

Common Threads in Neurodegenerative Disorders of Aging

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In recognition of the commonalities that exist among many neurodegenerative diseases of aging, two foundations devoted to finding cures for Alzheimer's disease (AD) and Huntington's disease (HD), joined forces at a "Think Tank," which brought together some of the world's leading authorities on human brain diseases, dementia, memory disorders, and aging. Keep Memory Alive, the foundation for the Lou Ruvo Alzheimer's Institute in Las Vegas, Nevada, partnered with the Hereditary Disease Foundation in sponsoring the workshop and in guiding its participants to consider not only the common pathogenic characteristics of these diseases and the challenges presented by those features, but also the common infrastructural and policy issues that must be addressed in order to accelerate drug discovery for these and other neurodegenerative diseases. The workshop focused on two interrelated pathogenic events: aggregation of misfolded proteins and mitochondrial dysfunction. The causes of both AD and HD have been attributed to both of these events, yet their relationship to one another remains unclear, raising two key questions: What is the role of mitochondria in causing protein misfolding, and how might misfolded proteins cause mitochondrial dysfunction? Although no consensus emerged on the precise role of these events in the pathogenesis of AD or HD, there was broad agreement that both processes are likely to play important roles and must be more fully investigated in a variety of *in vivo*, *in vitro*, and *in silico* models. Lest the workshop stray too far from the primary objective of both organizations; that is, improving the lives of patients and families affected by these diseases, participants in the scientific discussions were joined by a woman with AD and her husband. The couple spoke frankly and responded to questions from the scientists and clinicians in attendance about how the memory loss associated with AD has affected her day-to-day functioning and undermined her confidence in the ability to make decisions. They also focused attention on the important role patients and caregivers can play in the search for new treatments and on the need for scientists to pay close attention to patient experiences and what they reveal about pathogenic mechanisms.

Mitochondrial dysfunction in neurodegenerative disease

The evidence that mitochondrial dysfunction and oxidative damage play a central role in both aging and neurodegenerative diseases is strong¹. Several studies have demonstrated an age-related increase in deletions and point mutations in mitochondrial DNA. Such mutations could lead to impaired energy generation and increased levels of reactive oxygen species (ROS), which could cause cell damage. Animal studies indicate that reducing oxidative stress increases longevity.

In AD, longstanding data suggest a defect in the electron transport chain of mitochondria, with a decrease in cytochrome oxidase activity seen in both AD brain tissue and platelets from AD patients. Reduced levels of pyruvate and alpha-ketoglutarate dehydrogenases are also seen. In transgenic mouse models of AD, oxidative damage precedes deposition of A β , and administration of anti-oxidants significantly reduces A β production and A β plaque deposition. Soluble A β is seen in mitochondria as well as elsewhere in neurons; and amyloid precursor protein (APP) is seen in association with the outer mitochondrial membrane. *In vitro* studies show that the ABAD (A β -binding alcohol dehydrogenase) enzyme binds to A β and leads to increased production of ROS. ROS, in turn, modulate A β production. Blocking ABAD from binding to A β *in vitro* suppresses A β -induced apoptosis and free radical generation. In addition, transgenic mouse models of AD show abnormal expression of mitochondrial energy metabolism genes.

Despite the evidence suggesting mitochondrial involvement in the AD pathogenic process and a clear link between mitochondrial dysfunction and other neurodegenerative diseases, such as Parkinson's disease (PD), there was no consensus about the importance of mitochondrial dysfunction in AD; whether it is an upstream or downstream process; or whether it is a primary, secondary, or tertiary event. Resolving these questions will be critical to understanding whether interventions that target mitochondria will be useful in treating AD.

In HD, abundant evidence suggests an important role for mitochondrial dysfunction in the disease -- N-terminal aggregates of mutant huntingtin protein bind to the outer mitochondrial membrane, increasing susceptibility to the mitochondrial permeability transition (MPT) and reducing calcium uptake capacity. Mutant huntingtin also binds to and upregulates p53, leading to mitochondrial membrane depolarization. There is also a decrease in the activity of many mitochondrial enzymes in HD patients and animal models. Imaging studies of people with HD show multiple signs of mitochondrial dysfunction: reduced oxygen utilization, elevated lactate production in the cerebral cortex and basal ganglia, and a reduced ratio of phosphocreatine to inorganic phosphate that is CAG repeat dependent. Lymphoblasts from HD patients show abnormal membrane depolarization. Biopsy material from HD patients shows not only abnormal mitochondria but also impaired complex II-III activity in the basal ganglia. Another piece of evidence linking mitochondria and HD is that treatment of animals with the mitochondrial toxin, 3NP, results in selective spiny neuron degeneration and sparing of interneurons, mimicking the pathology seen in HD.

One possible mechanism for mitochondrial dysfunction in neurodegenerative disease is that excess mitochondrial fission may result in respiratory deficits, an increase in ROS, neuronal dysfunction, and cell death². In normal cells, mitochondrial fission is balanced by mitochondrial fusion to maintain mitochondrial and cellular function. Should AD, HD, or other neurodegenerative diseases be linked to increased rates of mitochondrial fission, this could be a useful target for intervention.

Proteins, protein folding, and aggregation

In both AD and HD, as well as in most other neurodegenerative diseases, insoluble aggregates of proteins, often referred to as amyloid fibrils, are found in the CNS^{3 4}. These protein aggregates all share certain structural characteristics, and it has been proposed that these diseases may also share common pathogenic mechanisms. The proteins themselves differ, as do the locations where aggregates are found. In AD, for example, extracellular aggregates called amyloid plaques are found throughout the cerebral cortex. The principal component of these plaques is A β , a small 4 kDa protein produced by proteolytic processing of the larger amyloid precursor protein. Also in AD, intracellular neurofibrillary tangles form from aggregates of phosphorylated tau protein; and sometimes, intraneuronal aggregates of the protein α -synuclein form what are called Lewy bodies. In HD, mutant huntingtin protein is found in different configurations both as intra- and extra-nuclear aggregates.

While extensive efforts have gone into defining the steps by which a protein such as huntingtin or A β aggregates, the process is still not well understood. Moreover the terminology used to describe the aggregation process has been applied inconsistently, resulting in considerable confusion surrounding the terms used⁵. The initial event appears to be misfolding of the monomeric protein such that it can form aggregates ranging from dimers to oligomers composed of 50 or more monomers. These oligomers proceed to form a variety of higher-order aggregates, including protofibrils and fibrils. The latter are typically 6-10 nm in diameter and of indeterminate length. They appear to be rigid and frequently form a mesh-work of interlaced or laterally-associated fibrils. The term protofibril has been applied to a variety of different pre-fibrillar microscopically detectable structure, including globular, curvilinear, and annular assemblies. The question as to which of these species are most important to the toxicity that causes these disease has yet to be resolved, as well as whether these different species are all formed on the assembly pathway of the mature fibril or off the pathway. It may be that all these structures are toxic to different degrees; however there is some evidence that the inclusion bodies that form in Parkinson's disease and HD may be protective, and that pre-fibrillar assemblies are the major toxic species in these diseases.

Linking aggregation with mitochondrial dysfunction

At least two mechanisms have been proposed for how aggregation might be linked to mitochondrial dysfunction, if indeed the two are linked. If they are linked, the question remains whether aggregation precedes mitochondrial dysfunction or vice versa. The toxicity of protofibrils may arise from interactions with mitochondrial membranes, resulting in depolarization and decoupling of the chemo-osmotic gradient. Another possibility is that protein aggregation and misfolding put a strain on cells' apparatus for clearing such aberrant forms, e.g., through ubiquitin-proteosomal-, chaperone-, or aggresome-mediated processes, and that breakdown of these systems leads to mitochondrial dysfunction.

The interaction of proteins and different aggregate forms with mitochondrial membranes may be critical to understanding how aggregates might cause mitochondrial dysfunction, but is currently poorly understood. Mitochondria have two very different membranes, an

inner membrane that is mostly protein and an outer membrane that is more hydrophobic, and there are important differences in the interaction of proteins with the two membranes. Binding of proteins to the membranes of mitochondria may alter important mitochondrial processes, such as fission, fusion, and apoptosis.

There is also evidence in HD that mutant huntingtin aggregates alter mitochondrial trafficking in cortical neurons, which could lead to insufficient mitochondria to meet energy demands at certain cellular locations, such as presynaptic nerve endings and dendrites; and which could prevent the transport of damaged mitochondria to locations where they could be repaired or degraded⁶.

The time is now for translation

Many workshop participants agreed that despite the number of unanswered questions, enough is currently known about AD and HD that the time for translation is now. For example, one possible approach would be to try to stabilize proteins in an aggregation-incompetent state, such as monomers, in order to prevent production of toxic species. Compounds that are able to stabilize the protein could be tested in animal models to assess efficacy. It might also be appropriate to test neuroprotectants that might target mitochondria, or to investigate the use of a ketogenic diet to slow the progression of neurodegenerative disease that in some way involves altered energy metabolism or oxidative stress. Perhaps a compound could be found that would make mitochondria fuse and that could be tested as a means of reversing the effects of mitochondrial fission. A treatment targeting the Nrf-ARE pathway (Nuclear respiratory factor bound to the antioxidant response element), which is involved in protection against oxidative stress, could also be beneficial for both AD and HD.

One of the barriers to developing new treatments is that none of the mouse models available fully recapitulate the disease, particularly AD. Models are needed that mimic not only the neurodegenerative aspects of the diseases but also the metabolic and other phenotypic characteristics that occur in affected people. Large-animal models are also needed to test therapies such as gene therapy and to provide an adequate number of mitochondria from discrete areas of the brain.

Another barrier is the lack of reliable biomarkers, particular pre-disease markers. One suggestion was to look for a marker of synapse loss, such as synaptophysin, which develops early in both AD and HD. Metabolomic studies using several different technological platforms may provide some useful biomarkers but are not well developed at this time. Structural imaging studies are perhaps the furthest along, and functional imaging may provide even more robust markers.

New techniques for answering long-standing questions

Participants in the workshop agreed that methods are needed that allow observation of protein assembly in a non-dissociated state, both *in vitro* (e.g., via mass spectroscopy) and *in vivo* (e.g., magnetic resonance spectroscopy, fluorescent probes); as well as dynamic monitoring of mitochondrial bioenergetics. New computational “*in silico*”

methods are also needed to develop models that can be tested *in vitro* and *in vivo*. Several methods are already available that could shed light on the processes involved in aggregation and mitochondrial dysfunction:

Steven Finkbeiner and colleagues have developed a robotic automated microscope system that allows monitoring of the fate of millions of individual living cells and intracellular proteins over time by returning to the precise cell multiple times⁷. Using conformational-specific antibodies, they can localize different species of aggregates dynamically, and the technique is adaptable to follow a large and growing number of molecular components in a cell simultaneously. The technique will allow monitoring of multiple related variables at the same time, for example, multiple aspects of mitochondrial function. A newer adaptation of the technology uses fluorescence resonance energy transfer (FRET) pairs and a spectral imaging system. This system provides information on the interaction of proteins but does not identify the proteins. David Nicholls and colleagues have developed an oxygen electrode that provides extensive information about the state and function of mitochondria⁸. The technique allows monitoring of the respiration of a small number of neurons on a coverslip placed in a thin closed perfusion chamber, providing precise and quantitative information on mitochondrial bioenergetics. The technique could be used to study cultured neurons from animal models as they become symptomatic and could also be applied to multiple other uses. It might also be used to test the hypothesis that there is indeed a link between mitochondrial dysfunction and aggregation. In addition, if the oxygen electrode could be developed into a nano-electrode, it could be used to study mitochondrial function in single cells in the intact brain.

Ella Bossy-Wetzel has developed techniques using time-lapse microscopy and 3D imaging to follow cells that have been transfected with different markers, and uses this technique to monitor mitochondrial fission in cells. Her lab is currently working on developing a high-throughput use of this technology to screen candidate drugs. The technique could also be used to detect oligomers and relate them to changes in mitochondrial morphology and to examine how mutant huntingtin, for example, modulates the activity of fission molecules.

Colleagues of Gary Fiskum and colleagues have developed tet-responsive transgenic mice that express an enhanced yellow fluorescent protein (eYFP) specifically within neuronal mitochondria (mito/eYFP). Once they are crossed with mice expressing mutant genes that are responsible for diseases like PD or HD, the resulting mice that express both genes will be valuable tools to study the interactions between mitochondrial alterations and congenital forms of neurodegenerative disease.

These technological developments offer new tools that can be broadly applied to study the processes of aggregation and mitochondrial dysfunction, as well as to investigate other targets in neurodegenerative disease. Moreover, it is essential to make biological and clinical connections, determining the relationship between neuronal dysfunction and death and clinical symptoms, particularly in the early stages of the disease. Presently,

most of what is known about the pathogenic processes involved comes from studies of people in the terminal stages of the disease.

Collaborations key to moving forward

The promise of these technologies will only be realized through collaborative research between investigators with a broad range of expertise. Participants at the workshop identified numerous possible experiments aimed at clarifying the role of mitochondria and misfolded proteins in AD and HD. These proposed studies included:

Identifying misfolded proteins and early aggregates in single cells to chronologically determine if they appear before the onset of other dysfunction.

Developing optical probes for investigating mitochondrial biochemistry in living cells in order to identify processes that predict cell fate, e.g., cell death.

Developing transgenic mice that express fluorescent proteins that can be used to identify mitochondria and protein aggregation in order to explore the relationship between protein aggregation and mitochondrial dysfunction both *in vitro* and *in vivo*.

Administering different species of aggregates to isolated mitochondria from different locations in the brain and determining the effects on both bioenergetic parameters and apoptotic activities, e.g., release of cytochrome c in response to proteins like Bax and p53.

Using time-lapse microscopy and biochemical approaches, investigate the effect of mutant huntingtin on mitochondrial fusion machinery by detecting oligomers and relating those to changes in mitochondrial morphology.

Using molecules that are mitochondrial protective and see if they protect against synaptic dysfunction.

Investigating the relationship between mitochondrial degeneration and mutant proteins involved in neurodegenerative disease by using Nicholls' oxygen probe combined with imaging of mitochondrial fusion, fission, and trafficking in primary cultures of neurons derived from double transgenic mice that express the mutant genes and eYFP within neuronal mitochondria.

Studying the time course of mitochondrial bioenergetics in an animal model and looking at mRNA expression of electron transport chain genes, cytochrome oxidase activity, cellular ATP, and ADP/ATP ratios.

Screening mitochondrial-targeted antioxidants in mice and cell lines.

Developing processes to label proteins for monitoring in a way that would not disrupt native folding. Several different types of molecules could be labeled in the same cell for FRET experiments.

Stabilizing oligomers and investigating the effect on pathology and phenotype in mouse models.

Investigating pathways *in vivo* in the double transgenic mice that express neuronal mito/eYFP and mutant HD using phosphorus NMR to look at a variety of processes, including cell morphology, total numbers, and distribution.

Perfecting the ability to culture neurons from adult transgenic animals and using a tet-inducible protein to follow aggregate formation and respiratory function using the oxygen electrode.

Investigating mitochondrial trafficking *in vivo* in mouse models of neurodegenerative diseases.

Identifying the toxic configuration of protein aggregates as well as the cells in which the protein is expressed and the fate of those cells.

Examining the dysregulation of neuronal circuitry in transgenic mouse models.

Studying the different kinds of mitochondria in the nervous system to determine if they explain the selective vulnerability of different types of neurons.

Exposing mice to sublethal levels of neurotoxins and then follow for a year or so (to simulate aging) and examine neuropathology.

In addition to these proposed studies, workshop participants suggested piggybacking on existing collaborative projects such as the biomarkers core of the Alzheimer's Disease Neuroimaging Initiative (ADNI), located at the University of Pennsylvania. This core responds to one of the most important requirements for many collaborations by collecting samples from people at risk of disease or who have developed the disease. John Trojanowski, Principle Investigator of the Core, noted that the Core could provide fluids for studies on AD and might also be open to some add-on projects, such as metabolomic studies or fluid banking for diseases other than AD, such as PD or HD. He added that ADNI fluids cannot be released without submission of a request, which is reviewed by an independent review body. All pertinent information about requesting ADNI fluids is available of the ADNI website: <http://www.adni-info.org/>. Other organizations are also banking tissue or fluids for biomarker studies for these diseases and may be amenable to collaborations. Trojanowski also offered access to the high-throughput screening core at Penn to investigators who wish to utilize their technology for AD and related studies. Similar Cores are also available and accessible at other academic medical centers, e.g., the Laboratory for Drug Discovery in Neurodegeneration (Harvard Center for Neurodegeneration and Repair).

Conclusions and next steps

The workshop concluded with many questions unanswered, but with seeds planted that may bear fruit in the coming years as collaborations develop between investigators representing different points of view and from different disciplines. Answers to the questions that were the focus of the workshop, i.e., the role of protein misfolding in mitochondrial dysfunction as well as the reverse, how mitochondrial dysfunction may contribute to protein misfolding, may shed light on some of the bigger unanswered questions relating to HD and AD: Why do these disease occur in late-life, and why are certain structures affected more than others?

The workshop was organized by the two advocacy organizations as a means of building synergy among investigators working on different neurodegenerative diseases, and to identify the barriers to progress and how these barriers can be overcome. The infrastructure and funding that already exists with these two organizations, as well as the new programs being developed at the Lou Ruvo Institute, offer a strong foundation that will facilitate the types of collaborative projects that emerged during this two day workshop, which is intended to be the first of many such “think tanks.”

The couple who spoke at the beginning of the workshop, both of whom had been involved in the aerospace program in the 1960s, compared the enthusiasm for finding a cure for AD to the effort to put a man on the moon. As with the space program, finding cures for neurodegenerative diseases will require the efforts and resources of many people and agencies, but more importantly, the creativity and vision of people who are able to join forces with experts from many other disciplines and imagine solutions that may seem unreachable.

Invited but could not attend:

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