosis (regulated by the E3 ubiquitin ligase Itch), such that activation of the DNA-damage response might be able to hold cell cycle-induced death (apoptosis) in check for prolonged periods [309,310]. The Guam ALS-PDC brain shows evidence of cell-cycle reactivation of neurons with tau pathology destined for degeneration [35,36,311]. The MAMac animal model [312–314] is also characterized by latent cell-cycle changes [315] and epigenetic changes in the rat hippocampus [316–319]. MAM disrupts the cell cycle presumably by inducing DNA damage via methylation of guanine (i.e., N-7 methyl or O^6-methyl) that inhibits DNA duplication during S phase [320] and disrupts neuroepithelial cells undergoing their final mitosis [321]. Some of the early changes induced by MAMac in somatic cells include nucleoprotein structural alterations, mitotic abnormalities, and induction of polyploidy [322] as well as retinoblastoma (*Rb*) gene mutations, which lead to the development of intraocular neoplasms [323,324]. Expression of the retinoblastoma gene is also altered in the prefrontal cortex of rats treated developmentally with MAMac [315] and in human neuroprogenitor cells (hNPCs) after acute treatment (24 h) with the genotoxin [325]. Studies with hNPCs indicate that the MAMac-induced cell-cycle changes are triggered by DNA methylation (Supplement Fig. S1), a finding consistent with the DNA methylation changes reported in the GD17 MAMac animal model of schizophrenia [312,316,326,327], which may occur before or after the appearance of motor systems in sporadic ALS and ALS-PDC (see Supplement Section 2.1). In addition to DNA methylation, histone deacetylase (HDAC I & II) activity was also reduced by MAMac in hNPCs (Supplement Fig. S2). The DNA methylation and HDAC changes observed in these recent *in vivo* and *in vitro* studies with MAMac suggest that the cycad genotoxin MAM, in contrast to L-BMAA, induces early epigenetic changes that coincide with both the DNA damage and cell-cycle changes.

Whether the MAM-induced DNA damage and/or epigenetic changes are the initial event(s) that trigger the cell-cycle changes is presently unknown, but conditional deletion of HDAC1 in transgenic mice suggests that epigenetic dysregulation (i.e., HDAC1 deficiency) is the initial event because it reduced DNA repair that led to the accumulation of DNA damage at the promoters of susceptible genes and their transcriptional repression [328]. Moreover, the 5xFAD transgenic mouse model of AD also exhibits impaired HDAC1 activity, elevated oxidative DNA damage and significant overlap with the genes downregulated in aged HDAC1 cKO mice with T cell-specific deletion of HDAC1. Since the repair of MAM-induced alkylated DNA damage is also regulated by HDAC [329] and the promoters of genes that contain guanine-rich sequences were targeted in HDAC1 cKO mice, similar genes might be equally targeted by MAM-induced DNA alkylation damage. This notion is consistent with the increased HDAC levels recently reported in the hippocampus of 20-week-old rats following prenatal (GD13) MAMac treatment [319], the reduced HDAC activity of MAMac-treated hNPCs and the ability of alkylated and oxidative DNA lesions to prevent the binding of transcription factors to their consensus sequences [289]. These studies suggest that MAM induces early and persistent epigenetic changes that could reduce the ability of the brain to repair alkylation or oxidative DNA damage, which would impair transcription of genes in cell-cycle and other neuronal pathways.

6.3. MAM Perturbs Brain UPP and Tau

Transcriptional mutagenesis has been proposed as the underlying mechanism for MAM neurotoxicity [289] and for the ubiquitin-proteasome pathway (UPP) dysfunction in Guam and Kii ALS-PDC brains [42]. Since MAM induces frameshift mutations in mammalian and other cells [297], this mutagen might plausibly induce frameshift mutations in ubiquitin (UBB) to produce the mutant UPP inhibitor UBB^{+1}. MAMac treatment of neonatal mice persistently perturbs the brain UPP and changes the expression of brain proteins that are also abnormally expressed in ALS-PDC [330]. The cerebellum of mice examined 1–19 days following a single subcutaneous dose of MAMac administered on PND3 showed persistent DNA damage, evidence of DNA strand breaks and, at 22 days, immunocytochemical evidence of 3R tau accumulation. Among the plethora of transcriptional changes, approximately 60% of UPP-related genes in the mouse cerebellum were dysregulated (mostly downregulated) relative to the cerebellum of control animals treated with saline [331]. MAMac treatment of M17 human neuroblastoma cells also resulted in a concentration-dependent increase in levels of 3R tau and phosphotau (AT8) [332], which compares with the cerebellar tau pathology in Kii ALS/PDC [38]. MAMac also induced DNA-damage-associated increased tau expression in rat neuronal cultures [167]. Previous studies have shown that the protein expression of tau, α-synuclein and ubiquitin is also altered in the brains of MAM-treated mice [333] and Guam ALS/PDC [334].

6.4. Timing of toxin exposure

Prenatal administration of MAMac to timed-pregnant rats (GD17) alters the modification of histone proteins during postnatal life; notably, the methylation (i.e., H3K4me3, H3K9me3, H3K27me3) and acetylation (i.e., H3K9ac) of histones are decreased in the prefrontal cortex of adults [317,335,336]. Histone dimethylation (H3Kme2) was significantly reduced in the prefrontal cortex of MAMac-treated PND15 and PND45 rats [335] whereas histone trimethylation and histone acetylation were reduced in adulthood (PND60 and 70), and the latter changes were associated with the decreased expression of glutamic acid decarboxylase (Gad1) in adulthood [336]. Abnormal histone-mediated epigenetic silencing of the *Grin2b* gene was associated with *N*-methyl-D-aspartate receptor hypofunction in the premotor cortex of juvenile rats treated gestationally on day E17 with MAMac, changes that may be related to cognitive impairment [317]. Perhaps a related epigenetic mechanism explains how gestational exposure to MAMac in the E17 rat induces postnatal, age-dependent GSK3β hyperactivity associated with significant reduction in dendritic spines, deficient long-term potentiation and facilitation of long-term depression in prefrontal cortex pyramidal neurons, together with working memory deficits [337]. Thus, histone methylation is an early event whereas histone acetylation is a late event following *in utero* treatment with MAMac.

The critical conclusion from these recent rodent studies is that time-specific *in utero* exposure to MAMac induces persistent changes in brain molecular function, including overexpression of tau kinase (GSK3β), that surfaces clinically during later life in the form of neurofibrillary degeneration [318]. Importantly, the epigenetic vulnerability of the developing rodent brain to MAMac, which spans other molecular

pathways such as the GABA transcriptome [338], are potentially heritable for generations. This principle has been demonstrated by the presence of aberrant methylation of transcription factor *Sp5,* coupled with enhanced dopamine neuron activity in the ventral tegmentum area in second (F2) and third (F3) filial generations of embryonic F1 rats treated with MAMac on E17 [316]. This study also raised the possibility of genetic alterations in E17 gonocytes of male or female embryos that contributed to the abnormal adult brain phenotype. Indeed, it has long been recognized that MAMac induces genetic alterations in various test systems in bacteria, yeast, plants, *Drosophila melanogaster*, and mammalian cells [339]. Moreover, adolescent F2 rats were more susceptible than controls to chemical (cannabinoid) challenge, an example of gene-environment interactions responsible for brain dysfunction [340]. In sum, the brain DNA damage and persistent epigenomic modifications produced by MAMac appear to modulate the expression of genes that regulate the cell cycle, neurodevelopment and contribute to neurodegeneration [331]. Based on recent studies with human neuroprogenitor cells described in the Supplement, there is no reason to expect a different outcome from human exposure to cycasin/MAM during a comparable critical period *in utero*, namely the second/third trimester when we propose cerebellar and retinal dysplasia also occurs.

There is also strong evidence that the neonatal rodent brain is sensitive to L-BMAA. A single subcutaneous injection rat pups with L-BMAA (50 and 200 mg/kg) on PND 9–10 produced deficits in spatial learning and memory in adult animals; a larger dose (600/mg) induced rapid neuronal cell death in the hippocampus and, to a lesser degree, in the retrosplenial and cingulate cortices [341]. Rats injected with 400 mg/kg L-BMAA on PND3–10 (especially PND 3) later developed behavioral and cognitive deficits, gait and postural abnormalities, with reduced neuronal density in the CA1 and CA3 regions of the hippocampus, dentate gyrus, caudate, putamen and anterior horn of the spinal cord, coupled with brain deposits of β-amyloid, α-synuclein and TDP-43 [254]. The rodent brain at PND1–3, which corresponds to 23–32 weeks of human fetal gestation, undergoes oligodendrocyte maturation, immune system development and establishment of the blood-brain barrier. A brain growth spurt peaks at PND7 (36–40 week human fetus) and, by PND21 (2–3 year-old infant), the brain has 90–95% of its adult weight, peak synaptic density and peak rate of myelination [342,343]. In sum, therefore, the developing rodent (PND3) appears to be most sensitive to L-BMAA at a time corresponding to the third human trimester (28–40 weeks), the reason for which has yet to be determined,

6.5. Molecular pathogenesis of ALS-PDC

MAMac reproducibly induces brain maldevelopment via DNA damage [187] and epigenetic mechanisms [316,335,336], but how this genotoxin induces chronic neurodegenerative disease is not understood. One possibility is that MAM perturbs the brain insulin-signaling pathway, as do the chemically related genotoxins streptozotocin (STZ) [344] and nitrosamines [345]. STZ, a glucosamine of *N*-methylnitrosourea, induces a rodent model of dementia characterized by progressive deterioration of memory, energy metabolism [346], and tau pathology [347,348]. The tau pathology in the STZ rodent model of dementia is preceded by early changes in the phosphatidylinositol-3-kinase (i.e., PI3K, phospho-Akt, GSK3β) insulin/IGF-signaling pathway [349]. STZ-induced hyperglycemia also triggered cell-cycle changes (e.g., cyclin D1) in the hippocampus of young adult mice (4-month old) following an intraperitoneal injection of the genotoxin [350].MAMac also significantly altered ($p < 0.05$) brain tissue levels of PI3K (phosphatidylinositol 3-kinase), pAkt (phosphorylated serine/threonine kinase Akt), and GSK3β (activated glycogen synthetase kinase 3β) in 90-day-old htau mice after neonatal animals (PND3) were administered a single injection of the cycad toxin [351]. Whether MAMac, like STZ, can produce an animal model of dementia when given by intracerebroventricular injection should be explored, as should the question of whether the associated pathological and behavioral changes are tied to the accumulation of DNA lesions and/or epigenetic changes (e.g., DNA methylation, modified histones).

A second possibility is that the persistent brain DNA lesions induced by MAM trigger transcriptional mutagenesis, a mechanism shown to produce abnormal proteins and transcripts following the placement of DNA lesions in the coding regions of genes [352,353]. In cells where repair of O^6-mG DNA lesions was reduced by the MGMT inhibitor O^6-benzylguanine (BG), approximately half of the transcripts contained a uridine misincorporation opposite the DNA lesion, and this was associated with changes in protein function. Recent studies also showed that BG increases O^6-mG DNA lesions in human neural stem cells (G.K., unpublished data), which might explain the latent effect of MAMac on the development of human neuroprogenitor cells into cortical neurons [325]. These MAM-induced effects on developing human neural cells were also associated with changes in the DNA methylation of genes involved in the differentiation of human neurons. Additional studies with human neural stem cells could provide clues as to the influence of DNA lesions and epigenetic changes that might contribute to the neurodegenerative changes observed in ALS-PDC. The recent development of human neural stem cells, neuroprogenitors, neurons and astrocytes from both healthy and PDC subjects from Guam [325,354] offers the opportunity to determine the underlying differences between neural cells of diseased patients and further assess the role of cycad toxins in ALS-PDC.

Lastly, DNA lesions may also persist in the promoter of genes (i.e., transcriptional disruption) resulting in altered expression of the associated proteins. Bonfanti and colleagues [355] showed that O^6-mG, one of the DNA lesions produced by MAMac, inhibited the binding of transcription factors to their cognate DNA sequences in a position-dependent manner. The O^6-mG lesion blocked DNA binding of NF-kB, Sp1 and SRF (*see* Keywords). Binding of NF-kB to its cognate sequence was also disrupted in a position-dependent manner by the presence of O^6-mG or 8-oxoG in DNA [290]. The importance of these observations is that DNA lesions within the promoter (vs. the coding) regions of genes may produce persistent up- or down-regulation of gene expression that is dependent upon both the sequence location and type of lesion. This may explain why NF-kB was a prominent hub in the brains of MAMac-treated mice, and why low doses of MAMac disrupted the expression of genes enriched in differentially methylated regions that regulate the differentiation of human neuroprogenitor cells into cortical neurons [325].

7. Relevance to other neurodegenerative disorders

Whether any genetic modifications arise as a result of exposure to cycad toxins has not been assessed, but the possibility certainly exists. Beyond Western Pacific ALS-PDC, the foregoing discussion is relevant to ALS with frontotemporal dementia (ALS-FTD), with which it has been compared [356,357]. Hexanucleotide expansion of *C9orf72* is found in 50–70% of familial and 15–20% of sporadic ALS-FTD cases [358,359] and has also been linked to atypical parkinsonian syndromes, including: Corticobasal Syndrome, Progressive Supranuclear Palsy (PSP), and Multiple System Atrophy [360], as has AD [361]. Based on the present examination of the link between MAM and ALS-PDC, it is logical to explore whether related neurodegenerative disorders associated with nucleotide repeats arise from the effects of exposure to environmental mutagens that interfere with DNA repair. Indeed, nitrosamines and hydrazines, which are related to MAM by chemistry and molecular mechanism, have been considered elsewhere for possible etiological links with ALS, PSP and AD [289,362].

The proposed genotoxic origin of ALS-PDC is broadly consistent with the suggested role of (mostly oxidative) DNA damage, cell-cycle dysregulation and abnormalities of DNA repair in related diseases, including ALS [156,363–370] and other neurodegenerative and psychiatric disorders [371,372]. This includes schizophrenia although there was no significant link with the later onset of ALS in an unbiased hospital record linkage study [373]. The genotoxic properties of cycad toxins suggest

relationships between Western Pacific ALS-PDC and the genesis of carcinogenicity, notably colon cancer [285,289]. Links between ALS and cancer have been reviewed recently [374] although an earlier record linkage study failed to find an association [375]. With respect to the association between brain pathology and skin abnormalities in ALS-PDC, it is noteworthy that multisystem proteinopathy may be linked with ALS, frontotemporal dementia, and Paget's disease [376]. In regard to type-2-diabetes mellitus, both positive and negative associations with ALS risk have been made [377–382]. These topics are addressed in the Supplement.

8. Conclusion

The foregoing has highlighted evidence supporting the proposal that cycad-derived MAM is the primary etiologic agent in Western Pacific ALS-PDC acting through a DNA-damage and epigenetic mechanism that subsequently leads to cell-cycle disturbances and associated pathological changes. The minor cycad toxin L-BMAA, together with the as-yet undefined endogenous molecules that MAM methylates, likely contributes to disease etiology based on the responses of rodents and primates that resemble clinical and neuropathological aspects of ALS-PDC. The chemistry and molecular mechanisms that MAM shares with those of nitrosamines and hydrazines have been discussed elsewhere in relation to possible roles for these widely distributed compounds in the etiology of sporadic forms of ALS, PSP and AD [289,362].

The action of MAM on the fetal nervous system results in persistent anatomical perturbations of the developing cerebellum and retina that appear directly relevant to the subclinical abnormalities described in cases of ALS-PDC on Guam and in Kii-Japan. Ectopic neurons are also found in the hippocampus of postnatal rats treated with MAMac [383]. Whether pathological changes in olfaction, skin and other organs in ALS-PDC are acquired before or after birth, they are also plausibly related to MAM exposure, as discussed in the Supplement. While this information is longstanding, it has not been appreciated before in large part because there has been insufficient communication between clinical and experimental scientists. Further, while scientists focused on cancer mechanisms have long understood the DNA-damaging effects of MAM and related genotoxicants, the significance of this property in perturbing the development and long-term maintenance of brain cells has not been widely appreciated. The neurology community has been focused on the genetic and infectious causes of neurodegenerative diseases while giving sparse attention to the role of exposure to potent environmental chemicals that act as slow toxins, a term first used >30 years ago to describe the putative role of cycad chemical(s) in ALS-PDC [137]. The fact that embryonic exposure to MAMac induces a reliable animal model of schizophrenia (*see* Supplement), demonstrates the genotoxic actions on the developing brain are, like many genetic disorders, delayed in their clinical expression. Similarly, epidemiological studies have shown that periods exceeding half a century may intervene between exposure to high-risk environments and the clinical onset of ALS-PDC. This observation directly challenges the statement that chemicals with neurotoxic potential do not induce long-latency disease and thus cannot be responsible for ALS-PDC [384]. Clearly, the search for environmental risk factors for disorders related to ALS-PDC must take into consideration early-life exposures as possible etiological factors [385]. Significant exposure to chemicals that induce a DNA-damage response in neurons merit the highest level of suspicion.

The assembled evidence raises the possibility that some cases of ALS-PDC may have a partly fetal origin arising from maternal exposure to cycad genotoxins, while others are traceable to exposure as infants, juveniles or adults. The human fetus in the second trimester is especially vulnerable to chemicals, which is consistent with the ability of MAMac to trigger cerebellar dysplasia, retinal dysplasia and a schizophrenia-like illness, all of which are described in ALS-PDC as discussed in the Supplement. Since ingested cycasin gives rise to MAM which, like L-BMAA, crosses the placenta [247,386], the fetus of a pregnant Guamanian or Japanese woman exposed orally to cycasin in food (Guam) or medicine/tonic (Kii-Japan), would have an increased risk for the development of CNS abnormalities, the hallmarks of which would persist throughout life. MAM and L-BMAA enter mother's milk [250,252,387], which would result in exposure of infants to these genotoxins. With acculturation to modernity, the declining use of cycad seed that resulted in exposure to cycad genotoxins would account for the progressive reduction of ALS-PDC in all three geographic foci of the disease.

Whether fetal exposure to cycasin can trigger a cascade of molecular events that culminate in ALS-PDC is unknown, but the epidemiology and long-latency between exposure and clinical onset makes clear that this disease was sometimes acquired in infancy/childhood (Kii-Japan) or adolescence (Guam) [61,65,388]. Also unknown is whether the timing or the accumulated dose of cycad genotoxins determines the delayed development of subclinical neurofibrillary pathology, clinical dementia, atypical parkinsonism or ALS. Elsewhere, we have suggested these represent four points on a increasing dose-response continuum, which accounts for why ALS generally affects younger subjects while dementia is mostly confined to the elderly [137]. Viewed from this perspective, ALS would have resulted from high doses of cycad toxin(s) that induced fatal motor neuron disease at a relatively early age while lower levels of exposure spared motor neurons, thereby allowing longer survival periods during which other phenotypes had time to develop and surface. The response of Guam-derived neural stem cells to cycad toxins at different stages of neuronal development might provide important insight as to timing and vulnerability to genotoxin exposure.

While the purpose of this review is to examine the pre-eminent role of environmental factors in the genesis of Western Pacific ALS-PDC, common exposure to which across generations has produced familial as well as sporadic examples of the disease, some authors have continued to insist on the role of an heritable component even though the disorder has all but disappeared. Given the genotoxic and epigenotoxic potential of MAM and formaldehyde, a common metabolite of both MAM and L-BMAA [290,389], systemic exposure to such compounds could potentially result in genetic and/or epigenetic changes, whether heritable or not. In addition to the carcinogenic and neurotoxic risks of overexposure to formaldehyde, it is also a physiological molecule that functions in association with the one-carbon cycle. Concentrations of endogenous formaldehyde are increased in the plasma of sporadic ALS [390], in the AD brain and primate models [391,392], the subject of which has been extensively discussed in relation to ALS-PDC [295]. Formaldehyde and epigenetics in AD is the subject of a recent paper [393]. Of note, occupational exposure to exogenous formaldehyde is reportedly a risk factor for ALS in Europe and the USA [394,395] and use of cigarettes, the smoke of which contains substantial amounts of formaldehyde and MAM-related nitrosamines [396,397], has been linked to increased risk for sporadic ALS [368,398–400], the pathogenesis of which has been proposed to be a multi-step process analogous to that of cancer [401]. While smoking rates among Chamorros traditionally have been high [402], a potential link with ALS-PDC in Guam or elsewhere has not been explored.

We conclude that the cycad chemicals MAM and L-BMAA are incriminated in the etiology of ALS-PDC. MAM, the common aglycone of cycad azoxyglycosides, is a major and perhaps principal player. MAM produces persistent DNA damage that perturbs cell-cycle control and probably drives neurons to attempt cell division, which fails, activates apoptosis, and culminates in cell death. While MAM is both neurotoxic and carcinogenic for organs such as the colon, BSS-BSSG is non-neurotoxic and a colon chemopreventive agent through its influence on the cell cycle [403]. A role for minerals in the etiopathogenesis of ALS-PDC is uncertain, but it is noteworthy that L-BMAA chelates divalent metal ions, notably Cu^{2+}, Zn^{2+} [404,405], which conceivably could perturb the enzyme activity of Cu-Zn superoxide dismutase, mutations of which underlie genetic forms of ALS [406] and protein misfolding has been reported in sporadic ALS, Parkinson's disease and supranuclear palsy [407].

Decades of study of Western Pacific ALS-PDC have provided important lessons for the investigation of related neurodegenerative disorders worldwide:

- geographic clusters (including conjugal cases) are unique opportunities for etiologic discovery;
- isolated clusters of the same neurodegenerative disease likely have a common etiology;
- disease phenotype may evolve clinically from young-onset ALS to late-life dementia;
- familial (as well as sporadic) disease can have a primary environmental etiology;
- exposure assessment should straddle the period from conception to symptom onset;
- etiologic clues are most likely to arise from intense study of young-onset cases;
- open-ended interviews as well as epidemiologic instruments generate testable hypotheses;
- experimental animal studies are critically important to test suspect environmental agents;
- knowledge of environmental etiology of disease can be used for primary disease prevention.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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SUPPLEMENTARY TEXT

1. ALS-PDC and MAM Developmental and Adult Abnormalities

There is evidence of subclinical and systemic disease in the Western Pacific Amyotrophic Lateral Sclerosis and Parkinsonism-Dementia Complex (ALS-PDC) that is consistent with the known effects of cycasin and/or its active metabolite methylazoxymethanol (MAM) in humans and/or animals (Supplemental Table 1).

Subclinical cerebellar and retinal pathology consistent with *in utero* second-trimester exposure to the cycad genotoxins is found in some cases of Guam and Kii-Japan ALS-PDC. Changes in mental function (schizophrenia) that precede and/or accompany the clinical appearance of motor dysfunction in sporadic ALS and Kii-ALS may also suggest early exposure to MAM, which is widely used to induce an established animal model of schizophrenia. Guam, Kii and sporadic ALS exhibit abnormalities of skin suggestive of spontaneous regenerative changes, an effect of cycasin/MAM that was used traditionally as a poultice of cycad seed pulp to promote skin repair in Guam and Papua-Indonesia communities impacted by ALS-PDC. Bone and skin abnormalities may be related phenomena. Other links with varying strength between ALS-PDC and cycasin/MAM include changes in olfaction, diabetes mellitus, birth defects, liver disease and cancer risk. MAMac induces epigenetic and cell-cycle changes in human neuroprogenitor cells that are also consistent with cell-cycle perturbation of neurons in ALS-PDC-related neurodegenerative disorders, namely ALS and Alzheimer disease (AD).

1.1. Cerebellum

The brains of some Japanese and many Guamanian subjects with ALS-PDC have multinucleated and ectopic Purkinje-like neurons in the cerebellum, with comparable developmental abnormalities of vestibular nuclei, occipital gyri and other areas of the brain [S1-3]. These long-neglected findings were confirmed in a recently published Japanese study of the cerebellum of 10 male and female Kii ALS-PDC subjects (aged 63-77 years) [S4]. Cases, but not age-matched controls, had plentiful cerebellar tau pathology together with dislocated and multinucleated Purkinje cells in the molecular layer and pathological changes in the dentate nucleus and cerebellum, the latter characterized by 3R and 4R tau pathology.

Comparable cerebellar dysplasia developed in early postnatal rodents following a single intraperitoneal injection of MAMac [S4-S18]. MAMac disrupted cell division and migration that resulted in tissue disorganization featured by ectopic and misplaced Purkinje and granule cells [S14, S19]. Neonatal administration of MAMac perturbed cerebellar development in rodents such that, at 21 days of age, granule cells were mixed with Purkinje neurons instead of forming layers [S14,S20]. Ectopic neurons were also found in the hippocampus of neonatal rats following administration of

MAMac during fetal development [S21]. Based on human cerebellar development [S22], migrating granule and Purkinje cells would be at risk for MAM-induced disruption from the human second trimester onwards.

The neurotoxic property of MAM involves the formation of O^6-methylguanine (O^6-mG) DNA lesions that are primarily repaired by O^6-mG methyltransferase (MGMT), an enzyme that is especially low in brain tissue such that these DNA lesions accumulate and persist [S23-S26]. MGMT transfers the methyl group from O^6-mG to a cysteine residue in its active site, and the enzyme is subsequently targeted for ubiquitin-related degradation [S27]. MAMac perturbs several brain protein networks, including transport (e.g., α-synuclein), cytoskeletal (e.g., β-tubulin, vimentin), and mitochondrial (e.g., Atp5b) proteins [S28]. Whereas transgenic mice lacking MGMT show increased susceptibility to MAMac, mice that overexpress MGMT are markedly protected from MAMac-induced cerebellar damage [S13]. These studies suggest that MAMac-induced DNA damage (i.e., O^6-mG, N7-mG) plays an important role in the neuropathological and behavioral changes observed in rodents.

1.2. Retina

Linear or vermiform retinal tracts were found in 53% (n=38 of 72) of Guamanians with ALS-PDC and 16% (n=85 of 531) of neurologically normal Guam subjects [S29-S31]. Described by North American investigators as Guam Linear Retinal Pigment Epitheliopathy (LRPE), this condition was thought to resemble ophthalmomyiasis interna [S31], which results from the parasitic activity of an intraocular botfly larva [S34, S35]. LRPE occurred in one or both eyes without visual impairment, remained unchanged for up to 24 years, and predicted and preceded the clinical onset of ALS-PDC [R33]. Steele [S32] followed 17 of 28 Chamorro subjects (34-65 years old) diagnosed with LRPE, 16 of whom were neurologically asymptomatic; 10 developed P-D 3 to 22 years after the retinal tracks had been recognized. A similar retinopathy was described in 4 of 12 male and female Kii ALS-PDC patients (mean age 64.3 years) and in one of 115 healthy residents (aged 20-89 years) [S36-38].

The retinal tracks seen in Guamanian and Japanese subjects with and without ALS-PDC may result from persistent focal or multifocal disorganization of the sensory retina due to developmental perturbation probably arising from *in utero* exposure to cycad toxins, notably MAM. MAMac methylates nucleic acids and proteins *in vivo* and thereby kills rapidly dividing neuroblasts [S39, S40], perhaps during their terminal cell division [S41,S42]. MAMac also modulates gene expression associated with melanogenesis in mouse brain [S43]. The experimental response to a single systemic dose of cycasin or MAMac is critically dependent on the stage of embryonic developmental, as well as the species and dosage [S44-S48]. The rat retina is susceptible to systemic MAMac between days E17 and PE5. The neuroblastic layer folds to produce rosettes that are tubular in cross-section and which persist as retinal tracts in adult life [S46]. Thus, systemic exposure to MAMac during certain

critical perinatal periods results in permanent dysplasia of retinal cytoarchitecture [S42]. This is consistent with the persistence of clinically insignificant retinal tracts in Guam and Kii-Japanese subjects. Studies are needed to determine whether retinal and cerebellar dysplasia coexist in ALS-PDC, as observed in animals treated perinatally with MAMac [S9,S47]. Since the retina of the rat at birth is equivalent in developmental stage to the human retina at 4–5 months of gestation [S49], this implies that human retinal dysplasia, as with cerebellar dysplasia, would also result from fetal MAM exposure during the second to third trimester.

1.3. Skin and Bone

Western Pacific ALS-PDC is a disease that extends beyond the nervous system and includes abnormalities of connective tissue. Dermatological changes in Guam ALS [S50] and Kii ALS [S51] reproduce those reported in sporadic ALS [S52-S54]. Collagen fibrils of unusually small diameter, together with increased amorphous material, are correlated with a sluggish return of stretched skin and resistance to bed sores. Japanese investigators concluded that cross-linking of skin collagen is affected in ALS, such that the pathway of skin collagen runs counter to normal aging, resulting in a "rejuvenation" of skin collagen [S55]. Noteworthy is that some of the pathological changes in the CNS of subjects with sporadic ALS may be mirrored in the skin, including the presence of epidermal TDP-43+ cells and FUS (fused in sarcoma) immunoreactivity [S56,S57], both of which are related to DNA damage control/repair.

Spencer [S58] reviewed the possible relationship between connective tissue abnormalities in Guamanians, their propensity to ALS-PDC, and their exposure to cycad toxins in food and medicine. In addition to an atypical skin in Guam ALS, there were reductions in cortical bone mass and subtle abnormalities in calcium and vitamin D metabolism in Guam ALS-PDC subjects [S59,S60]. However, the mean skull thickness of Chamorros was higher in females (but not males) who died with ALS or PDC compared with controls [S61]. The world's highest incidence of diaphyseal aclasis (multiple exostoses), a disorder featured by benign bony tumors (mostly of long bones) appearing in childhood (4-8 years of age), was reported among Guam Chamorros in 1958-59 [S62,S63] but never studied in relation to ALS-PDC. Cervical spondylosis was found in 30 Kii-ALS patients (48%), lumbar spondylosis in 7 (13%), ossification of the posterior longitudinal ligament in 4 (6.3%), and ossification of the yellow ligament in 4 (6.3%) [S64,S65].

Bone and skin disorders are reported in animals treated with cycad toxins. Dastur [S66,S67] found skin atrophy and collagen degeneration in a single young monkey with Betz cell and anterior horn cell degeneration associated with severe weakness and wasting of one arm that appeared after several months on a diet of cycad flour prepared Chamorro-style. Animals grazing on young cycad leaves or given cycasin develop progressive hindlimb weakness and cattle lose their bone-keratin horns and

keratinaceous hooves in the manner of a molt, in which dermal regeneration is active [S69-S70]. To induce more rapid skin repair and prevent infection, crushed cycad seed was traditionally used to treat tropical ulcers or pressed into open skin wounds in communities hyperendemic for ALS-PDC in Guam and Papua-Indonesia, respectively [S71,S72, *see also* 36:37 min:sec at https://vimeo.com/1621281]. Mammalian skin contains β-glucosidase that converts cycasin to its metabolite MAM [S73]. Taken in concert, these disparate observations suggest that certain cycad toxin(s) can induce marked changes in connective tissues.

MAMac modulates the canonical Wnt signaling pathway, which has a pivotal role in the regulation of cortical bone mass, with pathway activation in bone regeneration [S74, S75]. Wnt proteins (Wnt5a) regulate epidermal differentiation in adult skin [S76,S77]. MAMac also upregulates the *Acvr1b* gene, a member of the transforming-growth-factor-β family linked to skin epithelial cell proliferation and hair development [S43,S78]. While the molecular mechanism has not been established, MAM is chemically related to hydrazines and hydrazides that inhibit lysyl oxidase [S79, S80], a family of proteins that oxidize lysine residues in collagen and elastin, establish stable cross-linking of the extracellular matrix, and provide tensile strength and structural integrity to skin and bone [S81]. Inhibition of lysyl oxidase expression or enzyme activity triggers connective tissue disorders (osteolathyrism) and fibrotic diseases [S82]. Lathyrogens exert their maximal effect in immature animals where they can induce the formation of abnormally thin collagen fibrils [S80,S83], as described in Guam ALS-PDC and sporadic ALS.

1.4. Congenital Defects

Information on birth defects on Guam is sparse. In the 1950s, of children born to 17 women with ALS, two aged 41 and 37 years bore infants with anencephaly, and cleft palate and harelip, respectively [S84]. In the 1980s, Chamorro women stated that spina bifida was not uncommon [S85]. From 1970–1989, there were 121 infant deaths among 49,841 live births on Guam due to congenital anomalies including congenital heart disease, and diaphragmatic hernia, and anencephaly [S86]. Attribution of these birth defects to Agent Orange exposure was vigorously contested [S87]. No information on congenital abnormalities has been found for Kii-ALS/PDC.

Cycasin and MAMac cross the placental barrier, and MAMac has neuroteratogenic potential in several mammalian species [S44, S88]. A single intravenous injection of MAMac (20-23 mg/kg) to pregnant hamsters on the day 8 of gestation variably resulted in neural tube defects, hydrocephalus, microcephalus, cranioschisis, exencephaly, spina bifida, rachischisis, anophthalmia, microophthalmia, and oligodactyly in fetuses that were examined on day 12 of gestation [S44,S88].

2. ALS-PDC Comorbidities and MAM-Related Abnormal Functions

2.1. Mental Health

Prior or concomitant psychiatric illness (schizophrenia, psychosis, mood disorders) has been noted in ALS [S91-S96], and others have reported links between perinatal stress, schizophrenia and ALS [S98-S101]. Register-based nationwide studies have proven a higher occurrence of schizophrenia up to 1-5 years before and 2-5 years after ALS diagnosis [S102]. Motor dysfunction also occurs in schizophrenia [S103-S105]. Neuropsychological deficits, some of which were consistent with a diagnosis of schizophrenia, occurred prior to or after the onset of motor symptoms of Kii-ALS [S106]. A 32-year-old Japanese patient with Kii-ALS was documented to have had schizophrenic symptoms antedating the neurological picture [S107]. Kii ALS-PDC has been linked to oral medicinal exposure to toxins in cycad seed (*Cycas revoluta*) [S108,S109].

The subject of neuropsychological deficits in Guam ALS-PDC has not been addressed. Keith [S110] interviewed 15 Guam and 5 Saipan-resident Chamorros with schizophrenia (equal numbers of males and females, aged 24-59 years). In general, delusional content reflected Chamorro culture: magic, heredity and (often food-associated) poisoning. In the late 1970s, Chamorro descriptors for mental illness included: *malango* (sick in the head), *caduco* (crazy, talks bad words, preoccupied, forgetful) *atmariao* (insane). Chamorros were said to have little concern for the causation of *caduco* or *atmariao* and considered these disorders a fact of life. An exception was *chet not maipe*, a physical illness assumed to be caused by the supernatural powers of *taotaomona* and that should be treated by *suruhåna* (elderly male native healer who specialized in treating *chet not maipe*, "hot sickness"). Symptoms of *chet not maipe* include: fever, boils, blisters, marks on the skin, other bodily ills and some types of paralysis [S111]. Said in 1977 to be "less common than in the past," adolescent girls with *chet not maipe* with partial paralysis (considered to be akin to hysteria or conversion disorder) were treated with advice, skin potions, herbal remedies and prescribed magical ritual. Most *caduco* and *atmariao* behaviors were not associated with *chet not maipe.*

Schizophenia is widely modeled in rodents by *in utero* exposure to MAMac (>100 papers between 1998-2020). The MAMac animal model replicates changes both in mesolimbic dopamine function, which may contribute to the positive symptoms of schizophrenia, and to altered frontal cortical–limbic circuits thought to be associated with changes reminiscent of the negative and cognitive impairments of the human disorder [S112]. Schizophrenia-like deficits develop in the juvenile offspring of pregnant mice and rats treated with a carefully timed single dose of MAMac [S113-S117]. This is accompanied by a reduced volume/weight of hippocampal, entorhinal, parietal and prefrontal cortex and dorsal striatum, the first abnormalities associated with deficits in glutamatergic transmission and dopamine dysregulation in the prefrontal cortex and associated cognitive deficits

[S117-123]. Rats aged 4 months develop schizophrenia-like features following a single *in utero* injection of MAMac (i.e., embryonic day E17) as indicated by an MRI of enlarged lateral ventricles and altered cerebral blood flow [S124], much like that observed in the frontal or temporal lobes of Kii ALS-PDC brains [S106]. Adult rats that were exposed *in utero* to MAMac also exhibited a significant reduction in neuronal spine density as well as impaired working memory, which could be blocked by treatment with a glycogen synthase kinase 3β (GSK3β) inhibitor during the juvenile period [S125]. The age-dependent "hyperactive" GSK3β in this rodent model caused significant deficits in long-term potentiation and facilitated long-term depression in prefrontal cortical pyramidal neurons [S126]. GSK-3β (tau protein kinase 1) is implicated in the aggregation of hyperphosphorylated tau proteins into paired helical filaments that form NFTs in several neurodegenerative disorders, including ALS-PDC [S127-S129]. In sum, there are links between prenatal exposure to MAM and the latent onset of abnormal brain structure and function. For humans, the second trimester is a period of risk for brain changes that result in childhood schizophrenia [S113].

2.2. Epilepsy

Few data are available for the incidence of epilepsy on Guam, and the possibility of an association with ALS-PDC was never explored. Between 1960 and 1966, 122 Guamanians had their first episode of epilepsy, for an average annual incidence of 47.3/100,000, substantially higher than the corresponding figure for the population of Rochester, Minnesota, USA (29.8/100,000) [S130]. Other estimates of the annual incidence rate for afebrile seizures (30-35/100,000) and the prevalence of "active" cases (230-542/100,000) were within international norms [S131-S134]; however, in four survey villages, there was "severe underreporting of idiopathic epilepsy", defined as having no causal or associated factor other than family history, which had a high incidence in infancy, fell to moderate levels in childhood, rose again in adolescence and declined thereafter [S134].

MAMac administration to rat pups on gestational day 15 reduces the brain's seizure threshold, with evidence of spontaneous electrographic seizure activity and reduced potassium current function and expression for the Kv4.2 channel subunit [S135-S137]. Schwartzkroin and Wenzel [S20] proposed that MAMac-induced cortical heterotopic neuronal clusters are insufficient to determine the seizure-initiating process.

2.3. Olfaction

Olfactory dysfunction is among the first signs of ALS-PDC [S138]. Marked olfactory deficits, first reported in Guam PDC, were also present in Chamorro patients with ALS, atypical parkinsonism or dementia, and in some controls with or without possible sub-clinical ALS-PDC [S139,S140]. Olfactory deficits are also among the first signs of AD and idiopathic Parkinson disease [S141,S142]. The inability to distinguish the nature of olfactory dysfunction among Guam P-D, AD [S138] and

ALS patients [S140] suggests a common neurologic substrate and underlines the close relationship between ALS-PDC and the more familiar neurodegenerative disorders seen in the West.

MAMac perturbs brain and olfactory bulb development in albino rats [S11]. Related abnormalities were found in MAMac-treated young adult mice with brain levels of the DNA-repair enzyme MGMT comparable to those found in human brain. As described above, gene expression changes in mouse brain were examined 7 days after a single injection of MAMac (MAM_{early}) and 6 months later (MAM_{late}). Whereas six brain cell-signaling pathways (n = 4 or more genes per pathway) were common to MAM_{early} and MAM_{late} and three were unique to MAM_{late}, the latter was dominated by 28 (of a total of ~1300) modulated genes involved in olfactory transduction, including genes coding for olfactory receptors that were both upregulated (n=25) and downregulated (n = 3) [S78]. While caution is merited when comparing rodent and human data, these findings support the proposal that cycad toxin exposure is responsible for perturbation of olfaction in ALS-PDC.

2.4. Diabetes mellitus

Diabetes mellitus (DM) and dysglycemia appear to be more frequent among Western patients with ALS [S143] although an inverse association has also been reported [S144-S148]. On Guam, a 1974 study of carbohydrate metabolism in 110 persons with a diagnosis or suspicion of ALS-PDC found a considerably higher incidence of abnormalities than in the general population of the continental United States and the tropical Pacific area [S149,S150]. Glucose tolerance test results suggested abnormal carbohydrate metabolism in three-fifths of those with definite neurologic disease, of which 43% and 12% of ALS and P-D subjects met criteria for DM. A positive family history for DM in study participants was about twice the expected rate. A 1980 study found that age-specific rates for DM in Guamanians aged 45 years and over were 2-3 times greater than that of the general U.S. population, with Guam Chamorros (prone to ALS-PDC) having the highest rates of DM and related complications [S151], including hypertension [S153,S154]. A 1997 case-control study found DM in 44% of Chamorros with ALS-PDC (n=16) and 31% of neurologically normal Guamanian subjects (n=16), some of whom probably had preclinical neurodegenerative disease [S155].

Cycasin and MAM impair pancreatic cell function leading to β-islet cell destruction *in vitro*. Human islet cultures were less sensitive to MAMac than corresponding cultures of murine islets, and the cellular damage was associated with nitric oxide release and DNA alkylation (O^6-mG adducts) [S156]. The MAM-induced DNA damage was also ~3 times higher than that of murine islets treated with the potent genotoxin streptozotocin (STZ). Extensive pancreatic β-islet cell damage was noted in Old World primates chronically treated with oral or intraperitoneal cycasin [S157]. Notably, activity of the specific DNA-repair enzyme MGMT is low in β-islet cells as in post-mitotic neurons [S158]. These observations suggest there might be a link between the former dietary exposure to cycasin

among Chamorros and their contemporaneous high rates of DM. This would be consistent with the established relationship between DM and neurodegenerative disorders [S159] and with the induction of a murine model of sporadic AD by intracerebroventricular administration of STZ [S160] which, like MAM, methylates biomolecules via a common methyldiazonium ion [S160,S161] (*vide infra*). No significant changes were observed in β-islet cell function after 6 days of continuous treatment with a high concentration (1.0 mM) of L-BMAA [S156].

2.5. Liver disease, hepatic and other cancers

Most cancers in Pacific Island populations are linked to smoking, obesity, physical inactivity, poor nutrition or infections. Data for Guam are available only for infections. Between 1970 and 2012, the mean age of the 3,437 hepatitis cases on Guam was 40.7 ± 16.7 years old, of which one-third (34.7%) were Chamorros [S162]. This group had the highest cases of hepatitis A (5.2%) and hepatitis C (19.6%), whereas Micronesians had the highest cases of hepatitis B (23.4%). The difference in proportion of liver cancer cases among Guam ethnic groups was also statistically significant. Hepatitis C was the type of hepatitis most common among liver cancer cases (63.3% of viral hepatitis-associated liver cancer cases). Guam cancer incidence for the period 1998-2002 showed that Chamorros had high age-adjusted incidence rates for cancers of the mouth and pharynx (24.4 vs. U.S. 10.7), nasopharynx (13.9 vs. 0.6 U.S.) and liver (13.2 vs. 5.2 U.S.), and Filipinos living on Guam also had high age-adjusted incidence rates for cancers of the nasopharynx (5.1), and liver (9.6) [S163]. There are no studies of the type or incidence of cancer in ALS-PDC.

Soon after ingestion of cycad plant products, there is sudden onset of nausea and vomiting, enlargement of the liver, convulsions, loss of consciousness, and death or recovery [S164]. Hepatocellular damage resulting from acute cycad toxicosis is seen in domestic animals and cattle [S165-S167], and cycasin induces liver and neuromuscular disease in goats [S69]. The livers of rats showed extensive hemorrhage and necrosis 24-48 hours after a single dose of MAMac; however, by the 4th day the liver showed little pathology other than irregularities in the size and shape of the hepatocyte [S168]. Prolonged treatment with cycasin/MAMac produced hepatomas, renal and intestinal tumors in rats [S88,S169,S170] and, in non-human primates, hepatocellular and bile duct carcinoma, renal carcinoma and adenomatous polyps of the small intestine [S157, S171]. Unfortunately, the nervous system of these valuable animals was not examined in these cancer-focused experiments, and there are no studies of the type or incidence of cancer in ALS-PDC. There was an increased death rate from cirrhosis but not hepatoma in Japanese residents of Miyako island who had been forced to subsist for food on cycads 2-7 years earlier [S164]. While a cancer registry for Miyako islanders was formed, there was no long-term follow-up of survivors.

MAM has mutagenic and carcinogenic as well as neurotoxic potential mediated by specific patterns of DNA damage that has been discussed in detail elsewhere [S80, S163]. Simply stated, the outcome of treatment with MAMac appears to depend on whether the targeted cell is cycling and thus able to undergo mutagenesis and uncontrolled cell proliferation (cancer), has a limited potential for mitosis, or is post-mitotic (neurodegeneration). That neurodegeneration and cancer may be "two sides of the same coin" has been demonstrated experimentally [S43,S173]. Brains of single-dose MAMac-treated young adult mice with levels of the DNA-repair enzyme MGMT equivalent to those found in human brain displayed short-term (up to 7 days, i.e. MAM_{early}) changes in the expression of genes in cell-signaling pathways associated with Neurological Disease (n=159), Psychological Disorders (n=75), Cancer (n=114) and Genetic Disorder (n=212), coupled with changes in Physiological System Development and Function, namely: Nervous System Development and Function (n=64), Embryonic Development (n=22), Organ Development (n=14), and Skin and Hair Development and Function (n=11) [S43].

The involvement of brain-specific cell-signaling pathways, including transforming growth factor-β (TGF-β), wingless and proto-oncogene Int-1 (Wnt), and mitogen-activated protein kinase (MAPK), match those involved in the genesis of colon carcinogenesis induced by azoxymethane, which is mediated by its proximate metabolite MAM [S174-S176]. Whereas TGF-β and MAPK signaling is up-regulated and Wnt down-regulated in colon cells prone to MAM-induced mutagenesis, the Wnt-pathway tau kinase GSK3β appears to be upregulated in the brains of young adult mice following a single dose of MAM (MAM_{early}), leading to tau hyperphosphorylation [S43]. This interpretation is supported by the presence in mouse brain 6 months following an injection of MAMac (MAM_{late}) of elevated mitogen-activated protein kinases and increased caspase-3 activity, both of which are involved in tau aggregation and NFT formation typical of ALS-PDC and AD [S43].

2.6. Cell-cycle and Epigenetics

While neurons undergo terminal mitosis during their development and remain in a quiescent G_0 post-mitotic state under normal conditions throughout life, there is mounting evidence of aberrant neuronal cell-cycle reactivation in neurodegenerative disorders with tauopathy, including ALS-PDC [S177,S178]. Mature neurons in various brain regions of subjects with tauopathy activate DNA duplication without subsequent mitosis, which results in apoptosis [S179].

Preliminary studies have been undertaken to examine the cell-cycle network of human neuroprogenitor cells (hNPCs) treated *in vitro* with MAMac. DNA methylome analysis, which provides a picture of epigenetic regulation of individual gene expression, revealed marked differential methylation of key genes that regulate the cell cycle (Figure S1). MAMac in a concentration-

dependent manner also reduced hNPC histone deacetylase activity, which has a key role in the epigenetic regulation of gene expression (Figure S2).

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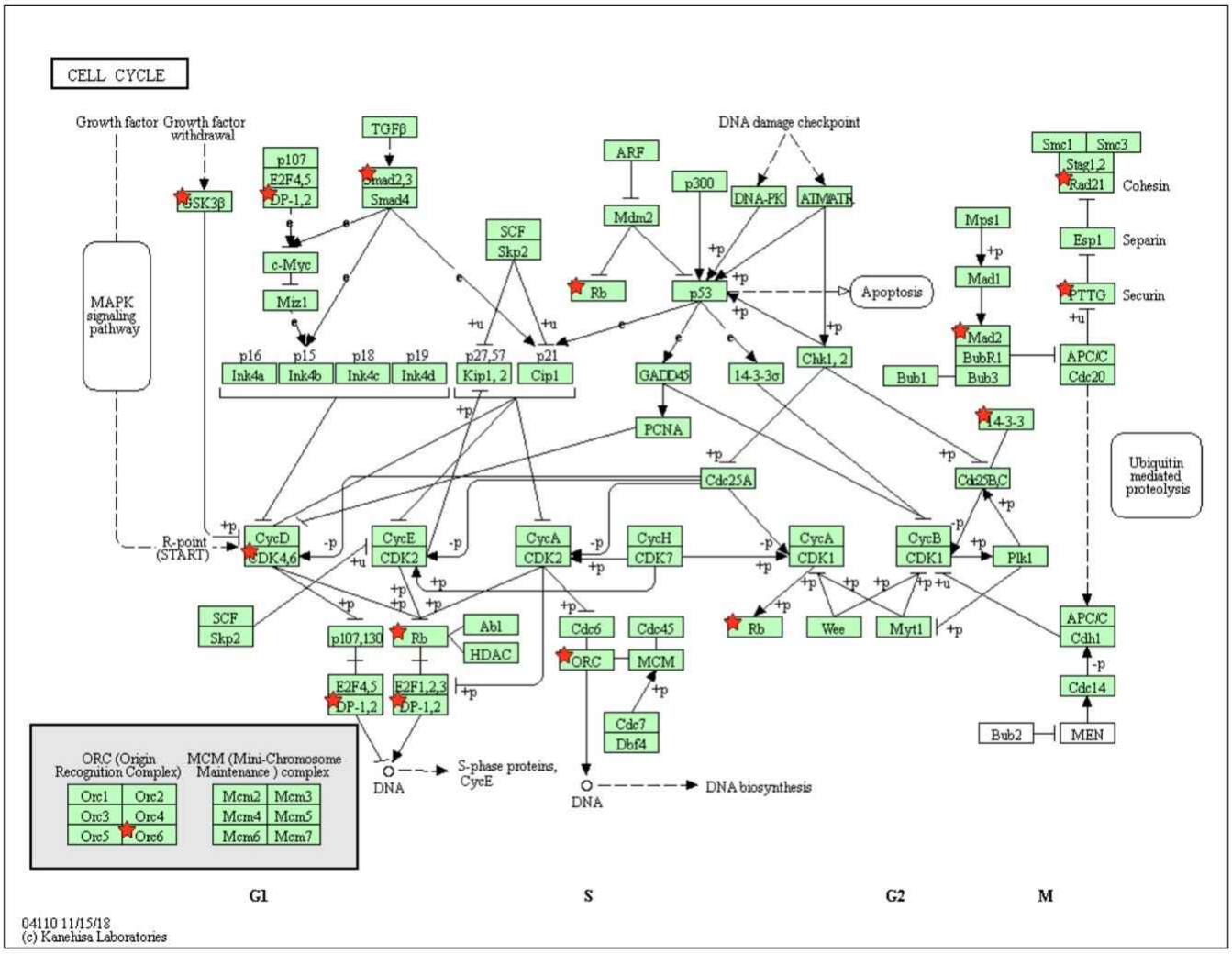
SUPPLEMENTARY TABLE

Associations between ALS-PDC and the mammalian toxic effects of cycasin/MAM acetate (MAMac)

ALS-PDC and MAM Developmental and Adult Abnormalities				
No.	**Tissue**	**ALS-PDC** Guam and/or Kii-Japan	**Cycasin/MAMac** Single dose to animal unless otherwise stated	**Comment/Related Information**
1.1.	**Cerebellum** (Guam and Kii)	Sub-clinical cellular disruption; ectopic neurons.	Developmental cerebellar dysplasia.	Human and experimental animal pathology matches.
1.2.	**Retina** (Guam and Kii)	Sub-clinical linear retinal pigmentary epitheliopathy.	Developmental retinal dysplasia.	Human and experimental animal pathology matches.
1.3	**Skin and Bone** (Guam and heavily studied in ALS in Japan)	Resistance to bed sores; loss of elasticity; subclinical connective tissue disorder; focal collagen regeneration.	Perturbed brain KEGG *Wnt* signaling pathway (skin). Affects skin and hair development and function. Perturbed brain KEGG *Wnt* signaling (bone).	Animals grazing on cycads shed horns and hooves. Cycad seed pulp used traditionally to speed skin repair (Guam and Papua-Indonesia). Former very high incidence of diaphyseal aclasis among Chamorros
1.4	**Congenital Defects** (Guam, not studied in Kii)	Anencephaly, and cleft palate and harelip (children born to 2/17 Guam women with ALS).	Neural tube defects, hydrocephalus, microcephalus, cranioschisis, exencephaly, spina bifida.	In the 1980s, Chamorro women stated that spinal bifida was not uncommon, but supporting data unavailable.
ALS-PDC Comorbidities and MAMac-Related Abnormal Functions				
2.1.	**Mental health** Kii and Guam	Schizophrenia prior to motor signs (1 reported Kii case). One patient with Guam P-D	Links with psychological disorders; MAMac models schizophrenia in rodents.	Schizophrenia before/after onset of motor weakness reported in and associated with sporadic ALS.
2.2.	**Epilepsy** Guam, not studied in Kii-Japan	1960-66. Mean annual incidence of epilepsy higher on Guam than on mainland; 1968. Similar incidence rates to international norms.	Lower threshold for epileptifom activity associated with cortical dysplasia.	Severe underreporting of cases when clinical data were used while ascertainment of symptomatic seizure disorders was nearly complete.
2.3.	**Olfaction** Guam, not studied in Kii-Japan	Marked olfactory deficits in all ALS-PDC phenotypes, and in possible subclinical cases.	Perturbed brain olfactory gene expression in mice.	Altered transcription of 28 olfactory genes detected 6 months after single MAMac dose.
2.4	**Diabetes mellitus** Guam, not studied in Kii-Japan	Elevated, perhaps related to ALS-PDC. Link with neuropathy of possible diabetic origin.	Perturbed brain KEGG insulin signaling.	Cycasin/MAMac *in vitro* impaired human and rodent pancreatic β-islet cell response to insulin and, at high concentrations, destroyed β-islet cells.
2.5.	**Liver disease, hepatic and other cancers** Guam, not studied in Kii-Japan	Co-morbidity uninvestigated. 1998-2002. High-incidence of liver, nasopharynx, mouth-pharynx tumors.	Perturbed brain KEGG cancer signaling in mice.	MAM is a DNA-damaging agent with acute hepatotoxic, neurotoxic and carcinogenic properties. Tumors of liver, kidney, esophagus and small intestine occur with prolonged primate treatment.
2.6.	**Cell-cycle and epigenetic changes** Guam and Kii-Japan	Neurons show mitotic activation (Guam) and reduced expression of GADD genes (Kii-Japan) (see Section 2.3 of main text)	Changes in cell-cycle network gene transcription and inhibition of histone deacetylase activity in human neuroprogenitor cells.	Aberrant re-expression of many cell-cycle proteins in vulnerable neuronal populations occurs in several neurodegenerative disorders



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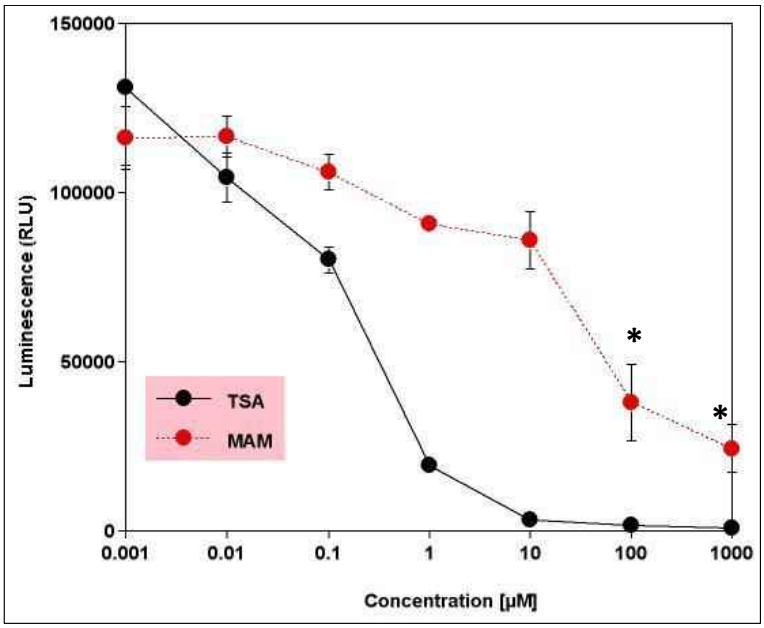
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Luminescence (RLU)
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Concentration [µM]