

Reverse Engineering Neurological Disease

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1. Abstract

The key problem for preventing the onset of the age-dependent neurological diseases (Alzheimer's, Parkinson's, and ALS) lies in knowing what factor(s) triggers the cascade of events leading to neuron cell death. Similarly, halting such cascades before they have done irreparable harm to the nervous system requires knowing the stages in the cascade in the correct temporal sequence. Without addressing and solving these problems, only palliative efforts are possible. A number of features of each of these diseases, and a consideration of the unusual features of an unusual variant, ALS-parkinsonism dementia complex (ALS-PDC) provide some clues to etiological factors that might be crucial. Epidemiological evidence from ALS-PDC suggests an environmental dietary neurotoxin as an early trigger for neurodegeneration. A murine model of ALS-PDC based on these findings has succeeded in duplicating all of the essential behavioral and pathological features of the human disease and provided insight into many stages in the neurodegenerative cascade. The insights gained from this model system will allow us to understand the sequence of events leading to initiation of disease end state, thereby providing a basis for future treatment strategies.

2. The Key Problems in Neurological Disease

The age-related neurological diseases, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS), are diagnosed only once significant behavioral deficits have been observed clinically. Alzheimer's disease involves the death of neurons of various regions of the cerebral cortex and the hippocampus and results in loss of cognitive functions such as memory and learning. In Parkinson's disease a part of the nigral-striatal system dies and the loss of dopamine containing neurons in the substantia nigra leads to loss of dopaminergic terminals that terminate in the striatum. This loss, in turn, impacts motor control leading to tremor and gait disturbances. ALS primarily involves the loss of spinal and cortical motor neurons, leading to increasing paralysis and death.

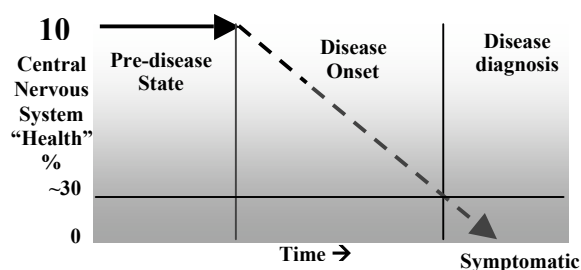


Figure 1: Schematic timeline of putative stages in sporadic forms of neurological disease. The schematic represents an idealized timeline from a condition of an early intact nervous system, particularly the neural subsets affected in the age-dependent neurodegenerative diseases Alzheimer's, Parkinson's, and ALS. The middle panel suggests that the diseases follow a linear decline, but the actual rate remains unknown (see Clarke et al., 2000). Clinical diagnosis occurs in most cases once behavioral symptoms have become overt. Between this stage and the death of the individual, it is believed that the majority of the neurons in the particular neural subset have or will die. The horizontal line represents the presumed 'threshold' level of neuron loss leading to clinical symptoms.

In each case, by the time clinical diagnosis is achieved, major damage has been done to the specific regions of the nervous system affected. Estimates of neuron loss vary, but may approach 60-70%, suggesting that neural compensation of surviving neurons occurs over long periods until some final threshold of functional neuron number is reached. Figure 1 is a schematic diagram illustrating the presumed stages of the various diseases, including the late stage of clinical diagnosis, some earlier stage involving the gradual loss of neurons, and some pre-disease state. Note for the purposes of this schematic that the rate of decline in the middle phase is drawn as a linear function, but other functions are actually more likely [Clarke et al., 2000]. Determining the actual rate of decline is an important experimental goal. The realization of this goal will have profound implications for understanding the mechanisms/processes underlying neuron loss, enhancing the prospects for early intervention thereby

preventing the final stages of each disease from being reached. We will discuss this point in more detail below.

It is important to note that in spite of conventional views that each of these diseases are totally distinct entities, considerable overlap occurs as a combination of symptoms, biochemical features, and in regions of the central nervous system showing neural degeneration. Such overlap has suggested to various investigators the possibility that each of these age-related diseases may share common mechanism/pathways leading to neural degeneration [Calne & Eisen, 1990], if not common etiologies. Further support for this notion is provided by observations concerning ALS-parkinsonism dementia complex (ALS-PDC), a disease complex first described for the islands of Guam and Rota in the Marianas [see Kurland 1988 for review] and similar to disorders described for the Kii Peninsula of Japan and for parts of New Guinea. Details about ALS-PDC and its implications for understanding the factors responsible for all age-dependent neurological disorders will be discussed below.

The confirmation of diagnosis for any of these diseases usually occurs only postmortem by histological measurements of neuron loss and identification of specific molecular markers. For example, Alzheimer's disease cortex and hippocampus show neuronal loss combined with the expression of the 'hallmark' features of the disease, amyloid plaques and neurofibrillary tangles (NFT) of abnormal tau protein. Treatment for such diseases is undertaken once preliminary diagnosis has been performed but, as noted, large-scale neural degeneration has already occurred. Treatments initiated at this stage have typically been largely palliative, offering only limited amelioration of symptoms and life extension. For Parkinson's disease, intense current interest focuses on stem cell injections as potential means to reverse the neural damage. However, such measures raise theoretical, practical, and economic problems that are difficult, if not impossible, to overcome. Briefly, chaos theory and the dynamic nature of the developing nervous system, the myriad problems associated with recreating neural circuits outside of normal developmental periods, and the costs associated with stem cell transplantation, make this technology as the 'cure' for Parkinson's disease at best a faint hope. Other prospective treatments of end stage neurological disease, e.g., estrogen, anti-oxidants, etc. all face the same ultimate problem of attempting to restore function to already destroyed neural circuits.

Given such problems, a conventional triage approach would focus attention on early stages in the disease: An ideal solution would be prophylactic, but would require that the cause of the disease be known. Next best would be to halt disease progression before irreversible damage had been done to regions of the nervous system, but this would require that the various stages of disease progression be known, so that targeted, rather than random, pharmacological therapeutics could be applied.

It is obvious that we are not in the position to be able to do either of the latter since we do not know what etiological factors cause these diseases nor do we have any clear idea of the early stages in disease progression. While many molecules and abnormal cellular processes can be identified postmortem in humans and in some animal models, it is equally possible that such events represent not causal factors, but rather molecules that are altered 'co-incidentally' but not pathologically, or molecules or processes that may actually be compensatory (successful or failed). Postmortem examination alone cannot distinguish between these possibilities, nor can it put the myriad affected molecules into any sort of temporal sequence from early stages of the disease to

final stages resulting in cell death. This fact alone leaves animal models as the most likely means to unravel the temporal sequence of events, a timeline that may prove crucial to successful future therapy. An effective model would have to mimic the essential behavioral and pathological stages in the disease including its progressive nature. It would be predictive, revealing features not found in previous human studies. In addition, the model would have to be induced in a

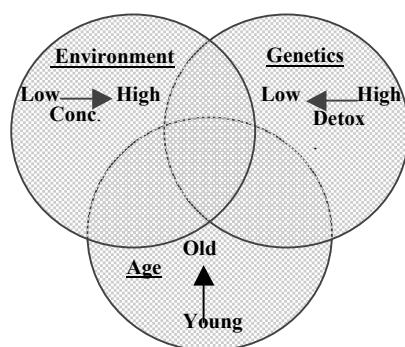


Figure 2: Potential synergies of causal and risk factors in sporadic neurological disease. The key causal factor(s) involved in such diseases are thought to reflect exposure to some toxin(s) that can arise from the environment or within the body and which may be synthetic or naturally occurring. This is represented by the set on the left side of the schematic. Range of toxicity effects run from left to right as low to high. Intersecting this is a set consisting of a propensity that could arise due to genetic polymorphism in efficiency of detoxification mechanisms (from right to left expressed as high to low). Genes with other features could also be involved (e.g., APOE alleles). The intersection of these two sets describes the individuals who may be at risk of developing the neurological disorder. Note that the intersecting region can increase or decrease depending on strength of either variable. Intersecting these two sets is the variable of age with risk factor increasing from young to old (bottom to top).

manner similar to that presumed to underlie the disease being studied. The model would also have to allow for the testing of potential therapeutics. Finally, the model would have to allow the stages of disease progression to be ‘template matched’ to presumably similar, but still unidentified, stages in the human disease.

The identification of the classes of potential causal factors is key to the discussion. In brief, these include ‘gain of function’ mutations, deletions, environmental toxins or a combination of epigenetic and genetic factors. While an early onset familial form of Parkinson’s disease has been identified to be linked to abnormal α -synuclein [Sharma et. al., 2001], it now seems obvious that the vast majority of cases are late onset and non-genetic [Tanner et al., 1999]. Alzheimer’s disease has early onset genetic factors involving abnormal tau proteins [McKeith et. al., 1996], but the incidence of this mutation in relation to the total Alzheimer’s population is small. Similarly, the ALS population has a familial form, a small fraction of which involves a heritable mutation of the gene

coding for superoxide dismutase (mSOD1) [Gaudette et. al., 2000]. For ALS, only 2 to 3% of all cases involve this mutation leaving the sporadic form accounting for virtually all others. The absence of a clear causal genetic component for the sporadic form of any of these diseases focuses our attention on potential etiological features in the environment, notably neurotoxins. Various examples of synthetic toxins have been documented [eg. Ballard et al., 1985] and numerous natural neurotoxins exist [Betarbet et al., 2000].

Although environmental neurotoxins, synthetic or natural, seem the most

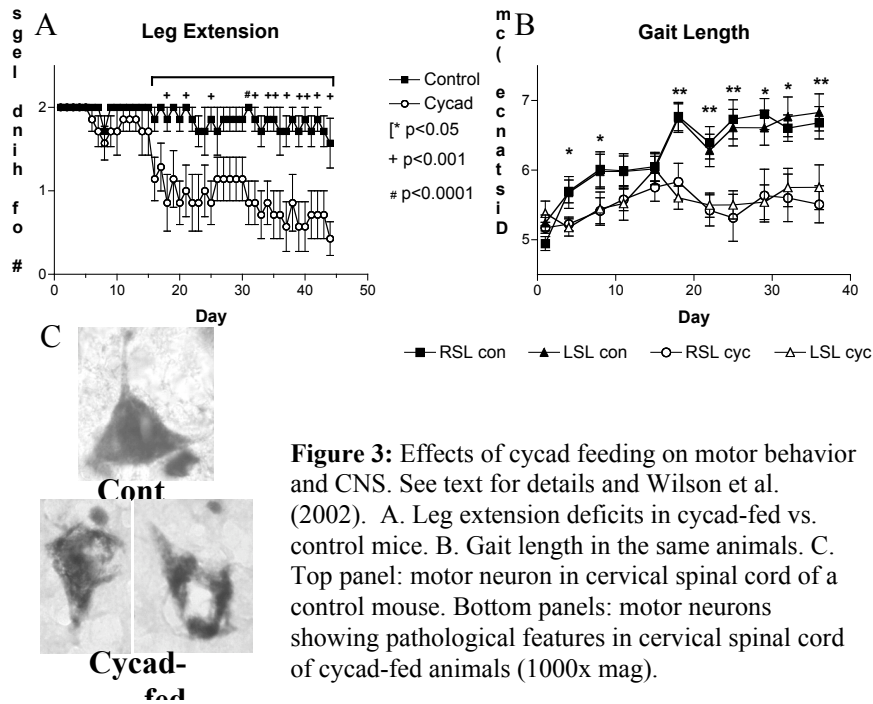


Figure 3: Effects of cycad feeding on motor behavior and CNS. See text for details and Wilson et al. (2002). A. Leg extension deficits in cycad-fed vs. control mice. B. Gait length in the same animals. C. Top panel: motor neuron in cervical spinal cord of a control mouse. Bottom panels: motor neurons showing pathological features in cervical spinal cord of cycad-fed animals (1000x mag).

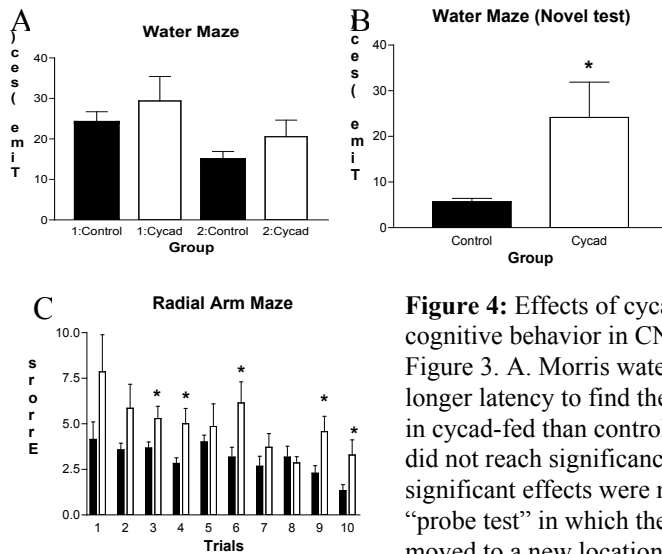


Figure 4: Effects of cycad feeding on cognitive behavior in CNS. Details as in Figure 3. A. Morris water maze showed longer latency to find the hidden platform in cycad-fed than control mice. These data did not reach significance, but highly significant effects were noted with a later “probe test” in which the platform was moved to a new location (B). C. Radial arm data showing significantly increased learning and memory deficits in cycad-fed mice.

likely causal factors leading to neurodegenerative changes in the nervous system, it will be equally clear that if any given toxin were the sole cause, larger fractions of the population would have to be afflicted. As this is not the case (although disease incidence may be on the increase [see Martyn, 1994]), it seems most likely that the age-related neurodegenerative diseases arise due to an intersection of exposure to an environmental toxin and a genetic susceptibility (see Figure 2). This susceptibility may take a number of forms, including genetic polymorphisms. For example, it might involve toxin handling (e.g., detoxifying enzyme expression), absorption (e.g., transporter proteins), or an interaction with the handling of related molecules (e.g., APOE alleles). In this view, the intersection of the sets comprised of environmental factor and genetic susceptibility can expand or contract depending on toxin concentration and/or duration and the relative levels of expression of the genetic factor. Age is also a critical variable, with increasing age involved in each of the disorders discussed here.

Various animal models of environmental toxicity leading to parkinsonism-like features have been described [Ballard et al., 1985]. Recently, Betarbet et al. [2000] demonstrated that rotenone, a natural pesticide, causes complex I mitochondrial damage leading to behavioral and pathophysiological outcomes in 50% of rats injected with the molecule.

Accepting that an animal model is the most likely means of identifying the stages of disease progression or for testing therapeutic approaches, it should be an important effort to identify animal models that could satisfy the above model criteria. In the following, we will describe a model that we believe satisfies these criteria and the data that support this view. The implications of this model for early treatment of pre-symptomatic neurodegenerative disease will also be discussed.

3. ALS-PDC and an Animal Model of This Neurological Disease

In the years after World War II, L.T. Kurland and various other investigators described in detail an unusual neurological disease complex, ALS-parkinsonism dementia complex (ALS-PDC). The disease could be expressed as a rather conventional form of ALS (termed 'lytico' or 'paralytico' by the Chamorro population of Guam), or as a form of Alzheimer's disease with strong parkinsonian features (parkinsonism-dementia complex or PDC, locally termed 'bodig'). The history of this disorder was believed by Kurland and other early investigators [for review, see Kurland, 1988] to be so unique that the disease could serve as a type of neurological "Rosetta Stone", the decipherment of which would unlock crucial clues to neurological disorders worldwide. Kurland and other neurologists identified a number of unusual features of ALS-PDC, including its (then) high level of incidence, the occasional overlap of symptoms of the neurological subsets, and often early age of onset. Epidemiology failed to find a genetic cause [Morris et al., 1999] and the main clue was the notion that a toxin contained in the seed of the cycad palm was the crucial factor [Kurland, 1988]. Kurland and others noted that the disease incidence peaked within several years of massive cycad consumption by the indigenous Chamorro population and dramatically declined when cycad consumption declined. In spite of this, the early enthusiasm that neurotoxins contained in cycad seed were causal to ALS-PDC stalled due to results showing that toxins identified early in the studies did

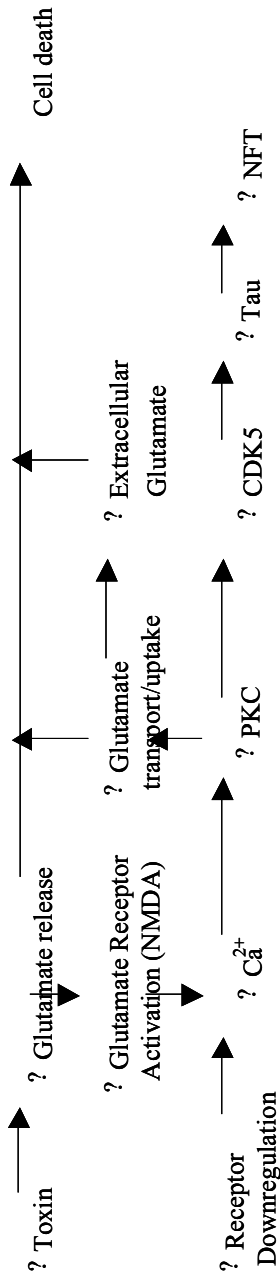


Figure 5: Preliminary outline of stages in the biochemical cascade leading to neuronal degeneration. See text. Small arrows indicate increases (↑) or decreases (↓) in amount or function. Big arrows indicate stages or pathways.

not remain in the flour of the cycad processed for consumption [Duncan et. al., 1990].

We recently reexamined the issue using quantitative isolation procedures combined with bioassays for neural activity and cell death [see Khabazian et al., 2002]. These studies identified the most toxic molecule contained in cycad as a sterol glucoside whose actions *in vitro* included the excitotoxic release of glutamate and increases in protein kinase activity. *In vivo* studies of mice fed washed cycad seed flour containing this sterol glucoside employed a battery of motor, cognitive, and olfactory behavioral measures that demonstrated a temporal sequence of behavioral deficits in all three systems [see Wilson et al., 2002]. In regard to motor neuron disorders (see Figure 3 and Wilson et al., 2002), the cycad-fed mice showed significant losses of the leg extension reflex (indicative of motor neuron dysfunction), had pronounced gait disturbances as well as losses of muscle strength and balance. Upon sacrifice, mice fed cycad

showed TUNEL and caspase-3 positive cells indicative of apoptosis in spinal cord, cortex, hippocampus, substantia nigra and olfactory bulb. Motor neuron numbers were decreased significantly in ventral cord and various regions of cortex showed significant thinning. These regions of neural degeneration were consistent with the observed behavioral deficits. Cognitive deficits were observed in spatial learning tasks (Morris water maze) and reference memory (radial arm maze) with corresponding neurodegeneration seen in regions of cerebral cortex and hippocampus [see Figure 4 and Wilson et al., 2002]. In addition, the olfactory system showed a significant loss of function accompanied by disrupted structures and cell loss in the olfactory glomeruli. In various regions, key molecules associated with neuronal degeneration were altered. These included elevation in tau protein and various protein kinases (notably PKCs and CDK5) [see Khabazian et al., 2002]; in addition, elements of the glutamatergic system were severely affected including a dramatic decrease in two variants of the GLT1 glutamate transporter (GLT-1 α and GLT-1B; also called EAAT2) [Reye et al., 2002] on astrocytes and a decrease in NMDA and AMPA receptor binding [Wilson et al., 2002b; Khabazian et al., 2002b]. Both effects were noted in regions of the CNS showing neural degeneration. The decrease of the GLT-1B transporter was quantified by Western blots and showed a primary loss of the 30 kDa cleavage product that could be partially restored by pre-treating the tested protein fraction with alkaline phosphatase. All of the features described above in our murine model are consistent with features of ALS-PDC, as well as key aspects of Alzheimer's, Parkinson's, and ALS, including a progressive development of neuronal dysfunction [see Wilson et al., 2002].

4. Reverse Engineering Neurological Disease

The data cited above have several key implications. First, the correspondence between the changes in behavioral outcome and histological indices of neurodegeneration in the animal model compared to ALS-PDC validates the hypothesis that ALS-PDC may be due to consumption of cycad toxins. Second, the overall similarities to many behavioral, biochemical, and pathophysiological outcomes in age-dependent neurodegenerative diseases suggests that similar mechanisms based on exposure to environmental neurotoxins may be common features of each. In regard to potential therapeutic treatment for early stage neurodegenerative diseases, the fact that we can mimic many of the essential characteristics of these diseases in a reproducible manner suggests that we can use this model system to create a timeline of neurodegenerative events. These events span the period from initiation (onset of exposure to an identified neurotoxin; see Khabazian et al., 2002] through the various stages of neural dysfunction culminating in neural cell death. By rerunning the experiment and sacrificing some animals at set time points, we should be able to create a detailed temporal sequence of molecular events leading to neural degeneration such that causal, co-incident, and compensatory molecules and events can be put into the correct sequence. The implications of the latter are that specific, targeted therapeutics could, in principle, be directed at particular abnormal biochemical events, ideally at early enough stages to prevent the final loss of neurons and neuronal function. Based on our data and studies in the literature, we have been able to begin this analysis as shown in Fig. 5. This schematic shows various putative stages of the neurodegenerative disease process, including the onset of pathological processes induced by the identified cycad toxin. In so doing, it

predicts the temporal order of some crucial events. For example, conventional notions have suggested that NFT are an early causal feature of neurodegeneration in Alzheimer's disease and ALS-PDC. In contrast, our model suggests that NFT are 'down stream' of events such as the down-regulation of the glutamate transporter.

Being able to place the molecular events into correct temporal sequence is a key feature of our emerging model system. In addition, as details of the sequence emerge, we will be able to plot the function of neural cell loss with respect to time and answer the question of the quantitative relationship between the loss of particular neurons and the decline in the functions they control. We will also be able to determine if the loss of neurons and the underlying biochemical modifications over time describe linear, sigmoidal, exponential, or other functions. The type of function defined has huge implications for potential therapies. For example, exponential declines in cell number may imply single pathological 'one hit' events in which cell loss is constant over time [Clarke et al., 2000]; in contrast, a sigmoidal decline would suggest that cell death occurs following cumulative long-term damage. The former is consistent with an excitotoxic mechanism of cell death and with the data cited above, but not with mechanisms based on oxidative stress as proposed by various researchers. As a preliminary attempt to address such issues, we have plotted leg extension data (measuring motor neuron function) from mice fed washed cycad vs. time and found an exponential decline (Fig. 6) that closely resembles data generated in various models of Parkinson's disease [see example in Clarke et al., 2000].

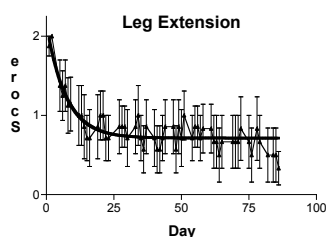


Figure 6: Loss of motor neuron function as measured by leg extension. The data represent the averaged results of 7 cycad-fed animals from the onset of cycad feeding to sacrifice. These data are from a different batch of cycad-fed animals than those shown in Figure 3. Note that the resulting curve is initially exponential but appears to reach a steady level for a period of time before showing a further decline (Curve fitting provides the equation $y=1.444*\exp^{(-0.138x)}+0.714$). These data are similar to data derived in a model of chemically-induced Parkinson's disease (Clarke et al., 2000).

5. Future Directions

The attempt to reverse engineer neurological disease using our model system is still in very preliminary stages. However, the success of this effort in establishing the correct temporal sequence of molecules involved in neurodegeneration cascades, the feedback and feed-forward loops involved, and the dynamical characteristics of what is seemingly an increasingly chaotic system

seems promising. Once the above issues have been resolved, we will have a means to attempt the next crucial task of matching the unseen features of evolving human neurological diseases to known features in the model system. This approach may offer the best hope of successfully treating neurological disease processes before they have done irreparable harm to the nervous system.

Acknowledgments

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