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Contributions of isolated Pacific populations to understanding neurodegenerative diseases

Dedicated in Honor of D. Carleton Gajdusek: Mentor, Colleague and Friend

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Abstract

Isolated human populations have provided a natural experimental laboratory for the ongoing study of human disease. In the mid-20th century a number of high-incidence foci of neurodegenerative diseases were brought to medical attention including kuru, amyotrophic lateral sclerosis, and parkinsonism-dementia. These foci were discovered in Papua New Guinea, West New Guinea, the Kii Peninsula of Japan, and in the Mariana Islands. The study of these diseases in isolated human groups has significantly contributed to our understanding of the cause and mechanisms of pathogenesis of these and related neurodegenerative disorders globally. This paper is dedicated to D. Carleton Gajdusek, a pioneer in the study of neurodegenerative diseases, whose decades of fieldwork and laboratory studies have led to numerous scientific discoveries that have reshaped our thinking and understanding about neurodegeneration.

Key words: *New Guinea, Guam, kuru, ALS, gene-environment, historical, mechanisms.*

Introduction

In 1956, after completing his visiting appointment with Sir Frank Macfarlane Burnet at the Walter and Eliza Hall Institute of Medical Research in Melbourne, Australia, D. Carleton Gajdusek set out on an unplanned expedition, initially to work with virgin-soil populations for measles and a child growth and development program among isolated Australian Aboriginals, and subsequently to New Guinea where, in 1957, he (Fig. 1), along with Australian medical officer Vincent Zigas, studied and prepared the first medical report on a focus of a mysterious new disease that would open up an entirely new field of research on human transmissible spongiform encephalopathies or misfolded protein disorders [39]. The latter unplanned detour, while traveling back to the National Institutes of Health in Bethesda, would launch his significant scientific career and 20 years later would result in the awarding of the Nobel Prize in Physiology or Medicine in 1976 for discoveries of new mechanisms for the origin and dissemination of infectious diseases.

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He wrote to Smadel from the field on March 15, 1957:

"*[...] I am in one of the most remote, recently opened regions of New Guinea (in the Eastern Highlands), in the center of tribal groups of cannibals, only contacted in the last ten years and controlled for five years – still spearing each other as of a few days ago, and cooking and feeding the children the body of a kuru case, the disease I am studying – only a few weeks ago. This is a sorcery-induced disease, according to the local populace, and that it has been the major disease problem of the region, as well as a social problem for the past five years, is certain. It is so astonishing an illness that clinical description can only be read with skepticism, and I was highly skeptical until two days ago, when I arrived and began to see the cases on every side. Classical advancing 'Parkinsonism' involving every age, overwhelming in females although many boys and a few men also have it, is a mighty strange syndrome. To see whole groups of well-nourished healthy young adults dancing about, with athetoid tremors which look far more hysterical than organic, is a real sight. And to see them however, regularly progress to neurological degeneration in three to six months, usually three, and to death is another matter and cannot be shrugged off. If psychological, we have the very best epidemic psychosis for study in the world, and the Institute of Mental Health should be most interested. If a heredo-familial disease, we have the highest incidence concentrated 'epidemic' ever seen, and the human geneticist should be 'on the ball.' If a post-encephalitic or toxic or allergic encephalitic epidemic of basal ganglia disease is here, then all medicine should be interested [...]*" [32, p. 50].

Fig. 1. D. Carleton Gajdusek and Vincent Zigas in the Kuru region, Eastern Highlands of Papua New Guinea with a young patient in the terminal stage of kuru. Photo circa 1957, courtesy of D. Carleton Gajdusek.

However, the discovery of this unique focus of neurodegeneration would not be the first among isolated Pacific people, nor the last one brought to the attention of the international biomedical community by Gajdusek. Subsequent to the discovery of kuru, in the late 1950s and early 1960s in then Dutch New Guinea, Gajdusek described a new focus of "motor neuron disease" among the Auyu and Jakai people of southern West New Guinea [31]. He would return again in 1974 and in later years to what was now known as Irian Jaya or West New Guinea to continue his research, culminating in several important papers on this focus, including its most comprehensive description and analysis in 1982 [38]. This discovery would bring a new dimension to the already existing knowledge of two other foci of high-incidence amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia (PD) in isolated Pacific populations (Fig. 2), one in the Kii Peninsula of Japan [51,118,119] and the other in the Mariana Islands [41,75]. These foci represented natural experimental models of diseases in Pacific populations that hold significant promise for understanding the mechanisms of neurodegeneration globally [41,42]. However, it would only be kuru among the Fore people of New Guinea that would lead relatively quickly to a significant new discovery. The three high-incidence foci of ALS and PD, while positioned to lead to discovery and major breakthroughs in neurodegenerative diseases, did not readily yield a cause or mechanisms of neurodegeneration in a disease that presumably occurred decades after the initial insult, be it genetic, toxic, or some combination of a gene-environment interaction [42].

What follows is an account of our understanding of ALS and PD in these previously high-incidence foci, interjected with thinking as to whether or not these natural experiments will have the impact they were perceived to originally have more than a half-century ago with the first systematic study of their high incidence [31,70,153].

Pacific Foci of ALS and PD

One of the most unique and interesting questions in our understanding of the cause of high-incidence neurodegenerative disease is how three foci of ALS and PD could represent the same neurodegenerative process and initiating causal factor(s) arising in three

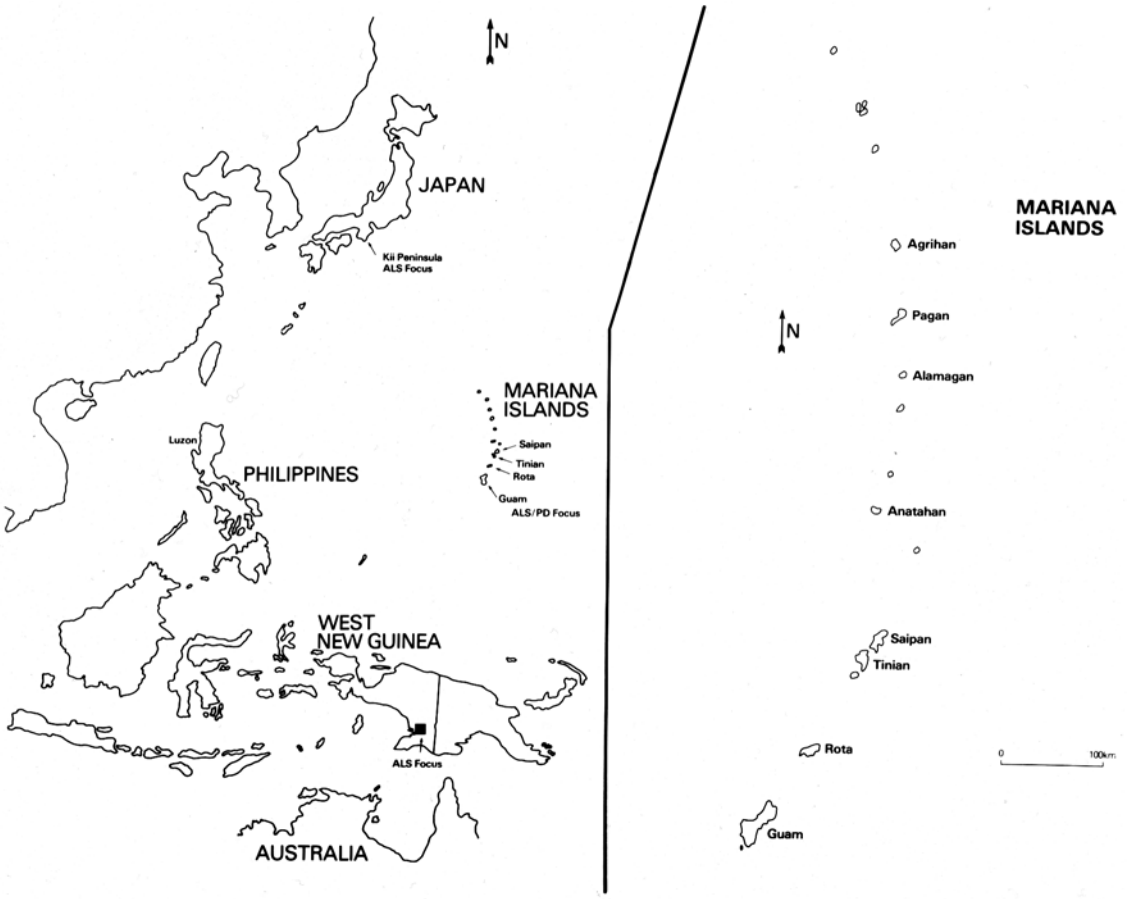

Fig. 2. Map showing the geographic location of the three previously isolated high-incidence foci of ALS and PD in the Western Pacific among the Auyu- and Jakai-speaking people of West New Guinea, among the Chamorros of the Mariana Islands and among Japanese in the Kii Peninsula of Japan.

geographically and genetically distinct isolated human groups, all in the Pacific basin, and all approximately on the same north-south longitude (Fig. 2). The three foci of ALS and PD are found in the Kii Peninsula of Japan, in the Mariana Islands and in southeastern West New Guinea (Fig. 2). ALS and PD in the Mariana Islands was first mentioned in the literature as early as 1875, followed shortly thereafter by the Kii Peninsula, with both foci extensively studied soon after the end of World War II [76,85,89,111]. The striking concentration of disease, the significant geographic and genetic isolation, and limited ecology of these foci have provided an unusual opportunity to study the natural history of ALS and parkinsonism to an extent that would not be possible in large cosmopolitan communities [40].

ALS in these foci is clinically the same as sporadic ALS described by Charcot more than a century ago [15]. It is a disease of the motor neurons and corticospinal tract, characterized by muscle weakness, progressive muscular atrophy, paralysis and spasticity [16,25,110]. Neuropathologically, Pacific ALS is similar to sporadic ALS, except that neurofibrillary tangles (NFTs) are a hallmark lesion in both brain and spinal cord (Fig. 4) [60,119]. By comparison, parkinsonism in these foci is characterized clinically by slowness of voluntary motor activity, muscular rigidity and tremor, and is usually accompanied by an early-onset progressive dementia [60,119]. Neuropathologically, PD is also associated with severe NFT formation and neuronal loss in a specific distribution in the brain (Fig. 4). Lewy bodies and senile plaques are rarely observed [60]. ALS and PD in

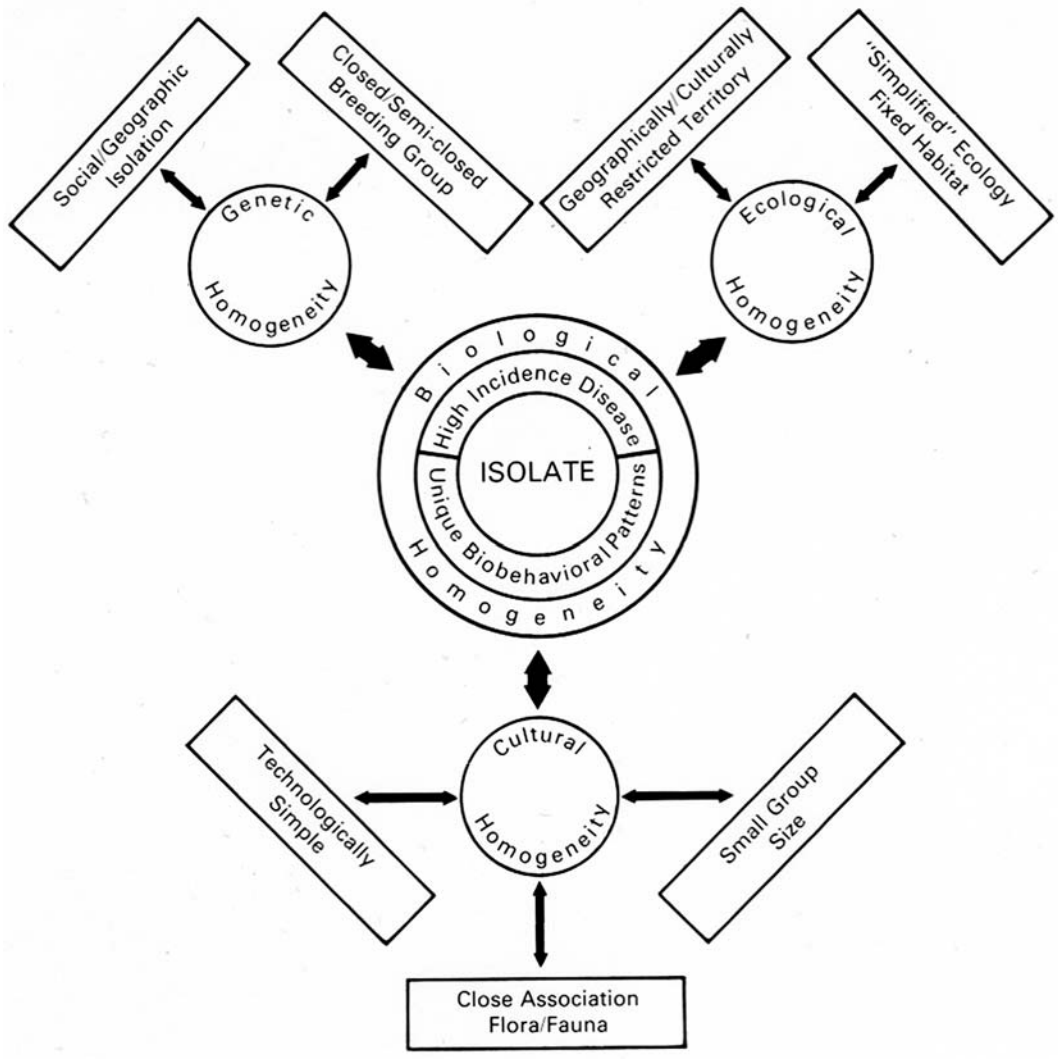

Fig. 3. A schematic representation of natural experimental models of disease in isolated human groups. In addition to their isolation, these groups provide a natural laboratory for study because of their genetic, ecological and cultural homogeneity.

these foci are often found in the same families, the same sibship and even in the same individual [16,25,31, 38,100,104,110,118,119]. They likely represent different clinical endpoints of the same disease spectrum. Both disorders are late-onset, uniformly fatal, and of unknown etiology. The hyperendemic foci of ALS and PD, all located in the western Pacific, represent natural experimental models of chronic degenerative disease that occur in different cultures, in different ecological zones, and among genetically diverse human groups (Fig. 3).

West New Guinea Focus

The most isolated and remote of the three foci of ALS and parkinsonism is that found among the Auyu and Jakai people living on the southern inland plain of West New Guinea. In 1960, while Gajdusek was searching other regions of New Guinea and Melanesia for kuru-like disease and while he was on the southern coast of West New Guinea, Dr. Blom, the Dutch medical officer at Kepi, and Dr. A.C. Janmaat, the Dutch medical officer at Tanahmerah, brought to his attention cases of a motor neuron disease [30,31]. In this region some

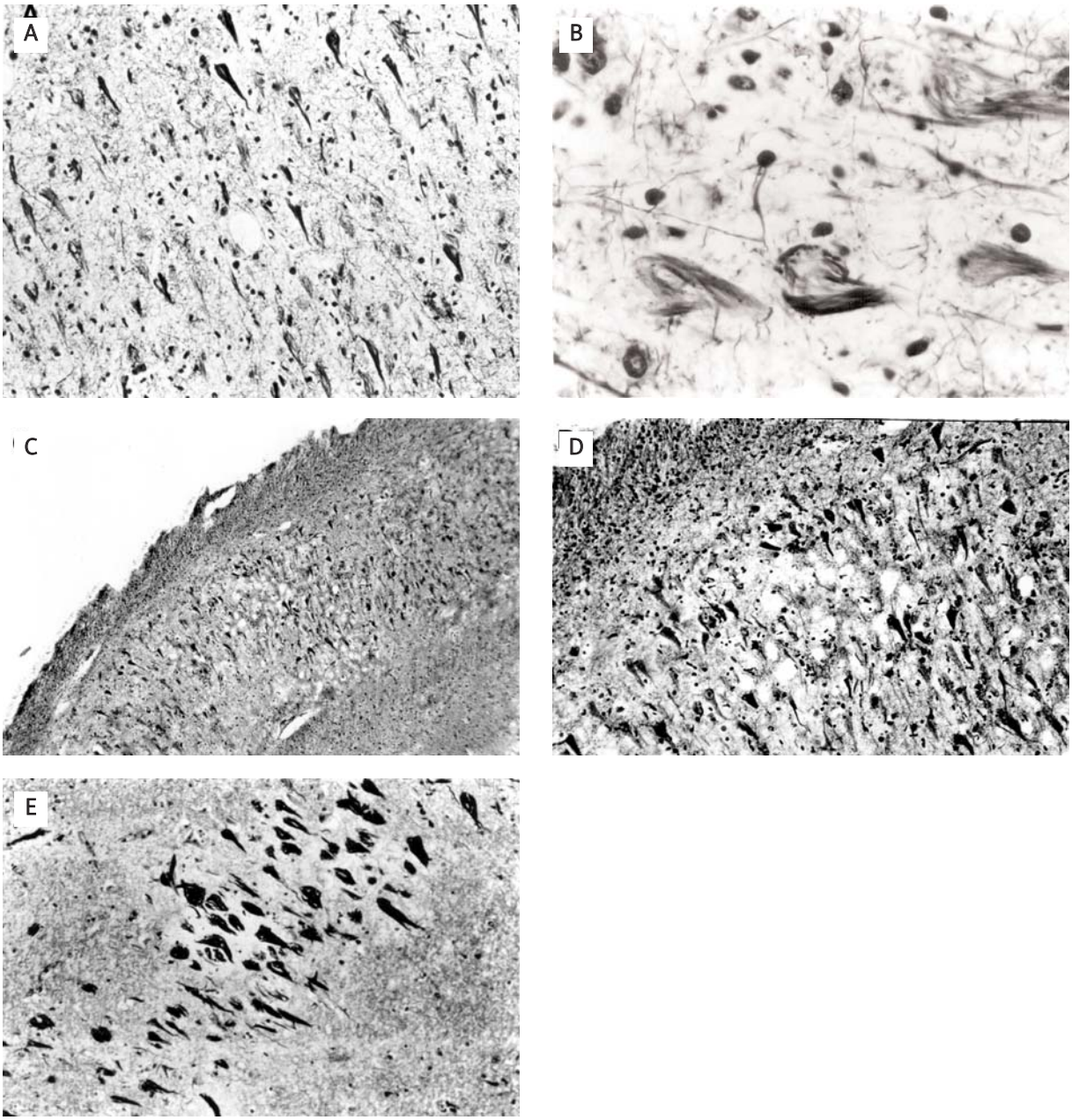

Fig. 4. Neuropathological changes in the hippocampus of Guam ALS and PD patients. A. NFTs are the hallmark characteristic change in the brain of Guam ALS patients (500 ×). B. In higher magnification, one sees the accumulation of fibrillary proteins in the neuronal perikarya (1000 ×). NFTs are also found in the spinal cord of ALS patients. C. Extensive NFTs in Sommers sector in a Guam PD patient (100 ×). D. In higher magnification (500 ×). E. Extensive NFTs in a Guam "control" patient, although there were no clinical symptoms of ALS or PD in this patient (500 ×). Seventy percent of Guamanians during the 1960s and 1970s had NFTs in their brain without clinical disease [1,17]. This control case represents the difficulty one has in identifying true controls on Guam. Do such individuals represent a pre-clinical disease state? Figure 10A-E are Bodian silver strained 6 µm paraffin sections.

Fig. 5. Map of the West New Guinea focus showing the affected Auyu and Jakai villages which are all located on the small rivers that originate in the flat coastal inland plain rather than along the longer, larger rivers. A survey by Gajdusek for cases outside this circumscribed area in neighboring populations and villages in all directions yielded no additional cases (photo courtesy of D.C. Gajdusek).

4000-5000 Auyu- and 9000-10,000 Jakai-speaking people were living around the lower Digul and Mappi Rivers (Fig. 5). The region had been visited by exploring missionaries in 1935, although even in 1960 they were still headhunters and cannibals [30,31]. The ALS/PD focus in West New Guinea is geographically limited, and neither ALS nor PD is found in the surrounding larger populations residing outside of the focal region (Fig. 5).

The ecology of the region is that of a low coastal plain covered by tropical rain forest lying 100-200 kilometers inland from tidal swamplands. Villages are located near rivers, which serve as the major avenue of transportation. The Auyu people live a semi-nomadic

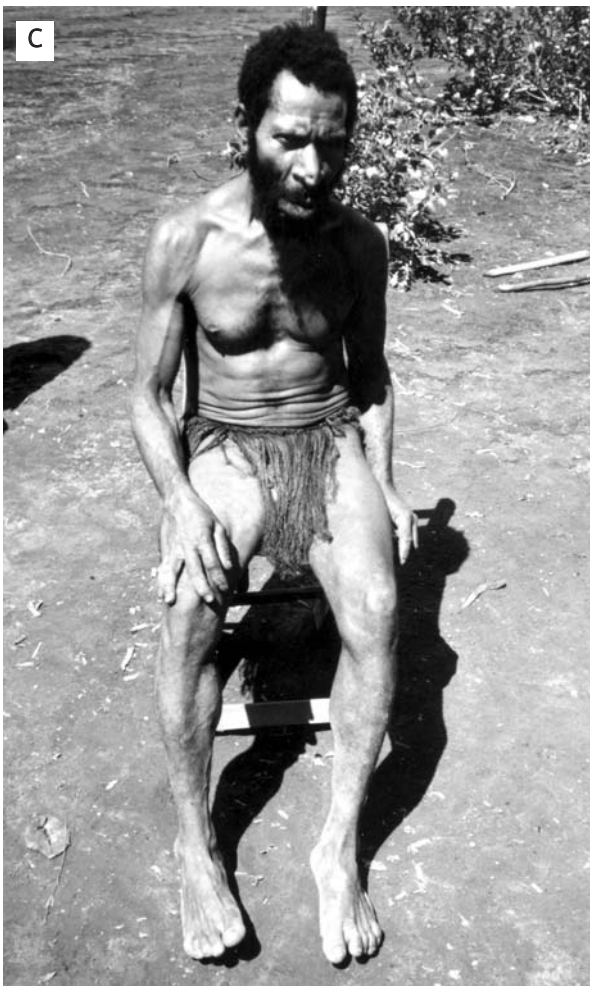

Fig. 6. ALS and PD in the West New Guinea focus. A. Two women with late stage ALS. Both women had diffuse fasciculations, were hyperrflexic and bradykinetic with significant weakness and atrophy. B. Two ALS and patients with severe upper body weakness and atrophy. The woman on the left was unable to hold her head upright because of muscle atrophy in her neck and shoulder girdle. Both patients also have fasciculations of the tongue and hyperrflexia in the lower extremities. C. A patient with advanced parkinsonism of five years duration. The patient showed no upper or lower motor neuron signs and had not yet developed dementia. He had a parkinsonian gait with moderate bradykinesia, tremor and rigidity along with blank facies. Photo courtesy of D. Carleton Gajdusek (DCG-74-WNG 44-8, WNG 15-11 and WNG 44-11 respectively).

subsistence-level existence that includes producing sago flour (a staple carbohydrate source), fishing, food-gathering, and, only more recently, some horticultural activity. The Jakai people, on the other hand, are more sedentary [30,31].

Originally, Gajdusek reported finding 13 cases of ALS among the Auyu and 7 cases among the Jakai along with at least one PD patient (Fig. 6). As in Guam, the PD patients were slower to be recognized. Among the Asmat and Tjitjak people who border the

Fig. 7. The fruit of Cycas micronesica has been processed and consumed to varying extents on Guam for many years, as it has in many places around the world. These fruit were collected on Guam in 1977 by one of us (RMG) and used for a series of studies on cycad toxicity.

Auyu and Jakai, no patients suffering from ALS- or PD-like symptoms were found [31]. Gajdusek originally described ALS as slowly progressive with an unusual incidence of "thin aesthetic hands in youths and adults who are otherwise normal" [31, p. 475]. He went on to say that there is a flattening of the thenar and hyperthenar areas of the hand, in some villages a condition that represented 10% to 20% of youths and young adults, which he felt preceded later components of the disease in older adults. Unlike Guam, where the male-to-female ratios were 2 : 1 for ALS and even 3 : 1 for PD, the sex ratio in the West New Guinea focus was near unity. The mean age at onset of ALS, however, was much younger in the West New Guinea focus than on Guam. Thirty percent of Auyu and Jakai cases were 30 years of age or younger, with a mean duration similar to Guam of 3.5 years. In 1982 after a series of expeditions over the preceding 20 years (in 1962, 1974, 1976, 1979 and 1980), Gajdusek and Salazar [38] summarized a total of 97 cases of ALS, 19 cases of PD and 18 cases of polymyeloradiculitis. Polymyeloradiculitis was found in close association with ALS and PD in the same households and villages, and on average had an onset some ten years earlier than ALS. The question raised was whether polymyeloradiculitis was part of the spectrum of ALS and PD in West New Guinea. Gajdusek and Salazar certainly thought so, but stated that the relationship of polymyeloradiculitis to ALS and PD was entirely speculative. The overall crude average annual incidence rate for ALS alone in this focus was 147 per 100,000 population, more than 100 times higher than rates in the continental United States and Western Europe, and the highest recorded level anywhere in the world [38]. In selected villages, the

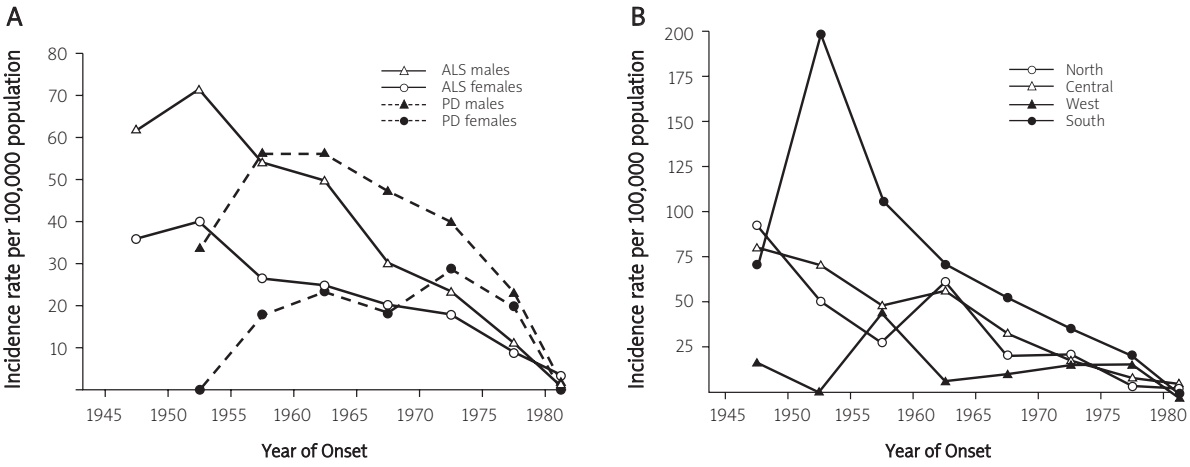

Fig. 8. Graphs showing the dramatic decline in the incidence (five-year average annual incidence rates) of ALS and PD among the Chamorro people of Guam. A. The original sex ratio was 2 : 1 and even 3 : 1 males : females, but by 1982 it was near unity. The last data points on the graph represent only partial data (two years) and probably under-represent the actual rate by 1982. B. The decline was most dramatic in the high-incidence southern region of Guam. The graph shown is for male ALS patients, but female ALS patients and PD males and females similarly declined during this same time period in each of the four geographic regions, but dramatically so in the previously isolated southern region of the island.

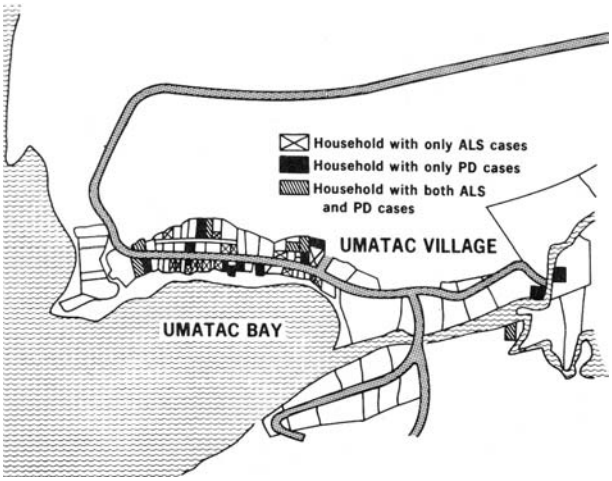

Fig. 9. Map of the village of Umatac, Guam showing, by 1975, the households that had ALS patients, PD patients or both ALS and PD. The map of the main street in Umatac village from Reed and Brody [108] incredibly demonstrates the huge concentration of cases in a village of approximately 600-700 people. The concentration and distribution of ALS and PD in high-incidence southern villages on Guam was one of the main factors that has led to the controversy surrounding the etiology of the disease. Do the distribution and high incidence represent a genetic, an environmental, or a genetic-environmental etiology? Gajdusek, based on the West New Guinea focus along with the epidemiological data from Guam and the Kii Peninsula, favored a "place" disease (environmental) phenomenon in all three foci.

prevalence rate exceeded 1,300 per 100,000 population, more than twice that of the highest-incidence village on Guam. Parkinsonism in the West New Guinea focus was found in association with the high incidence of ALS, and recognized during Gajdusek's second major survey of the area in 1974. It occurs with or without dementia and is much less common than ALS. Gajdusek remarked that ALS and PD had not spread to other villages within the affected area and indeed had dramatically declined in some villages by 1982, and by 1987, ALS had virtually disappeared among the Auyu and Jakai [Gajdusek, personal communication; 124].

Of the three foci, only the West New Guinea focus had no autopsies and thus no neuropathology for any cases of ALS or PD. Thus, the hallmark lesion found in the brain and spinal cord of ALS and PD patients from Guam (Fig. 4) and the Kii Peninsula of Japan could not be confirmed in the West New Guinea focus. The reason there was never an autopsy among the Auyu and Jakai was both interesting and unique. Gajdusek, some years ago, stated to one of us (RMG) that the lack of autopsy tissues was related to his fear that head hunting, which was under control because of missionary intervention, would again return if he or the Indonesian or Dutch physicians sanctioned the taking of "heads" (brain autopsies).

Potential mechanisms of neurodegeneration

Gajdusek felt strongly that the West New Guinea focus of ALS and PD was a "place" disease and that its rapid disappearance strongly implicated an environmental cause [33-36,38]. He noted that affected villages were all situated along rivers that originated in the flat coastal plain rather than along larger, longer rivers, such as the Digul and the Eilanden that originate in the Central Highlands of West New Guinea. He mentions in his article that the Indonesian government had moved people in a number of the affected villages (the entire village) from the smaller rivers to the larger ones for economic development purposes (Gajdusek, personal communication), a point that was confirmed by Ben Van Oers, the Catholic missionary working in the region from the 1960s for 20 years (van Oers, personal communication). Figure 5 clearly demonstrates the point, and shows the geographic location of affected villages on the small rivers in a circumscribed area rather well. Gajdusek long believed and later found that red clay soil of the Auyu and Jakai gardens were poor in calcium and rich in iron and bauxite (aluminum), along with the affected villages drinking exclusively from shallow dug wells in such soils [38]. He saw it as analogous to deficiency syndromes such as goiter and cretinism that depended on local geological conditions [37]. People living on the larger rivers were free of ALS and PD, as were people, such as the Asmat, who lived on tidal swamplands nearer the coast [33-36,38].

The metal hypothesis

As early as 1961, Kimura and colleagues established the high incidence of ALS in the Kii Peninsula of Japan and noted that nearly 20% of the adult po-

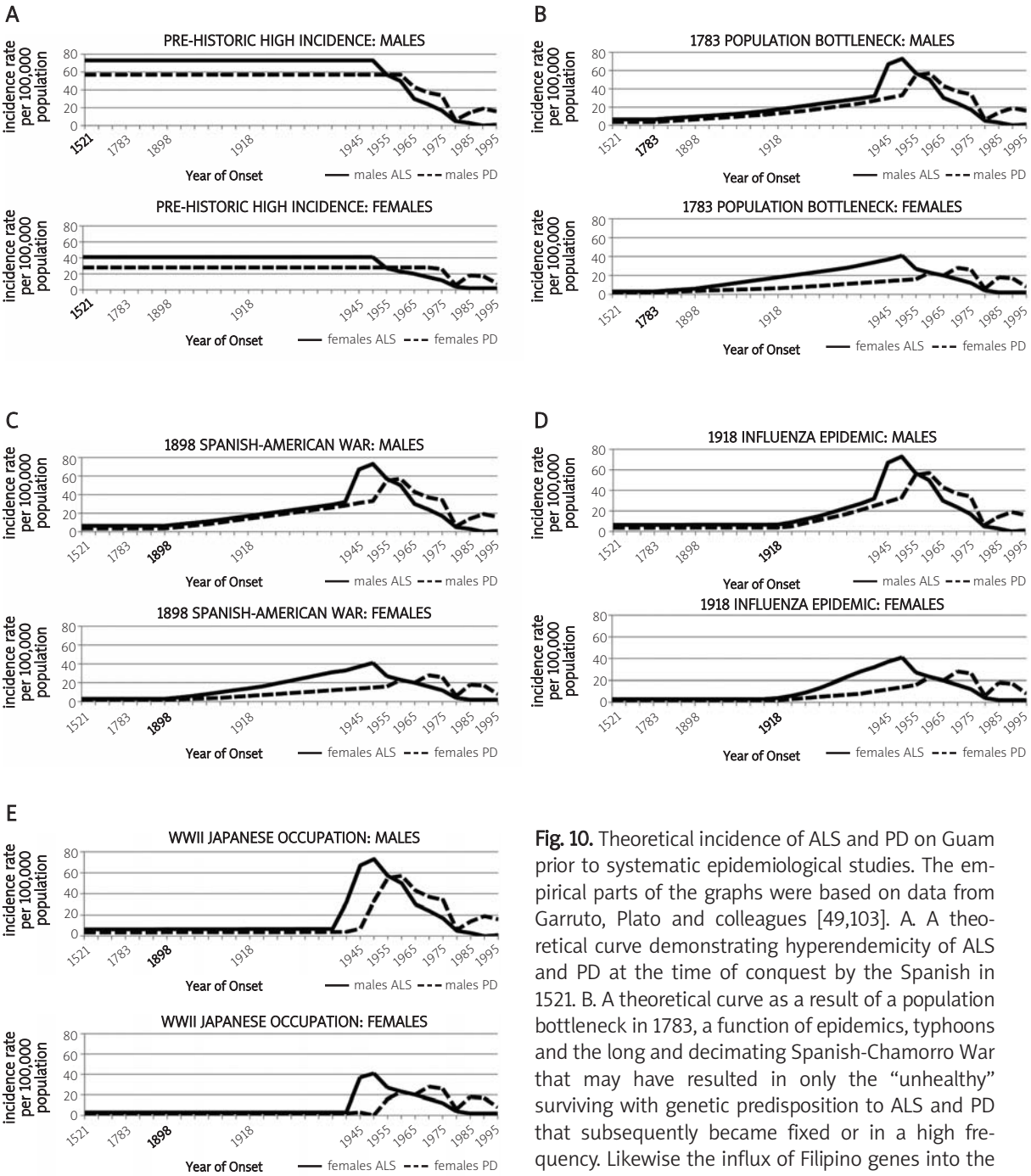

Fig. 10. Theoretical incidence of ALS and PD on Guam prior to systematic epidemiological studies. The empirical parts of the graphs were based on data from Garruto, Plato and colleagues [49,103]. A. A theoretical curve demonstrating hyperendemicity of ALS and PD at the time of conquest by the Spanish in 1521. B. A theoretical curve as a result of a population bottleneck in 1783, a function of epidemics, typhoons and the long and decimating Spanish-Chamorro War that may have resulted in only the "unhealthy" surviving with genetic predisposition to ALS and PD that subsequently became fixed or in a high frequency. Likewise the influx of Filipino genes into the population may also have been important at this time with one third of all Chamorro females marrying Filipino men to bolster the population [99,125,130]. C. A theoretical curve illustrating an initiating event at the turn of the 20th century with the takeover of Guam by the Americans as a result of the Spanish-American War. D. A theoretical curve as a result of the 1918 influenza epidemic on Guam. E. A theoretical curve of an initiating event during World War II.

pulation examined had neurological symptoms that included hyperreflexia, "tendency of muscular atrophy", extremity weakness and mild dysarthria [70]. They also observed that ALS occurred in areas where river and drinking water were low in minerals. Over the next decade, Yase and colleagues working in both the Kii Peninsula and on Guam began to postulate a possible mechanism for the involvement of manganese and other metals in these disorders [146,149,152]. Ensuing studies seemed to suggest that a deficiency in calcium was somehow linked to the disease process [139]. In all three foci, reports of low environmental levels of calcium and high level of aluminum began to appear [38,43,48,65,118,119,144,147]. On Guam, for instance, the soil in the high-incidence southern region of the island was found to be composed of a shallow red clay (laterite), like that found in the West New Guinea focus, which overlies volcanic rock and contains oxides and hydroxides of aluminum (40%), iron (20%) and silicon (1%) [29,94,132]. During this same time, biochemical disturbances in calcium and vitamin D metabolism were reported in 37% of Guamanian patients with ALS and PD [143], a study that greatly extended earlier results [140]. Chronic animal models were largely supportive of the toxicity of aluminum and of the impact of low calcium diets [summarized in 41,128,138].

In 1968, Yase and colleagues [149] had determined, by neutron activation analysis, elevated levels of mainly manganese, but also of calcium and aluminum, in brain and spinal cord tissues in ALS patients from the Kii Peninsula of Japan. These studies were repeated using neutron activation analysis and X-ray microanalysis [65,150] and the presence of aluminum in the central nervous system (CNS) was confirmed and extended in 1975 using graphite-furnace atomic absorption spectrometry on brain tissue from several patients with Guamanian ALS and PD [133]. These elevated levels of aluminum were not found to be present in brain tissue from patients with Creutzfeldt-Jakob disease and other neurodegenerative disorders, suggesting that brain destruction alone did not elevate brain aluminum [133]. With the advent of major technical and analytical breakthroughs, Ikuta and Makifuchi [62], and subsequently Daniel Perl, in collaboration with Gajdusek, reported the intraneuronal accumulation of aluminum in NFT-bearing neurons in brain tissues of Guamanian patients, using X-ray microanalysis and energy-dispersive spectrometry [95]. Two years later, in 1984, a then novel application of computer-controlled X-ray microanalysis and wavelength-dispersive spectrometry was used to produce chemical images of the hippocampus and subsequently of the spinal cord of Guam ALS and PD patients [43]. These chemical images demonstrated the co-localization of calcium (7200 ppm), aluminum (500 ppm) and later silicon (3000 ppm) in NFT-bearing neurons and in the walls of cerebral blood vessels [41,43,46,47,98] (Fig. 12). The co-localization of these elements in neurons has been repeatedly confirmed by other techniques, including secondary ion-mass spectrometry (which also demonstrated the deposition of these elements in oligodendrocytes), laser microprobe mass analysis, particle-induced X-ray emission analysis, X-ray fluorescence and electron energy loss, by different investigators in different laboratories worldwide [78,96,98, 148,150,151]. The abnormal deposition of calcium, aluminum and silicon also occurs in neurologically normal Guamanians with NFT formation, but not in those free of NFTs [98].

We believe, as did Gajdusek, that the discovery of aluminum and silicon (and perhaps other elements, such as iron), in combination with calcium, disrupt neuronal function, form mineral complexes in NFT-bearing neurons, and lead to eventual cell death. Our working hypothesis over more than a quarter century, based initially on the earlier work of Yase and colleagues, is that a basic defect in mineral metabolism in susceptible individuals in these high-incidence foci, provoked by chronic nutritional deficiencies of calcium and magnesium, leads to enhanced gastrointestinal absorption of aluminum and the deposition of calcium, aluminum and silicon in neurons. The infusion of these metals disrupts neuronal function, forms intracellular mineral complexes, such as hydroxyapatites or aluminosilicates, and induces the accumulation of hyperphosphorylated fibrillary proteins in perikarya and neuronal processes, resulting in neuronal death and cascading neuronal degeneration. A quarter century later, additional support for the role of metals in Guam ALS and PD has been reported by Hermosura and colleagues [57-59], as discussed under the GxE heading below. Furthermore, a metabolic defect, with resultant deposition of these elements in the CNS, may occur *in utero*, during infancy, childhood, adolescence or even later, but long before onset of clinical disease. Later intake of calcium and subsequent correction of the problem is unlikely to prevent

or reverse the metal accumulation or resulting neuronal damage in the CNS. The exact mechanisms for such a process, however, are as yet unknown [41].

The cycad hypothesis

Perhaps no other scientific issue involving these disorders has caused more disagreement than the suggestion that cycad or its neurotoxic derivative β-N-methylamino-L-alanine (BMAA) could be the cause of ALS and PD in the Western Pacific. The fruit of *Cycas micronesica* (Fig. 7) has been used on Guam as an occasional food source for at least 150 years [111]. Safford [115] states that on Guam it was "a staple of the aborigines before the discovery of the island" (p. 71), as it has been in many parts of the Pacific, in Southeast Asia, and on the Indian subcontinent [12]. In the 1960s, Whiting [136] and Kurland [73] suggested that an unknown neurotoxin in cycad fruit might be the culprit in these neurological disorders. There was early evidence that cycad was toxic to chickens on Guam [115] and the fruit and leaves were toxic to foraging domestic animals, producing a hindlimb paralysis in Australia [55]. However, subsequent research over the ensuing decade was unable to establish a causal link with ALS and PD. In a case-control study, Reed and Brody [108] found no significant difference between the groups in the frequency of eating cycad on Guam at that time of their study or in the past, and no difference between patients and controls in the consumption of fruit bats. Although Vega and Bell [134] had isolated BMAA from cycad fruit and Polsky and colleagues [105] had shown it to be neurotoxic to chicks, rats and mice when given acutely and in high concentration, the latter group concluded that it was unlikely to be a major factor in the development of neurological disease in humans. Other toxicological studies using cycad were both positive [22] and negative [45,60, 120,145].

The cycad hypothesis was resurrected when Spencer and colleagues [123] reported that cynomolgus monkeys (*Macaca fascicularis*) fed huge subconvulsive doses (average 250 mg/kg body weight) of synthetic BMAA daily by gavage developed neurological symptoms and limited pathological changes in motor neurons 2 to 12 weeks later. The near maximum content of total BMAA in fresh cycad fruit is about 1 g/kg [23], so each monkey would have to ingest the equivalent of 42 kg of unwashed cycad for 12 weeks [50]. Assuming that humans are similarly susceptible, a 70-kg person would need to ingest 17.5 kg of unwashed cycad seeds per day or approximately 1,500 kg over the same 12-week period. Previous studies also demonstrated that washed cycad, as traditionally prepared on Guam to elute water-soluble toxins, contains only trace amounts of free BMAA [23,24]. This indicates that the amount of washed cycad necessary to produce clinical disease would have to be orders of magnitude higher than the estimated 1,500 kg of unwashed cycad estimated above. However, only free BMAA and not the protein-bound fraction of BMAA was considered in these studies. Further studies also indicated that BMAA does not readily cross the blood-brain barrier [24; Duncan, personal communication]. Recently, a new study found that eating cycad was a risk factor for dementia, mild cognitive impairment and PD on Guam [8].

As stated above, cycad has traditionally been eaten and used as a poultice in many regions of India, Southeast Asia and the Pacific basin [12], yet no unusual prevalence of ALS or PD has ever been described in these regions, even when specifically looked for in cycad-eating populations [36,72]. Spencer and colleagues [122] in a letter to *Science* stated that their original *in vivo* study of BMAA orally administered to cynomolgus monkeys falls short of a model of ALS/PD and that BMAA is probably not the cause of these western Pacific disorders, but that some other as yet unidentified toxin in the cycad fruit may still be responsible [123].

By the early 1990s, the cycad hypothesis again had been abandoned, the second time in its 40-year-long history of suspected involvement in these high-incidence Pacific foci of ALS and PD. More recently, a new rendition of the cycad hypothesis emerged, proposed by Cox and Sacks [20]. They suggested that while processed cycad used as flour for tortillas by the Chamorro people was unlikely to yield significant toxicity, other parts of the Chamorro diet were [5]. Cox and Sacks [20] proposed that biomagnification (bioaccumulation) of BMAA by cycad-eating fruit bats, which were a sought-after delicacy on Guam, could have resulted in ingestion of high levels of cycad toxins, without any consequences to the fruit bat and with onset of ALS and PD decades after exposure during childhood, adolescence or as young adults. There is, however, no precedent in all of neurotoxicology and neuropharmacology for a decades-long delay in onset after organotoxin exposure [36]. When

cycad does produce a motor neuron-like disease, as in foraging cattle, the onset is acute or subacute with no long-term progression after exposure ceases [55]. Thus, Gajdusek's argument against cycad as a cause of ALS and PD seems as strong today as it was a quarter-century ago. Cox and Sacks [20] suggested that with the disappearance of fruit bats that were hunted to near extinction by the Chamorro people, there was an associated decline in Guam ALS and PD. Interestingly, our original curves of the decline of ALS and PD which Cox and Sacks used in their argument [49] does not appear to match their curve for the decline in the fruit bat population [82]. Subsequent studies by Cox and colleagues found that BMAA occurred at much higher levels when protein bound than as free BMAA, with the implication that protein-bound BMAA would be released through the digestive tract at some point after ingestion [91]. Cox and colleagues reported that the brains of two Guam patients with ALS and PD had both free and protein-bound BMAA, as did Canadian Alzheimer's disease patients, but not Canadian controls [90]. Finally, Cox and colleagues reported that diverse taxa of cyanobacteria produce BMAA naturally [19], although Rosén and Hellenäs [112] could not. Indeed on Guam the roots of *C. micronesica*, the fruit from which the flying foxes incorporate in their diets, were infected with cyanobacteria [19]. Since cyanobacteria appeared to be the culprit, rather than the cycad *per se*, the implication was that it could potentially explain not only ALS and PD, but neurodegenerative problems such as Alzheimer's disease worldwide [83,90]. Indeed, Cox and colleagues reported finding BMAA in Canadian patients with Alzheimer's disease as well as in Guam ALS and PD [91].

Recent studies of BMAA and of the cycad hypothesis are equivocal. Several studies suggest that BMAA is neurotoxic and plays a role in neurodegeneration [10,79], while other studies suggest selective neuronal injury [107], or the role of steryl glycosides [80], or a need for more research [68,93]. Also, there are arguments against the Cox and Sacks hypothesis [63,82, 126], and some studies show no effect of BMAA [21,80, 117]. Finally, one study reported no BMAA in the brain of Guam PD and ALS patients [86], directly contradicting the earlier reported results of BMAA in the brains of Guam ALS and PD patients [90]. Recently, a Norwegian study of BMAA in serum and brain tissue from Guam ALS and PD patients found no BMAA in either patients or controls (T. Flaten, personal communication). Attempts to confirm these results are underway.

Gajdusek felt very strongly that cycad and its associated toxins had nothing to do with ALS and PD in these Pacific foci [36]. As stated earlier, one of us (RMG) went to the Netherlands to interview Ben van Oers, who lived among the Auyu and Jakai people of the West New Guinea focus of ALS and PD as a missionary between 1960 and 1980. Van Oers recalled no use of cycad by the Auyu and Jakai, although he did state that in the affected villages where he worked, Auyu did eat fruit bats, like many groups throughout New Guinea, but that the Jakai, their affected neighbors, did not, as it was taboo for them to do so. Thus, flying foxes would not seem to be implicated in ALS and PD in the West New Guinea focus as the high incidence of ALS and PD affected both the Auyu and Jakai, who live as neighbors in the same circumscribed area of West New Guinea (Fig. 5). Van Oers said that the fruit bats fly from brackish water areas up the large rivers at night to feed on the many fruit trees, including papaya, but he did not know if they fed on cycad. The biggest change both he and Gajdusek observed in the 1960s was the introduction of new foodstuffs that were brought in by the oil companies who were involved in the economic development of the region [Gajdusek and van Oers, personal communication; 38]. Thus, the rapid decline in ALS and PD in the West New Guinea focus, like that on Guam (Fig. 8), is consistent with a major environmental change as a cause of these diseases. With new foodstuffs rapidly introduced in the affected villages living on smaller rivers, and the outright relocation of some villages by the Indonesian government, the etiology of the West New Guinea focus remains consistent with Gajdusek's argument for the metal hypothesis and a "place disease" phenomenon [38] and would seem to contradict the Cox and Sacks hypothesis and the arguments raised by Spencer and colleagues [124].

Genetic studies

Since the early 1950s there has been an intense search for a genetic cause of ALS and PD, especially on Guam. The initial observation of familial clustering on Guam led in 1957 to the development of a case-control registry with a prospective follow-up of all first-degree relatives (parents, siblings and offspring) and spouses, the latter serving as unrelated household controls [99]. A quarter-century later these ongoing

studies confirmed that parents, siblings and spouses of patients had a significantly increased risk of developing disease compared with relatives of controls, but that offspring of patients did not, probably because they had not yet reached the age at risk. The increased risk among spouses of patients who acted as non-genetic controls confirmed a household effect and the rapidly declining disease on Guam, likely as a result of increased modernization and introduction of new foodstuffs, supported the hypothesis that exogenous factors were involved in the etiology of ALS and PD [49,102,103,142]. By 2002, offspring of index patients having reached the age of risk showed a significantly increased risk compared to offspring of controls. Thus, 40% of probands had at least one affected first-degree relative, with many families having more than one affected relative [101]. These long-term studies were led by Chris C. Plato, who also developed a pedigree for the entire village of Umatac [100], the highest-incidence village on Guam (Fig. 9). Other intensive genetic studies followed the development of the Plato ALS/PD registry, including calculation of inbreeding coefficients, segregation analysis, identification of genetic markers including blood groups, serum proteins, red cell enzymes, HLA antigens, immunoglobulin allotypes and dermatoglyphics [7,44,61, 100,109]. Familial clustering also occurred in the Kii Peninsula focus as well as in the West New Guinea focus [38,119]. However, all failed to provide a satisfactory genetic explanation, and the continuing rapid decline in the disease implicated a major environmental factor [49,101] (Fig. 8).

With the discovery of a mutation in the superoxide dismutase (SOD) gene as a risk factor for familial ALS in North America [113] an effort to look at not only SOD but also other nuclear DNA markers for Guam ALS and PD ensued. Interestingly, no mutations were found in any of the six exon regions of the SOD 1 gene, nor in SOD 2 in Guam patients [28]. Furthermore, mutations in the tau gene, whose protein was a prominent component of the hallmark NFTs in these disorders, were pursued. But tau proved not to be a major gene, at best only a minor gene in Guam ALS and PD [88,106]. Schellenberg and colleagues returned to the tau gene several years later, genotyping single nucleotide polymorphisms (SNPs) in a larger sample, and concluded that genetic changes in the "tau region" contributed a small risk to Guam ALS and PD, but were unlikely to be the cause [129]. Interestingly, Morris and colleagues [88] conducted a genome-wide analysis but could not identify a single gene locus, either major or minor, involved in Guam PD, confirming a purely environmental or a gene-environment interaction as the most likely model of ALS and PD. Gajdusek, while acknowledging that subtle and complex genetics underlie most diseases, felt the high-incidence foci of ALS and PD were clearly caused by a major environmental factor and felt it was not productive (at least at the time) to search for any underlying associated genetic mechanisms.

Gene-environment (GxE) interaction

In a major study in 1993, Joan Bailey-Wilson and Chris C. Plato and colleagues [4] pursued an updated analysis of the Guam patient-control relative registry cited above [101,102,104] using cumulative lifetime risks of developing ALS and PD in the Chamorro population. For the first time, it was demonstrated that the best-fit model based on 40 years of empirical data was one involving a gene-environment interaction. Although not a new idea in the evolving thinking about the neurodegenerative process in these disorders [51], the timing was right with significant advances in molecular genetics to rethink and test an interactive gene-environment model for these Pacific paradigms [42]. Clearly, neither pure genetic, nor pure environmental lines of evidence and inquiry seemed sufficient, obtainable or convincing enough by themselves, and thus active pursuit of a testable GxE interaction model ensued.

Also in the early 1990s, a great deal of attention and research effort in the neurosciences concentrated on the mechanisms of cell death in neurodegeneration and the role of reactive oxygen species (ROS) in diseases such as Alzheimer's, Parkinson's and ALS [14,26,27,121]. ROS was found to initiate a process of neuronal instability and mitochondrial dysfunction, if uncontrolled, through a series of regulatory mechanisms. Thus, studies in addition to SOD began to appear to directly link mitochondrial dysfunction with neurodegeneration [14,18,77,114]. Certain metals, such as calcium, magnesium, aluminum, copper, iron and zinc, some of which were implicated in Guam ALS and PD, contribute significantly to the formation of ROS. With a lack of much success in finding major genes responsible for ALS and PD in the nuclear genome, a search ensued for inherited and somatic mutations in the mitochondrial genome. The search led to the discovery of a number of inherited and

somatic mutations in the light-strand (LSP) and heavy-strand promoter (HSP) transcriptional control region and their associated transcription factor A (TFAM) binding sites in the mitochondrial genome of Guam ALS and PD [81]. A GxE risk factor model was developed that overall showed a significant increased risk in ALS or PD patients compared to Guam controls. Thus, somatic or inherited LSP mutations might result in decreased transcription and replication of mitochondria in Guam patients, and contribute mechanistically to neuronal degeneration, possibly through metal-induced neurotoxicity and disruption of metal ion regulation [81]. Recently, Kazuno and co-workers [69] found two non-synonymous closely linked mtDNA polymorphisms that alter both mitochondrial pH as well as intracellular calcium levels. These polymorphisms could also be mechanistically important in Guam ALS and PD, a possibility we are currently exploring.

At about the same time, Hermosura and colleagues discovered a nuclear mutation in the calcium/magnesium membrane ion channel transient receptor potential melastatin 7 (TRPM 7) gene, in a subset of Guam ALS and PD patients [57-59]. The channel is a bi-functional protein involved in homeostatic regulation of intracellular calcium and magnesium and trace metal ion concentration. The discovery of the novel mutant channel was shown to have decreased functionality under low cellular levels of magnesium. Effectively, the defective channel would potentially decrease the cellular import of magnesium, causing lipid peroxidation leading to increased oxidative stress and production of ROS [18,57-59]. Or alternately, low extracellular magnesium may lead to an influx of extracellular calcium into neurons.

In a second study, Hermosura and colleagues investigated the TRPM 2 calcium ion channel gene that is highly expressed in the brain, especially in neuronal and microglial cells in the hippocampus among other regions [67,72,97,116]. They discovered, again in a subset of Guam patients, a mutation in the TRPM 2 gene that produced a heterozygous variant that activates in response to H_2O_2 and adenosine diphosphate ribose (ADPR), but that in the presence of physiological concentrations of extracellular calcium is quickly inactivated. There is a decreased influx of calcium into the cell in response to H_2O_2, increasing ROS and its downstream processing, potentially leading to neuronal death and the cascade of neuronal loss in neurodegeneration [57]. Thus, with mutations in the mitochondrial LSP region [81], coupled with mutations in TRPM 7 and 2 [57-59], and the purported environmental triggers in Guam ALS and PD [41,42], we have proposed a novel gene-environment interaction model of Guam ALS and PD that represents a start in the evolution of our thinking regarding neurodegeneration in these Pacific foci, and a significant contribution to the ongoing research effort to understand how complex GxE interactions contribute to not only the pathogenesis of ALS and PD foci in the western Pacific, but to neurodegeneration globally.

One of the most important and intriguing aspects of ALS and PD in these foci, as stated earlier, is the unusual accumulation of fibrillary proteins in a variety of lesions. While proclaimed a tauopathy by some, the discovery of yet another protein, TDP-43, in ubiquitin-positive, tau-negative inclusion bodies, originally in ALS, but later in frontotemporal lobar dementia (FTLD)-U with motor neuron disease and in FTLD with motor neuron inclusion bodies without clinical disease, is most intriguing [6,64,66,92,137]. The discovery of TDP-43 in patients with Guam ALS and PD [52,56,84] links FTLD-U with ALS and with Guam ALS and PD with a common pathogenic mechanism of neurodegeneration. TDP-43 is a nuclear protein that appears to repress transcription and enhance exon skipping (alternative splicing) [3,11], but its physiological function is presently unknown. Recently, TDP-43 has been found to be an mRNA neurofilament light (NFL) as well as a DNA binding protein and likely contributes to the formation of skeins of neurofilament in the brain and spinal cord of ALS patients [127]. TDP-43 inclusions do not co-localize with tau in Guam ALS and PD [52,56,84], and TDP-43 positive inclusions in the brain were not found in Guam controls. Thus, TDP-43 positive inclusions discriminate patients from controls much better than tau pathology [52]. Of note, non-Chamorros with Guam ALS and PD were TDP-43 positive as well as NFT tau positive [52], a fact that may have etiological implications. To what extent the expression of, or mutations in, the TDP-43 gene might impact the etiology of ALS and PD in these high-incidence foci is as yet unknown.

Lessons learned

In our view, the primary weakness of the metal hypothesis is that there are no mechanistic details put forth to understand in what forms metals are

ingested by susceptible individuals in a pre-disposing environment, how they are absorbed in the gut, and how they cross the blood brain barrier, and enter into neurons causing neuronal dysfunction. Many studies demonstrate that metals such as calcium, aluminum, silicon and iron, among others, are found intraneuronally in NFT-bearing neurons in Guam and Kii Peninsula patients and that *in vivo* experimental models show deposition of metals in neurons with associated clinical signs and neuropathological lesions. However, until we understand exactly how such deposition occurs, the exact conditions under which it occurs, and which individuals are susceptible, the hypothesis is unlikely to be fully embraced.

The primary weakness of the cycad and BMAA hypothesis, unlike the metal hypothesis, is not so much in its general mechanisms for accumulation, as the Cox and Sacks hypothesis is endearingly elegant and simplistic. However, supporting evidence for cycad toxicity, directly or through biomagnifications of BMAA, is lacking, as is the confirmation of BMAA deposition in the CNS of patients with ALS and PD. Animal models of BMAA are also conflicting [68], and if toxic, we do not know, as with metals, the biochemical mechanism from ingestion (or absorption) to cascading neuronal degeneration and death. Until these issues are resolved, the scientific community will likely not embrace the cycad/BMAA hypothesis for ALS and PD in these foci, let alone cyanobacteria/BMAA as the cause of Alzheimer's disease globally.

The weakness of an infectious hypothesis, which remains a possibility, but was not discussed as one of the major contending hypotheses above, is that an infectious agent has never been isolated from the tissues of ALS and PD foci patients; nor has ALS or PD ever been transmitted to experimental animals over the past 50 years [9,53,54,71,87,135]. There is some concern, of course, that an epidemic, such as influenza in 1918, may have resulted in a cohort of post-encephalitic Parkinson's disease (see below), but no evidence exists in support of this hypothesis.

Finally, our biggest gap may be in understanding who is susceptible to ALS and PD, be it those exposed to some toxin, those who are genetically susceptible, or genetically susceptible individuals who are also exposed. The latter is the easiest of all concepts to understand, but the most difficult to prove, even in today's high-tech molecular environment. Yet, in our opinion, it is this avenue that is likely to bear the most fruit, and is perhaps a primary reason we have failed to resolve the cause of the ALS and PD in these Pacific foci to date. The ALS and PD research road was unlike that traveled in kuru research, which had such obvious (in hindsight) routes of exposure (cannibalism), with a robust and indestructible agent (prion), and a genetic susceptibility that likely had everything to do with age at onset. One might also acknowledge a bit of luck with kuru, although Gajdusek always said he never believed in "luck". With ALS and PD in these Pacific foci we are unfortunately destined to take the long road to a final solution.

One of the most important aspects for understanding ALS and PD in these foci is to think about them within a historical context. For ALS and PD on Guam and in the Kii Peninsula of Japan, there is at least a century-long historical record available to us and about a half-century-long historical perspective available for the West New Guinea focus. In order to decipher why there was such an incredibly high incidence of disease in these isolated, genetically and culturally homogeneous groups, each living in small ecologically circumscribed areas, certain historical information becomes important. For example, are there post-contact written records by missionaries, explorers or indigenous or colonial administrators that shed some light on the health and disease of these isolated groups? Are there particular events in the historical record that may have created a genetic predisposition to ALS and PD? Does the historical record support any of the current or past etiological hypotheses? Have we systematically reviewed all past studies in the context of the historical record to determine if our thinking and conclusions about etiological questions are sound? And finally, what studies have yet to be done to provide answers to our remaining questions?

Using Guam as the best studied of the three foci as the example, Figure 10 shows the theoretical incidence rates of ALS and PD on Guam as impacted by historical events prior to systematic epidemiological studies. At the time of the first systematic studies [2,75,131,153], the disease likely was already in decline (Fig. 10A). Each of the four theoretical curves (Fig. 10B-E) is potentially useful in thinking about etiological possibilities such as cycad and BMAA, toxic metals or epidemic infections (such as influenza). For example, ingestion of cycad was likely used prior to 1875 [111] and likely at the time of Spanish conquest [115], as it has been used indigenously by many populations in Asia and the Pacific

basin. Guam ALS and PD may have been known and recognized in 1875 as a heritable disease, especially in the high-incidence southern region of the island, but was never commented on by the Spanish prior to de la Corte in 1875 [111]. Even then, the record is conflicting, as de la Corte says "endemic illnesses which have become hereditary and produce elephantiasis illness, often causing premature aging and short life" [111, p. 101]. One also wonders if in the 18th century a population bottleneck, as a result of the Spanish-Chamorro War, epidemics and typhoons [13,99,141], could have led to an increased frequency of a susceptibility allele(s) for ALS and PD in the small remaining population of about 1,500 Chamorros from an initial population at the time of conquest estimated to be 50,000 to 100,000. Alternatively, could the impact of the 1918 influenza epidemic on Guam have produced the later high frequency of ALS and PD, since the most susceptible birth cohorts were born prior to 1920 [49]?

Gajdusek's contributions to the understanding of neurodegenerative diseases through studies of focal disease in isolated human groups is legendary and a landmark in the history of science. We who follow, and who have spent decades in search of answers like our mentors, are simply running out of time, not only with respect to the disappearance of disease in these foci, but as a result of the limitations of our own mortality. Yet there exist ample pathological specimens both fixed and frozen from patients and controls in two of the three foci (Guam and the Kii Peninsula of Japan), along with supporting clinical and pathological records to assist scholars from around the world to continue to pursue ongoing questions of etiology and mechanisms of pathogenesis, an understanding of which have obvious consequences for our understanding of neurodegenerative diseases worldwide.

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