TIME COURSE OF NIGRO-STRIATAL NEURODEGENERATION: A NOVEL TOXIN-INDUCED MODEL SHOWING NEUROPATHOLOGICAL FEATURES OF PARKINSONISM

by

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Abstract

Amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC) is a neurodegenerative disease characterized by features of amyotrophic lateral sclerosis, parkinsonism, and a dementia reminiscent of Alzheimer's disease. Occurring primarily on the island of Guam, it has been linked epidemiologically to the consumption of seeds from the cycad palm *Cycas micronesica*. To further investigate the 'cycad hypothesis' of ALS-PDC, a cycad model has been developed in which mice are fed cycad as prepared by the indigenous people of Guam. Initial studies have shown that cycad-fed mice develop motor, cognitive and neuropathological changes similar to those of ALS-PDC. This study was designed to assess changes related to parkinsonism by investigating motor dysfunction and neuropathological changes related to dopamine in the basal ganglia. Cycad-fed animals demonstrated dysfunction in several motor tests, including the de Medinaceli gait length task. However, it was not possible to confirm that the motor phenotype was a result of dysfunction of the basal ganglia because of the potentially confounding effect of motor neuron involvement. Neuropathological analysis revealed a slow, progressive change in striatal dopaminergic integrity as evidenced by decreases in striatal tyrosine hydroxylase and dopamine transporter immunoreactivity as well as a compensatory increase in dopamine D2 receptor levels. Analysis of apoptotic cell death showed the presence of activated caspase-3 dopaminergic cells in the substantia nigra pars compacta which was accompanied by astrogliosis. Notably, these neuropathological changes persisted despite the cessation of cycad feeding. In a subsequent experiment, measurement of striatal dopamine levels failed to detect a difference in cycad-fed animals, although this may be due to the fact that the tissue might have been collected
before any significant neuropathological changes had occurred. Taken together, the results of this study provide further support for the ‘cycad hypothesis’ of ALS-PDC and show that features of parkinsonism are present in the cycad model.
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Abbreviations

AD..................Alzheimer's disease
ALS..................Amyotrophic lateral sclerosis
ALS-PDC.............Amyotrophic lateral sclerosis-parkinsonism dementia complex
BMAA..................β-methylamino-L-alanine
BSSG.................β-sitosterol-β-D-glucoside
CNS..................central nervous system
DA....................dopamine
DAT..................dopamine transporter
DAergic................dopaminergic
EIA...................enzyme immunoassay
GFAP..................glial fibrillary acidic protein
IHC..................immunohistochemistry
IR.....................immunoreactivity
LB....................Lewy body
MPTP..................1,2,3,6-methyl-phenyl-tetrahydropyridine
NFT...................neurofibrillary tangle
NMDA..................N-methyl-D-aspartate
6-OHDA...............6-hydroxy-dopamine
PBS...................phosphate-buffered saline
PD....................Parkinson's disease
PDC..................Parkinsonism-dementia complex
PFA..................paraformaldehyde
SG....................sterol glucoside
SN.....................substantia nigra
SNpc..................substantia nigra pars compacta
SNpr..................substantia nigra pars reticulata
TH....................tyrosine hydroxylase
TUNEL.................terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling
Introduction

Nearly two centuries ago, James Parkinson described the core clinical features of what would become known as Parkinson's disease (PD). His famous essay of 1817 on "The Shaking Palsy" began a quest to understand and treat this disorder, a search that continues to the present. PD is a progressive neurodegenerative disorder characterized clinically by bradykinesia, rigidity, resting tremor, gait disturbance and postural instability. Other clinical features may include the loss of facial expression, olfactory dysfunction, micrographia and cognitive changes including depression and dementia (Cummings 1988; Lang and Lozano, 1998a). The cardinal neuropathological feature of PD is the loss of dopamine (DA) neurons in the substantia nigra (SN), and their axons, which project to the striatum. A second typical feature is the presence of intracytoplasmic inclusions, Lewy bodies (LBs), in nigral and extranigral neurons (Braak and Braak, 2000; Forno 1996; Lang and Lozano, 1998b). It is the second most common neurodegenerative disease, after Alzheimer's disease (AD), affecting at least 1% of the population above the age of 55 (Hoehn and Yahr, 1967). However, PD is only one form of parkinsonism. Parkinsonism is a broad category defined by any combination of six specific motoric features – tremor at rest, bradykinesia, rigidity, loss of postural reflexes, flexed posture and the freezing phenomena (Fahn 2003)

The current standard treatment for parkinsonism is DA replacement. Drugs such as L-dopa do increase DA levels and thus temporarily alleviate symptoms, but this treatment is palliative and not curative: the loss of the dopaminergic (DAergic) cells is neither halted nor reversed. Furthermore, use of L-dopa is associated with the development of side effects, such as dyskinesias, that are often severe and debilitating.
Newer therapies include the transplantation of stem cells, which are intended to mature into functional DAergic neurons and replace cells that were lost to disease. This replacement has significant therapeutic implications but does not address the questions of how and why the DAergic neurons degenerate. That is, replacement therapy is compensatory, not curative. A truly curative treatment would involve targeting vulnerable cells before significant degeneration has occurred and enabling survival mechanisms or, ideally, identifying and removing the factors responsible for cellular vulnerability and dysfunction, thereby preventing disease. Therefore, it is imperative that we develop a comprehensive understanding of the etiological bases and subsequent neuropathological mechanisms underlying the behavioural symptoms of PD so that effective therapeutic strategies may be developed.

Approximately 5-10% of PD patients have a familial form with an autosomal-dominant pattern of inheritance (Olanow and Tatton, 1999). Recently, the potential genetic bases of PD attracted considerable attention with the identification of gene mutations responsible for the familial form of the disease (Gasser et al. 1998; Leroy et al. 1998; Matsumine et al. 1997; Polymeropoulos et al. 1997). However, the majority of PD cases are sporadic and although they may be multifactorial in origin, there is no clearly identifiable genetic component. This is supported by the well known twin study by Tanner et al. (1999) who report that for patients with disease onset after 50 years, there is no difference in concordance between monozygotic and dizygotic twins. If one assumes a critical contribution of genetic factors, the concordance rate of the disease would be expected to be greater in monzygotic twins, who are genetically identical, than in dizygotic pairs. Human epidemiological studies have implicated a variety of environmental causes in the
development of PD including residence in a rural environment and related exposure to herbicides and pesticides (Tanner 1992). Further support for an environmental factor in PD came from a set of patients exposed to the toxin 1,2,3,6-methyl-phenyl-tetrahydroxypiridine (MPTP). MPTP is a byproduct of the illicit manufacture of a synthetic meperdine derivative. Drug addicts who took MPTP developed a syndrome that strikingly resembled PD, both clinically and pathological (Langston et al. 1983). Although no MPTP-like factor has been identified in PD patients to date, the effects of MPTP intoxication further support the possibility that environmental factors have a role in PD etiology.

Given the relationship between environmental toxins and the risk of developing PD, a variety of animal models have been developed using toxin exposures. These models have been used to study toxin-induced neuropathological processes, to understand changes in neurocircuity induced by DAergic cell loss, as well as to suggest potential therapeutic strategies. Recently, our laboratory has developed a novel model of neurodegeneration based on exposure to toxins in the cycad seed, obtained from the cycad palm *Cycas micronesica*, which is endemic to the island of Guam.

**Amyotrophic lateral sclerosis-Parkinsonism dementia complex**

Amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC) is a complex neurodegenerative disorder characterized by symptoms of motor neuron disease, parkinsonism, and dementia that closely resemble more classical forms of ALS, Parkinson’s disease (PD), and Alzheimer’s disease (AD). Occurring primarily on the
island of Guam in the South Pacific, ALS-PDC has been epidemiologically linked to the consumption of cycad seeds, a traditional food of the indigenous people of Guam, the Chamorros.

**History of ALS-PDC**

Guam was first recognized as having a remarkable concentration of neurodegenerative disorders in the early 1950s. Shortly after the Second World War, a US Navy pathologist serving on Guam presented the first formal report of a high incidence of ALS among the indigenous Chamorro population (Zimmerman 1945). Initially, only ALS was recognized to be highly prevalent on the island, but early surveys of the native population soon revealed a large population of cases with predominantly parkinsonian symptoms and dementia (Hirano et al. 1961). Mulder, Kurland, Hirano, and colleagues referred to the collection of these symptoms as amyotrophic lateral sclerosis – parkinsonism dementia complex (ALS-PDC) (Hirano et al. 1961; Hirano et al. 1961b; Kurland et al. 1954).

Following the identification of this unique cluster of neurodegenerative disease, teams of neurologists, pathologists, and epidemiologists became interested in understanding Guamian neurodegeneration in the hopes that it would provide insight into understanding neurological disease worldwide. It was also imperative to understand ALS-PDC in its own right, given that between the early 1950’s and 1980’s, nearly 25% of adult deaths among the Chamorros were due to ALS and PDC (Kurland 1994). By 1980-89 the incidence of ALS had decreased to 7.5/100,000/year which is still significantly higher than that in the US (2.3/100,000/year) or Japan 0.6/100,000/year but is less than that in earlier decades on Guam (Okumura et al. 2003).
Clinical Features of ALS-PDC

ALS-PDC garnered considerable attention because its component features so closely resembled the primary neurodegenerative disorders occurring throughout the world. The similarities were immediately apparent upon examination of patients and later postmortem studies revealed similar neuropathological changes underlying the observed clinical symptoms. The following sections summarize the clinical and neuropathological manifestations of ALS-PDC and compare them to features of ALS, PD and AD.

ALS is a fatal paralytic disease characterized by loss of spinal cord motor neurons resulting in progressive muscle weakness and atrophy. Hyperreflexia, fasiculations and spasticity are also typically present. Disease onset is insidious; an increasing paralysis leads to death on average 3 years after diagnosis. Guamian ALS is similar to classic ALS in its form and presentation (Kurland and Mulder 1954; Rodgers-Johnson et al. 1986). As in classic ALS, the most common features at diagnosis are atrophy and muscle weakness. Despite a slight increase in the average age of onset and disease duration (Elizan et al. 1966), from a clinical perspective Guam ALS and classic ALS are nearly identical disorders (Elizan et al. 1966; Rodgers-Johnson et al. 1986).

As the name implies, PDC can manifest with parkinsonian features and cognitive decline reminiscent of Alzheimer's dementia. The parkinsonism aspect of the disease includes the classic features of tremor, rigidity, and bradykinesia (Hirano et al. 1961a). PDC is often associated with a disturbance of speech and gait apraxia (impairment of the
ability to execute complex coordinated movements). The motor dysfunction also includes markedly impaired fine motor movements as well as facial masking with 'reptilian' stare and infrequent blinking. At death, 85% of patients have developed bradykinesia, 75% rigidity and 65% tremor (Elizan et al. 1966; Rodgers-Johnson et al. 1986). While these proportions of symptoms are somewhat different than classical PD, in which tremor is a hallmark feature, the other classic features of parkinsonism are present.

The dominant cognitive feature of PDC is a progressive mental deterioration not unlike that of AD. Hirano and colleagues (1961a) note that in many PDC patients an “organic mental syndrome” is the dominant clinical feature, while the parkinsonian syndrome is less prevalent. In a group of 72 patients with PDC, 29% initially presented with dementia and only later did some develop features of parkinsonism (Elizan et al. 1966). In other cases, parkinsonian symptoms occurred initially with dementia developing later (Elizan et al. 1966). Dementia is therefore considered to be a ubiquitous feature of PDC, eventually appearing in all patients who initially present with parkinsonian features.

As described by Elizan et al. (1966), the most frequent signs of dementia at initial presentation are memory deficits and disorientation with regard to time, place and person. Difficulty with simple calculations and reasoning become increasingly severe with disease progression. In addition, personality changes, including apathy, irritability, and aggression, have been noted in one third of patients (Elizan et al. 1966). Olfactory deficits similar to those observed in AD and PD are present in virtually all PDC patients (Doty et al. 1991).
The clinical distinctions between ALS and PDC become less clear given that patients often express features of both ALS and PDC, in varying combinations and severities. In a study of 104 cases of ALS, Elizan and associates (1966) observed that 5 patients subsequently developed the total clinical picture of PDC, whereas 27 of the 72 previously mentioned PDC cases eventually developed ALS. Taken together, this suggests that ALS and PDC on Guam are not entirely distinct disease entities, and instead points towards a common pathology and etiology.

**Neuropathology of ALS-PDC**

Just as Guamanian ALS is similar to classic ALS from a clinical perspective, the spinal cord pathology of the Guamanian variant of ALS is also comparable to the more classic ALS found throughout the world (Hirano et al. 1967). The typical neuropathological features of ALS are loss of spinal and cortical motor neurons that innervate skeletal muscle resulting in muscle weakness and atrophy. The notable differences in Guamanian ALS include an abundance of neurofibrillary tangles (NFTs) composed of the microtubule-associated protein Tau throughout the CNS (Rodgers-Johnson et al. 1986). Tau regulates the assembly and stability of microtubules in a manner dependent upon its level of phosphorylation (Brich et al. 2003). Interestingly, abnormally high levels of hyperphosphorylated Tau are associated with various neurological pathologies including AD (Lee et al. 2001) and to a lesser degree PD (Ishizawa et al. 2003).

Further differences are present in other CNS regions of Guamanian patients with ALS. In a study of ALS patients lacking symptoms of PDC, there was significant neuronal loss and
presence of NFTs in the hippocampus - a pathology normally associated with AD and dementia - in 46% of the cases (Rodger-Johnson et al. 1986). Additionally, a more recent study found a reduced uptake of 6-fluorodopa in the striatum of Guamanian patients with ALS, a sign of DAergic cell loss that is the hallmark pathology of PD (Snow et al. 1990). While these changes are not features of classical ALS, their presence could indicate preclinical, concomitant PDC that has not progressed to a clinically-detectable point (Snow et al. 1990). This is in keeping with numerous studies of neurodegenerative disease demonstrating that significant neuron loss occurs before clinical symptoms appear. For example, the symptoms of PD only become apparent when more than 50% of nigral DA neurons are lost (McGeer et al. 1988) and for ALS, some estimates of spinal alpha motor neurons loss at diagnosis approach 70% (Arasaki and Tamaki, 1998).

The neuropathological changes in PDC are also similar to those observed in PD and AD, with a few exceptions. As previously mentioned, PD is characterized by a loss DA-containing neurons in the substantia nigra pars compacta (SNpc) and their terminals which project to the striatum. Similar changes have been documented in the nigral-striatal system in PDC. Not only is there a dramatic loss of DAergic cells in the SNpc (Hirano et al. 1961b), but Snow and colleagues (1990) have shown there is a dramatically reduced uptake of 6-fluorodopa in the striatum of patients with PDC: further evidence of a PD-like lesion. However, one of the hallmark pathologies of PD (albeit, a critized one: Calne and Eisen, 1989), LBs (intracellular inclusions of the aggregated protein α-synuclein), is not ubiquitous in PDC. In contrast to PD, in which virtually all patients display such α-synuclein pathology (Calne and Eisen 1989) only 37% of PDC patients with PDC show
such pathology (Forman et al. 2002). (It is due to this distinct lack of Lewy bodies, as well as differences in clinical manifestation, that the parkinsonian aspect of PDC is not considered to be true PD but rather a form of parkinsonism.)

With regard to neuropathological features of the dementia component of PDC, there is marked cortical atrophy, and NFTs are present in numerous brain regions including the hippocampus, entorhinal cortex, basal forebrain, and the neocortex (Hirano et al. 1961b; Kurland 1994), all of which is similar to the neuropathological changes observed in classical AD (Hyman et al. 1984). The ultrastructure and immunohistochemical profile of the NFTs appears to be identical to that in AD (Kurland 1994). However, there are differences in the pattern and distribution of NFTs. In PDC, there is a strong predilection for tangles in cortical layer 3, whereas in AD they are mainly found in layer 5 (Hof et al. 1994). Additionally, NFTs are present to a greater degree subcortically in PDC than in AD, where they primarily occur in cortical regions (Hirano et al. 1961b). Our understanding of the role of NFTs in dementia and neurodegeneration is furthered by a study of the occurrence of NFTs in Chamorros who died without any clinically detectable neurological disease. Chen and associates (1981) found that 57% of Chamorros between the ages of 40 and 59 years, and 95% of Chamorros older than 60 had extensive NFTs. These results could either mean that these people died before the symptoms of PDC were expressed at clinically-detectable levels, or that NFTs are merely a background feature unrelated to neurodegenerative disease in the Chamorro population. We believe that the former view warrants further consideration given that the extent of NFT formation in neurologically intact Chamorros was often equal to what is seen in endstage Alzheimers disease (Perl et al. 2003).
The coexistence of features of Guamian ALS and PDC within individual patients provides additional evidence of a common underlying etiology and pathogenesis. Furthermore, the clinical and pathological similarities between Guamian ALS-PDC and the classical forms of ALS, PD, and AD suggest that an understanding of the etiology and progression of Guamian neurodegeneration might shed light on neurodegenerative disease throughout the world.

Etiology of ALS-PDC

During the initial investigations into the etiology of ALS-PDC, investigators had hope that straightforward causal factors would be readily unearthed. For example, the Chamorro population was relatively homogeneous in genetic background, facilitating genetic analysis and identification of genes implicated in the disorder (Plato et al. 2003). In spite of this, detailed genetic surveys and analysis failed to identify a genetic basis for the disease (Reed et al. 1975; Lilienfeld et al. 1994). Further evidence against a genetic etiology came from studies of a Chamorro population living on the nearby island of Saipan (80 miles north of Guam). They had virtually the same genetic background as Chamorros on Guam, yet there was no evidence of an increased incidence of neurodegenerative disease, a finding that is strongly indicative of an environmental agent having an etiological role (Yanagirhara et al. 1984; Lilienfeld et al. 1994). The apparent lack of a genetic basis of the disorder is in keeping with observations regarding the classic forms of ALS, PD, and AD in which the majority of cases do not show a familial pattern of inheritance.
Investigators therefore rapidly focused on potential environmental toxins, screening hundreds of potential factors, including the ionic (mineral and heavy metal) composition of soil and ground water, native food products and industrial materials associated with military activity. Most of these environmentally-based hypotheses were discredited, although a few gained some support through further investigations.

One hypothesis that has garnered support for some time is that ALS-PDC is triggered by nutritional deficiencies of calcium and magnesium, which leads to secondary hyperparathyroidism that then facilitates the entry of calcium and toxic heavy metals, such as aluminum, into the brain (Yanagihara et al. 1984). However, this was later refuted by a study that showed that ALS-PDC patients have no indications of abnormalities in calcium metabolism, have normal parathyroid hormone levels, and have levels of heavy metals in blood and urine samples that are statistically similar compared to that of controls (Ahlskog et al. 1995). Further work demonstrated that monkeys fed a low-calcium, high-aluminum diet develop neurodegenerative changes comparable to those of early ALS and PD in the spinal cord, brainstem, substantia nigra and cerebrum (Garruto et al. 1989). Some of the neurons in these affected areas accumulate aluminum at concentrations 200 times that of unaffected cells (Perl et al. 1982). However, an examination of a large number of NFT-bearing and non-NFT-bearing neurons from deceased AD patients revealed no significant difference in aluminum content (Markesbery et al. 1990). Furthermore, despite early studies suggesting low environmental levels of calcium and high levels of aluminum (Garruto et al. 1980), Zolan
and Ellis-Neill (1986) report adequate calcium and magnesium content of water and food grown in soil near areas of the highest prevalence of ALS-PDC.

In the search for a different environmental etiology, Kurland and others (1988) were led to investigate the consumption of a local food product: cycad. Cycad consumption parallels the occurrence of ALS-PDC in several respects. Firstly, cycad has been consumed by the Chamorros as a dietary staple, but the level of consumption rose to far greater than normal levels during WWII as a famine food as a result of the harsh conditions during the Japanese occupation of the island in the 1950s. In support of the cycad hypothesis, the incidence of ALS-PDC peaked within several years of the war and declined dramatically as cycad consumption lessened during the post-war years, during which Guam was largely ‘westernized’ and cycad became a less significant part of the Chamorro diet (Kurland 1988). Secondly, as previously noted, there is a distinct absence of ALS-PDC on the nearby island of Saipan, where cycads had been removed from the island and the Chamorros of Saipan only very rarely consumed cycad products (Kurland, LT. Personal communication). Kurland and colleagues (1994) therefore reasoned that the striking differences in the incidence of ALS-PDC on these two islands could be a result of cycad consumption. Following these observations, cycad was considered by many to be the key etiological factor in ALS-PDC, sparking a flurry of investigations into the biology of cycads and the identification of cycad toxins.

Cycad Toxins

As noted above, ALS-PDC has been linked epidemiologically to the consumption of
cycad, although the causal toxin(s) has yet to be conclusively determined. A variety of species of this palm-like tree are found throughout the world. For as many as several thousand years, cycad seeds, stem, roots, and leaves have been exploited as a traditional source of dietary starch by various indigenous populations around the world. Since seeds are a more renewable source than stems, these are the most commonly ingested part. Traditional processing renders the seeds into flour, which is then made into such products as “fadang” tortillas.

Cycad contains toxic bioactive compounds, such as the azoxyglycosides cycasin and macrozamins, and the non-protein amino acid, \(\beta\)-methylamino-L-alanine (BMAA) that must be removed before ingestion. Traditional processing techniques involving washing have therefore been developed to remove these substances. While this removes toxins that are water-soluble, water-insoluble toxins remain. Since the former are associated with more acute illness, it was assumed that, because washing prevents such acute toxicity, it rendered cycad safe for ingestion. That is, water-insoluble toxins whose clinical effects are not seen until many years, or even decades, after exposure would not have been easily linked to cycad consumption. It is therefore not surprising that although traditionally-prepared cycad has had many of its toxins removed, the ingestion of processed cycad products is still associated with the development of Guam’s unique neurodegenerative disorder, ALS-PDC (Kurland 1988).

Although cycasin, macrozamins, and BMAA have all been shown to be toxic, and despite the fact that early research pointed to these compounds as causal agents in the development of Guamian ALS-PDC, it is unlikely that these are in fact the neurotoxins
causally responsible for this disease. The major evidence against this link is that traditional washing of cycad effectively removes virtually all traces of these water-soluble compounds. Thus, if we are to explain the apparent link between cycad consumption and the development of ALS-PDC, we must re-examine the so-called ‘cycad hypothesis’ and look for (neuro)toxins which remain unchanged by the traditional preparation. That is, the toxin(s) must be insoluble in water, able to survive normal cooking temperatures, and sufficiently lipophilic to be able to cross the blood-brain barrier.

Recent work in our laboratory has shown that several sterol glucosides (SGs), present in processed cycad seeds, may be at least partly responsible for the neuronal damage underlying the symptomatology and neuropathology of ALS-PDC. Experiments using cortical wedge preparations and assays for lactate dehydrogenase (LDH) activity in cortical slices revealed biological activity and cell death, respectively. When the most active fractions were analyzed, it was found that they contained significant amounts of three (SGs): beta-sitosterol-beta-D-glucoside, campestral or dihydrobrassicasterol beta-D-glucoside, and stigmasterol beta-D-glucoside (Khabazian et al. 2002).

Further supporting our hypothesis that these SGs are the neurotoxic agents in washed cycad, both isolated and synthesized SGs provoke similar cellular reactions, as shown by Khabazian and colleagues (2002). In cortical wedge preparation they both give depolarizing responses that can be selectively blocked by the NMDA receptor antagonist D-AP5 and the noncompetitive antagonist MK-801. Although SG exposure leads to a significant release of glutamate, it does not compete with either glutamate or NMDA in competition binding assays. Exposure to NMDA or SG fractions changes the expression
of some (CDK-2, PKC-beta) but not all (Erk-1, Rsk-1, Cot, PKB-2) protein kinases. Taken together, these results suggest that cycad’s SG content is both neuroactive and neurotoxic, thus causing it to be considered a potential causal agent in the development of ALS-PDC.

A murine model of ALS-PDC

Given the work from our laboratory showing the neurotoxicity of processed cycad seeds, along with the correlation between cycad consumption and ALS-PDC we have continued investigating the ‘cycad hypothesis’. Although it has been shown repeatedly that animals, such as laboratory rodents and primates, are susceptible to cycad’s water-soluble toxins (azoxyligosides: Sanger et al. 1972; BMAA: Spencer et al. 1987), the crucial in vivo experiments involving the ingestion of processed cycad had been neglected. For this reason, we performed the critical in vivo experiments that would definitively link cycad consumption to ALS-PDC: feeding mice washed cycad. Such experiments provide us with an animal model of the human situation, in which cycad is ingested only after extensive processing, and would establish cycad as having a causal role in ALS-PDC.

The cycad used to induce ALS-PDC in mice was prepared in the same way as done by the Chamorros on Guam (i.e., washed, dried, ground into flour, and mixed with water to form a dough). Cycad constituted approximately 1/4 of their daily intake by weight and control mice were fed pellets identical in weight and similar in nutritional content made of commercial grade processed white flour. Initial studies identified changes in both motor and cognitive function in cycad fed mice (Wilson et al, 2002) as well as
neuropathological changes including cell loss in the spinal cord and in various cortical and hippocampal fields. (Wilson et al. 2002; Wilson et al. 2004)

The objective of the current study is to determine whether there are also specific features of parkinsonism in the cycad model, including both motor and neuropathological changes similar to those of parkinsonism, including PDC and PD. Given that this study will be examining neurochemical changes associated with parkinsonism, the following section provides a brief overview of the anatomy and changes in neural circuitry underlying the motor dysfunction.

The Basal Ganglia: Direct and Indirect Pathways

The basal ganglia have been implicated in a wide variety of motor functions, including the planning, initiation and execution of movements, the performance of learned movements and sequencing of movements (Martin et al. 1994). The basal ganglia consist of four main structures: the striatum (caudate nucleus, putamen), the pallidum (external and internal segments of the globus pallidus and ventral pallidum), the subthalamic nucleus and the SN (pc and pr). The striatum is the input structure of the basal ganglia, receiving afferents from the entire cerebral cortex, the thalamic nuclei, and midbrain serotonergic and DAergic systems. The output structure of the basal ganglia consists of the internal segment of the globus pallidus and the SNpr, which project to the medial and ventral thalamic nuclei, the deep layers of the superior colliculus and the reticular formation. Of particular relevance to the motor changes associated with PD, the various thalamic nuclei that are innervated by these output structures project to different cortical
areas of the frontal lobe, including motor, premotor and prefrontal cortical areas (Obeso et al. 2002)

The input (striatum) and output structures (internal segments of the globus pallidus and SNpr) of the basal ganglia are connected to each other by means of two pathways, a “direct” and “indirect” pathway (Martin et al. 1994) (Figure 1A). The direct pathway consists of co-localized GABA/substance P/dynorphin-containing striatopallidal (striatum to internal segment of the globus pallidus) and striatonigral (striatum to SNpr) projections. The indirect pathway is comprised of the sequence of the GABA/enkephalin containing striatopallidal (striatum to external segment of the globus pallidus), the GABA-ergic pallido-subthalamic (external segment of globus pallidus to subthalamic nucleus), and the glutamatergic subthalamo-pallidal (sub thalamic nucleus to internal segment of the globus pallidus) projections. At the level of the output structures of the basal ganglia, these direct and indirect pathways have opposite effects on the GABA-ergic neurons that project to the thalamic nuclei, the superior colliculus and the reticular formation. A ‘balance’ between these two striatal output pathways appears to be essential for the normal regulation of movement. In PD, loss of striatal DA leads to enhanced activation of the indirect pathway, with increased excitation of the internal segment of the globus pallidus/SNpr. This results in decreased facilitation of cortical motor areas and subsequent development of akinesia and bradykinesia that is exhibited in PD (Obeso et al. 2002; Wichmann and DeLong, 1993).

Although there are many similarities between the anatomy of the basal ganglia in human and rodents, there are a few differences which deserve consideration. First, in humans as
well as non-human primates, the caudate nucleus and putamen are separated from each other by the internal capsule where as in rodents, the internal capsule is not well developed making it difficult to differentiate these structures. As a result, in the rodent these structures together are referred to as the caudate-putamen. Second, in humans, unlike in rodents, the globus pallidus is subdivided into an internal and external segments. In rodents, the homologues of these structures are not immediately adjacent to one another and thus have been named differently. The globus pallidus is the homologue of the internal segment of the GP and the entopeduncular nucleus is the homologue of the external segment of the GP. Despite the spatial separation of these structures in rodents, they maintain comparable functional properties.

The Basal Ganglia: Dopamine

The primary source of DA innervation to the striatum is the SNpc. The DAergic terminals form symmetric synaptic contacts primarily with the necks of dendritic spines of spiny projection neurons in the striatum (Smith et al. 1994). The head of these spines receive input from terminals of glutamatergic neurons originating in the cortex. This anatomical arrangement enables DAergic input from the SNpc to modulate striatal responses to the excitatory cortical input at the head of the spine (Yung et al. 1995).

DA has opposing effects on the direct and indirect pathways (Gerfen 1992). Activation of the dopamine D1 receptor (D1) has a stimulatory effect on the direct pathway, whereas activation of the dopamine D2 receptor (D2) has an overall inhibitory effect on the indirect pathway. Normally, the overall effect of DA release within the striatum is to
reduce inhibitory basal ganglia output to the thalamus, leading to increased activity of the thalamocortical projection neurons, thus facilitating movement (Figure 1A). In PD, a compromised DAergic pathway leads to net stimulation of the indirect pathway, resulting in a greater than normal excitation of the globus pallidus internal segment and SNpr. This increases the GABAergic output from the internal segment of the globus pallidus and the SNpr to the thalamus (DeLong 1990) ultimately resulting in a reduction of cortical activation, accounting for the classic motor symptoms of PD (Figure 1B).

In addition to changes in motor function, a valid model of parkinsonism should include underlying neurochemical changes comparable to those observed in the human condition. As already described, a decrease in striatal DA is critical, as well as DAergic cell loss in the SN with concomitant terminal loss in the striatum which may be reflected by decreases in both tyrosine hydroxylase (TH) and dopamine transporter (DAT). Postsynaptic changes may also be present. Several studies have shown that the postsynaptic D2 receptor is upregulated in PD (Ichise et al. 1999); a compensatory mechanism aimed at restoring striatal DAergic integrity (ie. the indirect pathway).
Figure 1.

Neurocircuitry of the normal basal ganglia (A) and the changes associated with Parkinson's disease (B). Inhibitory connections are colored red (-) and excitatory connections are colored green (+). Note that in Parkinson's disease, both the direct and indirect pathways are affected such that there is a net increase in the activity of the GPi/SNr resulting in increased inhibition of the thalamus and thus decreased input to the motor regions of the cerebral cortex. D1R and D2R = dopamine receptors, STN = Subthalamic nucleus, GP = Globus pallidus, SNr = Substantia nigra pars reticulate, SNpc = Substantia nigra pars compacta.

A. Normal basal ganglia

B. Parkinson's disease
Hypotheses

The current study has been designed to determine whether the consumption of cycad leads to behavioural and neuropathological features of parkinsonism in mice.

Specifically:

1. Cycad consumption will induce a gait disturbance, as measured by decreased stride lengths; a task typically used to assess basal ganglia function in rodent models.

2. Cycad consumption will induce concomitant changes in striatal markers of DAergic integrity as evidenced by decreases in levels of striatal DA, TH and DAT.

3. Cycad induced disruption of striatal DAergic integrity will be compensated for by specific upregulation of the D2 receptor.

4. Cycad consumption will induce apoptotic DAergic cell loss in the SNpc as evidenced by immunostaining for both TH and activated caspase-3 as well a decrease in the number of TH positive cells.

5. Given that olfactory dysfunction is a prominent feature of PD as well as PDC, cycad consumption will induce pathological changes in the olfactory bulb as evidenced by decreases in TH and the presence of astrogliosis.
Methods

Feeding Protocol

Adult CD-1 male mice were used in all experiments. All animals were solitarily housed in a room maintained at a temperature of 22°C with a 12/12 light cycle. Cycad seeds received from Guam were washed in water 7 times over 7 days (each wash lasting 24 hrs). Seeds were then dried, ground into flour and pellets weighing approximately 0.5g (1/6 – 1/4 of diet by weight) were prepared using a small amount of water. Mice were fed one pellet in the morning after having their regular chow removed. In the afternoon their regular chow was returned and uneaten pellets were weighed to track the total amount of cycad eaten per animal. Control animals were fed pellets prepared from commercial grade processed white flour.

In experiment 1 (Figure 2A), mice were 3 months old (weighing 30 g) at the onset of the study. They were fed cycad for 100 days. In order to study the progression of neuropathological changes, groups of mice were sacrificed at 75, 100 and 175 days following the initiation of cycad feeding (the last group of mice were placed on a regular chow diet for the last 75 days). There were 10 animals/group/timepoint.

In experiment 2 (Figure 2B), mice were 4 months old at the onset of the study. They were fed cycad for 100 days at which point they were euthanized for histological analysis of apoptosis. There were 4 animals/group.
Figure 2.

Schedule of cycad-feeding and tissue collection for the three experiments from which tissue was obtained for histological and biochemical analysis.

A. Experiment 1.

B. Experiment 2.

C. Experiment 3.
In experiment 3 (Figure 2C), mice were 4 months old at the onset of the study. They were fed cycad for 75 days after which they were euthanized for biochemical analysis of striatal DA and immunohistochemical markers of striatal DAergic integrity. There were 7 animals/group.

**Behavioural analysis**

Gait length, a measure of basal ganglia function, was measured by the de Medinaceli pawprint test (de Medinaceli *et al.* 1982). Briefly, paint was applied to the hind paws and the animals were allowed to walk through an enclosed track (approx. 60 cm long, 10 cm wide). The average stride length was measured from the pawprints left on paper lining the track. Testing occurred twice per week throughout the feeding protocol and each animal received 2 trials per testing session.

The leg extension reflex reflects motor neuron integrity (Barneoud and Curet, 1999). Mice were scored on a five-point non-continuous scale while held upside down by the tail (4 = complete extension of both legs to 0 = complete retraction). Each trial lasted 5 seconds. Animals received 2 trials per testing session.

The wire hang task assesses muscle strength, a measure that may be affected by either/both motor neuron integrity (Sango *et al.* 1996) and/or parkinsonian changes associated with basal ganglia (Blanchard 1995). Mice were subtended by their forepaws on a string 30 cm above base (wire hang). The latency to fall was recorded for 3 trials (maximum per trial = 60 sec) and results averaged.
For all behavioural tasks, testing occurred twice per week in the mornings between 9:00 and 12:00. During each task, the animal testing order was randomized for each testing session. Repeated measures ANOVA was used to assess overall statistical significance and an unpaired, two-tailed student t-test was used to assess specific differences between groups for each data point.

**Immunohistochemistry**

Mouse CNS was obtained from both cycad-fed and control groups following perfusion with 4% paraformaldehyde under halothane anaesthesia, post-fixed overnight, cryoprotected in 30% sucrose, frozen on dry-ice cooled isopentane and coronal sections cut on a cryostat at 30 μm. All sections were slide mounted. Care was taken to ensure sections from both diet groups were present on each slide to minimize the possibility of subtle changes in staining to effect group differences. Following a 5 minute rinse in PBS with 0.3% Triton-X, sections were blocked for 1 hour with either 5% goat or rat serum. Primary antibody incubations were performed overnight at 4°C. Primary antibodies included anti-tyrosine hydroxylase; 1:500 (Chemicon), anti-dopamine transporter; 1:500 (Chemicon), anti-dopamine D1 receptor; 1:100, anti-dopamine D2 receptor; 1:250 (Chemicon) and anti-glial fibrillary acid protein (GFAP); 1:1500 (Calbiochem), anti-activated -casapse-3; 1:250 (Promega). Biotinylated secondary antibodies were obtained from Vector Laboratories and used according to the suppliers specifications. Staining was visualized via the ABC method (Vector Laboratories) using diaminobenzidine as the chromogen. Sections were dehydrated in alcohol, cleared in xylene and coverslipped with
Entellen. TH stained sections used for stereological analysis were Nissl stained with 1% thionin prior to mounting.

Immunofluorescent methods were used for TH and GFAP analysis in the olfactory bulb and SN as well as for the TH and activated caspase-3 double labelling experiment. Primary antibodies were used at the same concentration, but were detected using fluorescent secondary antibodies (Molecular Probes). Following application of the secondary antibody (30 min, 1:200), sections were rinsed extensively, counter stained with DAPI and coverslipped using Vectashield (Vector Labs). All microscopy was performed using a Zeiss Axiovert II microscope with Axiovision software.

Semi-quantitative densitometry

Striatal-ir (TH, D1 and D2) was quantified using a high performance CCD camera and NIH Scion imaging software to measure optical density. Briefly, the striatum (approx. bregma 0.80) was circumscribed on low magnification images using NIH Scion Image and optical density measurements were then taken using the optical density tool of this software. Non-specific background staining was subtracted from each measurement. Multiple sections from 4 cycad-fed and 4 control mice were used.

GFAP and DAT-ir in the striatum, and TH and GFAP immunoreactivity in the olfactory bulb were quantified by a slightly different method as described by Pittman et al. (2003). 40x magnification images were obtained with a Ziess Axiovert microscope and staining intensity was quantified by the histogram tool in Adobe Photoshop. For any selected area
of pixels from a digital photograph, this function can determine the mean gray scale value (0-255, where 0 is black and 255 is white) and the number of pixels that are darker than a specified grayscale value. All images were converted to grayscale and a mean minimum grayscale value was determined for immunolabeled varicosities and processes. All pixels darker than this minimum value would subsequently be counted as positive immunostaining. The mean minimum grayscale value was determined by finding the mean grayscale value of immunolabeled varicosities or processes from several different sections from control brains. This value was then used to make pixel counts on tissue from both the control and cycad-fed animals. The number of pixels that had a value less than, i.e. darker than, the minimum acceptable grayscale value was recorded for each field within a brain section. The count for each field was used to calculate a mean striatal pixel count of immunopositive labeling for each section. Multiple section were used from 4 control and 4 cycad-fed animals. For the assessments of olfactory bulb pathology, photomicrographs were taken from the outer, glomerular layer of the olfactory bulb, a region containing DAergic neurons.

For all densitometric measurements values from each animal were averaged to generate 1 value per animal so as to not artificially increase the sample size. For densitometric measurements involving tissue collected from 2 or 3 different timepoints, a 2-way ANOVA was used to assess effects with respect to feeding-group or time from the initiation of cycad-feeding. Duncan posthoc tests were used to assess specific differences within feeding groups or between timepoints. For densitometric measurements involving tissue from only 1 timepoint, unpaired two-tailed student t tests were used to compare the means.
TUNEL Assay

DNA fragmentation was detected using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Multiple sections from 5 control and 5 cycad-fed animals were used in this fluorescent assay as directed by the manufactures of the assay kit (Oncogene). TUNEL fluorescence was observed using a Zeiss Axiovert II microscope with Axiovision software.

Stereological cell counts

Stereological analysis was performed to measure the total volume of the SNpc, as well as the numerical density (Nv neurons per mm$^3$) and total number of TH positive neurons on tissue collected at day 175 during experiment 1 (N=5/group). Nucleated, process-bearing, TH-positive cells in the SNpc were counted on every 5th 30 μm section collected through the SN. The boundary between the ventral tegmental area and SNpc was defined as a line extending dorsally from the medial boundary of the cerebral peduncle.

The total volume of the SNpc and the SNpr was measured using Cavalieri’s direct estimator (Gundersen et al. 1988a). Sections were examined with an Olympus BH-2 compound microscope (4x planapochromatic objective) with a Bioquant TCW98 image analysis system. The outline of the SNpc and the SNpr was traced and its area was measured in um$^3$. Total volume was calculated from the equation $V = \sum A \times P \times T_h$ where
A is the sum of the area measurements, P is the periodicity of the section samples and Th is the section thickness.

The numerical density of TH-positive neurons was determined using the optical disector method (Gundersen et al. 1988b). Five sections equally spaced along the caudal to rostral extent of the SNpc were selected for analysis and were examined using a 100x oil immersion planapochromatic objective. For all sections, the focusing plane was set 4 microns below the top of the section and nuclei of TH positive neurons were counted if they came into focus as the focusing plane moved down through 10μm of section thickness in the z axis plane. The Nv of neurons was calculated using the equation $Nv = \sum Q / \sum (A \times H)$, where $Q$ is the sum of neurons counted, $A$ is the area of the section and $H$ is the dissector height, which is equal to the distance traveled in the Z axis plane (10 μm).

Estimates of neuronal Nv and total SN volume were used to calculate the total number of SNpc TH positive neurons in the right hemisphere of each animal using the formula $T = Nv \times V$ where $T$ is the cell number, $Nv$ is the numerical density of TH stained cells and $V$ is the volume of the SNpc. The statistical significance of each measure was assessed using an unpaired, two-tailed student t-test.

**Dopamine Enzyme Immunoassay**

Striata were dissected and suspended in 15 volumes of 0.01N HCl. Each sample was sonicated and the DA enzyme immunoassay (EIA) was performed according to the
manufacturers instructions. Briefly, following DA extraction from the tissue, the samples were placed in a 96 well microtitre plate supplied with the kit. DA is prebound to the solid phase of these plates. During the reaction procedure acylated DA from the sample and solid phase catecholamine compete for a fixed number of antiserum binding sites. When the system is in equilibrium free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase catecholamine is detected by an anti-rabbit IgG-peroxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm with the amount of antibody bound to the solid phase catecholamine being inversely proportional to the catecholamine concentration of the sample. All samples and standards were run in duplicate and quantification was made by reference to calibration curves made with DA standards supplied with the kit. DA content was expressed as a function of sample protein (ie. ng DA/g protein) which was determined using the Lowry Method (Lowry et al. 1951). Statistical significance was assessed using an unpaired, two-tailed student t-test.

Results

Experiment 1. Behavioural features of cycad toxicity

Deficits on the gait length task, as revealed by the De Medinaceli gait length test, occurred in a biphasic manner (Figure 3). Cycad-fed animals initially displayed a significantly shorter gait length (-8%) following 15 days of cycad consumption; this deficit remained statistically significant for an additional 15 days after which time the
performance of cycad-fed animals improved resulting in both groups having comparable gait lengths. Following 55 days of cycad consumption cycad-fed animals again exhibited significantly lower gait length compared to control animals (-15%). However, rather than a consistently decreased gait length compared to their previous performance gait length over time, the cycad-fed animals had drops in their gait length at days 50, 110 and 150 and otherwise showed increases in performance, but not as great as those of control animals. The difference between cycad-fed and control animals persisted for almost 100 days (from day 55 through to day 150) despite the cessation of cycad feeding at day 100. There was no significant difference between groups for the last two trials (day 160 and 173) however this was not due to a decrease in the gait length of cycad-fed animals but rather an unexpected decrease in the performance of control animals. A repeated measures ANOVA analysis showed there was an overall significant difference with respect to group \( [F(1,48) = 4.2; \ p=0.04] \), day of testing \( [F(19,912) = 14.1; \ p<0.001] \) and that there was a significant interaction between these variables \( [F(19,912) = 5.6; \ p<0.001] \).

In cycad-fed mice, leg extension deficits (-27%) developed by day 60 (Figure 4A), followed by difficulty with the wire hang task (-30 %) by day 100 (Figure 4B). Deficits on the wire hang task remained for the course of the experiment while leg extension
Figure 3.

Time course of gait length changes following cycad consumption. Cycad feeding began on day 0 following several days of baseline training and stopped on day 100 at which time the mice were returned to regular chow. The grey line marked with triangles and the black line marked with squares represents the average gait length of control animals and cycad-fed animals respectively. Significant differences between control and cycad-fed animals, as determined by t tests, are marked with stars (*) (p > 0.05). All values are presented as the mean ± SEM.
Figure 4.

Wire hang (A) and leg extension reflex (B) performance in cycad-fed animals. All values are presented as mean ± SEM. In the wire hang task, cycad-fed mice performed consistently worse than control mice from approx. day 75. In the leg extension cycad-fed mice began showing progressive deficits at day 50, however there was an apparent recovery of function at day 120. All values are presented as the mean ± SEM.
showed substantial recovery of function: cycad-fed mice returned to baseline around day 115 (-0%) and were indistinguishable from controls for the remainder of the experiment.

**Experiment 1. Neuropathological features of cycad toxicity: Basal Ganglia**

In the striatum of cycad-fed animals there was a 26% decrease in TH-ir at day 175 (Figure 5). Two-way ANOVA, using group (cycad-fed, control) and number of days from the beginning of cycad-feeding (day 75, 100 and 175) as independent variables, showed that there were significant differences with respect to both feeding group \[F (1,18) = 20.2; p<0.001\] and day of sacrifice \[F (2, 18) = 18.5; p<0.001\]. Posthoc analysis showed that TH-ir at day 175 in cycad-animals was significantly different than that at both day 75 (p<0.001) and day 100 (p=0.006). The only significant difference between control and cycad-fed animals occurred at day 175 (p<0.001).

Two-way ANOVA analysis of DAT-ir showed that there were significant differences with respect to both the feeding group \[F(1, 25) = 18.2; p<0.001\] and day of sacrifice \[F (2, 25) = 3.5; p=0.04\] (Figure 6). In the striatum of cycad-fed animals there was a comparable decrease (21%) in DAT-ir at day 175 (p<0.001) (Figure 3A, B) that was preceded by a 13.5 % decrease at day 100 (p=0.02). (Figure 3C). Note that there was a significant difference in gait length at day 100 but not at day 175.

D1 receptor IHC showed no significant differences between the feeding groups \[F (1,18) = 0.55; p=0.47\] or among the three timepoints \[F (2, 18) = 1.44; p=0.26\] (Figure 7A) as
Figure 5.

Striatal tyrosine hydroxylase immunoreactivity. (A) TH-IR of control and (B) cycad-fed animals 175 days following the initiation of cycad feeding. (C) Histogram of differences in TH-IR at the 3 timepoints of the feeding paradigm in experiment 1. There is a 26% decrease at the last timepoint (p< 0.001). All values are presented as the mean ± SEM.

Figure 6.

Striatal dopamine transporter immunoreactivity. (A) DAT-IR of control and (B) cycad-fed animals 175 days following the initiation of cycad feeding. (C) Histogram of DAT-IR at the 3 timepoints of the feeding paradigm in experiment 1. There is a 13.5% decrease in cycad-fed animals at day 100 (p=0.020) and a 21% decrease in cycad-fed animals at day 175 (p< 0.001). All values are presented as the mean ± SEM.
revealed by a 2-way ANOVA. Conversely, D2 receptor analysis revealed a progressive increase in immunoreactivity throughout all three timepoints. (Figure 7B). The 2-way ANOVA showed significant effects with respect to feeding group \([F (1, 24) = 43.3; p<0.001]\) and day of sacrifice \([F (2, 24) = 4.6; p=0.02]\). Posthoc analysis confirmed a significant 20% increase in D2R-ir in cycad-fed at day 175 \((p<0.001)\) compared to control animals although the 7% increase at day 75 \((p=0.07)\) and the 9% increase at day 100 \((p=0.08)\) were not significant. Note that the increases at day 75 and day 100 correspond to points at which there was a significant difference in gait length between control and cycad-fed animals and that the increase at day 175 (20%) corresponds to a point at which there was no significant difference in gait length.

While there was no increase in GFAP-ir (a marker of astrogliosis), in the striatum of cycad-fed animals (Figure 8A) there was a significant 16% increase \((p=0.02)\) as shown by a student t-test in the SN of cycad-fed animals (Figure 8B,C). Individual astrocytes displayed marked morphological changes; including darker staining as well as withdrawn and thickened processes, indicative of active astrogliosis (Figure 8B,C inset). Double immunofluorescent staining for GFAP and TH confirmed the increased GFAP-IR occurred in the region of the SN (Figure 8D,E).

In experiment 1, examination of apoptosis by activated caspase-3-ir failed to detect apoptotic cells in the substantia nigra. However, a small number of TUNEL positive cells were observed in the region of the SN (Figure 9). Quantification of TUNEL positive cells proved impossible given the small number of labelled cells as well as a
Figure 7.

Striatal dopamine receptor immunoreactivity. (A) There are no significant differences in D1 receptor IR within any of the groups. (B) D2 receptor immunoreactivity. There is a progressive increase in D2 receptor IR: day 75, 7.5% increase (p=0.07); day 100, 9.3% increase (p=0.08); day 175, 20% increase (p<0.001). All values are presented as the mean ± SEM.
Figure 8.

GFAP immunoreactivity in the striatum and substantia nigra. (A) Histogram of GFAP IR in the striatum and substantia nigra at 175 days following the initiation of cycad-feeding. There is no significant difference between control and cycad-fed animals in the striatum, however there is a 16% increase in GFAP-IR in the SN of cycad-fed animals (p=0.02). Micrographs of GFAP-IR in the SN of (B, D) control and (C, E) cycad-fed animals. The insets contain high magnification micrographs of individual astrocytes. Note the marked change in morphology and intensity of staining. (Figure 8D, E) Double immunofluorescent staining for GFAP and TH confirmed the increase in GFAP-IR occurred in the region of the SN. All values are presented as the mean ± SEM. Scale bars: b/c=30 μm, inset=5 μm, d/e=100 μm.
Figure 9.

TUNEL staining in the substantia nigra. (A) Cycad-fed and (B) control animals. (scale bar = 10μm). The blue label is DAPI (nuclei) and the green label is the reaction product of the TUNEL assay.
limited amount of tissue. A repeat experiment employing double staining techniques (to confirm that the TUNEL positive cells were indeed dopaminergic) was unable to identify TUNEL positive cells, possibly due to tissue damage caused by extended storage.

To quantify DAergic cell loss in the SN of cycad-fed animals, stereological methods were used to determine the total number and numerical density of TH positive neurons in the SNpc as well as the volume of the SNpc and the SNpr on tissue collected at day 175 during experiment 1 (Table 1). No significant differences were found although there was a trend towards cell loss in cycad-fed animals (7% less TH positive neurons in cycad-fed animals, p=0.29).

Table 1.

Substantia nigra volume measurements and stereological cell counts of TH positive neurons. There are no significant differences between any of the measures.

<table>
<thead>
<tr>
<th></th>
<th>Cycad-fed mice (N=5)</th>
<th>Control mice (N=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNpc volume (mm$^3$)</td>
<td>0.228 ± 0.012</td>
<td>0.238 ± 0.008</td>
</tr>
<tr>
<td>SNpr volume (mm$^3$)</td>
<td>0.593 ± 0.023</td>
<td>0.614 ± 0.018</td>
</tr>
<tr>
<td>Nv (TH$^+$ neurons)</td>
<td>16212 ± 785</td>
<td>16587 ± 283</td>
</tr>
<tr>
<td>Total TH$^+$ neurons</td>
<td>3683 ± 211</td>
<td>3951 ± 112</td>
</tr>
</tbody>
</table>

Values are given as the mean ± SEM
Experiment 1. Neuropathological features of cycad toxicity: Olfactory bulb

To determine whether comparable neuropathological changes were present in the olfactory system, which is also affected in PD and PDC, TH and GFAP-ir was measured on olfactory bulbs collected at days 75 and 175. Two-way ANOVA showed significant changes in TH-ir with respect to feeding group \([F (1, 12) = 29.1; p<0.001]\) but not with respect to day of sacrifice \([F (1, 12) = 0.19; \ p=0.66]\) (Figure 10). Posthoc analysis of TH-ir surrounding olfactory glomeruli showed decreases in cycad-fed animals at both time points; day 75 (-12%, \ p=0.006) and day 175 (-7%, \ p=0.019). Notably, the TH decrease in the striatum was not detected until day 150.

There were also significant changes in GFAP-ir. Two-way ANOVA showed significant changes in GFAP-ir with respect to feeding group \([F (1,16) = 14.0; p=0.002]\) but not with respect to the day of sacrifice \([F (1,16) = 1.25; \ p=0.27]\) (Figure 11). Posthoc analysis confirmed a significant increase in GFAP-ir at day 175 (+7 %, \ p=0.02) but not at day 75 (p=0.20).
Figure 10.

TH immunoreactivity in the glomerular layer of the olfactory bulb. (A) There were significant decreases at both day 75 (p=0.006) and day 175 (p=0.019). Micrographs of TH-IR (red) at day 175 of (B) control and (C) cycad-fed animals. The blue stain (DAPI) is specific to nuclei. All values are presented as the mean ± SEM. Scale bar=100μm
Figure 11.

GFAP immunoreactivity in the glomerular layer of the olfactory bulb. (A) Cycad-fed mice show significantly more GFAP IR in the glomerular layer of olfactory bulbs compared to controls at day 75 (p=0.20) and day 175 (p=0.02). Micrographs of GFAP-IR (green) in the glomerular layer at day 175 of (B) control and (C) cycad-fed animals. The blue stain (DAPI) is specific to nuclei. All values are presented as the mean ± SEM. Scale bar = 100 μm.

A. GFAP - Glomerular Layer

B.

C.
Experiment 2. Apoptotic cell death in the SNpc

Degenerating cells as indicated by TUNEL staining and activated caspase-3-ir have been identified in other experiments from our laboratory employing cycad toxicity (Wilson 2002, 2005). To determine whether these degenerating cells are indeed DAergic, doubling labelling was used to identify cells in the SN positive for both activated caspase-3 and TH (Figure 12). Multiple double positive neurons were observed.

Experiment 3. Striatal DA

To determine whether cycad feeding results in striatal DA deficiency, striatal DA was quantified using an enzyme immunoassay (EIA). Because there was no tissue available from the initial set of animals used in experiment 1 (figure 2A), striatal tissue was obtained from a subsequent experiment in which mice were exposed to a similar cycad feeding regime (figure 2C). The tissue was obtained at a point when cycad-fed animals had a 10% shorter gait length compared to control animals. Note that this deficit is not as pronounced as the maximal deficit exhibited by the cycad-fed animals in the primary study. The EIA failed to detect a difference in striatal DA (p=0.91) (Figure 13).

To determine if there were changes in striatal markers of DAergic function in this set of tissue IHC analysis of TH, DAT and D2 receptor were performed. None of these markers were significantly different in cycad-fed animals; however, there were non-significant decreases in TH (-13.4 %, p=0.3), DAT (-8.1 %, p=0.08) and an increase in D2 receptor (10.4 %, p=0.08) as assessed by unpaired two-tailed student t-tests (Figure 14).
Figure 12.

Caspase-3 immunoreactivity in TH positive cells in the SN in tissue obtained from experiment 2. (A) Cycad fed animals (20x). (B) Control (scale bar=40μm). The bottom panel contains high magnification images of double stained neurons; (C) Cycad and (D) control animals (scale bar=10μm). DAergic cells as indicated by TH-IR are green and activated caspase-3-IR is red. Colocalization of TH and capsase-3 IR produces a yellow/orange color. Note the lack of colocalized staining in control animals (B and D).
Figure 13.

Striatal dopamine content in control and cycad fed animals. There was no significant difference between these groups (p=0.91). All values are presented as the mean ± SEM.

![Graph showing dopamine content comparison between control and cycad-fed animals.](image)

Figure 14.

Striatal TH, DAT and D2R immunoreactivity in cycad fed animals. This tissue was from the same animals striatal DA measurements were obtained from. There were no significant differences between control and cycad-fed animals for any of the DAergic markers. All values are presented as the mean ± SEM.

![Graph showing immunoreactivity comparison between control and cycad-fed animals.](image)
Discussion

The cycad model

The characterization of this model began with an analysis of cycad-induced changes in motor function. Because ALS-PDC is associated with changes in the nigro-striatal system as well as the spinal cord a battery of behavioural tests were used. The gait length task is highly sensitive to striatal DA deficiencies and thus appropriate for assessing motor changes related to parkinsonism (Fernagut et al. 2002). The wire hang task measures upper arm strength and is used to assess motor dysfunction in models of PD as well as ALS (Liebetanz et al. 2004; Blanchard 1995). Finally, the leg extension task was used to assess the integrity of spinal cord reflexes that are mediated by motor neurons, a measure related to ALS (Liebetanz et al. 2004; Barneoud and Curtet, 1999).

Given the multiple motor systems affected in ALS-PDC, and the effects multiple motor systems may have on single measures of motor dysfunction, it is difficult to relate changes in motor function to specific neural subsystems. For example, performance on the wire hang task may reflect dysfunction in either spinal cord or basal ganglia. Similarly, motor neuron dysfunction may be reflected in performance on the gait length task, even though it is commonly used to assess integrity of the basal ganglia (Liebetanz et al. 2004). Because the leg extension task involves spinal cord reflexes, it is less likely that failure of other motor/neural systems will affect this test.
In this study, cycad-fed animals exhibited changes in all three of the motor tasks. The first indications of dysfunction were apparent in the gait length task (Figure 3). The biphasic appearance of gait length dysfunction, although unexpected, is interesting because an understanding of the neurochemical changes underlying initial dysfunction and subsequent recovery and may shed light on early compensatory mechanisms, possibly uncovering targets for therapeutic intervention. Alternatively, given that the feeding paradigm did not involve feeding a known toxin, of known concentration (there may be variability between batches of cycad flour) it is possible that some batches were more toxic than others and therefore the initial phase of gait disturbance was induced by a particular toxic batch of cycad.

The second phase of the apparent gait length disturbance (day 55-150) is atypical compared to the appearance of gait length changes previously described in the cycad model (Wilson et al. 2002; Wilson et al. 2005) as well as in other chronic models of PD (Petroske et al. 2001) in that the gait length of cycad-fed animals did not gradually decrease over time. Rather than a continuous decrease in gait length, drops in cycad-fed animal performance occurred sporadically (day 50, 110, 150) whereas control animals showed consistent increases in gait length. This effect is not due to differences in growth as both groups had reached, on average, comparable and stable body weights. These results highlight one of the problems associated with long-term longitudinal behavioural analysis in that animals may become accustomed to the task and behave differently over time, distinct from changes induced specifically by motor-related pathology. In addition, this study did not include measures of stress or emotionality, potential variables that may also effect performance. The differences in performance on this task may also reflect
changes in motor learning, a function mediated by striatal DAergic systems (i.e., the longer stride length of the controls may indicate that they learned to perform the task more quickly) (Smiley et al. 2003; Ogura et al. 2005).

Cycad-fed mice also exhibited a deficit in leg extension (Figure 4A), indicative of motor neuron dysfunction. This change emerged at approximately the same time as the gait length disturbance. Although it is unlikely that other neural systems (i.e., DAergic/basal ganglia) will effect the leg extension reflex, it is possible that spinal cord dysfunction will be reflected in a decreased gait length, making it difficult to determine whether the dysfunction of the basal ganglia underlies the gait length change (Leibetanz et al. 2004, Nagata et al. 1998).

The wirehang, although not specific to either motor neuron or basal ganglia dysfunction, was the last measure to exhibit significant deficits (Figure 4B). This suggests it is not as sensitive as the other two tasks in detecting changes in motor function. Notably, the deficit in this task persisted throughout to the end of the study despite the cessation of cycad feeding at day 100, suggesting that the pathology underlying the wirehang dysfunction is not a transient effect of cycad feeding, but rather reflects permanent damage to the CNS.

These behavioural results clearly indicate that cycad toxins induce motor dysfunction, but to further understand the neuropathological changes underlying this dysfunction, as well as to determine whether the gait length disturbance is a parkinsonian feature caused by
DAergic deficiency, an extensive immunohistochemical and neurochemical analysis of markers related to parkinsonian changes was performed.

Loss of nigral DA neurons and the corresponding loss of DA-containing neuronal terminals in the striatum are well recognized as hallmark features of PD. Similar histopathological changes are observed in PDC (Snow et al. 1990; Hirano et al. 1961b). In this study, TH-IR in the striatum, was decreased only on day 175, indicating either a downregulation of TH in striatal DAergic terminals, or an actual loss of DAergic terminals (Figure 5). These results were unexpected given the significant behavioural deficits apparent in the gait length task at days 75 and 100, but not at day 175. Because there is no correlation between decreases in TH-IR and the gait length dysfunction this data does not support the hypothesis that a decrease in striatal TH underlies the cycad induced gait length disturbance in this model.

The dopamine transporter is another marker relevant to parkinsonian changes in the basal ganglia. The major physiological role of DAT is the termination of neurotransmission by rapid reuptake of DA from the synaptic cleft into presynaptic terminals, and it is thought to control the intensity and duration of DAergic neurotransmission by setting the concentration of DA in the extracellular space (for Review see Uhl 2003). Numerous DAT imaging studies have shown a correlation between overall striatal DAT binding and global measures of disease severity and parkinsonian motor handicap, with a progressive decline of DAT binding with increasing disability; in early PD, striatal DAT is reduced 30 to 50% (Marek et al. 1996; Ichise et al 1999) and greater reductions are observed in more advanced PD (Seibyl et al. 1995; Benamer et al. 2000).
In the current study, the progressive decrease in DAT (day 100 and 175) is comparable to early stages of human PD as well as to the MPTP and 6-OHDA animal models of parkinsonism, and further supports the notion cycad toxins produce neuropathological changes in the basal ganglia (Figure 6). A variety of studies have implicated DAT downregulation as an early compensatory mechanism in animal models of PD, thereby reducing DA uptake from the synapse and resulting in a greater availability of DA to receptors at the synapse (Zigmond et al. 1990; Fisher et al. 2004). Similar changes have been observed in human PD, as evidenced by a decreased level of DAT mRNA per DA neuron (Uhl et al. 1994) and by imaging studies showing a greater reduction in the binding potential of [11C]methylphenidate (DAT marker) compared to [11C]dihydrotetrabenazine ([11C]DTBZ; vesicular monoamine transporter type 2 marker) (Lee et al. 2000). By comparing the relationship between changes in DAT-IR and TH-IR (Figure 15) it becomes apparent that at day 100 of this study, DAT-IR is significantly lower than TH-IR. If striatal DAT and TH-IR are reduced proportionally as a result of DAergic terminal loss, then a greater loss of DAT, as observed at day 100, may indicate specific downregulation of DAT as a potential compensatory mechanism. This can be confirmed by quantifying transcripts of DAT; a compensatory downregulation of DAT would be shown by a reduced amount of DAT mRNA/remaining DAergic neuron.
Figure 15

Relationship of neuropathological changes in cycad-fed animals. Note the progressive increase in D2 receptor-IR, and the progressive decreases in TH and DAT-IR. At day 100 DAT shows a greater reduction in IR than TH, suggesting the decrease in DAT may, in part, reflect a compensatory mechanism.
As with the TH data, it is difficult to determine whether the decreases in DAT-IR have a role in the gait disturbance as this occurs before there is a detectable change in DAT-IR. Furthermore, the greatest decrease in DAT-IR occurs when there is no difference in gait length. If the decreased DAT were a compensatory mechanism, rather than just an artifact of terminal loss, it is tempting to speculate that the lack of difference in gait length for the two data points immediately prior to sacrifice (day 175) is due to recovery of function due compensatory decrease in DAT that restores striatal DAergic integrity. However, this hypothesis becomes less tenable considering the lack of difference was due entirely to a decrease in performance of controls rather than an increase in the performance of cycad-fed animals.

Given that gait length disturbances are observed in animal models of ALS (Nagata et al. 1998; Liebetanz et al. 2004) together with the data showing the leg extension and gait length differences emerge at approximately the same time without concomitant changes in TH or DAT-IR, a likely explanation is that motor neuron, rather than basal ganglia dysfunction underlie the gait disturbance. In this view, in this model gait length alone is not a valid indicator of basal ganglia dysfunction. As the gait length deficit progresses, there is a corresponding decrease in DAT (day 100) implicating the DAergic systems in the gait length dysfunction. With regards to the leg extension task, there is an apparent recovery of function on the leg extension task on day 117 which persists through to the end of the study (day 175). During this time there is an improvement, although not a full recovery in performance on the gait length task until the day 160. This provides further evidence that the gait disturbance observed in this study is related to motor neuron dysfunction. A possible explanation for the partial, though incomplete recovery in gait
length function as compared to the leg extension task, is that by this point in the model basal ganglia dysfunction has some role in the gait length deficit. This notion is supported by the significant decreases in TH-IR and DAT-IR apparent at day 175.

Therefore, even though the gait length disturbance in this study may be related to motorneuron dysfunction, the progressive decreases of presynaptic markers of striatal DAergic integrity (TH and DAT) suggest the pathology observed thus far may be an early feature of a parkinsonian syndrome that has not yet fully developed. Future studies are aimed at feeding cycad for a longer time period, to determine whether long-term cycad exposure is able to generate a model that more closely mimics the end stage of human parkinsonism as evidenced by massive, if not total loss of striatal TH and DAT.

To investigate whether other markers related to postsynaptic DAergic function were affected, further IHC analysis was performed for the DA receptors D1 and D2, the principle subtypes of DA receptors in the striatum. The upregulation of the D2 receptor observed in the cycad model is consistent with numerous in vivo and in vitro imaging studies of human PD demonstrating increased D2 binding levels as well increased D2R protein levels. (Lee et al. 1978; Guttman et al. 1985; Ichise et al. 1999) (Figure 7B).

There was a progressive increase in D2 receptor levels from day 75 through to day 175 in the cycad model. This parallels findings in both humans and animals in which D2 receptor upregulation is present early in the disease process (Bezard et al. 2001; Doudet et al. 2002), perhaps even prior to significant symptom onset.
There is currently no consensus with regard to the changes of the D1 receptor in PD; studies of D1 receptor status in PD are often contradictory (Laihinen et al. 1994; Shinotoh et al. 1993; Turjanski et al. 1997). No change in the quantity of striatal D1 receptors was observed in the cycad model (Figure 7A). However, given the uncertainty of the status of the D1 receptor in PD, these results neither support nor negate the validity of the cycad model.

Given that a key neuropathological feature of PD as well as PDC is loss of DAergic neurons in the SN, a variety of methods were used to detect cell loss in this study. There is a significant body of data suggesting DAergic cells are lost through an apoptotic mechanism, although the evidence is not unequivocal. Studies using both the TUNEL assay and morphological indicators of apoptosis have either succeeded (Mochizuki et al. 1996; Anglade et al. 1997; Tatton et al. 1998; Tompkins et al. 1997) or failed (Banati et al. 1998; Wullner et al. 1999) to identify apoptotic neurons in patients who died with PD.

The TUNEL assay detects DNA fragmentation; a typical feature of programmed cell death. However, TUNEL alone is not sufficient to confirm cell death through an apoptotic mechanism as several studies have shown that a variety of methodological factors may effect this assay (for review see Tatton 2003). For example, prolonged post-mortem delays before fixation, or prolonged fixation may increase non-specific DNA labelling (Nishizaki et al. 1999; Tamura et al. 2000). Therefore, studies have also investigated the molecular pathways of apoptotic processes in human PD as well as animal models of parkinsonism. The caspase family of proteases have a critical role in the apoptotic process. Caspase-3 is normally expressed as an inactive proenzyme which is activated
upon cleavage by a variety of upstream effectors. Upon activation, caspase-3 cleaves the inhibitor of caspase-activated DNase (ICAD) to generate caspase-activated DNase (CAD), which fragments nuclear DNA (Nagata 2000). Further support of an apoptotic process in PD has come from several studies that have used IHC methods to show increased amounts of activated caspase-3 and Bax, another pro-apoptotic protein, in PD. (Hartmann 2000; Tatton 2000).

In experiment 1, IHC methods for activated caspase-3 failed to detect apoptotic cells in the SNpc whereas analysis using the TUNEL assay identified a limited number of TUNEL positive cells in the SNpc (Figure 9). Given the methodological problems associated with the TUNEL assay, additional tools were used to assess cell loss. Stereological methods designed to quantify TH positive cells in the SNpc also failed to detect a significant difference in cell number, neuronal density of the SNpc or volume changes in the SNpc and SNpr (Table 1). However, recent studies from our lab have measured decreases in SN volume using magnetic resonance microscopy (Wilson et al. 2004) as well as apoptotic profiles in the SNpc indicating cycad toxins induce cell death in the basal ganglia (Wilson et al. 2002; Wilson et al. 2003).

To further investigate the possibility of DAergic cell death in the cycad model, tissue from experiment 2 was used for additional studies using IHC methods to detect activated caspase-3. Following the detection of activated caspase-3 positive cells in the region of the SNpc, double staining methods to identify cells immunoreactive for both activated caspase-3 and TH were used to determine whether the apoptotic cells were DAergic. Multiple double-positive cells were identified, demonstrating that cycad toxicity can lead
to DAergic cell death in the SNpc (Figure 12). Given these results, a possible explanation for the differing results in experiments 1 and 2 is that in experiment 1 the neurodegenerative process was not as advanced as in experiment 2. In this view, the neurodegenerative process in experiment 1 was limited to changes in DAergic terminals in the striatum whereas in experiment 2 the neurodegenerative process had progressed to include cell loss in the SNpc. This is in agreement with a variety of studies that have shown changes in DAergic terminals are observed prior to cell loss in the SNpc (Dauer and Przedborski, 2003).

Despite the lack of quantifiable DAergic cell loss in experiment 1, there was a profound increase in GFAP-IR in the SN of cycad-fed mice that was absent in the striatum (Figure 8). An astroglial reaction is generally described at neuropathological examination in the SN of patients with PD and has been confirmed quantitatively using GFAP protein immunostaining (Damier et al. 1993). Animal models of parkinsonism generally exhibit an astroglial response in the SN, and occasionally in the striatum as well (Muramatsu et al. 2003). Multiple studies have shown that GFAP is upregulated in astrocytes in regions of traumatic brain injury, as well as in ischemic or excitotoxic cerebral lesions, and as such is often used as a marker of CNS ‘damage’ (Araki et al. 2001; Gordon et al. 1997; Kornyei et al. 2000; O’Callaghan et al. 1990; Reinhard et al. 1988). Astrocytes respond particularly to pro-inflammatory cytokines, such as IL-1α and TNF-α and it is believed that these cytokines participate in astrocyte activation following CNS injury (Giulian et al. 1988), thus astroglial reaction in the cycad model, as well as in human PD implicate inflammatory processes in the disease process.
The astrogliosis observed in the cycad model provides further evidence that the basal ganglia is specifically affected by cycad toxins. It also suggests there is an active pathological process occurring in the SN that could be generating striatal dysfunction. However, the significance of the astrocytic response in neurodegenerative disease is not yet fully understood.

In the MPTP model, astrocytic activation parallels the time course of DAergic cell death in the SNpc and GFAP expression remains upregulated even after most of DAergic neurons have died due to MPTP intoxication (Czlonkowska et al. 1996; Kohutnicka et al. 1998; Liberatore et al. 1999). These findings suggest that the astrocytic reaction occurs after neuronal cell death (Przedborski et al. 2000), which is supported by the finding that blockade of MPTP uptake into DAergic neurons not only prevents SNpc neuronal cell death but also up-regulation of GFAP (O’Callaghan et al. 1990). Additional studies have shown that stereotaxic infusion of IL-1α into the SN of rats increases activation of astrocytes, an effect associated with protection of DAergic neurons in the SNpc against 6-OHDA toxicity (Saura et al. 2003). It has also been shown that astrocytes improve the survival and phenotypic expression of mesencephalic neurons in culture while decreasing the risk of apoptotic death of these neurons (Sortwell et al. 2000). Taken together these findings show that astrocytes may have a neuroprotective role in the cycad model as well as in human PD.

An interesting feature of this model are the neuropathological changes occurring in the olfactory bulb (Figures 10, 11). The olfactory system is one of the non-motor systems
severely affected in both PD (Doty et al. 1988) and PDC (Doty et al. 1991) although the cellular and molecular mechanisms underlying the dysfunction are unclear. The olfactory dysfunction occurs early in the disease process, often preceding cognitive and motor deficits. As such, olfactory testing has been proposed as a screening tool to identify those in early stages of the disease process. Furthermore, because it is one of the earliest detectable changes, some have suggested that an understanding of its etiology may help identify causative factors of PD (Wolters et al. 2000; Berendse et al. 2001).

The pathophysiological basis for olfactory dysfunction in PD has not been fully elucidated. Neuronal loss and Lewy bodies in the anterior olfactory nucleus occur early in the neurodegenerative process (Pearce et al. 1995). Severe neuropathological changes affecting the amygdala, which are believed to be involved in olfaction, have also been reported. A recent study has reported a significant increase of TH positive cells in the olfactory bulb of patients with PD (Huisman et al. 2004). This is a counterintuitive result given that a hallmark feature of PD is the loss of DA and DA-releasing cells. Animal data have revealed an inhibitory effect of DA on transmission between olfactory receptor and mitral cells in the olfactory glomeruli (Duchamp-Viret et al. 1997; Hsia et al. 1999). Although these findings need to be confirmed, they are in keeping with several clinical properties of hyposomia in PD, in particular its lack of correlation with L-dopa responsiveness and independence of disease severity (Katzenschlager et al. 2004).

In contrast to the findings of Huisman et al (2004), studies of the MPTP mouse model report a non-significant decrease of DA content concomitant to minimal, though significant decreases in TH-IR (Mitsumoto et al. 2005). However, given that human
parkinsonism induced by MPTP exposure does not include indications of olfactory
dysfunction, the MPTP model seems an unlikely one for studies of olfactory dysfunction.
Because the cycad model is based on exposure to a toxin which induces olfactory
dysfunction in human patients, it meets etiological validity requirements. Furthermore,
because there are preliminary indications of neuropathological changes in the olfactory
bulb of cycad-fed mice this model would seem to be useful for further studies into the
etiological and pathophysiological bases of olfactory dysfunction. Of critical importance
will be to show functional changes in olfactory function concomitant to neuropathological
changes in this model.

Because the predicted effects of a loss of TH in the striatum are decreased levels of
striatal DA, an analysis of striatal DA levels at the 3 time points (day 75, 100 and 175)
would clarify the role DA insufficiency has in the observed motor dysfunction, as well as
lend support to the cycad model as a valid one for studies of parkinsonism. Unfortunately,
experiment 1 was not initially designed to include tissue collection for DA quantification
so tissue was obtained and analyzed from a subsequent study (experiment 3). Despite the
10% reduction in gait length of the cycad fed animals in this experiment, there was no
detectable decrease in striatal DA (Figure 13). There may be several explanations for
this. First, as already noted, the gait length deficit may be a result of motor neuron
dysfunction rather than basal ganglia dysfunction in which cases a difference in striatal
DA would not be expected. To further examine this possibility an IHC analysis of
DAergic integrity was performed to enable a comparison of the integrity of the basal
ganglia between this group of animals and those described in the initial timepoint study.
The lack of significant differences in TH, DAT and D2R-IR supports the notion that the
gait length dysfunction in these animals is not a result of DA insufficiency and thus a decreased measure of striatal DA is not expected (Figure 14).

Summary of the cycad model

This study has shown that in mice, cycad consumption leads to disturbances in the basal ganglia comparable to some of the changes observed in human parkinsonism, including PDC. Neuropathological features of parkinsonism were observed, including changes in both pre and postsynaptic markers of DAergic function, thus validating this as a neurodegenerative model with features of parkinsonism as well as lending further support to the cycad hypothesis of ALS-PDC. There were also indications of apoptotic cell death of DAergic neurons in the SNpc, although quantification of cell loss failed to detect significant cycad related changes. Behavioural changes reminiscent of parkinsonism were also observed; however, it was not possible to determine with confidence whether the changes in gait length were a result of basal ganglia or motor neuron dysfunction.

A particularly interesting feature of the cycad model are the neuropathological changes occurring in the olfactory bulb. Although functional correlates to these changes have not yet been identified, an understanding of the etiological and pathophysiological bases of the changes may provide insight into causative factors and disease mechanisms in early parkinsonism and human PD.
The neuropathological deficits appear progressively in the cycad model, over a period of weeks-months. This is an important feature of the model as many animal models of neurodegenerative disease, such as the alpha-synuclein over-expression model of PD (Kirik et al. 2002) or the 6-OHDA lesioning of the SN model of PD (Oiwa et al. 2003) rely on disease induction that is relatively sudden and/or severe and are generally concerned with states that correspond to late or even end-state phases of the disease. In contrast, our model mimics the human situation quite closely: daily consumption of processed cycad constituting a small but significant proportion of total dietary intake proves the gradual emergence of ALS-PDC. The slow, progressive nature of the cycad model is therefore well suited for studies of the rate, type and extent of disease progression as the subjects move from normal CNS state, through preclinical neurological damage to stages that mimic points at clinical diagnosis and ultimately, end state. As figure 15 demonstrates, the realistically slow appearance of neuropathological changes enables a study of the relationships between various markers of DAergic function.

It should be noted that the effects of cycad intoxication are not always the same. For example, in the initial set of tissues analysed in this study, no quantifiable evidence of apoptosis or volume changes in the SNpc was obtained, whereas previous studies (Wilson et al. 2002; Wilson et al. 2003) have clearly shown this. Differences have also been observed in the degree, and order of appearance of motor dysfunction as evidenced by different motor tasks. There are several potential explanations for this variability in the cycad model. As previously discussed, the clinical picture of ALS-PDC is not simple; there is variation both in the degree and order of appearance of its various components. Therefore, it is not unexpected that a model of ALS-PDC also displays such variation.
Although the source of such variation is unclear, it may reflect genetic differences that effect an organism's ability to respond to toxic insult or it may be due to the potential heterogeneity of cycad and its toxins; current studies are aimed at understanding how the content of cycad seeds varies with regard to season and location of harvest.

As previously discussed, recent work in our laboratory has identified sterol glucosides as a neurotoxic component of cycad that may be at least partly responsible for the neuropathological changes observed in the cycad model. Although we are still working towards a complete timeline of the neurodegenerative changes induced by SGs, several aspects of their neurotoxic action are clear. In vitro studies have shown that sterol glucosides induce the excitotoxic release of glutamate (Khabasian et al. 2000), a process numerous studies have implicated in parkinsonian pathogenesis (Beal 1998; Golembiowska et al. 2002; Rodriguez et al. 1998). Furthermore, in vivo studies have shown that cycad consumption produces decreases of the glial glutamate transporter (GLT-1b) in various brain regions including the striatum (Wilson et al. 2003); a change that may result in decreased clearance of glutamate from the synapse, resulting in increased glutamate signalling.

A variety of in vitro and in vivo studies have shown that an excessive stimulation of glutamate receptors by agonists can lead to neuronal damage and death (Olney et al. 1971); a process referred to as excitotoxicity. Excitotoxicity appears to be mediated by a massive influx of calcium ions resulting in activation of calcium ion dependent enzymes including protein kinase C, phospholipase A, phospholipase C, calcium/calmodulin dependent protein kinase II, nitric oxide synthase and various proteases and nucleases (for review see Blandini et al. 1996). Excitotoxic cell death may be a direct result of
activation of these calcium dependent pathways. Alternatively, damage induced by excessive stimulation of glutamate receptors may lead to secondary changes such as mitochondrial dysfunction, glutathione depletion and free radical formation which may either kill the cell directly or further increase the risk of cell death by making neurons even more vulnerable to excitotoxicity. There is recent preliminary evidence that oxidative stress, free radical production and heat shock responses are present in the cycad model; changes which are also associated with PD (Jenner 1994; Coyle et al. 1993; Beal 1998). Therefore, current studies are aimed at determining the significance of excessive glutamate stimulation in the cycad model as well as whether cycad toxins induce oxidative stress through other mechanisms (ie. via activation of glial cells).

Etiological relevance of the cycad model

Given that most people are not exposed to cycad and its toxins, it is important to determine if similar compounds are present in our environment. An interesting link between cycad toxins, parkinsonism and Helicobactor pylori infection has recently been described which may make more relevant the identification and mechanisms of cycad toxins (Schulz et al. 2005).

The bacterium H. pylori has gained notoriety over the past two decades as having a causal role in a variety of human diseases including chronic gastritis, peptic ulcer disease, gastric cancer, and primary B-cell mucosa-associated lymphoid tissue
lymphoma of the stomach (Suerbaum et al. 2002). In a study of the lipid composition of \textit{H. pylori}, Hirai et al. (1995) identified 3 cholesterol glucosides (CGs) – cholesteryl-\(\alpha\)-Dglucopyranoside, cholesteryl-6-O-tetradecanoyl-\(\alpha\)-D-glucopyranoside and cholesteryl-6-O-phosphatidyl-\(\alpha\)-D-glucopyranoside – accounting for approximately 25\% of the total lipid content. CGs have been identified in a variety of strains obtained from diverse geographical locations, suggesting that their presence in \textit{H. pylori} is a characteristic feature of the species (Haque et al. 1996). Of interest to our studies of neurodegenerative disease is the similarity in structure of the sterol glucosides identified in cycad and the cholesterol glucosides present in \textit{H. pylori} (Figure 16).

Figure 16.

Chemical structure of the putative neurotoxin \(\beta\)-sitosterol \(\beta\)-D-glucoside isolated from cycad seeds (A) and the cholesterol glucoside (cholesteryl-\(\alpha\)-Dglucopyranoside) present in \textit{H pylori} (B).
A link between gastric ulcer and parkinsonism was first reported in the early 1960s by Strang and Shwab, who independently described an increased prevalence of gastric ulcers in their parkinsonian patients (Schwab 1961; Strang 1965). Subsequent studies have examined whether *H. pylori* infection has a causal role in the development of parkinsonism. While parkinsonian patients are three times more likely to test positive for *H. pylori* seropositivity than controls, their siblings are also more likely to be both seropositive and affected by parkinsonism (Charlett *et al.* 1999). These effects could be alternatively explained by genetic factors or by an environmental insult early in life. A common pathogenic factor in the home environment is also suggested by the fact that spouses of parkinsonian patients often display symptoms of parkinsonism (Kirollos *et al.* 1993). An environmental etiology for parkinsonism has been suggested by twin studies showing similar concordance rates for mono- and dizygotic twins, suggesting a minimal genetic involvement (Tanner *et al.* 1999).

There are a variety of hypotheses regarding the mechanism by which *H. pylori* infection may be linked to parkinsonism (for review see, Dobbs *et al.* 2000). However, we propose an alternative mechanism by which *H. pylori* infections could have a causal role in parkinsonism. Given our *in vitro* and *in vivo* work demonstrating the neurotoxic potential of SGs and the structural similarities between cycad’s SGs and *H. pylori*’s CGs, we hypothesize that cholesterol glucosides arising from *H. pylori* infection may act as neurotoxins, promoting the degeneration of DAergic neurons of the SN. We are currently examining the links between CGs and parkinsonism by administering CGs and SGs to mice. Results of these studies will be compared on the basis of behavioural and
neuropathological data to those in which cycad’s SGs were administered. Similarly, the behaviour and neuropathology of mice chronically infected with *H. pylori* will be examined. Further epidemiological studies could complement this work and suggest to what extent the animal work can be extended to human populations.

**Future Directions**

Although the cycad model shows potential as a model of parkinsonism, there are several features it has yet to reproduce. First, although there are strong immunohistochemical indications of a DA deficiency in cycad-fed animals, the evidence is indirect; to confirm there is a loss of DA it must be measured directly by either EIA or HPLC methods. Given that the cycad model shows progressive neuropathological changes, cycad feeding should be continued for a longer period of time to determine whether cycad can induce a dopamine deficiency comparable to that in human disease. Second, the DA lesion in human PD patients is severe; at diagnosis it is estimated there is a 70-80% reduction of striatal DAergic nerve terminals and a 50-60% loss of neurons in the SNpc (Bernheimer *et al.* 1973; Agid *et al.* 1991). Therefore, a comprehensive model of PD should also exhibit comparable changes. Third, as one of the hallmark features of PD is the presence of α-synuclein containing LBs, a rigorous model of PD should exhibit such structures at some point in the progression of pathological changes. In the current study an attempt was made to identify changes in the distribution of α-synuclein, however the antibody proved unreliable and positive staining could not be confirmed.
Given the neuropathological changes in the olfactory bulb, it is important to identify functional correlates of such pathologies. The olfactory deficit in PD and PDC involves defects in both odor detection and odor identification/discrimination (Doty et al. 1988; Ahlskog et al. 1998), therefore tasks assessing these functions should be utilized in the cycad model. Olfactory sensitivity and odor discrimination may be measured by a simple method developed by Tillerson et al. (2002).

Considerable work has yet to be done to fully elucidate the mechanisms of cycad-induced neuropathology. As already discussed, cycad toxicity may promote excitotoxic processes. Cycad toxins may also have other effects either primary to, or secondary to excitotoxicity. PD is also characterized by a marked inflammatory response and there is recent preliminary evidence that there is also a microglial response in the cycad model. Therefore it would be important to determine the identity, and mechanism by which cycad toxins induce an inflammatory response. In addition to excitotoxicity, oxidative stress and inflammation, there is evidence of mitochondrial dysfunction in human PD as well as in some animals models (Dauer and Prezdborski, 2003; Betarbet et al. 2002) which may result in an abnormal generation of reactive oxygen species. Targets of oxidative damage may include components of the electron transport chain in mitochondria, setting up a positive feedback loop of electron transport chain inhibition with increased production of reactive oxygen species and further mitochondrial dysfunction. Alternatively, cycad toxins may interfere with the electron transport chain directly, similar to the mechanisms of MPTP and the pesticide rotenone. Current studies are examining the effects of cycad toxins on mitochondrial respiration and on the generation of reactive oxygen species to further define the mechanisms of cycad toxicity.
Further work should be performed using putative toxins isolated from cycad seeds as using a known toxin, of a known concentration, will improve the consistency of the behavioural and neuropathological changes observed in the cycad model. A greater understanding of the specific molecule, or molecules responsible for such changes will be useful for *in vitro* studies to further investigate the mechanisms of cycad toxicity. Furthermore, positive identification of specific toxins is important in that similar molecules may be present in our environment. Ultimately, it is hoped that identification of specific environmental toxins responsible for neurodegenerative processes will facilitate our understanding of the etiological bases and subsequent neuropathological mechanisms underlying the behavioural symptoms of PD so that effective and truly curative therapeutic strategies may be developed.
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