Environmental tauopathies

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1 Abbreviations

3-NP	3-Nitropropionic Acid
AD	Alzheimer's Disease
AGD	Argyrophilic Grain Disease
ALS	Amyotrophic Lateral Sclerosis
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AP	Atypical Parkinsonism
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BMAA	β-Methylamino-L-Alanine
BSSG	β-Sitosterol β-d-Glucoside
C/EBP	CCAAT-Enhancer-Binding Proteins
CBD	Corticobasilar Degeneration
CCCP	Carbonyl Cyanide m-Chlorophenylhydrazone
CHIP	Carboxyl Terminus HSP70/90 Interacting Protein
CHOP	C/EBP Homologous Protein
CSF	Cerebrospinal Fluid
DAergic	Dopaminergic
DMA	Dendrite-Morphogenesis-Abnormal
EAAs	Excitatory Amino Acids
ER	Endoplasmic Reticulum
FTD	Frontotemporal Dementia
Gd-PDC/PSP	Guadeloupean Parkinsonism Dementia Complex/Progressive Supranuclear Palsy
GSK-3B	Glycogen Synthase Kinase 3 Beta
HDAC6	Histone Deacetylase 6
IGF1	Insulin-like Growth Factor-1
L-BOAA	Beta-Oxalylamino-L-Alanine
LAT1	L-type Amino Acid Transporter 1
Lrrk2	Leucine-rich repeat kinase 2
LSP	Lipoprotein Signal Peptide
MAM	Methylazoxymethanol
MAP	Microtubule Associated Protein
MAPT	Microtubule-Associated Protein Tau (Gene)
MD	Mariana's Dementia
mGluR5	Metabotropic Glutamate Receptor 5
MPP+	1-Methyl-4-Phenylpyridinium
MRI	Magnetic Resonance Imaging

NDDs	Neurodegenerative Diseases
NFTs	Neurofibrillary Tangles
NMDA	N-Methyl-D-Aspartate Receptor
PD	Parkinson's Disease
PDC	Parkinsonism Dementia Complex
PHF	Paired Helical Fragments
PiD	Pick's Disease
PP1, PP2A and PP2B	Tau Phosphatases
PP2AC	Catalytic Subunit of Protein Phosphatase 2A
PSP	Progressive Supranuclear Palsy
RBD	REM-sleep Behavior Disorder
REM	Rapid Eye Movement
ROS	Reactive Oxygen Species
Ser262	Serine Residue Number 262
SG	Sterol Glucosides
SNO	Supranuclear Ophthalmoplegia
SPECT	Single-photon Emission Computed Tomography
Src	Steroid Receptor Coactivator
SRSF2	Serine/Arginine-Rich Splicing Factor 2
TDP-43	Transactive Response DNA Binding Protein *43* kDa
Thr 231	Threonine Residue Number 231
TRPM	Transient Receptor Potential Cation Channel, Subfamily M
TRPM2	Transient Receptor Potential Cation Channel, Subfamily M, Member 2
TRPM7	Transient Receptor Potential Cation Channel, Subfamily M, Member 7
Tyr307	Tyrosine Residue 307
UMN	Upper Motor Neuron
UPR	Unfolded Protein Response
WWII	World War Two

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3 Summary

Title: Environmental Tauopathies
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Neurodegenerative diseases, of which tauopathies form a major class, are increasing in prevalence worldwide. Tauopathies are characterized by the presence of abnormal neuronal and glial inclusions that are composed predominantly of the microtubule associated protein, tau. Several foci of increased incidence of tauopathies have been identified that have been attributed to environmental neurotoxins. Exposure to inorganic or organic toxins (such as β-Methylamino-L-Alanine and annonacin) through water, food and traditional medical practices is the suspected etiology but it remains to be proven whether these are sole sufficient factors. These progressive and uniformly fatal diseases are characterized clinically by various combinations of dementia, parkinsonism and motor neuron disease. This review attempts to summarize the currently available information on small geographic clusters of endemic environmental tauopathies that have been identified in the Chamarros of Guam, inhabitants of the Kii Peninsula in Japan, members of the Auyu and Jaqai groups in Papa New Guinea, and residents of Guadeloupe. Possible unifying neurodegenerative mechanisms are explored in relation to sporadic tauopathies worldwide.

Keywords: ALS/PDC; annonacin; atypical parkinsonism; cycads; L-BMAA; lytico-bodig; tauopathy

4 Introduction

The search for the primary etiology of neurodegenerative diseases has largely revolved around genetic studies, a perspective that has been strengthened by the identification of mutations associated with Huntington's disease, familial Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). Despite years of extensive investigation into the role of genetic causation, the discovered mutations have thus far managed to account for no more than 10% of the ever-increasing neurodegenerative disease burden. While it is likely that future genetic discoveries will account for some of the remaining 90% of cases, it is at least equally likely that environmental factors such as neurotoxins play an important role in the development and increased incidence of neurodegenerative disease. Perhaps most likely of all is a scenario in which these diseases are due to multiple gene-environment interactions mediated by epigenetic mechanisms. In the quest for a greater understanding of neurodegenerative disease, several foci of increased incidence of tauopathies, a subset of neurodegenerative diseases, have been identified that are attributed to environmental neurotoxicant exposures. This review attempts to summarize the information that is currently available on small geographic clusters of these environmentally caused tauopathies.

Tauopathies are a heterogenous class of neurodegenerative diseases (NDDs) characterized by the presence of abnormal neuronal and/or glial inclusions that are composed predominantly of the microtubule associated protein, tau. Defined clinicopathologically, this group encompasses more than 25 recognized entities (Murray et al., 2014) that have historically been subdivided into primary (those diseases in which tau inclusions are believed to be the main cause of the disease) and secondary (tau inclusions occur but are not believed to be part of the primary process) tauopathies. A newer subclassification, based on biochemical structure, divides tauopathies into three groups according to the predominant tau isoform found in the inclusions, that is: 4R tauopathies (including progressive supranuclear palsy (PSP), corticobasilar degeneration (CBD), Huntington's and argyrophilic grain disease (AGD)), 3R tauopathies (Pick's disease) and 3R+4R tauopathies (for example, Alzheimer's disease (AD)) (Dickson et al., 2011). Clinically, tauopathies are characterized by various combinations of parkinsonism, dementia, and motor neuron disorders. While some tauopathies have clearly been shown to be of genetic origin, the majority appear to occur sporadically, either as a result of environmental factors

alone or in combination with genetic susceptibility. Elucidating the cause of these clusters of “environmental” diseases would provide models for sporadic tauopathies and potentially lead to the discovery of new therapeutic interventions.

5 Tau Protein

5.1 Physiological Tau

Tau protein was first purified from porcine brain and characterized as a member of the Microtubule-associated protein (MAP) family. It is distributed extensively throughout neurons and found at lower levels in astrocytes and oligodendrocytes. Under physiologic conditions it is relegated largely to the distal end of axons (Huang et al., 2015). In humans, tau has six isoforms (ranging from 352-441 amino acids in size) that are the products of alternative splicing from a single, 16 exon gene, Microtubule-Associated Protein Tau (*MAPT)*, localized on chromosome 17q21. *MAPT* is over 50 kb in size with two haplotypes, H1 and H2, and multiple variants of each (Lee and Leugers, 2012). The differences among the isoforms are due to alternative splicing of exons 2, 3 and 10, which results in the presence or absence of 29- or 58-amino acid long inserts (0N, 1N, or 2N) in the amino-terminal (N-terminal) region, as well as an additional 31-amino acid repeat (3R or 4R) in the carboxy-terminal (C-terminal) region. Isoforms that include exon 10 are referred to as 4R while those that exclude exon 10 are referred to as 3R. 1N3R and 1N4R are the most abundant forms (Lee and Leugers, 2012). A relatively constant ratio of 3R/4R isoforms is maintained in the normal brain. The major known function of tau is to bind to microtubules and it has been shown that 4R isoforms exhibit a 40 times higher affinity for microtubule binding than 3R isoforms (Goode and Feinstein, 1994). The diversity of isoforms is increased by post-translational modifications, in particular, phosphorylation. Tau contains 85 serine (Ser), threonine (Thr), and tyrosine (Tyr) potential phosphorylation sites. Approximately 10 phosphorylation sites can be detected on soluble tau purified from a normal brain (Sun and Chen 2015). Tau is highly soluble in water and exhibits stability under acidic conditions and in high temperatures. Overall, tau is a basic protein although the N-terminal is acidic and the C-terminal is neutral. The asymmetry of charge is likely important for microtubule interactions, folding, and aggregation (Wang and Mandelkow, 2015). Normal tau is natively unfolded and has little tendency for aggregation. The tau molecule tends to change its global conformation to form a shape similar to a paperclip, in which the N-

terminal, C-terminal and the repeat domains all approach each other under physiological conditions. Truncation of tau prevents this formation and may promote aggregation (Wang and Mandelkow, 2015).

5.2 Pathological Tau

To date, the pathological finding in every known tauopathy involves hyperphosphorylated tau protein. Tau normally contains 2-3 moles of phosphate per mole of the protein whereas pathological tau proteins in AD brains have been found to be 2-3 times more hyperphosphorylated (Iqbal et al., 2015). Hyperphosphorylation (particularly at residues Ser262 and Thr231) significantly decreases the binding affinity of tau for microtubules. The resultant instability of the microtubules leads to their depolymerization, which directly affects axoplasmic transport, a function essential to neuron survival. The dissociated tau missorts to the cell body as well as becoming more diffusible (Huang et al., 2015). Small quantities of missorted tau appear to have the ability to influence normal tau protein to release microtubules and missort, eventually leading to pathological tau aggregation and ultimately, death of neurons (Huang et al. 2015). The protein subunit of neurofibrillary tangles (NFT) consists mostly of hyperphosphorylated tau which, is apparently inert and neither binds to tubulin nor promotes it assembly into microtubules (Iqbal et al., 2009). It is the subject of an as yet unanswered debate which form of tau (aggregated misfolded/fibrillar, soluble hyperphosphorylated/mislocalized, or both) is in fact the most toxic (Šimić et al. 2016). Many studies have shown a direct correlation between the number and density of NFTs and the severity of symptoms in AD. On the other hand, recent findings have shown that NFT containing neurons can survive up to 20-30 years (Huang et al., 2015), leading to the suspicion that they may not be the major toxic player. The most recent data suggests oligomerized forms of tau as the true source of toxicity (Huang et al., 2015) as they have been shown to appear early in the disease course and are highly toxic in *in vitro* and *in vivo* experiments. Complicating matters even further is the implication of several post-translational modifications leading to tau toxicity, which include: glycosylation, *O*-GlcNAcylation, acetylation and abnormal truncation. Additionally, a common factor in several tau toxicity cascades involves the activity of the major tau phosphorylation regulation enzymes, the tau phosphatases (PP1, PP2A and PP2B). The activity of these phosphatases has been found altered in several tauopathies (Huang et al., 2015).

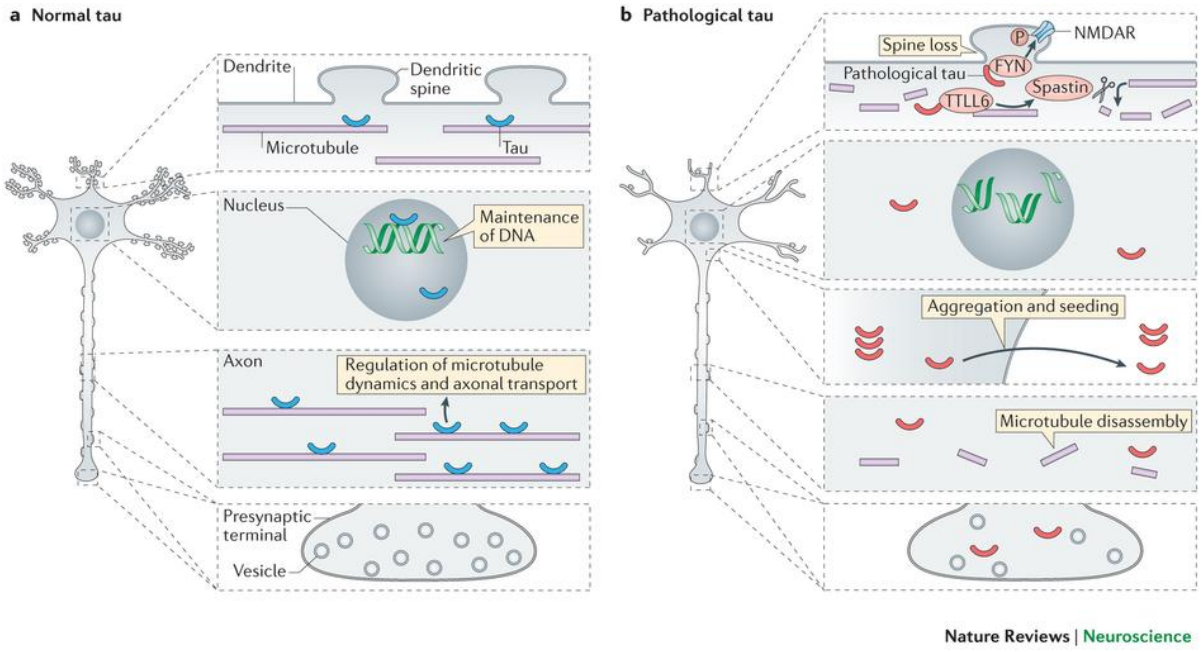

Figure 5-1: Normal and Pathological Tau Proteins.

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6 ALS/PDC of the Western Pacific

6.1 ALS/PDC/MD on Guam- "Lytico-bodig" disease

6.1.1 Epidemiology

Following the American recapture of Guam from the Japanese in 1944, army neurologist Zimmerman described a high incidence of amyotrophic lateral sclerosis (ALS) on the island. The first published reports came in 1952 and 1953 when army physicians Koerner and Arnold described a high incidence of ALS among the Chamarros on Guam, which was soon confirmed and expanded on in several studies by Kurland, Mulder and others (Koerner, 1952; Kurland and Mulder, 1954, 1955). The incidence of ALS on Guam peaked in the 1950s at 200/100,000 per year, 100 times higher than elsewhere in the world (Bradley and Mash 2009). Soon after, a form of atypical parkinsonism often associated with dementia was also identified by Mulder, and later shown by Hirano to be a unique disease entity in high prevalence amongst the Chamarros on Guam (Plato et al., 2003). It was linked epidemiologically with ALS and shown to be a different phenotypic expression of the same disease, which became known as amyotrophic lateral sclerosis/parkinsonism- dementia complex (ALS/PDC). In some villages, one-third of adult deaths were due to

ALS/PDC (McConnell, 2004). It was known as lytico-bodig (idiotic and lazy in the Chamarro language) disease by the locals and references to it have been found in documents dating back to the late Spanish period (1700-1800) as well as in death certificates from the early 1900s (Bradley and Mash, 2009).

Rather early, it was recognized that ALS/PDC occurred with a high prevalence in certain Chamarro families and with an uneven geographical distribution on the island. The highest incidence was found in the village of Umatac (10 times higher than elsewhere on Guam). Zhang and collaborators (1990) reported that the age-adjusted incidence there was 273/100,000 in men and 222/100,000 in women while a mere 4 miles away it was 38/100,000. The identification of clustering within families initially prompted a search for a genetic cause. A house-to-house survey showed that more than 60% of cases were concentrated in a single pedigree. The disease was more prevalent in men, a fact that led researchers towards an explanation involving an autosomal dominant gene with 50% penetrance in women. Subsequent segregation analyses by Reed and colleagues found a much weaker familial association (Bradley, 2009) and Bailey-Wilson et al. (1993) concluded that purely genetic (either Mendelian dominant or recessive) or environmental hypotheses could be rejected. Instead, they suggested a two allele major locus hypothesis coupled with an unidentified environmental exposure. A significantly increased risk of developing ALS or PD among parents, siblings and surprisingly, spouses of patients (but not among relatives of controls), was demonstrated. Offspring of patients showed no increased risk (Plato et al. 1986). Additionally, genetically identical Chamarros on the island of Saipan, only 80 miles away, did not have ALS/PDC (Ince and Codd, 2005) and the disease appeared in a genetically divergent population of Filipino migrants to Guam after several years of exposure.

In a 1981 study, Gajdusek et al. reported finding nine cases of ALS and two cases of PDC in Filipino migrants to Guam and ten cases of ALS and six of PDC in part-Filipino patients born on Guam. They had spent an average of 17 years on Guam before the onset of the disease. Of the four cases autopsied, the findings were somewhat equivocal: two showed changes consistent with Guamanian ALS but two showed only the classical pathology of sporadic ALS. It was determined that Filipinos had a rate of developing ALS that was six times that of the continental USA but still only 50% the rate of Chamarros living on Guam. The second generation of Filipino

migrants did not have a higher risk of developing neurodegenerative disease. Also, it was found that Chamarros who moved to California after at least 18 years of exposure to Guam maintained an elevated risk (four times the rate of US citizens) for over three decades but their children showed no increased risk (Ince and Codd, 2005). These findings, supplemented by reports of a rapidly declining disease incidence, pointed towards the role of an environmental exposure.

However, in 2005 the first evidence for a genetic component came from Hermosura et al. who reported a mutation in the transient receptor potential melastatin 7 membrane channel in a subset of Guam ALS/PDC patients. The channel is involved in intracellular calcium and magnesium regulation. A subsequent study implicated *TRPM2* (Hermosura and Garruto, 2007). *TRPM7* mutations were not found in ALS/PDC cases in the Kii peninsula (Hara et al., 2010) ruling out the mutation as the putative cause. Lynch et al. (2008) presented a gene-environment model, that showed significantly increased risk associated with a group of eight variables (age and gender, village, NFTs, metals, β-Methylamino-L-Alanine (BMAA), *TRMP7* mutation, family history, somatic Lipoprotein Signal Peptide (*LSP)* mutation) in which genetic susceptibility combines with an environmental exposure or exposures in order to produce the disease. The most recent genetic study on the subject (Steele et al., 2015), found only 20% of patients had any identifiable pathogenic mutations, again confirming the relevance of environmental factors.

In response to reports of a rapidly declining incidence of ALS/PDC, Plato et al. (2003) published a report that attempted to identify all cases of ALS and PDC reported on Guam between 1940-1999 in order to determine whether the reported changes in incidence rates, age at onset, sex ratio, and duration of the disease were accurate. They identified a total of 929 ALS/PDC cases. Of these, 436 (278 males and 158 females) were diagnosed as ALS and 493 (312 males and 181 females) diagnosed as PDC. They established that the incidence of ALS peaked in both males in females in 1950-54. Starting in the late 1950s, the incidence declined steadily until 1980-84 when the rates were 4-5/100000. The incidence of PDC in males peaked in 1960-64 and declined until 1980-84 and then increased slightly. PDC in females increased until 1970-74 and then declined sharply until 1980-84. After 1980, ALS incidence stabilized at 3/100000 and PDC increased to 18-19/100000.

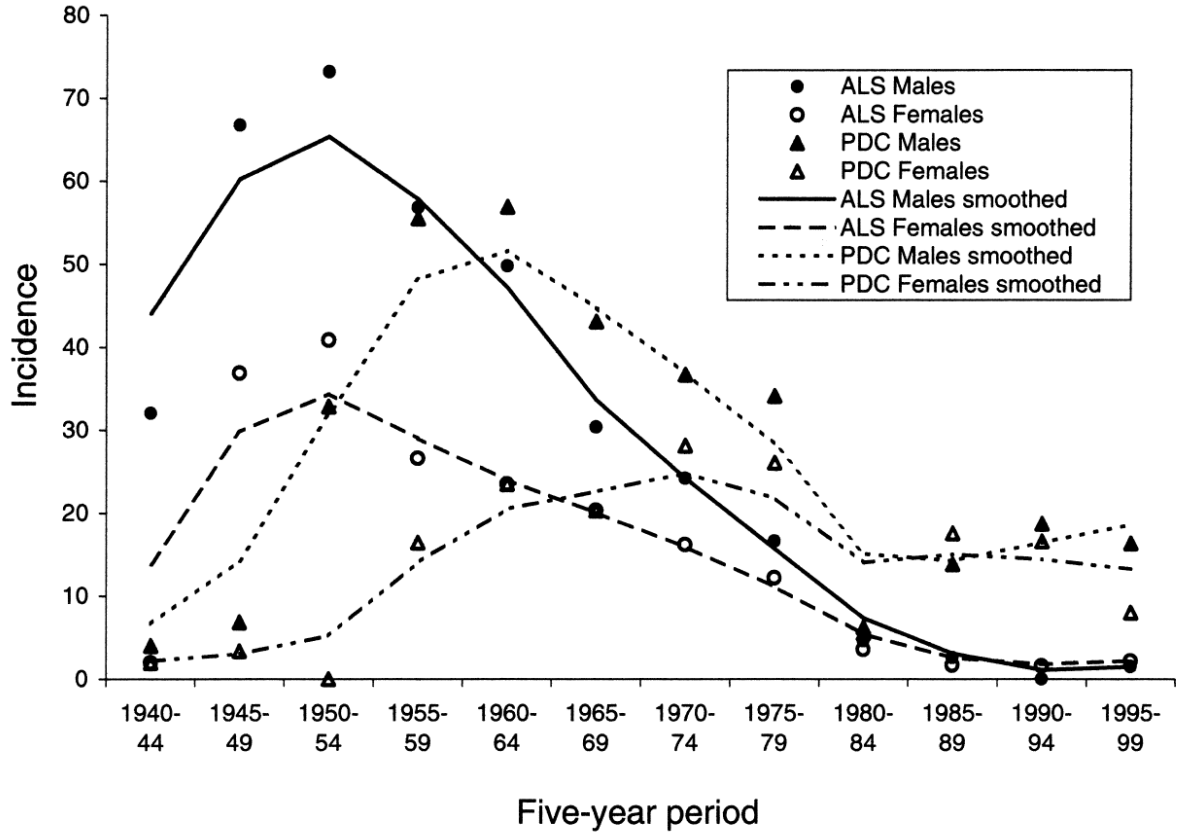

Figure 6-1 Incidence of ALS and PDC on Guam from 1940-1999

Reprinted by permission from Oxford University Press: Plato CC[1], Garruto RM, Galasko D, Craig UK, Plato M, Gamst A, Torres JM, Wiederholt W. Amyotrophic lateral sclerosis and parkinsonism-dementia complex of Guam: changing incidence rates during the past 60 years. Am J Epidemiol. 2003 Jan 15;157(2):149-57.

While the incidence has seemingly declined drastically, some authors report a continued high incidence. An island wide survey of Chamarros aged 65 and above was carried out, in which a high prevalence of dementia was identified (Steele and McGreer 2008). This indicates that the disease may have again changed phenotypes, possibly due to decreased exposure to the causative agent. As it is highly implausible that a gene could have been removed from a population in such a short period, especially within a relatively stable genetic group, searches for an environmental cause became a focus of future investigations. Environmental factors on Guam had changed rapidly because of modernization and westernization. Changes in residence, housing, nutrition, water supply and toxic exposures all occurred and could possibly have accounted for the decrease in exposure to the source of the disease (Plato et al., 2003). In a cohort study, Reed et al. (1987) found that of 23 variables, only preference for traditional Chamarro food was associated with increased risk. Two other case control studies, conducted by Reed and Brody, identified “growing up on farms, frequent contact with animals, and eating home-

grown food as well as raw fish all together were significantly associated with PDC" (Caparros-Lefebvre and Steele 2005).

The results of the epidemiological studies discussed above suggested an environmental toxin with a long latency period. The search for this toxin divided into two major lines of research: organic toxins and inorganic toxins. The indigenous cycad was identified early by locals as a likely cause, and put forward by Whiting as a hypothesis in 1963. Chamarros knew it was toxic and employed special measures to render it suitable for consumption. Researchers that sought an inorganic toxin focused their attention on a hypothesis related to an imbalance in water mineral content. They thought that such an imbalance may cause parathyroid dysfunction, which by affecting calcium and magnesium metabolism, would lead to increased uptake of heavy metals and eventual neurodegeneration (Garruto et al., 1990). There was also a handful of researchers that looked into the possibility of an unidentified slow viral agent given the similarity of the neuropathological findings those to postencephalitic parkinsonism (Spencer et al., 2016). However, attempts by Gajdusek to transfect the disease from human to monkey were unsuccessful and led to the abandonment of the infectious hypothesis (Spencer et al., 2016).

6.1.2 The Cycad Hypothesis

The cycad hypothesis was originally put forth by Marjorie Whiting in 1963 (Steele and McGreer, 2008). Cycads are gymnosperms considered to be living fossils because they have been present since the Mesozoic era. There are 185 species described, most of which are endemic. They are frequently cultivated as exotic ornamentals and also provide food for humans, particularly during times of famine. Cycads synthesize and store a number of toxic active ingredients including Methylazoxymethanol (MAM), cycasin, sterol glucosides, macrozamin, neocycasin and BMAA. Botanist Knut Norstog observed that cycads endemic to Guam produce pollen with particularly high concentrations of cycasin and BMAA and hypothesized an aerosolized route of exposure. Cycad seeds had been recognized to be poisonous and Chamarro people went to great lengths to remove the toxins. They cut open the seed and removed the gametophyte, which they then split, sliced or grated before soaking in at least three changes of water over several days (Banack and Murch, 2009). Three food items related to cycads in the traditional Chamarro diet

include: fadang (tortillas), fadang pilota (dumplings) and fanihi (flying foxes). In 2007, a group of biostatisticians led by Borenstein conducted an in depth population study of the Chamarro people, statistically showing that picking, processing or eating fadang in young adulthood presents the highest associated risk of developing ALS/PDC (Borenstein et al., 2007. They did not find a significant relation to the consumption of flying foxes. In support of this hypothesis it was found that the marked decrease in the incidence of ALS/PDC in Guam paralleled the decreased use of cycad flour and the near extinction of fruit bats (Bradley and Mash 2009). Studies have implicated several toxins (discussed in the next chapter) in cycads as potential causes of ALS/PDC but due to a lack of success in producing an animal model, all remain unproven.

Figure 6-2: Cycads and Fruit Bats on Guam

Reprinted by permission from John Wiley and Sons: Steele, JC. Parkinsonism-Dementia Complex of Guam. Movement Disorders 2005; Vol.20: Suppl. 12, pp S99-S107.

6.1.3 Cycad Toxins

6.1.3.1 L-BOAA

It is present in *C. circinalis* and decreases mitochondrial enzymatic activity and elicits marked inhibition of mitochondrial complex I. It has a strong affinity for glutamate receptors, especially α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic

Acid (AMPA) receptors, making it excitotoxic. Data indicates that it produces vacuolization, axon and dendrite swelling in neurons in the thalamus, and to a lesser extent, in the hippocampus, cortex, and cerebellum (Ross, 1989).

6.1.3.2 L-BMAA

Following the discovery that Beta-Oxalylamino-L-Alanine (BOAA) was responsible for the upper motor neuron (UMN) disease lathyrism, researchers looked for BOAA in cycads and ended up discovering β-N-methylamino-L-alanine (BMAA) (Bradley and Mash 2009). BMAA is a non-protein amino acid with the molecular formula $C_4H_{10}N_2O_2$ and a molar mass of 118.13 g/mol. The naturally occurring isomer is L-BMAA (Vega, 1968). Non-protein amino acids are potent toxins that help plants to protect themselves against predation and function as nitrogen storage molecules (Bradley and Mash, 2009).

In the 1980's, Spencer et al. demonstrated that administering 100-315 mg BMAA/kg daily for twelve weeks to macaques caused symptoms very similar to ALS/PDC. It was the first successful *in vivo* experiment involving primates, but the animals demonstrated only acute/subacute, reversible toxicity and not the chronic form characteristic of Guamanian ALS/PDC (Bradley 2009). Duncan and colleagues rejected the BMAA hypothesis because they found extremely low concentrations of BMAA in their own experiments. Moreover, they reported that the dose used in Spencer's experiments was orders of magnitude higher than humans would be likely to ingest from flour. They calculated the worst-case daily exposure to be 0.36 mg/kg and cumulative exposure to be 10.8 mg/kg BMAA per kilogram of bodyweight per month (Duncan et al., 1990).

The BMAA hypothesis was revived when Cox et al. demonstrated that the traditional diet of Chamorros contained high levels of BMAA through consumption of fruit bats (3 mg per gram of meat, 3 mg per 250 mL of broth), cycad flour, and possibly the meat of pigs and other animals that forage on cycad seeds (Bradley, 2009). Flying foxes had commonly been eaten by indigenous people on ceremonial occasions (Cox and Sacks 2002). The local custom was to cook the bats by boiling them in their entirety in coconut milk (Ince and Codd, 2005). This was identified as a core part of Chamarro culture, particularly in the villages of Umatac and Inarajan which, had the highest incidence of ALS/PDC. Furthermore, the greater frequency of

bat consumption by men corresponded to the higher rate of ALS/PDC in the male population.

Two species of flying foxes, *Pteropus tokudae* and *P. mariannus* were at one time very common on Guam. They were known to consume up to 2.5 times their body weight in fruit and nectar from cycads each night, and like all bats, accumulate lipophilic toxins in their fat. Analysis of museum bat specimens showed a mean concentration of 3,556 μg BMAA/g (Cox et al., 2002). In 1900, Guam had more than 50,000 bats and by 1958 the number had declined to 3000. The decline in population resulted from their popularity as a food substance combined with greater access to firearms in the 20th century. *P. tokudae* was hunted into extinction by 1978 and *P. mariannus* reduced to less than 100 animals by 1974 (Cox and Sacks, 2002).

It was calculated that a human of 70 kg eating two fruit bats of 500 g each would ingest 28 mg/kg of BMAA per kilogram of bodyweight (Cox and Sacks, 2002). In other words, the dose of BMAA from eating one bat would be equivalent to consuming about 1014 kg of cycad flour (Ince and Codd, 2005), which happened to be close to the doses used in Spencer's experiments on macaques. Unfortunately, subsequent *in vivo* studies in which mice were orally dosed for periods of eleven weeks or 30 days showed no change in behavioral, neurochemical, or neuropathological findings (Al-Sammak et al. 2015).

The BMAA hypothesis gained momentum for a third time as a result of studies by Cox and colleagues (2003) when they found that BMAA originates from symbiotic cyanobacteria resident in specialized coralloid roots of *Cycas micronesica* (37 μg/g) and proceeded to provide a mechanism for biomagnification in ascending trophic levels of the Guam ecosystem. Subsequent studies identified that more than 95% of cyanobacterial species produce BMAA (0.3 μg BMAA/g) (Ince and Codd, 2005), which then accumulates in cycads. The cycad seed sarcotesta was found to contain 9 μg BMAA/g and the outer skin of the sarcotesta contains 50 times that of the roots (Ince and Codd, 2005). Cycad seeds are then eaten by flying foxes (3556 μg/g BMAA) and eventually through consumption of the foxes, reach the Chamarro people. Each organismic level is associated with an increase of two orders of magnitude in BMAA concentration (Cox et al., 2003).

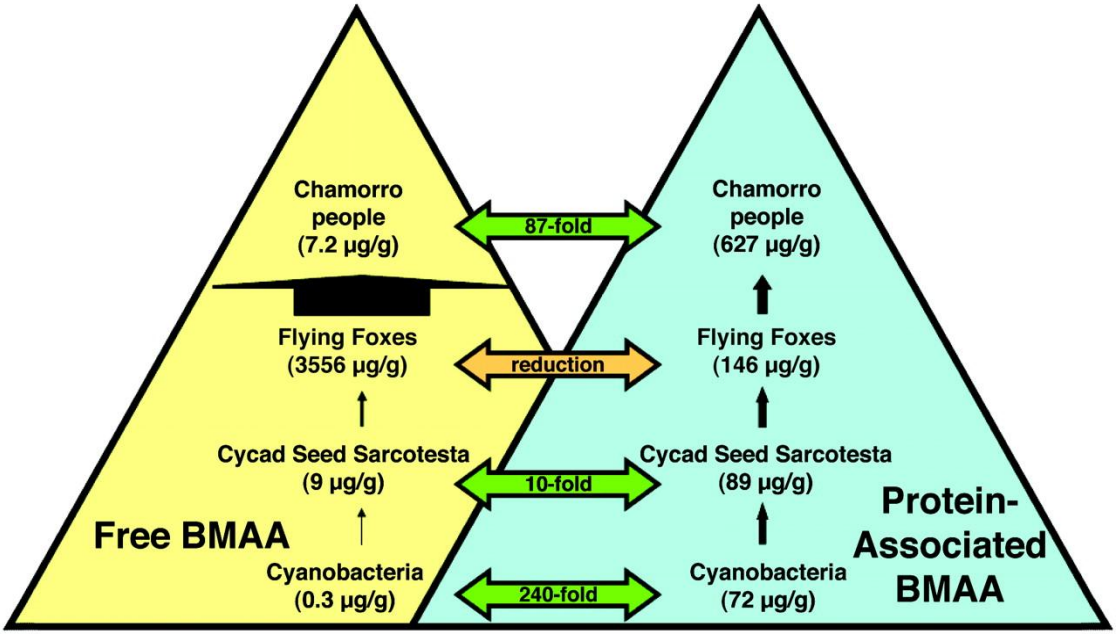

Figure 6-3 Biomagnification of BMAA

Susan J. Murch et al. PNAS 2004;101:12228-12231

In support of the BMAA bioaccumulation theory, Banack et al. (2010a) found a significant link between consumption of foxes and disease status, especially in those individuals who reported consumption of moderate to high numbers of fruit bats. Banack, Cox, and Murch then analysed hair samples to determine exposure to BMAA and whether it correlated with clinical symptoms. While BMAA was detected in the hair of villagers who had consumed flying foxes, cycads, pigs, deer or land crabs, the presence of BMAA in hair was not directly correlated to symptoms of disease (Banack et al., 2010a). BMAA was not present in the hair of the control group (non-consumers of flying foxes etc.). The presence of BMAA in the hair of villagers who consumed one of the proposed BMAA- containing foods did indicate some level of exposure but clinical symptoms would not likely be present for several years (Banack et al., 2010a).

6.1.3.2.1 Protein-bound BMAA

While early experiments had only measured free BMAA concentration, it was discovered that BMAA occurs at much higher levels in the protein fraction of cycad flour than in the free form (Bradley and Mash 2009). Murch et al. (2004) showed that the brains of Chamarro people dying of ALS/PDC contain protein bound BMAA an

average of 100 (10-240) times the concentration of the free form. They found that the brains of all autopsied ALS/PDC patients contained BMAA (five out of six contained free BMAA and six out of six contained protein bound BMAA at concentrations ranging from 0.8-5 mM). In addition to demonstrating a larger accumulation of BMAA than had been previously measured, it indicated that one possible mechanism of BMAA toxicity is through its misincorporation into proteins.

Misincorporation of BMAA into proteins could account for the long latency period between toxin exposure and disease onset. Murch and colleagues (2004) proposed that an endotoxic BMAA reservoir must be present. Pharmacokinetic studies in rats have shown that only 10% of BMAA is excreted into urine and feces and the fate of 90% of the BMAA is unknown. Murch et al. (2004) hypothesized that low dose, long term exposure leads to BMAA accumulation in tissue proteins and that, as proteins are slowly catabolized, the BMAA is released causing neurotoxicity. The peak incidence shortly after WWII may have resulted from starvation on the island of Guam during the Japanese occupation, which led to increased catabolism of protein with a marked increase in toxicity.

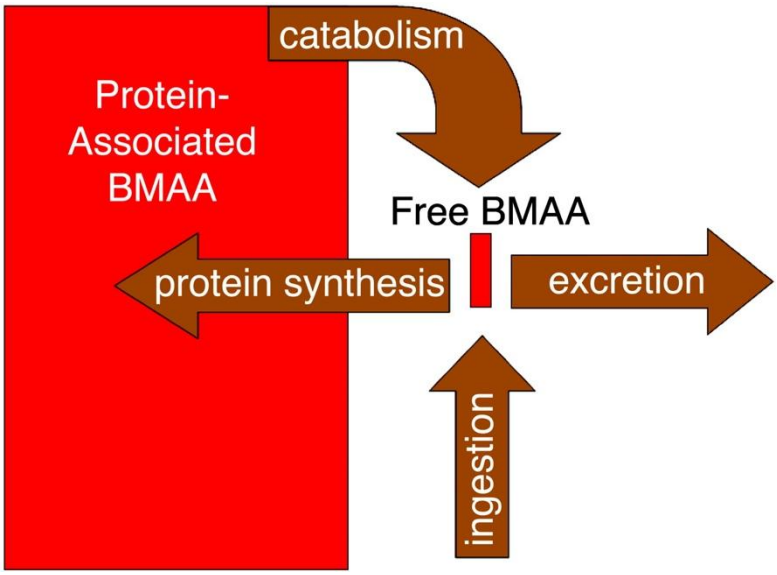

Figure 6-4 Endotoxic Reservoir of BMAA. *According to Susan J. Murch et al. PNAS 2004;101:12228-12231*

One example of possible BMAA-protein misincorporation is related to melanin. BMAA has been shown to selectively bind to synthetic melanin and to melanin containing cells of the retina and brain of rats and mice. BMAA appears to be

incorporated into melanin during synthesis and may be linked to the degeneration of the substantia nigra in PD. This, combined with the retinal toxicity of BMAA suggest a BMAA link between PDC and pigmentary retinopathy (Bradley, 2009), an unusual retinopathy that occured in 52% of Guamanian ALS/PDC patients (compared with only 16% of controls) (Cox et al., 1989). Steele and collaborators (2015) carried out a prospective study over twenty years that followed 239 Guamanian Chamarros in order to assess whether there is an association between linear pigmentary retinopathy and ALS/PDC. They found that of the patients who had been diagnosed with this retinopathy, 30% developed ALS/PDC (versus 2% of those without pigmentary retinopathy). Pigmentary retinopathy has also been seen in cases of ALS/PDC on the Kii Peninsula and may mark the etiologic event (Ludolph et al., 2009). Interestingly, the genetic variant first connected with ALS/PDC (Transient Receptor Potential Cation Channel, Subfamily M) was originally described in connection with metastatic melanomas (Banack and Murch, 2009). Identification of the cause of this retinopathy could provide insight into the pathogenesis of ALS/PDC on Guam and elsewhere.

6.1.3.2.2 Animal model of chronic BMAA toxicity

Cox et al. (2016) recently reported the first successful chronic BMAA toxicity animal model. Anti-tau antibody AT8 (to pSer202 and Thr205) positive tangles and neuronal processes as well as sparse β-amyloid plaque–like deposits were observed in the brain tissues of vervets dosed with BMAA. The NFT were found in the perirhinal, entorhinal, motor, frontal, temporopolar and occipital cortices as well as the amygdala. However, no inclusions were seen in the hippocampus. The lack of NFT in the hippocampus could possibly be accounted for by differential neuronal sensitivity to glutamate excitotoxicity. In the replication experiment, chronic BMAA exposure over 140 days again led to hyperphosphorylated tau deposits and NFT formation in all BMAA-fed vervets. There was a clear dose-response relationship between dietary exposure and density of NFTs. Even with low dose cohorts, protein bound BMAA within vervet brain tissues reached concentrations consistent with the Guam disease. The distribution of NFT and their relationship to dose exposure in the temporal lobe is similar to Braak 1 early stage AD pathology. That is to say, a high density of NFT was observed in the transentorhinal region and no profound clinical symptoms were observed in any of the vervets. This is similar to Braak I early stage

AD pathology in which the pathological changes are largely confined to the pre-alpha layer of the transentorhinal region and there are no cognitive symptoms because the threshold of brain changes required to cause symptoms has not yet been reached (Braak and Braak, 1995).

6.1.3.2.3 BMAA hypothesis controversy

Opponents of the BMAA hypothesis question the relevance of the findings on several grounds. Firstly, the result of BMAA content analysis in cycad flour was found to be similar in both high and low prevalence villages (Duncan, 1990). Snyder and Marler questioned the foundation of the cyanobacterial biomagnification hypothesis when they found that BMAA may be produced by cycads without cyanobacteria (Snyder and Marler 2010). Additionally, some authors have reportedly been unable to detect any free or protein bound BMAA in the brains of either Chamarros or controls (Montine et al. 2005; Snyder et al., 2009). On the other hand, a comprehensive review of animal models of BMAA neurotoxicity by Karamyan and Speth (2008) found that the results of almost all *in vivo* BMAA studies proved its neurotoxicity in regards to its effects on motor neuron function. Several studies have shown neurotoxicity involving hyperexcitability, inability to extend legs, dragging gait, myoclonus and convulsions after a single dose, administered in animal models, intraperitoneally, orally, and intracerebroventricularly (Al-Sammak, 2015). The sum of the evidence suggests that BMAA is at least partially responsible in the case of ALS/PDC.

6.1.3.2.4 Pharmacokinetics

After being consumed orally, 80% of ingested BMAA passes from the gut into the blood stream. It then crosses the BBB either by facilitated diffusion or transport by a specific transporter, ie. LAT1 (Boado et al., 1999). Several studies have demonstrated poor uptake of BMAA into the adult rodent brain (Duncan 1991; Smith 1992; Karlsson 2009; Xie, 2013) but high uptake into various brain regions in fetal/neonatal mice. This correlates well with epidemiologic data suggesting that the toxic exposure occurs in childhood. Mash and colleagues recently showed that the uptake of L-BMAA into the brain differed considerably from that of other tissues. In the brain, the maximum concentration was found to occur around seven hours after ingestion, compared to 0.5-2 hours for muscle, heart liver and kidney. Moreover, the

half-life of BMAA in the brain was longer (25 h) compared to other organs (10-15 hours). The BMAA was preferentially taken up in the hippocampus, basal ganglia and cerebellum of rats (Bradley and Mash 2009). Al-Sammak et al. (2015) observed that the median lethal dose in mice was 3 mg/kg body weight and the lowest observed effect level was 2 mg/kg body weight. Histopathologic lesions were not seen in brains, suggesting a biochemical lesion.

6.1.3.2.5 Proposed Mechanisms of Toxicity

BMAA is believed to exert its effects through multiple mechanisms including excitotoxicity via glutamate receptors, oxidative stress and protein misincorporation. The multiple mechanisms of action could be responsible for the wide variety of disease phenotypes.

- **The glutamate hypothesis**

  Shortly after the isolation of BMAA from cycads, it was shown to have neurotoxic and neuroexcitatory properties (Polsky et al., 1972). As glutamate is the main excitatory neurotransmitter in the CNS, and there was a postulated link between glutamate and sporadic cases of ALS (patients had shown elevated levels of plasma and CSF glutamate levels), several studies were performed in an attempt to determine the effect of BMAA on glutamate receptors. In general, excitatory toxicity results from exposure to excitatory amino acids (glutamate and aspartate) stimulating glutamate N-Methyl-D-Aspartate (NMDA) receptors, thereby causing excessive intracellular calcium ion accumulation and motor neuron death (Bradley and Mash, 2009).

  Unlike other excitatory amino acids (EAAs), BMAA lacks a characteristic terminal side-chain acidic moiety important for potential excitatory toxicity, which prompted the suggestion that its effects may be indirect (Vyas and Weiss, 2009). Neuronal depolarization by BMAA has been shown to be bicarbonate dependent through electrophysiological studies (Vyas and Weiss, 2009). Physiologic bicarbonate ions react with BMAA in a covalent reaction between CO2 and an unprotonated amino group that results in the formation of a carbamate (Vyas and Weiss, 2009). This carbamate shares a structural similarity to glutamate and is likely to be the toxic agent since BMAA is

toxic only in the presence of bicarbonate (Bradley and Mash, 2009). In this form, BMAA competes for binding at various types of glutamate receptors. Binding of the carbamate then leads to shifts in ion concentration involving an increase in intracellular sodium and calcium and a decrease in potassium. Increases in intracellular calcium disrupt mitochondrial function leading to release of ROS. Cytochrome–c is then released from mitochondria leading to apoptosis (Chiu, 2011).

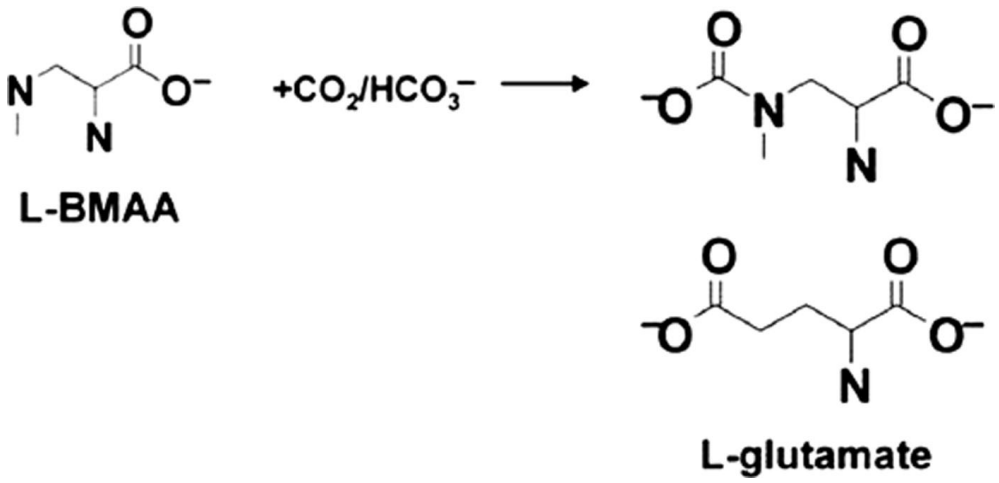

Figure 6-5: BMAA reacts with bicarbonate to form a β-carbamate that may simulate glutamate

Weiss JH. BMAA--an unusual cyanobacterial neurotoxin. Amyotroph Lateral Scler. 2009;10 Suppl 2:50-5. doi: 10.3109/17482960903268742.

BMAA causes changes in cholinergic and glutamatergic transmission due to a decrease in glutamatergic receptors. Experiments in rats shows that BMAA affects the monoaminergic neurons in the substantia nigra, increases the output of dopamine and decreases noradrenaline levels in the hypothalamus. BMAA also inhibits 3h-glutamate binding to metabotropic and strychnine-insensitive 3H-glycine binding site. It strongly stimulates polyphosphoinositol hydrolysis through a mechanism involving metabotropic receptors (Bradley and Mash 2009).

Insight into the mechanisms of BMAA toxicity has been achieved through experiments utilizing antagonists of various glutamate receptors. BMAA has been shown to be a weak agonist at NMDA receptors requiring milimolar concentrations for lethal effects on neurons (Vyas and Weiss, 2009). Concentrations of 3mM cause the

death of entire cortical neuron populations but widespread neurodegeneration induced by BMAA is largely blocked (80%) by selective NMDA antagonists (Weiss 1989). Spinal motor neurons appear to be especially sensitive to BMAA. Mouse spinal neurons show specific toxicity at doses as low as 30 μM (Liu 2010). BMAA produces postsynaptic vacuolization and neuron death that can be prevented by NMDA receptor antagonists and open-channel blockers (Bradley and Mash 2009). The acute convulsant effects of BMAA can be attenuated by antagonists of both NMDA and AMPA receptors for glutamate.

- **Oxidative Stress**

  Closely intertwined with excitatory toxicity is increased oxidative stress. Nunn and Ponnusamy postulated that BMAA toxicity might be due to its conversion to methylamine, which increases oxidative stress, lowers free pyidozal-5'-phosphate and causes selective depletion of AA from brain cells (Bradley and Mash 2009). Additionally, dimers or polymers of BMAA have the ability to chelate metal ions from solution resulting in the formation of reactive oxygen species (ROS). Couratier et al. suggested that neuronal degeneration in ALS could be initiated at the level of glutamate-kainate receptors and is possibly due to a combination of direct action on NMDA receptors and activation of mGluR5 and/or oxidative stress induction (Couratier et al., 1993). Studies have demonstrated that BMAA (3mM, 3h) inhibits the cysteine/glutamate antiporter that mediates cysteine uptake, leading to a depletion of glutathionine thereby increasing oxidative stress (Liu et al., 2009). It also activates AMPA-kainate receptors causing the selective death of motor (cholinergic) neurons. Concentrations as low as 30 μM cause motor neuron death and concentrations of 10 μM enhance neuronal death in a synergistic manner (Liu et al., 2010). Chronic infusion of blockers of calcium-permeable AMPA channels markedly reduced the loss of spinal motor neurons in rats. Low BMAA exposures trigger selective neuronal injury via AMPA/kainate receptors, which can be blocked by a broad spectrum of glutamate blockers, thus

strengthening the feasibility of the BMAA hypothesis because toxicity occurs at low levels (Vyas and Weiss, 2009).

- **Misincorporation into proteins**

  Due to the structural similarity to the amino acid alanine, it has been hypothesized that the misincorporation of BMAA into brain proteins leads to protein misfolding or conformational change (Banack et al. 2010). Dunlop et al. (2013) showed evidence that BMAA competes with L-Serine for protein synthesis in mammalian cells in culture resulting in apoptotic cell death and providing a possible mechanism to explain why in some tissues, BMAA is mostly “protein associated” and can only be released by hydrolysis or proteolysis. Karlsson et al. showed that BMAA was distributed to all brain areas in neonatal rats and was also associated with proteins, especially in the hippocampus (Karlsson, 2015). Okle et al. studied the non-excitotoxic effects of low BMAA concentrations using neuroblastoma cells lacking a NMDA receptor and found a close association between BMAA and proteins but were unable to confirm misincorporation. They concluded that there are two independent mechanisms of toxicity according to the dose of BMAA used: high dose (≥ 1mM, 48 h) excitotoxicity leads to increased ROS and protein oxidation, while a low dose (≥ 0.1 mM, 48 h) of BMAA leads to a dysregulation of protein homeostasis with resultant ER stress. This correlates with the report of Spencer et al. (1987), who observed more than one mechanism of toxicity in macaques: early (2-12 weeks) motor toxicity after a high dose of BMAA and late (>13 weeks) behavioural change and extrapyramidal signs after smaller doses. Misincorporation of BMAA into proteins provides a mechanism in which BMAA could initiate misfolding, which could lead to the accumulation of aggregate-prone proteins in neurons (Main et al., 2016).

- **ER stress**

  The accumulation of misfolded proteins in the ER causes ER stress within cells; the mechanisms that deal with this stress are known as the unfolded protein response (UPR) (Main et al., 2016). Current data strongly suggest that the ubiquitin proteasome system (UPS) plays

a central role in the clearance of abnormally folded and oxidized proteins. Failure of the UPS or protein structural changes via ROS damage can lead to the inability of neuronal cells to degrade ubiquinated proteins, contributing to the formation of inclusions and neurodegeneration (Okle, 2013). Interestingly, tau itself has been linked with impairment of the UPS (through inhibition of HDAC6) (Šimić et al., 2016). Okle et al. (2013) noted that low dose BMAA (≥0.1 mM, 48 h) increased protein oxidation, ubiquination, 20S proteasomal and caspase 12 activity, expression of the ER stress marker CHIP, and enhanced phosphorylation of elf2a protein. On a similar note, Main et al. showed that BMAA at low levels (0.5 mM, 24-48 h) does not cause an acute toxicity in neuroblastoma cells but it does increase the expression of the ER stress marker, C/EBP homologous protein (CHOP) and increases the activity of the pro-apoptotic enzyme caspase-3. They also observed an increase in the activity of the lysosomal cysteine proteases cathepsin B and L, which are markers of the accumulation of proteins in the lysosomal system. Interestingly, they were able to prevent the proteotoxic effects using co-treatment with L-serine, implying that the proteotoxic changes initially resulted from incorporation of BMAA into proteins (Main et al., 2016).

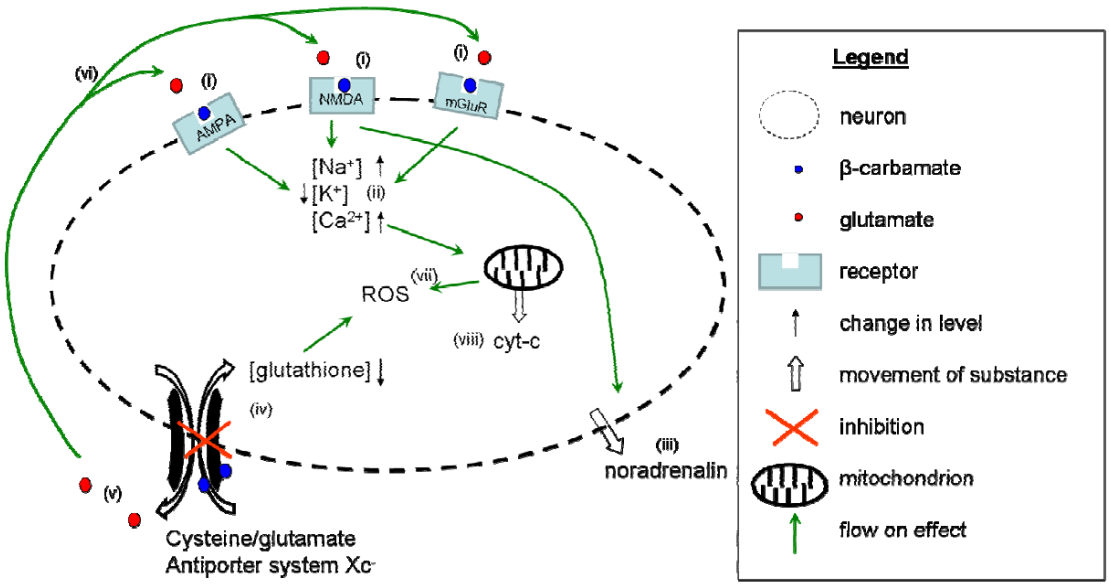

Figure 6-6: Mechanism of BMAA toxicity

Chiu, AS., Gehringer, MM., Welch, JH., Neilan, BA. Does α-Amino-β-methylaminopropionic acid (BMAA) play a role in neurodegeneration? Int. J, Environ. Res. Public Health 2011; 8:3728-3746.

6.1.3.2.6 BMAA effects on Tau

Only one study to date has focused exclusively on the effects of BMAA on tau protein. Arif et al. identified a decrease in PP2A activity associated with an increase in inhibitory phosphorylation of its catalytic subunit PP2Ac at Tyr 307 and abnormal hyperphosphorylation of tau in brains of patients with ALS/PDC. Subsequent studies have been performed to determine the effect of BMAA on PP2A activity in mouse primary neuronal cultures and metabolically active rat brain slices. The results showed that BMAA significantly decreased PP2A activity, leading to an increase in tau kinase activity resulting in tau hyperphosphorylation at PP2A favourable sites. By blocking the mGluR5 receptor and Src, the abnormal phosphorylation of tau is prevented. It was shown by co-immunoprecipitation that BMAA dissociates PP2Ax from mGluR5 making it available for phosphorylation at Tyr307 by Src (Arif et al., 2013).

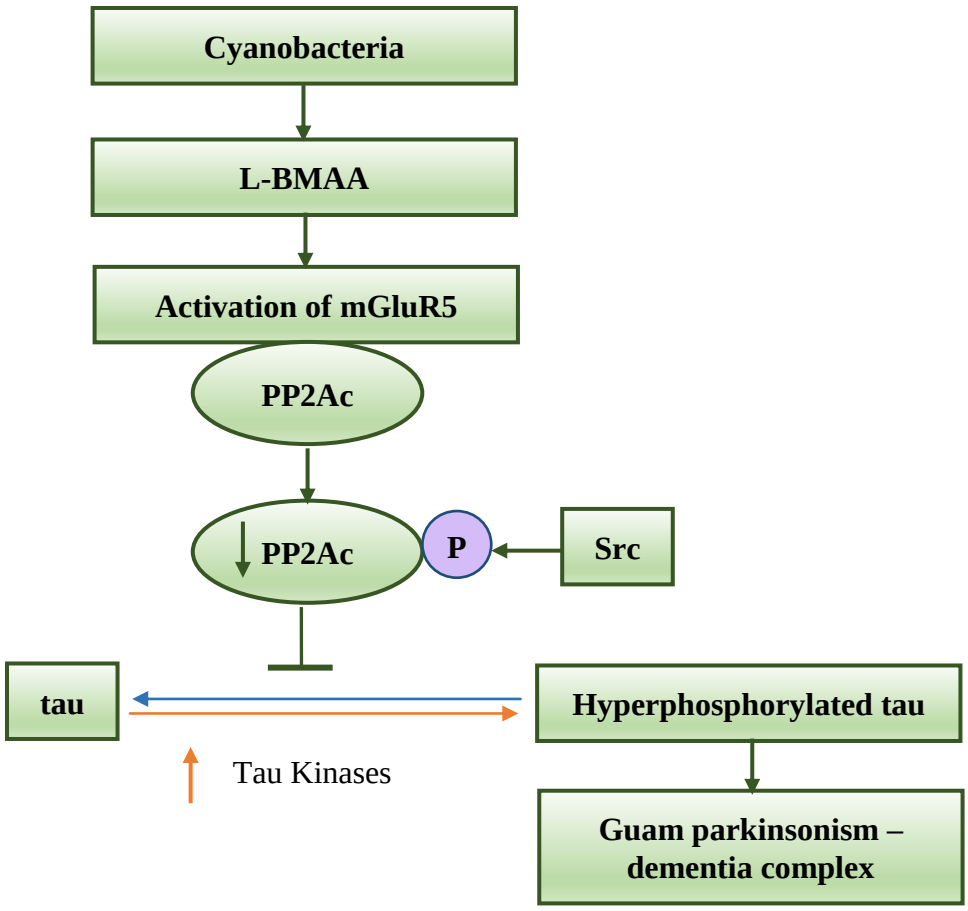

Figure 6-7 BMAA effect on tau proteins.

- **Relevance beyond the Western Pacific?**

Cyanobacteria are ubiquitous and potential exists for widespread human exposure through aerosolization and biomagnification in the food chain. BMAA has been detected in many fish species and aquatic plants as well as open water indicating possible human exposure (Al-Sammak, 2015). Interestingly, cyanobacteria have been found in the human gut and although they are usually a small component, an imbalance could have possible relevance for developing neurodegenerative disease (Brenner, 2012; Klingelhoefer and Reichmann, 2015 ; Ley et al. 2006).

In support of the hypothesis regarding possible global implications of cyanobacterially-produced BMAA, a cluster of ALS with an incidence about 25 times higher than the expected rate in the USA was recently identified around a lake in New Hampshire and is hypothesized to be the result of cyanobacterial blooms that produce BMAA (Banack, 2015). Banack and colleagues tested carp brain, muscle, and liver as well as filtered aerosol for BMAA and demonstrated a positive result and thus possible cause for sporadic ALS. This supported earlier findings in which brains of two Canadian AD patients from the Vancouver brain back tested positive for BMAA at similar concentrations to those found in Chamarros (Murch et al., 2004). Pablo and Mash confirmed and expanded on these findings through their studies on brains from Miami with proven ALS/AD. They found BMAA in the protein fraction in 49/50 AD/ALS brains at a similar level reported by Murch et al. and importantly, no BMAA in controls (Chiu et al., 2011). Mash and colleagues also performed studies on the brains of Parkinson's disease patients and found levels of BMAA similar to those found in previously autopsied ALS/AD. These studies prove exposure to BMAA exists outside the Western Pacific foci and provides a possible etiologic link to sporadic neurodegenerative disease.

Interestingly, a two fold increase in the incidence of ALS in deployed US military involved in several wars has been identified raising the question of whether BMAA is present in the dust of cyanobacterial- containing desert crust (Bradley and Mash, 2009). Additionally, studies have reported the coexistence of ALS and PDC in 4 patients in the Czech Republic (Farnikova et al., 2010) as well as a six fold increased incidence of ALS in Italian soccer players, prompting questions about sporadic, genetic, or possibly similar environmental exposures to BMAA outside of the Western Pacific. It could be informative to

conduct research on possible cyanobacterial/BMAA sources in all of these cases.

6.1.3.3 Sterol Glucosides

Among the opponents to the BMAA hypothesis are Shaw and colleagues who showed that pure BMAA fed to mice over 30 days caused no sign of toxicity (Shaw et al., 2009). A combination of the knowledge that Chamarros were aware of the toxicity of cycads and carefully washed the seeds to detoxify them as well as findings that BMAA content of the flour was the same in high and low prevalence villages led Shaw and colleagues to search for water insoluble toxins. They identified a new group of neurotoxins in cycad seeds through chemical extraction of washed cycad flour. Three primary β-D-glucosides were isolated that showed neurotoxic effects in vitro: β-Sitosterol β-d-Glucoside (BSSG) (the most abundant but least potent neurotoxin), campesterol and stigmasterol (SG) (Shaw et al., 2009). They observed that mice exposed to BSSG and SG on a daily basis for 15 weeks developed a pattern of toxicity similar to that found in ALS/PDC, which included progressive deficits in motor, cognitive, and olfactory functions, neuron loss in the spinal cord, nigrostriatal system, cortex, hippocampus, and olfactory bulb.

Sterol glucosides occur in many plants and animals and it has been hypothesized that autosynthesis, *H. pylori*, *mycoplasma* and other bacteria are additional possible sources of exposure. Especially interesting on a global scale is a possible link between *H. pylori* and parkinsonism. Schultz et al. (2006) hypothesized that cholesterol glucosides arising from *H. pylori* infection may act as neurotoxins, promoting the degeneration of DAergic neurons, in a similar fashion to that thought to link cycad consumption and ALS-PDC.

Several authors including Karamyan, Speth, and Spencer criticized the sterol glucoside hypothesis because glucosides are normally present in high concentrations in the ordinary human diet. Moreover, some of them have been marketed as health supplements (Bradley, 2009). Shaw and colleagues believe that, since cycads contain a higher amount of sterol glucosides than most other plants, it is a dose-related toxicity. Additional criticism involves the inability to remove both cycasin and protein bound BMAA by the methods Shaw and colleagues employed for washing the cycad flour.

6.1.3.4 Cycasin

Cycasin is a member of a family of naturally occurring azoxyglycosides and is the most abundant toxin by weight in Cycad (4% w/w). It has been shown to be a weak cyanogen compound that releases minor amounts of hydrogen cyanide following hydrolysis by β-glucosidases in the alkaline environment of the small intestines. The active toxic metabolite is the potent alkylating agent MAM. Most of the MAM formed by this reaction is degraded to nitrogen gas, methanol and formaldehyde, particularly in the acidic conditions of the stomach (Barceloux, 2009). Samples of cycad flour tested in Guam contained 10 times more cycasin than BMAA on average and those samples containing the highest cycasin content correlated with high prevalence villages (Kisby, 1992). Spencer et al. (2016) suggest that perhaps by acting as a slow toxin, cycasin is the main causative agent of ALS/PDC, with BMAA playing an agonistic role.

Support for this hypothesis has been demonstrated by the occurrence of pathological changes comparable to ALS/PDC after administration of MAM to neonatal mice. MAM disrupts neuronal migration in the developing rodent brain resulting in ectopic neurons in the cerebellum, which are similar to those found in some ALS/PDC patients (Spencer, 2016). Proteomic studies show that a single injection of MAM produces persistent DNA damage, and disrupts the expression of proteins and genes that regulate the neuronal cytoskeleton, protein degradation, and mitochondrial function. It appears that MAM targets distinct networks in the developing brain and that the adult brain is relatively insensitive to its effects (Spencer et al., 2015). Another possible link between cycasin and ALS/PDC is related to its effects on pancreatic islet cells. MAM damages human islet cells, which is consistent with the 44% incidence of diabetes amongst ALS/PDC patients on Guam (Spencer et al., 2016).

Cycasin and MAM readily cross the BBB through a Na+-dependent glucose transporter (SGLT) (Spencer et al., 2015). MAM produces DNA damage by methylation leading to N^7-mG and O^6-mG DNA lesions. The immature human brain has a low capacity to repair alkylation-induced DNA damage. DNA lesions cause changes in typically “cancer associated” genes such as *P53* perhaps indicating that modulation of those genes can cause conditions other than cancer in non-cycling cells. Spencer (2016) hypothesizes that persistence of O^6-mG DNA lesions can lead

to changes in the CNS that result in a predisposition to neurodegenerative disease later in life. DNA damage or epigenetic-mediated mechanisms are thought to contribute to tau pathology through the ability of MAM to induce latent tau phosphorylation in mice. It has been demonstrated that MAM increases glutamate-stimulated *MAPT* mRNA expression and cell loss *in vitro*. As part of an increased transcription response, increased MAPK and caspase-3 activity have been noted, both of which have been documented to be involved in tau aggregation and NFT formation. In studies by Kisby et al. (2011), a single dose of MAM produced early DNA damage, followed by an increase in cortical and hippocampal levels of tau isoforms, tau oligomers and phospho-tau species 3 months later. Moreover, the pathology was preceded by early changes in brain cell signaling proteins, PI3 kinase, phospho-Akt, and GSK-3β (Kisby et al., 2011). Moreover, MAM caused the increased presence of genes associated with olfaction, possibly accounting for the change in olfaction status that is one of the earliest signs of many types of neurodegenerative disease (Spencer et al., 2016; Klingelhoefer and Reichmann, 2015).

MAM is tumorigenic in the periphery, particularly to the intestines. The Wnt/β-catenin signalling pathway appears to be common to perturbations of function in both brain tissue and colonic epithelium (Kisby et al., 2011). In a murine colon cancer model, the MAM treated mice display elevated β-catenin levels and decreased activity of GSK3β (a multifunctional serine/threonine kinase that participates in insulin-dependent glycogen synthesis). Over expression of GSK3β resulting from age-related negative regulation of Wnt signalling promotes excessive tau phosphorylation in brain tissue, a key feature of pathological aging and AD (Spencer et al., 2016).

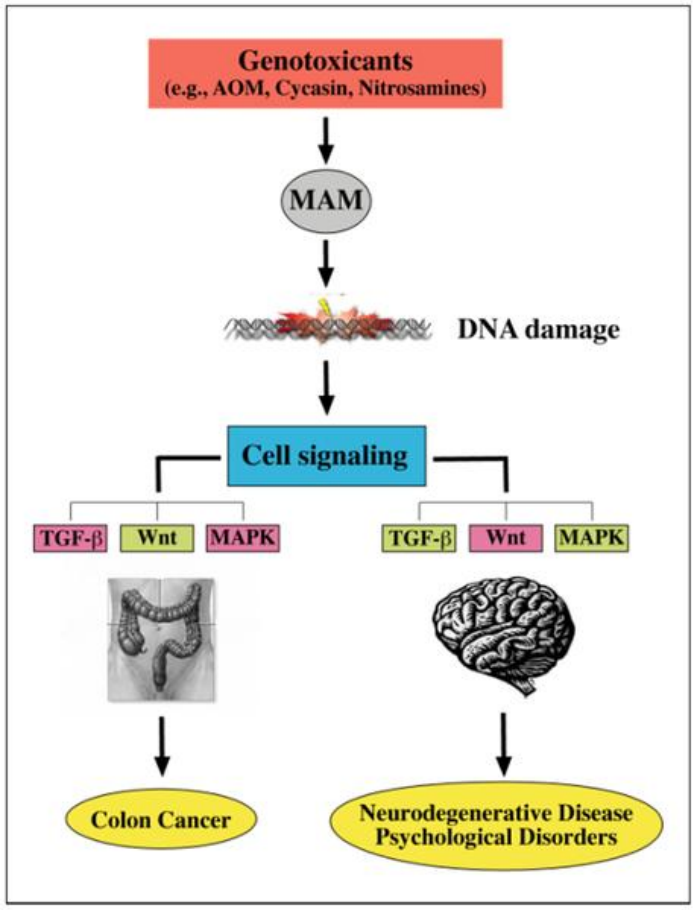

Figure 6-8: Proposed relationship between MAM-induced cancer and brain disease

Kisby GE, Fry RC, Lasarev MR, Bammler TK, Beyer RP, Churchwell M, et al. The cycad genotoxin MAM modulates brain cellular pathways involved in neurodegenerative disease and cancer in a DNA damage-linked manner. PLoS ONE 2011; 6:20911; DOI:10.1371/journal.pone.0020911.

6.1.3.5 Rotenone

A hypothesis of neurotoxicity that has never been tested in Guam but could be linked with ALS/PDC involves exposure to the pesticide, rotenone. Rotenone is known to cause parkinsonism and for many centuries the Chamarros used plants containing this compound to paralyze fish and shrimp in rivers and ocean shallows. In the village of Umatac, which has the highest incidence of PDC, people remember pounding the roots and leaves of *Derris elliptica* and *Derris trifoliate* into a mash that they spread over the surface of the sea in a large area (Caparros-Lefebvre et al., 2006). The use of this method of trapping fish ended shortly after WW II. It could be that exposure in the past caused ALS/PDC after a long latency. Support for this is given by Greenmyre and collaborators who developed a model of chronic toxicity after exposure (Greenmyre et al., 2001), and by the surveys of Filipino migrants who developed ALS/PDC and denied eating cycads but were heavy consumers of locally caught fish.

6.1.4 Inorganic Toxins

The other major branch of research has involved a search for inorganic toxins. Studies by several different groups revealed the only environmental factor directly correlated with each of the three Western Pacific foci was a deficiency in calcium and magnesium in the drinking water, coupled with high levels of aluminium and iron. On Guam, the high incidence area is covered with a red-clay soil containing high levels of aluminium (40%), iron oxides (20%) and silica (1%) and similar foci exist in the other two areas (Garruto et al., 1990). The involvement of alkaline earth metals has been supported by findings that 20-46% of Guamanian and Kii Peninsular patients exhibited biochemical disturbances of calcium and vitamin D metabolism. Studies of Guamanian children and adults also suggested a lower bone mass in the general population (Garruto et al., 1990). This complements the findings of calcium, aluminium and silicon in the brain and spinal cord tissues from patients with ALS/PDC (Malette et al., 1977). Additionally, defects in calcium and vitamin D metabolism have been reported in sporadic cases of ALS. The deposition is hypothesized to interfere with axonal transport by disrupting the cytoskeleton and altering normal catabolism resulting in excessive accumulation of neurofilament. The deposition is likely to occur long before the onset of clinical symptoms.

Taniguchi et al. (2013) reported cataleptic behavior in mice (equivalent to PD in humans) fed a low calcium/magnesium diet, which was transiently relieved by administration of L-DOPA and amantadine. It has been reported that accumulation of pollutant metals such as manganese and aluminium is likely caused by metabolic changes due to a lack of protective factors. A reduction in blood calcium levels has been found to enhance aluminium absorption and aluminium has been shown to cause lesions similar to those found in ALS/PDC. Exposure to arsenite was shown to result in a significant increase in the phosphorylation of several amino acid residues in tau. Arsenite induced tau phosphorylation did not however, involve glycogen synthase kinase-3 or protein phosphatase-1 or -2 (Giasson et al., 2002).

Interestingly, extremely high levels of manganese have been found in *Pandanus* trees in Guam, a tree that once had a strong role in Chamarro culture as a source of food, fibre and medicine. Manganese has been suspected to play a role in ALS/PDC since 1965 (Denton et al., 2009). Normally, manganese is biologically unavailable in soil and rocks because of its 4+ valence, but under wetland conditions it may be reduced to Mn2+ and make its way into biological resources. Manganese

poisoning is symptomatically similar to PDC. Reported cases of manganism have been confined almost exclusively to occupational exposures involving inhalation of dust.

6.1.5 Neuropathology of ALS/PDC

On the basis of on the basis of NFT similarity and the coexistence of both phenotypes in 7% of cases, Hirano and Zimmerman reached the conclusion that the ALS/PDC syndromes represent a spectrum of a single disease. The intensity of NFTs was less in ALS than PDC and showed a typical anatomical distribution in the motor system but the cytopathology and molecular pathology were reportedly different from classical ALS. In ALS/PDC spinal cord NFT pathology is present both in anterior horn motor neurons and also in other areas of the spinal white matter including the dorsal horn (Ince and Codd, 2005). The tangles share the same profile of tau biochemistry as the lesions present in other brain regions. Some authors published papers showing that classical ALS pathology (non-tau ubiquitinated inclusions) is also a feature of Lytico (Ince and Codd, 2005). In a neuropathology study by Winton and colleagues (Winton et al., 2006) of nine PDC brains and eight Chamarro controls, widespread accumulations of insoluble, hyperphosphorylated tau similar to the PHF of AD were found throughout the gray and white matter. Western blot analysis showed widespread tau pathology in the neocortex, cerebellum and medulla that was confirmed by the identification of multiple types of tau-positive neuronal and glial inclusions using immunohistochemical methods. A high degree of tau pathology in the hippocampus and entorhinal cortex of controls was also detected. It differed from AD in that there was abundant glial tau pathology including astrocytic plaques, inclusions and oligodendritic coiled bodies. The NFTs were preferentially localized to layers II and III (supragranular layers) of the neocortex and were also observed in the spinal cord. Hof et al. also reported that most of the NFTs were located in the supragranular layers rather than the infragranular layers where they commonly predominate in AD. Neuropil threads have been inconsistently observed in PDC. The presence of α-synuclein deposits more closely resemble frontotemporal dementia (FTD) tauopathies than AD (Winton et al., 2006). Biee-Scherrer et al. identified the tau isoform as a triplet of 3R and 4R isoforms similar to that found in AD. Transactive Response DNA Binding Protein *43* kDa (TDP-43) was found in dystrophic neurites as well as in neuronal and glial inclusions and Lrrk2 has

also been implicated in ALS/PDC (Miklossy et al., 2008). Interestingly, Anderson et al. (1979) and Oyanagi et al. (1994) found a significant level of NFTs in asymptomatic Chamarros (Steele and McGreer, 2008), which raised the question whether this represents an early asymptomatic form of the disease or whether Chamarros are simply prone to form NFTs for some reason.

6.1.6 Clinical features of ALS/PDC

ALS is known locally on Guam as lytico (idiotic). Parkinson's Disease is known as bodig (lazy). Both are uniformly fatal, usually within 5 years. Guamanian ALS is clinically identical to sporadic ALS while the clinical features of PDC are highly variable. As noted by Steele (2005), typical symptoms and signs of the disease include generalized slowing, difficulty maintaining voluntary motor activities and marked diminution of movements. Early symptoms include stooped posture, slurring of speech and slowing of gait. As the disease progresses, flexor rigidity becomes more prominent, particularly in the trunk and neck. The rigidity begins proximally and is symmetrical in the upper extremities. Fixed, rigid catatonic postures have been also seen in some patients. By the late stage of the disease, generalized rigidity and immobility are usually pronounced. Tremor is present in many patients but never completely incapacitating. The tremor is localized to the hands and tends to lessen as the disease progresses due to increased rigidity. Patients show tremor at various moments (rest, action, and with changes of posture) that can be increased by movement or excitement and sometimes disappears with rest. In approximately one third of patients, the tongue is affected by tremor. Eyelid, jaw and head tremor are also sometimes seen. Mental changes with memory impairment and disorientation are common and often accompanied by personality changes and mood disorders. Comprehension and simple reasoning worsen with disease progression and patients exhibit increased confusion, and apathy. Dementia is often the dominant and sometimes the initial symptom. Few patients exhibit the classic pill-rolling tremor, micrographia, festinating gait, propulsion, retropulsion, or other features associated with PD (Steele, 2005). A PSP-like syndrome is a common finding in many cases of PDC/ALS (Steele, 2005), as is CBD (asymmetical apraxia, rigidity, limb dystonia,) and primary progressive aphasia.

Interestingly, 44% of Guamanian patients also exhibited diabetes. It has been hypothesized that impairment of glucose metabolism in the brain occurs much earlier than the clinical symptoms of neurodegenerative disease, and may lead to decreased *O*-GlcNAcylation of tau. As *O*-GlcNAcylation has been shown to be in inverse correlation to tau protein phosphorylation, this state is believed to lead to tau hyperphosphorylation resulting in a cascade leading to eventual neuron demise.

6.1.6.1 Marianas dementia

In 1987, Schoenberg performed surveys in three villages on Guam to assess prevalence rates and make a comparison with those measured 35 years before. Fifty-five cases of neurodegeneration in a population of 3,576 residents were identified (Steele, 2005). Interestingly, 19 of these cases showed only symptoms of dementia with a relatively late age of onset of 73. It was unsure whether this dementia was part of the spectrum of ALS/PDC or AD. It has been given a special designation: Mariana's dementia MD). Further research was conducted from 1997-2000 in which 194 Chamarros were tested in an attempt to differentiate between cortical and subcortical forms of dementia. It was determined that MD was present in eighty three patients; ninety were diagnosed with PDC; eleven had parkinsonism alone; and ten were diagnosed with ALS. MD was indistinguishable from PDC, and also met the inclusion criteria for the diagnosis of AD. There were too few autopsy studies to determine if the histopathology was that of PDC or AD or a combination of both (Steele, 2005) but it raised the possibility of a phenotypic switch similar to the one that had occurred earlier involving ALS to PDC.

6.2 ALS/PDC in Japan- "Muro disease"

6.2.1 Epidemiology

A second focus of ALS/PDC was identified 2400 km away from Guam in Japan in the 1960s through an epidemiological survey conducted by Kimura and Kase (Yoshida et al., 1998). The incidence of ALS in this genetically and ethnically distinct population in the mountainous regions along the southern coast of the Kii Peninsula was found to be approximately 100 times higher than that of the rest of Japan. Two districts, Hohara (Mie prefecture) and Kozagawa (Wakayama prefecture) were identified as the epicentre (Kuzuhara and Kokuba, 2005). Interestingly, the first description of the disease 'Ashi-nae' (meaning paralysis of the legs) dates back to a

book written in the mid-17th century (Yoshida et al., 1998). A more recent survey by Kuzuhara and colleagues showed an ALS incidence rate of 49.4 per 100000 between the years of 1990-1994, which didn't differ substantially from the rate during 1960-1979 despite reports of a declining incidence and phenotypic switch from ALS to PDC (Kaji et al., 2012). The crude prevalence rate for all ALS/PDC in 1998 was 801 per 100,000 (334 PDC, 334 PDC+ALS, 133 ALS) (Kuzuhara, 2001). After a temporary decline in the 1980s it is evident that high prevalence rates continue (Kuzuhara, 2005).

6.2.2 Etiology

Possible causes including genetics, infection, and environmental factors have all been considered but none has been proven. One important hypothesis is related to a change in environmental factors to account for the dramatic decline in incidence and change in phenotype. The most likely suspect was an improvement in minerals in food and drinking water, as a consequence of increased westernization. Yase (1972) proposed that chronic nutritional deficiencies of calcium and magnesium and relative excess of aluminium resulted in abnormal accumulation in neurons and caused motor neuron degeneration. However, in subsequent studies serum calcium and parathyroid hormone levels have been found to be within normal limits in Hobara as well as on Guam. A recently published study by Kihira et al. (2015), analyzed scalp hair from ALS patients and controls for transition metals and found significantly elevated levels of zinc, vanadium, manganese, and aluminum. They hypothesize that long-term exposure to these metals may trigger neuronal degeneration through induction of oxidative stress, especially in patients with genetic vulnerabilities. Another study showed Kii ALS patients have elevated levels of urinary 8-hydroxydeoxyguanosine, a marker of DNA oxidation (Morimoto et al., 2009).

In an attempt to extend the cycad hypothesis to the Japanese cases, Spencer et al. (1987) performed two field studies in the Mie prefecture, which led to identification of the presence and use of cycads (*C. revoluta*). They reported a story of a young girl who played with cycad seeds every year that had been collected by her grandmother and died at the age of 25 from ALS. She had used them as marbles, and had likely ingested the seeds orally. She was healthy until the age of 18 when she complained of backache and calf spasms. The mature seed of *C. revoluta*

was also stocked by some pharmacies that filled prescriptions written by traditional folk medicine practitioners (Spencer et al., 1987).

Despite evidence of cycad use, the Japanese have largely pursued a genetic course in their research due to a greater than 70% familial occurrence. Environmental causes have effectively been ruled out in the minds of many researchers because of a case report of an 82-year-old Japanese man who had lived 28 years on Guam and who developed PD despite his extreme exposure to the traditional Chamarro lifestyle (Kuzuhara, 2005). Furthermore, the report of the development of PDC in the child (born in Kyoto) of a man born in an endemic region who died from ALS helped to steer the focus towards a genetic cause (Kaji, 2012). Despite these cases and extensive genetic analysis, a monogenic cause of the disease has not yet been identified. Current thinking is that the disorder may be autosomal dominant with poor penetrance, indicating multifactorial inheritance.

6.2.3 Neuropathology

The neuropathological findings are identical to those of Guam ALS/PDC in terms of distribution but differ in density of NFTs (Mimuro et al., 2007). Macroscopically, the brains of ALS cases are unremarkable while those of PDC or combined cases show marked atrophy. The substantia nigra and locus ceruleus show loss of pigmented neurons in all cases (Mimuro et al. 2007). Kii PDC is characterized by marked cortical atrophy and neuronal loss of the anterior portion of the frontal and temporal lobes as well as widespread NFTs and neuropil threads, with the highest density in the hippocampal formation and frontal neocortex (Itoh et al., 2003). Single-photon emission computed tomography (SPECT) shows decreased cerebral blood flow to the frontal and temporal lobes (Kuzuhara and Kokubo, 2005). The findings are similar to those of AD but differ in the paucity of senile plaques as well as being preferentially distributed in layers II and III of the cortex vs. layers V and VI in AD. The NFTs are more widespread than those of AD, occurring throughout the CNS, including basal ganglia, brain stem and spinal cord (Mimuro et al., 2007). Hyperphosphorylated tau proteins show a triplet band pattern (60, 64, and 68 kDa) consisting of all 6 isoforms, confirmed using immunohistochemistry (and identical to AD PHF tau). Ultrastructurally they are composed of twisted PHF with a diameter of 8-20 nm, twisted at a periodicity of 80 nm, again similar to AD PHF (Itoh et al. 2003;

Kuzuhara 2005). As in Guam, it has been determined that the similar topographical distribution of NFTs in ALS and PDC cases represent a single tauopathy (Mimuro et al., 2007).

6.2.4 Clinical Symptoms

The clinical symptoms of Kii ALS are indistinguishable from sporadic ALS in other parts of the world while Kii PDC is unique but identical to Guam. In cases with overlap, PDC usually precedes the development of ALS symptoms (Mimuro, 2007). The most characteristic cognitive deficit of Kii PDC was abulia/apathy (Shindo et al., 2014). Although sharing many similarities with Guam ALS/PDC, the disease in Kii differed by predominantly affecting females (Kuzuhara and Kotubo, 2005). As additional evidence that ALS/PDC in the Western Pacific foci is the same disease, a pigmentary retinopathy was identified in 33.3% of patients with Kii ALS/PDC believed similar though milder, to the retinopathy found in Guamanian PDC.

6.3 ALS/PDC in Papua New Guinea

A third focus of ALS/PDC was reported in New Guinea amongst the indigenous people of south West Papua and Irian Jaya, Indonesia. Gajdusek first reported a high incidence amongst the Auyu and Jakai linguistic groups and later conducted village surveys between 1974-81 that revealed an ALS incidence more than 10 times higher than either the Guamanian or Kii foci and more than 100 times higher than elsewhere in the world (Spencer et al., 2005). The age of onset was lower (33 years for ALS, 43 years for PDC) compared to cases on Guam but otherwise similar. Gajdusek believed that the cause was most likely environmental and of natural origin due to the occurrence of the disease in a hunter-gatherer tribe that was not exposed to man-made chemicals. He was amongst the authors who proposed that a mineral deficiency triggered a hyperparathyroid response to facilitate uptake of minerals that incidentally promoted the uptake of aluminium, manganese, and iron as bystanders, thus leading to neurodegeneration. The hypothesis has been challenged by Ahlskog and colleagues whose own study of Guamanian patients showed normal levels of parathyroid hormone, calcium, vitamin D, arsenic, cadmium, aluminium, copper, iron, mercury, lead and zinc in blood, hair, and nails (Spencer et al., 1987). The clinical and epidemiological picture mirror the situation in Guam and the Kii Peninsula but neuropathological evidence is lacking.

From 1987-1990 Spencer and colleagues carried out field studies in 6 villages, including the village of Bosuma, which had the highest ever reported prevalence of ALS (1,300/100,000) (Spencer et al., 1987). During the field studies, they made the discovery that cycad seed/pulp was widely used as an effective topical treatment for skin lesions (Spencer et al., 1987). Cycad seed materials have been experimentally demonstrated to speed skin repair in rodents. Also, bedridden ALS Guamanian patients are known to be extremely resistant to bedsores. The traditional use of cycads as a poultice involved: crushing the scrapings of the starchy, toxic inner portion of a raw seed by hand, immersing it in the milky poisonous exudate and then applying it directly to the lesion by means of a strap to hold it in place. It was replaced daily until the lesion healed. Spencer reported a case of a 29-year old man with ALS who recalled using the preparation for a month as taught by his mother who died from ALS at age 50 (Spencer et al., 1987). The surveys also revealed a decline and switch of ALS cases to PDC cases, from a ratio of 20:1 in 1963, to 5:1 in 1970, to 2:3 in 1990, reminiscent of Guam and Japan. Spencer considers that the lack of change in the water supply makes the mineral hypothesis highly unlikely compared to the cycad seed use hypothesis. The decline in prevalence over the years could be associated with the decreased use of cycad because of the change to a village-based lifestyle, which incurred fewer skin injuries as well as disappearance of the childhood skin disease of yaws. Additionally, the development of agriculture probably resulted in a better nutritional state. Proponents of the mineral hypothesis criticized cycad involvement as cycads have been used as medicine in many other populations and surveillance has failed to reveal any additional foci of high incidence (Garruto et al., 1990).

7 Atypical Parkinson’s on Guadeloupe

An increased frequency of atypical parkinsonism was identified on the Caribbean island of Guadeloupe in 1996 by Caparros-Lefebvre and colleagues (1999, 2002) in which the usual ratio of idiopathic to atypical Parkinson’s was found to be reversed. Atypical Parkinson’s (AP) has been identified as being 3.4 times more common on Guadeloupe than idiopathic PD with only one third of the patients reporting the classic symptoms of idiopathic PD. Atypical Parkinson’s syndromes account for about 20% of the total number of cases of parkinsonism throughout North America and Europe while idiopathic PD accounts for approximately 70% of all cases. Cases of AP differ

from idiopathic Parkinson's disease in that they are L-DOPA-resistant (in the long-term), tend to evolve rapidly, and are often associated with postural disorders, supranuclear gaze palsy, pyramidal or cerebellar signs, alien hand syndrome, severe ideomotor apraxia, early autonomic failure and early cognitive decline. Among these syndromes, PSP and CBD are the most common. Others include multiple-system atrophy, diffuse Lewy bodies disease, frontotemporal dementia, Guam PDC, and Gd-PDC/PSP. It has been well established that tau abnormalities play a crucial role in several parkinsonian disorders . One of the six regularly screened polymorphisms in the *MAPT* gene has been confirmed to be associated with sporadic Parkinson's disease (Kara et al., 2012).

7.1 Epidemiology

Guadeloupe has approximately 422,000 inhabitants of whom 80% are of African or mixed origin, 15 % are Indian and 5% are Caucasian (Caparros-Lefebvre, 2005). Cases of atypical PD have been identified in all ethnic groups suggesting an environmental etiology. As in Guam, cases of atypical PD showed an unequal geographic distribution. They were concentrated in three places (Le Moule, Le Lamentin-Sainte-Rose, Marie-Galante) that account for a relatively small part of the population. One third of patients presented with possible/probable PSP and were diagnosed with Guadeloupean Progressive Supranuclear Palsy (Gd-PSP). The prevalence of PSP taking into account only the identified cases was determined to be 14/100000, an almost three fold increase over the prevalence found elsewhere in the world. One third of patients were found to be unclassifiable according to standard diagnostic criteria and designated Guadeloupean Parkinsonism Dementia Complex (Gd-PDC).

Figure 7-1 Map of high incidence villages Guadeloupe

Reprinted by permission Elsevier Limited (Caparros-Lefabvre, D., Steele, J. Atypical Parkinsonism on Guadeloupe, comparison with the parkinsonism-dementia complex of Guam, and environmental toxic hypotheses. Env. Toxicology and pharmacology 2005; 19:407-413.)

Caparros-Lefebvre hypothesized a connection between consumption of soursop fruit/and or tea and indeed, a greater number of patients presenting with atypical parkinsonism reported consuming fruit and infusions of the leaves of *Annona muricata* (soursop, corossol) (OR 8.3) (Caparros-Lefebvre and Elbaz, 1999). In an attempt to clarify the role of *A. muricata*, Lannuzel and colleagues carried out a cross sectional survey on 69 patients and 88 controls matched for sex and age. Consumption of fruit and infusions was evaluated and expressed as fruit-years and cup-years, and then converted into milligrams of annonacin. The consumers were divided into groups of low consumers and high consumers. In the control groups, 14% reported high consumption of fruits and 19% high consumption of infusions, compared to 76% of Gd-PSP and 74% of Gd-PDC. The estimated ingested amount in the patient groups was almost 5 times higher than that of controls (150:33 g) (Lannuzel et al., 2007). Also, the doses consumed were about 20 times higher than doses used in previous experiments that induced widespread neuronal degeneration in rats over a period of 28 days. The seeds were determined to contain the highest level of neurotoxin and there was a correlation with seed consumption and severe, rapid progression of disease (Caparros-Lefebvre

and Lees, 2005). Interestingly, six patients showed improvement in their gait disorders, bradykinesia, and rigidity upon stopping consumption of *Annonceae* suggesting a component of reversibility to the disease.

About 50% of the atypical patients were not high consumers of *Annonceae* suggesting that additional factors, environmental, genetic, or otherwise, might affect the vulnerability of patients to the neurotoxins. One suspected cause is organochlorine derivatives that were once widely used in Guadeloupe as pesticides; however, no epidemiological data is available. A genetic component has been proposed, but less than 4% of the patients have positive family histories of neurodegenerative disease. Additionally, a large study of the PSP-associated H1 subhaplotype showed no association with atypical parkinsonism in Guadeloupe (Cazumat, 2008).

In support of the *Annonceae* neurotoxin hypothesis, clusters of AP have also been reported in New Caledonia (Angibaud, G et al., 2004) and in patients of Caribbean origin in London known to consume Annonceae (Chaudhuri, K et al. 2000). In a study in New Caledonia, Angibaud and collaborators, found that the frequency of atypical parkinsonism was elevated (accounting for approximately 50% of total cases), and that again, the ratio was similar between ethnic groups. They also showed that 73% of atypical parkinsonism patients were regular consumers of *Annonaceae* as opposed to 39% of the patients with idiopathic PD. Chaudhuri identified a four fold increased risk of atypical parkinsonism in Carribean/East Indian immigrants to the UK prompting a search for genetic and/or neurotoxic etiology.

7.2 Toxins in Annona muricata

Annonaceae originated in Central America and were brought by Spanish navigators to the Antilles and Pacific Islands. They are widely used as alimentation and medicine (cardiac, digestive sedation, impotence) in tropical areas of the world but like many plants, contain insecticidal substances essential to their survival that are found in the roots, seeds, bark, fruit, stems, and leaves. Two major classes of toxins have been suspected to play a role in Gd-PSP/PDC: alkaloids and acetogenins.

7.2.1 Alkaloids

Five water-soluble alkaloid toxins have been isolated from *A. muricata*: four benzylisoquinoline alkaloids and one aporphine alkaloid. They are both substrates and

inhibitors of monoamine oxidase (inhibit dopamine reuptake), and some are inhibitors of complex I of the mitochondrial respiratory chain (Coleman et al., 1996). 1-benzyl-1,2,3,4-tetrahydroisoquinoline alkaloids have previously been shown to induce PD in an animal model (Matsushige et al., 2012). Reticuline and coreximine are the most abundant toxins by weight and have shown dopaminergic toxicity in cell culture studies (Lannuzel, 2008). In support of the role of alkaloids as potential causes of Gd-PSP/PDC, high concentrations of endogenous benzyltetrahydroisoquinolones have been detected in the CSF of some parkinsonian patients (Caparros-Lefabvre and Elbaz 1999). It is possible that the mechanism of cytotoxicity is mediated through glutamate or free radicals (Caparros-Lefabvre and Elbaz, 1999). Annonaine showed the most neurotoxicity of all the alkaloids against catecholaminergic cell lines by reduction of tyrosine hydroxylase activity. The concentrations of alkaloids required to cause cell death are relatively high and unlikely to be reached in the brains of patients, making their role in the causation of Gd-PSP/PDC less likely than that of the other class of soursop toxins, acetogenins.

7.2.2 Acetogenins

Anonaceous acetogenins are potent lipophilic inhibitors of complex I of the mitochondrial respiratory chain (Escobar-Khondiker et al., 2007) that are exclusively produced by annonaceous plants. They constitute a unique class of polyketides and are the most potent mitochondrial inhibitors known to date. Thirty-four acetogenins have been isolated from the leaves of *A. muricata*. Annonacin makes up the majority (approximately 70%) of all acetogenins found in extracts (Champy, 2005). Annonacin was shown to readily cross both the BBB and the neuronal cell membrane in several experiments (Champy, 2004 et al., Lannuzel et al., 2003). As acetogenins are not actively exported from the brain parenchyma via P-glycoprotein systems in the BBB (Oberlies et al., 1997) and because they are highly lipophilic, it can be assumed that they accumulate within the brain.

Annonacin, the major acetogenin in *A. muricata*, is 1000 times more toxic than reticuline and 100 times more toxic than 1-Methyl-4-Phenylpyridinium (MPP+) to cultured mesencephalic neurons (Lannuzel et al., 2003). A 30 nM concentration killed most DA neurons in midbrain cultures (Lannuzel, 2008). Its widespread effects can be explained by the fact that, unlike MPP+, it is not a substrate of the dopamine transporter

(Lannuzel, 2008). When fed to rats systemically over 28 days, annonacin penetrated the BBB and reduced adenosine triphosphate (ATP) levels by 44% in the cerebral cortex (which was not severely lesioned) and caused neuronal cell loss and gliosis in the brainstem and basal ganglia with a PSP-like distribution. Interestingly, there were no measurable behavioral changes in the same study (Champy, 2004). However, the degree of cell loss was less than 45% (Champy, 2004) and in parkinsonian patients it is generally accepted that 70% of dopaminergic neurons must be lost before motor symptoms become apparent (Bernheimer et al., 1973). It is estimated that the equivalent to the experimental dose could be attained in humans by regular consumption of soursop within one year.

7.2.2.1 Effects of annonacin on tau

Escobar-Khondiker et al. (2007) performed experiments in which cultured striatal neurons were treated with annonacin for 48 hours. They noted that beginning at a concentration of 50 nm there was concentration dependent neuronal cell loss and accumulation of tau in the cell bodies. They also noted that incubation with annonacin resulted in an increase in tau protein levels that was unaffected by both inhibition of phosphatases and incubation with alkaline phosphatase to dephosphorylate tau. In order to assess the level at which the upregulation of tau was occurring, they tested tau mRNA levels and found that they were not increased. The increase in tau must therefore regulated at a posttranscriptional level. Additionally, they found that annonacin does not induce the aggregation of tau, shown by a lack of thioflavin S staining, but does cause the somatic accumulation of tau and tau-tagged mitochondria. After incubation with annonacin, mitochondria started to move strictly toward the soma as opposed to control cells in which they moved randomly, in both anterograde and retrograde directions. Annoncacin was also seen to cause fragmentation of microtubules. By transfecting the cultures with NADH-quinone oxidoreductase (protecting the cells against ATP depletion by annonacin) it was shown that complex I inhibition is responsible for the redistribution of tau. Also, stimulating anaerobic glycolysis prevents tau redistribution. To rule out the possibility that ROS are responsible for the redistribution of tau the cultures were pretreated with NAC or trolox to reduce ROS, which didn't prevent cell death or redistribution of tau. Other ATP depleting toxins such as MPP+, 3-Nitropropionic Acid (3-NP), and Carbonyl Cyanide m-Chlorophenylhydrazone (CCCP) cause similar tau pathology but at much higher

concentrations (Escobar-Khondiker et al., 2007). Additionally, truncated forms of tau in vitro were found to cause mitochondrial fragmentation while full-length tau caused mitochondrial elongation (Sun and Chen, 2015). It has also been shown that inhibition of complex I results in the upregulation of the splicing factor SRSF2, which increases the expression of 4R tau in neurons (Wang and Mandelkow, 2015) possibly indicating a mechanism for the occurrence of a 4R-tauopathy.

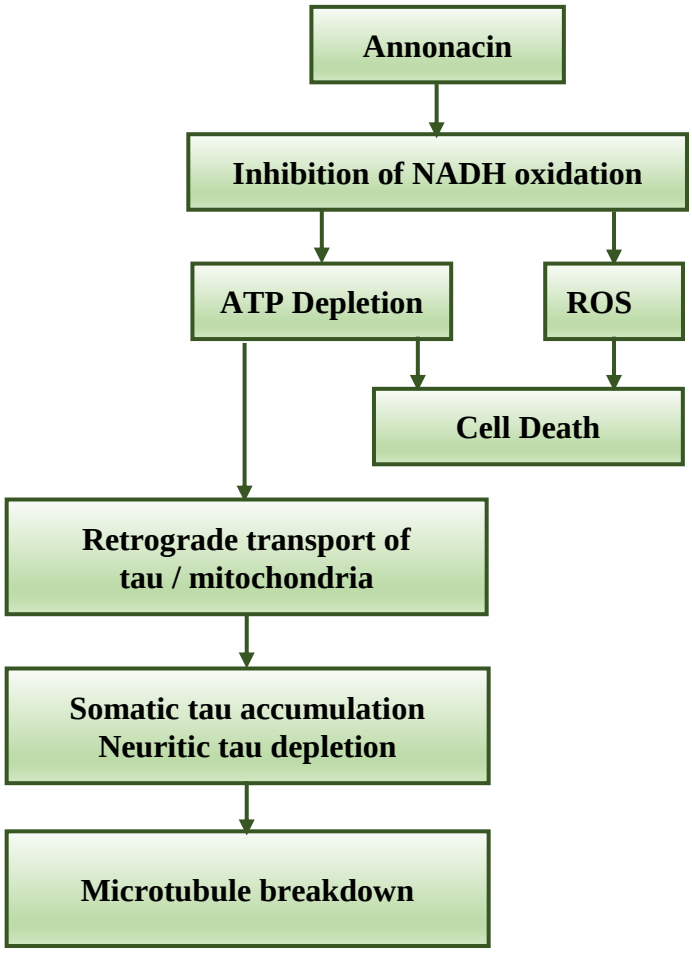

Figure 7-2 Annonacin mechanism of toxicity

7.2.2.2 Inhibitors of mitochondrial complex I

Mitochondria are involved in energy production, calcium buffering and apoptosis signaling pathways. It appears that localization and transport defects of mitochondria are important causes of disease (Schapira, 2010). Different tau isoforms influence mitochondrial localization within neurons in different ways and may change axonal mitochondrial content and/or transport (Stoothof et al., 2009). Free-radical mediated damage is a common cause of secondary mitochondrial mutations. The respiratory chain is an important source of free radicals and mtDNA is vulnerable to damage because it lacks a histone coat and has minimal repair facilities. Perhaps not

coincidentally, the normal human striatum is the site of the most age-related free radical damage in the human body (Schapira, 2010). Complex I inhibition, in addition to reducing ATP production, also increases free radical production, setting off an amplification cycle that leads to secondary mutations and further impairs mitochondrial function (Schapira, 2010).

Although Guadelopean parkinsonism is the only tauopathy in which mitochondrial toxins are suspected to play a major role in causation, complex I inhibition is likely to be important in multiple neurodegenerative disorders. It is estimated that 25% of Parkinson's disease patients have a significant defect in complex 1 (Schapira, 2010). Defects in complex I have also been identified in platelets of some Parkinson's patients (Schapira, 2010). Complex I consists of of 45 polypeptide subunits, with a total mass 980 kDa. It has an L-shaped structure with the hydrophobic component in the inner membrane and the hydrophilic arm projecting into the matrix. Based on the suspected association of *A. muricata* (a member of the family of mitochondrial complex I inhibitors) and Gd-PSP/PDC, several authors have tried to link other neurotoxins with the same mechanism of action to sporadic tauopathies. Hollerhage and colleagues investigated 24 different compounds that were reported in the literature to be inhibitors of complex I. They were grouped according to antagonism of ubiquinone, semiquinone, or ubiquinol as type A, B, or C respectively. They tested 22 natural substances and two synthetic substances. All compounds were found to be moderate to highly lipophilic and inhibited complex I with moderate to high intensity. After 48 hours, all substances induced neuronal cell death and also caused phosphorylated tau to redistribute from the axon to the somatodendritic compartment. They found that the redistribution was maximal at half the concentration with maximum toxicity and at a two- fold dilution lower, little or no tau redistribution occurred (Hollerhage et al., 2009). The specific binding site of these compounds does not appear to affect their biological effects. The redistribution of phosphorylated tau protein is not a specific result of complex 1 inhibition but also by other mitochondrial toxins that lead to ATP depletion by different mechanisms of action. Therefore, a broad array of environmental compounds (ie. pesticides such as fenazaquin) leading to energy depletion might be important etiologic factors in sporadic tauopathies. Microorganisms (myxobacteria, *Streptomyces spp.*) produce many highly toxic complex I inhibitors and as their natural habitat is soil, there is a high probability that many plants are contaminated the possibility of colonization of the human body exists (Hollerhage, 2009).

7.3 Clinical Symptoms

The clinical picture of Gd-PSP/PDC is a unique combination of levodopa-resistant parkinsonism, tremor, hallucinations, Rapid eye movement (REM) sleep behavior disorder (RBD), fronto-subcortical dementia and myoclonus. Two phenotypes (PSP and PDC) have been defined according to the presence or absence of oculomotor signs. The two groups appear to constitute a single disease entity. The presence of several unusual symptoms (hallucinations, dysautonomia, REM sleep behaviour disorder and myoclonus) distinguish Gd-PS/PDC from classical PSP. A study by Aspartis et al. (2008) showed a high prevalence of a very specific type of myoclonus among patients. It was a small amplitude, asymmetrical/unilateral, arrhythmic, rest- or posture-associated tremor of the distal part of the upper limb that did not impair motor function. The cortical origin of the myoclonus was demonstrated by the detection of a short-latency pre-myoclonic positivity in the sensorimotor cortex. This cortical myoclonus likely results from the disinhibition of the motor cortex secondary to basal ganglia dysfunction or intrinsic cortical pathology due to tau accumulations (Aspartis et al., 2008). A third possibility is that glial cells containing tau inclusions, interfere with regulation of the excitability of pyramidal neurons (Aspartis, 2008).

A second difference between classical PSP and Gd-PSP is in regard to the type of oculomotor disturbance. In classical PSP, lesions in the brainstem and cerebellum cause reduced vertical saccade velocity, marked saccade hypometria and severely saccadic smooth pursuit (Aspartis et al., 2008), whereas in Gd-PSP the disturbance is predominantly in the cortex and affects saccade latencies and error rates. This is in accordance with the severity of cognitive impairments and with the distribution of neuropathological findings (Caparros-Lefebvre et al., 2002).

In 2007, Lannuzel et al. (2007) attempted to define the characteristics of the clinical expression of Gd-PSP/PDC. Dementia was observed in 92% of PSP and 100% of PDC patients compared to 52-74% of classical PSP and involved frontal lobe dysfunctions including: slowness of thought, perseveration, primitive reflexes and apathy. Interestingly, both Guamanian and Guadeloupean patients often have preserved verbal comprehension up to a few days before death, unlike in AD (Caparros-Lefebvre et al. 2002). Parkinsonian symptoms were symmetric at onset in 39% of Gd-PSP and 47% of Gd-PDC and a bilateral, postural tremor was the most frequent initial symptom in 45% of Gd-PSP and 43% of Gd-PDC (Lannuzel et al.,

2007). Postural instability was extremely common in both Gd-PSP (82%) and Gd-PDC (70%). The response to levodopa was absent or minimal in 96% of Gd-PSP and 76% of Gd-PDC. Rigidity is most pronounced in the axial muscles in Gd-PSP and in the limb muscles of Gd-PDC. Supranuclear gaze palsy was partial in 80% and complete in 12% of Gd-PSP patients. Urinary incontinence and orthostatic hypotension occurred about 4 years after disease onset. Many Gd-PSP (59%) and PDC (52%) patients experienced hallucinations (most often visual).

RBD involving insomnia, dream enactment, and violence during sleep was found in 78% of Gd-PSP (43% reported the disorder several years before disease onset) as opposed to 13%- 33% of patients with classical PSP (Lannuzel 2008 and Cochen de Cock et al., 2007). The high prevalence of this disorder occurring in a tauopathy when it has usually been associated with synucleinopathies, suggests that the location of the lesions rather than the protein that forms them is important for the development of RBD (Cochen de Cock et al., 2007).

7.4 Neuroimaging of Gd-PSP/PDC

Magnetic resonance imaging (MRI) scans were abnormal in all patients with atypical parkinsonism and showed widespread atrophy of both the cerebral cortex and the brainstem. Atrophy was most pronounced in the frontal and temporal lobes in PSP and in the frontal and parietal lobes in PDC and was largely symmetrical (Lannuzel et al., 2007). Tectal atrophy was observed in 1/3 of PDC and PSP patients but mesencephalic or cerebellar atrophy was observed only in PSP. Interestingly, mesencephalic atrophy was not directly associated with disease duration. The lateral and third ventricles were frequently enlarged in both PDC and PSP. An MRI study by Lehericy and colleagues showed widespread grey matter atrophy in the cortex in Gd-PDC, , whereas the midbrain was relatively spared. Structural white matter abnormalities have been observed in the occipital and temporal white matter, as well as the splenium and the cerebellum. There were widespread diffusion abnormalities in the cortical and subcortical areas. PSP patients had fewer inferior frontal and medial temporal changes, and increased midbrain and superior cerebellar peduncle changes. These differences correlate with the difference in symptoms between classical PSP and Gd-PDC (Lehericy, 2010). The pattern of oculomotor abnormalities differs as well. The

presence of cortical lesions and minimal midbrain involvement provides a strong anatomical correlate to clinical and electrophysiological findings mentioned earlier.

7.5 Pathology

In patients with oculomotor signs, neuronal loss was found to predominate in the substantia nigra and the striatum (Lannuzel, 2008) and was associated with intense gliosis. Non-DAergic neuronal populations were also affected: frontal cortex, palladium, sub-thalamic nucleus and pontine nuclei. Extensive accumulation of tau was observed in neuronal and astrocytic processes but NFT were rare. Gel electrophoresis detected a major doublet of pathological tau, at 64 and 68 kDa similar to that found in PSP. Tau positive threads were the most abundant lesions. Astrocytic tufts were virtually absent as compared to classic PSP.

7.6 Comparison with Guam

In the year 2000, Steele and Caparros-Lefebvre compared Guamanian and Guadeloupean cases of atypical parkinsonism. They agreed that the diseases were very similar to one another. The pathological findings identified in 4 Guadeloupe cases is compatible with classical PSP with an electrophoretic profile of tau proteins in a 64-69 kDa doublet. In Guamanian PDC the abnormal protein was identified as a triplet of pathological tau components at 60, 64, and 69 kDa. However, McGreer identified additional forms of tau in PDC including a 64-69 kDa doublet (Caparros-Lefebvre and Steele, 2005). There were a few differences found in the pathology of Gd-PSP/PDC and Guamanian PDC. The Guadeloupean disease showed an abundance of threads in white matter and a paucity of NFT in the hippocampus compared to Guamanian PDC. The fruits of the Annonaceae family are also commonly eaten on Guam and investigators have begun to evaluate their possible role in PDC in a case-control study (Caparros-Lefebvre and Steele, 2005).

8 Progressive Supranuclear Palsy in Northern France

In 2015, Caparros-Lefebvre et al. reported the first known geographical cluster of PSP. They describe a cluster of PSP in Northern France at a hospital serving the population of the towns of Wattrelos and Leers. For most of the 20th century, this area was a centre for chromate and phosphate ore processing, textile dyeing, and

tanning. There is a significant amount of industrial waste remaining close to residential areas. The observed to expected ratio of PSP incidence was 12.3 with a mean age of onset of 74.3 years. The initial symptom involved gait/balance in over one half of patients. None of the patients were related. Western blots revealed a typical tau 4R doublet. The tau H1 haplotype occurred in 95% of cases. Arsenic and chromium are possible suspects.

Progressive supranuclear palsy (PSP) or Steele-Richardson-Olzsewski syndrome is a member of the 4R tauopathies that affects neurons and glial cells. Although the H1c haplotype is the strongest genetic factor associated with it, it is not thought to be sufficient to cause the disease and so, most cases occur sporadically (Golbe, 2014). The only known risk factor is lower educational attainment (Lefebvre, 2015). The incidence of PSP in developed countries is 1.2/100,000 per year and the prevalence is 5-6/100,000. The clinical diagnostic criteria are both sensitive and specific but the final diagnosis must be confirmed by pathohistological analysis.

PSP is characterized by parkinsonism, early postural instability and falls, vertical supranuclear-gaze palsy, pseudobulbar symptoms, and cognitive decline. The average age of onset is between 60-65 yr with fairly equal distribution between sexes. The median time to death is 5.9 years. Prevalence is 6/100000. The clinical hallmark is supranuclear ophthalmoplegia (SNO), initially affecting saccadic eye movements in the downward direction. The first symptoms of PSP are usually postural instability and falls. Cognitive changes begin in the first year and dysarthria and bradykinesia are the most common problems. In PSP there is accumulation of tau isoforms that contain four microtubule binding domains (4R) in the 64 and 69kDa isoforms. Microtubule function is perturbed and interferes with axonal transport. Impairment of mitochondrial function and oxidative damage have been suggested to play an important role in the pathogenesis. Free radicals induce oxidative stress, particularly membrane lipid peroxidation, inhibiting the dephosphorylation of tau. Neuropathologically, it is characterized by neuronal loss and gliosis consistently affecting mainly the globus pallidus, the subthalamic nucleus, and the substantia nigra. NFTs, pretangles, threads and tufts can be detected.

9 Conclusion

Although the geographic clusters of environmental tauopathies in Guam, Japan, Papua New Guinea, and Guadeloupe account for a relatively small proportion of cases of tauopathies worldwide, understanding their pathophysiology could provide vital clues to the mechanism of sporadic tauopathies elsewhere in the world. Some of the neurotoxins described in conjunction with environmental tauopathies have a world wide distribution, particularly L-BMAA and complex I inhibitors, that could be important factors in several neurodegenerative diseases. Understanding the effects of such neurotoxins can lead to improved animal models as well as potential therapeutic targets.

In summary, while research into the genetic components of disease is important, it is also vital to explore the suspected large role of environmental factors in order to understand and diminish the rapidly increasing burden of neurodegenerative disease.

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A literature search was carried out using the database PubMed using the search terms "environmental tauopathies", “ALS/PDC", "environmental neurotoxins", "Guadeloupe atypical parkinsonism”, "annonacin tauopathy”, and “cycad tauopathy”

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12 Biography

Cari Green was born in Winnipeg, Canada on August 9, 1978. She graduated with a dual diploma in academics and commercial art at Dauphin Regional Comprehensive Secondary school in 1996 and went on to complete a Bachelor of Music (Honours) degree at Brandon University with a major in piano and minors in fine art and philosophy. She moved to Toronto in 2000 to study Dance in the BFA program at York University and then worked internationally in Beirut and Paris for the next 9 years before beginning medical school in Zagreb in 2010. Cari's main field of interest is neuroscience and she plans to complete a PhD after graduating from medical school.