

## **Enabling Technologies for Alzheimer Disease Research: Seventh Bar Harbor Workshop, 2007**

By Gabrielle Strobel

Last August, Alzheimer disease researchers met with colleagues from other scientific fields and with foundation and NIH representatives in Bar Harbor, Maine, for the seventh annual workshop on Enabling Technologies for Alzheimer Disease Research. The participants' goal was to identify current knowledge gaps that block progress toward a deeper understanding of AD and therapy development, and to identify opportunities for bridging these gaps. The workshop focused on three areas. An update on recent research advances set the stage for an intensive focus on the AD risk gene ApoE, and for a discussion of the emerging role of lipids. The second session examined new thinking in protein folding, and the third session introduced technical advances in imaging of mouse and human tissue, as well as in-vivo imaging. A separate discussion grew out of recent advances toward a better understanding of the clinical observation that some people with AD have epilepsy-like seizures. The report below begins with a brief summary of major advances in AD research over the past 2 years. It then summarizes 2 days of presentations and discussion around the three main topics, and concludes with a list of research priorities culled from the prior proceedings. Readers will find a broad description of how the big questions in AD research are changing, as well as ideas to update their own studies. As always, comments are welcome.

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### **Part 1: Overview**

First, an overview of advances in the past year and new research questions they raised.

- Progranulin and TDP-43: The discovery of mutations in the gene progranulin gave a genetic identity to a large fraction of FTDL-U. This is a common form of frontotemporal dementia that is not caused by tau mutations and is sometimes confused with AD in the clinic. The progranulin discovery adds haploinsufficiency of a growth factor involved in regulatory signaling of the cell cycle, motility, and injury response to the range of underlying causes for age-related dementia. Soon after, TDP-43 proved to be the major constituent of the pathogenic inclusions seen in progranulin-related FTDL-U. TDP-43 is a nuclear protein of poorly understood function. It also occurs in nuclear and cytoplasmic inclusions of other neurodegenerative diseases, including some cases of AD and all cases of sporadic ALS. Together, these twin discoveries are driving a realignment of the clinico-pathological-genetic delineations of neurodegenerative protein deposition diseases. Both genes offer new areas for mechanistic study. For details, see AD/PD meeting progranulin report; AD/PD meeting TDP-43 report.

- AD genetics: Duplication of gene loci on chromosome 21, encompassing the APP gene, were found to cause autosomal-dominant familial AD with cerebral amyloid angiopathy in six different families (Rovelet-Lecrux et al., 2006; 2007). As have earlier reports of  $\alpha$ -synuclein triplication causing early-onset Parkinson's (Singleton et al., 2003), this finding supports the hypothesis that changes leading to elevated expression of a pathogenic, aggregation-prone protein increase its concentration past its point of solubility and in this way drive down the age of disease onset. A separate discovery in LOAD genetics pointed to a cell biological way of increasing A $\beta$  levels over time (Rogaeva et al., 2007). AD-associated variants of the sortilin-related receptor (SORLA) appear less able to perform the receptor's proposed function of trafficking APP towards recycling endosomes and away from amyloidogenic processing in BACE-containing late endosomes. This finding resulted from a large collaborative effort involving sharing sample sets of 6,000 genotyped patients. The search for pathogenic polymorphisms has since expanded to analysis of samples from some 10,000 patients, and the gene is estimated to be a risk factor in up to 10 percent of LOAD patients. SORLA has a weaker population effect than ApoE4 but confers risk independently of ApoE4 (SORLA Alzgene page).
- A physiological substrate for a preferred AD drug target, the  $\beta$ -secretase BACE, was discovered (Willem et al., 2006). The enzyme appears to cleave the protein type 3 neuregulin-1 during peripheral myelination early in life. It is unclear whether this cleavage participates in myelination in the adult CNS, but drug developers pursuing BACE inhibition will watch for potential demyelinating effects.
- The search for structural information on  $\gamma$ -secretase made initial progress with EM images of purified complex that visualize a globular, hollow chamber to 120Å resolution (Lazarov et al., 2006). Atomic-scale X-ray crystallography structures of this 19 transmembrane-domain complex remain a future goal.
- A new method in human CSF measurement has advanced mechanistic research into A $\beta$  accumulation during the years prior to AD. By continuously monitoring the fractional concentration of isotope-labeled forms of A $\beta$  in research volunteers over a 36-hour period, researchers have found a way to determine in real time how much A $\beta$  is generated and cleared per hour. In a first study in healthy people, about 7.6 percent of total CSF A $\beta$  turned out to have been newly made every hour, and 8.3 percent degraded (Bateman et al., 2006). This makes it possible to ask whether FAD mutation carriers indeed overproduce A $\beta$ , whether individuals with late-onset AD have a problem with A $\beta$  production or clearance, how relative concentrations of A $\beta$ 40 and 42 change prior to clinical disease, and how CSF A $\beta$  in humans responds to anti-amyloid therapies.
- The question of how A $\beta$  causes toxicity has generated intense interest in synaptic biology. One study found that local overproduction of A $\beta$  in hippocampal slices decreases the number of GluR2 subunits of AMPA-type glutamate receptors in the postsynaptic membrane, and that the internalization of these receptors leads to a reduction in dendritic spine number (Hsieh et al., 2006). Another study similarly found that A $\beta$  oligomers added to slices decrease both the response of glutamate receptors to glutamate and the density of dendritic spines (Shankar et al., 2007). Studies largely concur that A $\beta$  oligomers induce a state similar to long-term depression at the post-synapse.

- Analysis of how the nervous system loses function in mouse models of amyloid buildup is broadening the importance of the tau protein. A recent study showed that halving the amount of tau protein in APP-overexpressing lines protected the mice from known learning and memory deficits. Tau reduction changed neither the amount of A $\beta$  nor neuritic dystrophy around amyloid plaques, but it did prevent important functional deficits. This implies that tau somehow mediates the soluble amyloid toxicity that impairs brain function; neuronal dysfunction induced by excitotoxins also required the presence of tau (Roberson et al., 2007). An emerging notion from this work is that tau might disturb cortical and hippocampal networks in AD. A follow-up study showed that APP- overexpressing mice have non-convulsant seizures, as do some AD patients, and that removing tau eliminates these seizures (Palop et al., 2007; see ARF news, Q&A, commentary). Mechanisms remain to be explored. Possibilities include a new function of tau, or effects secondary to its role in axonal transport. Network dysfunction in AD, as opposed to neuronal loss, is a budding research area that implies new treatment options.
- Amyloid imaging is able to visualize the buildup of amyloid in the brain of still-healthy carriers of FAD mutations years before AD symptoms are expected to set in (Klunk et al., 2007). Ongoing PIB imaging studies in community-based aging cohorts and in people with MCI are geared toward documenting and quantifying amyloid more broadly throughout the prodromal phase of AD, and exploring its usefulness for differential diagnosis. Amyloid imaging in mouse models has recently become possible, as well. (For news and commentary, see Maeda et al., 2007; Pike et al., 2007; Villemagne, 2007; ARF related news story; Johnson et al., 2007.)
- Finally, some experimental treatments have reached phase 3 trials. This includes Alzhemed (first phase 3 trial has failed), Flurizan (phase 3 completed enrollment), AAB-001 antibody, and  $\gamma$ -secretase inhibitor LY450139 (phase 3 trials in planning). An ADCS phase 3 trial of IvIg, an off-the-shelf pooled antibody preparation used for various immunological conditions, is also in planning.
- No company-sponsored novel tau-based compounds are known to have entered clinical trials. A recent academic animal study has focused attention on a widely used immunosuppressant drug, FK506, as a possible candidate for suppressing early pathogenesis and inflammation in tauopathies including Pick's, FTDP-17, but also perhaps AD. FK506 prevented microglial activation and subsequent tau pathology in a tauopathy mouse model (Yoshiyama et al., 2007). Plans are underway for small-scale preventive testing in carriers of tauopathy mutations. Ensuing discussion of trial failures at the workshop reinforced the point that experimental drugs often are given too late in the disease to be able to have a detectable effect.

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## **Part 2: ApoE**

Apolipoprotein E (ApoE) was first identified in the 1970s as playing a role in regulating plasma cholesterol levels, and in genetic hyperlipoproteinemias. ApoE is produced in the CNS, where it may be involved in the process of repair following neuronal injury. In 1993, its gene became a focus of AD genetics with the discovery of the increased risk borne by ApoE4 carriers and decreased risk found in ApoE2

carriers. Fourteen years later, hundreds of genetic studies have established incontrovertibly that this 34 kDa glycoprotein has by far the strongest impact of all genetic risk factors for LOAD and for cerebral amyloid angiopathy (CAA) known to date. ApoE4 drives down age of onset of AD, acting most strongly in a person's fifties and sixties. Two E4 alleles increase risk 10-fold in men, 12-fold in women. ApoE remains understudied, in part because its complex association with lipids make its brain biochemistry challenging. ApoE is probably not directly related to APP or its processing machinery, so isoform-specific differences in its physiological function may hold independent clues to how ApoE changes neuronal function to lead to AD.

Previous research has focused on ApoE's interaction with the A $\beta$  peptide, coining the term "pathological chaperone." It is established that ApoE affects assembly, clearance, deposition, and toxicity of A $\beta$ , but no consensus mechanisms have emerged. Overall, human ApoE4 may be less efficient than E2 and E3 at clearing secreted A $\beta$  from extracellular spaces and preventing its adverse effects on synapses, and at the same time it more strongly facilitates A $\beta$  deposition. It is unclear which forms of A $\beta$  ApoE modulates in human brain, but it is clear that different human ApoE isoforms somehow regulate A $\beta$  conformation differently, (see ARF related news story; Holtzman et al., 2000).

This report below focuses on new research since then. Some groups examine how ApoE relates to the best-characterized biomarkers known to date as a function of age, which remains the strongest overall risk factor for LOAD. Basic science opportunities have opened up around ApoE receptors and lipidation, and some established lines of investigation are ready for translational efforts.

### **ApoE Interaction With Biomarkers**

ApoE genotype appears to affect age-related changes in some CSF markers such that people who carry an ApoE4 allele are more likely to develop a pathogenic marker profile that predicts AD. For example, CSF A $\beta$ 42 and tau/phospho-tau are candidate predictive markers for AD. Meta-analysis has established that CSF A $\beta$ 42 levels are reduced by about half in AD, and a less marked drop occurs already in amnesic MCI/incipient AD. Total tau and p-tau levels in CSF increase two- to threefold in AD. A high tau/A $\beta$ 42 ratio is widely thought to put a person at high risk of developing AD within the next 3 years. Adding ApoE to this background, a cross-sectional study of 184 community volunteers ranging from 21 to 88 years of age who were cognitively normal at enrollment suggests that ApoE influences age-related changes in CSF A $\beta$ 42, though not A $\beta$ 40 or tau. In people without an ApoE4 allele, CSF A $\beta$ 42 levels rose slightly until midlife and then edged down in old age. By contrast, people with an E4 allele had a much steeper decline after age 60 (Peskind et al., 2006). Among ApoE4 carriers, men and women had different trajectories. Men carrying an E4 allele had a slow linear decline in CSF A $\beta$ 42 starting in their twenties, while women had an increase until their mid-fifties and then a sharp drop. These time lines mirror age-related changes in testosterone in men and estradiol in women. Furthermore, the ratio of tau/A $\beta$ 42 was similar for all participants until age 60. After that, it became highly variable. In some people the ratio stayed stable, but in ApoE4 carriers it tended to become high enough to predict AD, and some converted during the follow-up period (Li et al., 2007; also Fagan et al., 2007). This suggests that ApoE4 accelerates pathogenic A $\beta$  deposition starting already in mid-life. The mechanistic underpinning of interactions between ApoE4, gonadal hormones, and A $\beta$ 42 remains unknown, but

these data in normal people imply that primary prevention efforts should begin before age 50.

### **ApoE Receptors**

Both ApoE and A $\beta$  occur in different conformations, and the particular conformation determines their activity. ApoE subserves numerous functions in the brain, yet isoform-specific and AD-related functions of ApoE4 in human brain remain elusive. In brain, ApoE occurs as lipoprotein particles released by astrocytes. The neuronal and glial surface receptors for these particles include members of the LDL receptor gene family, such as LRP, VLDLR, LDLR, ApoER2, MEGF7, and others. The functional conformation of ApoE receptors in brain cannot be assumed from their conformation on peripheral cells, nor from overexpression by clonal cell lines. Both the conformation of ApoE and the context and conformation of the CNS receptors are thought to produce unique affinity profiles that are not understood. Tools needed in this area include ApoE receptor antibodies that distinguish one receptor family member from another, or that detect conformational differences within a specific receptor.

The receptors mediate ApoE signaling, and some also mediate its endocytic uptake. Both ApoE signaling and uptake require more study. One current hypothesis for how ApoE and A $\beta$ 42 interact focuses on isoform-specific effects of ApoE receptor-mediated metabolism of ApoE and A $\beta$ . Studies using a co-culture system of neurons overlaid on glial cells that secrete human knock-in ApoE isoforms have suggested that ApoE4 and A $\beta$  oligomers act synergistically to exert neurotoxicity (Manelli et al., 2006). Further experiments since then suggest that ApoE receptors mediate this toxicity. One hypothesis ripe for investigation is that ApoE4 influences intraneuronal trafficking of oligomeric forms of A $\beta$ , leading to its accumulation in vesicular compartments and toxicity, whereas ApoE2 and 3 preferentially facilitate lysosomal degradation of A $\beta$ . On a broader note, the group agreed that research on A $\beta$  oligomers, on this and other questions, would benefit from a clarification of the nomenclature and various preparations used in the literature (see commentary).

In evolution, LDL receptors developed about a billion years ago, whereas ApoE arose some 600 million years later, suggesting that the ligand evolved separately and made use of an existing set of endocytosing receptors to get its lipid cargo (such as cholesterol and other lipids) into neurons. For their part, neuronal ApoE receptors serve the function of importing glia-secreted lipoprotein particles and relieving neurons from having to synthesize membrane components themselves. But importantly, the ApoE receptors also have other functions that evolved prior to ApoE transport, for example, signaling to the nucleus and to synaptic proteins. One hypothesis holds that ApoE might interfere with pathways normally routed through ApoE receptors by their cognate receptors, and in this way contribute to AD pathogenesis.

Mechanisms remain largely unknown, but fundamental knowledge about ApoE receptor signaling in neuronal function is growing. For example, the ApoE receptors VLDLR and ApoER2 regulate binding and signaling of their cognate ligand reelin. Mice lacking these receptors have neurodevelopmental effects, and humans with mutations in VLDLR suffer mental retardation (Boycott et al., 2005). ApoER2 is present at the postsynaptic density, where it associates with NMDA receptor

complexes. Specifically, a particular splice form of ApoER2 in mice is able to enhance LTP and is needed for learning and memory. Activity appears to regulate the requisite ApoER2 splicing. Reelin, which is secreted by interneurons in adult neocortex and hippocampus, clusters VLDLR and ApoER2 receptors and induces src kinase signaling. This signaling leads to a PSD-95-mediated phosphorylation of NMDA receptor subunits and, in a separate pathway, also to inhibition of the tau kinase GSK3 $\beta$  (Beffert et al., 2005). Furthermore, the same ApoE receptors that are needed for LTP are also important for survival of cortical neurons in adult brain (Beffert et al., 2006).

One hypothesis worth testing is that ApoE might interfere with reelin's effect on the NMDA receptor. Direct tie-ins with AD pathways do not exist yet, but regulation of NMDA receptor subunits is becoming a point of convergence. Separate research has shown that A $\beta$  dampens the excitability of excitatory synapses by downregulating NMDA receptor components, leading to an LTD-like state. Opposing that, ApoE tends to tune up the excitability of the same neurons by signaling through ApoE receptors and PSD-95 to NMDA receptors. In the same vein, A $\beta$  tends to downregulate dendritic spines, whereas ApoE upregulates them. Rigorous isoform-specific effects, or relationships among A $\beta$ , reelin, and its competitive ligand ApoE, in the same model system of neuronal excitability have not been found yet. However, it is known that hAPP-transgenic mice contain fewer than normal reelin-positive pyramidal neurons in their entorhinal cortex (Chin et al., 2007). The study of ApoE receptors in synaptic plasticity and neuronal survival offers opportunities for understanding how ApoE influences AD risk with approaches outside of the classic AD pathologies. Note, however, that since the Bar Harbor conference was held, a study has linked APP processing to ApoE receptors by reporting evidence that the intracellular APP stub AICD suppresses LRP1 transcription and indirectly affects ApoE and cholesterol levels (Liu et al., 2007).

### **ApoE Lipidation**

ApoE occurs in the body not as a free protein but packaged with cholesterol and phospholipids in HDL-like particles. In extracellular spaces, ApoE particles influence the transport, clearance, and conformation of A $\beta$  in ways still unknown. Therefore, understanding the protein's lipidation may yield clues to AD pathogenesis and therapeutic approaches. Research into whether the composition of these lipoprotein particles affects AD pathology is beginning, but the field needs more exploration.

Cardiovascular research has shown that lipidation of HDL occurs in part through the transmembrane protein ABCA1, which transports cholesterol and phospholipids onto nascent apolipoprotein particles. ABCA1 protein occurs in the cell membrane of CNS neurons and glia, and the gene has come up in genetic association studies of AD risk (see Alzgene). Crosses of human APP-transgenic mouse strains with ABCA1 knockout mice indeed have low ApoE and low HDL levels in blood, CSF, and brain. Analysis of these mice showed that when ABCA1 does not lipidate astrocyte-secreted ApoE normally, the HDL particles are rapidly degraded, suggesting that ABCA1 affects HDL levels in the central nervous system (see ARF related news story). But surprisingly, subsequent crosses of different lines of APP-transgenic mice to ABCA1 knockout mice showed more severe amyloid pathology in the brain parenchyma and blood vessels than did the APP transgenics alone. This would suggest that poorly lipidated HDL particles are fibrillogenic (see ARF related news story). By that logic,

elevated ABCA1 expression would be predicted to increase ApoE lipidation and decrease A $\beta$  deposition, and ongoing research suggests this is indeed the case. In PDAPP mice overexpressing ABCA1, ApoE-containing HDL particles in the CNS are larger and contain more lipid, and the mice have fewer fibrillar amyloid deposits in cortex and hippocampus. This work indicates that the lipidation of ApoE influences where and in which conformation A $\beta$  aggregates. This area of research has stimulated translational interest in agonists of the transcription factor LXR. These agonists can induce ABCA1, but at present remain unsuitable for chronic treatment in humans (see Koldamova and Lefterov, 2007; Cao et al., 2007; Riddell et al., 2007; Lefterov et al., 2007; Narlawar et al., 2007).

Next, this line of investigation could ask whether, and how, changes in ABCA1 levels influence ApoE clearance in CNS or A $\beta$  metabolism. Mouse studies could assess whether ABCA1 influences A $\beta$ -related learning and memory abnormalities. Mechanistic studies could tackle how ABCA1 mediates ApoE lipidation and how that changes ApoE-A $\beta$  interactions. Whether ABCA1 affects the size of lipoprotein particles differently depending on if astrocytes express ApoE2, 3, or 4 is also unknown.

Lipid metabolism can become disturbed with aging. For example, it is possible that physiologically regulated molecules such as ABCA1 and ApoE shift the brain's lipid environment with age. Mature amyloid fibrils are increasingly viewed as a less toxic form of A $\beta$  aggregate than soluble oligomers. Rather than being stable, plaques are increasingly thought to have an "off-rate," whereby a certain fraction of fibrils dissolve to release oligomers into the surrounding neuropil. Since hydrophobic forces drive A $\beta$  aggregation, the question arises if elevated brain triglycerides might alter the equilibrium between plaques and oligomers/protofibrils. Biochemical experiments published this month in an open-access article suggest that adding lipids to mature A $\beta$  fibrils releases "reverse oligomers" that are much more toxic to cultured neurons than are the mature fibrils. Different types of lipid can achieve this effect, and they do so with different kinds of amyloid made of A $\beta$ , prion, or tau proteins. Typically, the lipids release oligomers of the A11 conformation (Kayed et al., 2003). Furthermore, stereotactic injection suggests that these "reverse oligomers" are toxic in vivo and impair memory in mice. If this initial data is replicated and expanded, it would suggest that plaques can turn into sources of highly toxic oligomers when their lipid environment changes, perhaps as a function of age. It would suggest that plaques are not inert end stages of pathology but can act as reservoirs of toxicity (Martins et al., 2007).

### **Other ApoE Mechanisms**

A long-standing debate in the field revolves around the question of whether human neurons crank up production of ApoE in response to injury or in the course of aging. Many investigators have found that ApoE expression is largely exclusive to astrocytes, but a knock-in model has reported ApoE expression in hippocampal neurons after injury with kainic acid (e.g., Xu et al., 2006). Related research has shown that fragmentation of ApoE occurs in transgenic mice expressing ApoE in neurons, and human AD brain contains ApoE fragments in an isoform- and region-specific manner, as well. Cell culture studies indicate that the fragments impair the cytoskeleton and mitochondrial function. The enzyme responsible for this ApoE cleavage is thought to be a serine-like protease with chymotrypsin-like properties. The

protease has so far eluded research efforts to identify it. Proteases are particularly amenable to drug development, and the relevance and site of action of the ApoE-cleaving activity will become clear once it is found (for detailed coverage, see ARF conference report. The relative contributions to neuronal toxicity of oligomeric A $\beta$  and ApoE fragments are unclear. There was consensus that AD may well develop for different reasons in different people. A person with two ApoE4 alleles, especially if they sustained repeated mild concussions in contact sports or accidents, may develop AD partly due to ApoE toxicity, whereas a person with a presenilin mutation will develop AD primarily as a consequence of A $\beta$  overproduction.

### **Translational ApoE Research**

Given ApoE's overriding genetic influence on AD risk and onset, much more effort is warranted in bridging the gap between basic research and the clinic (Refolo and Fillit, 2004). Besides LXR agonists (see above), therapeutic approaches under study in academia and industry include the following:

- A research program at Gladstone Institute at the University of California, San Francisco, has tested small molecules for their ability to disrupt an intramolecular interaction between structural domains in ApoE4 alone, which results from a single amino acid difference between ApoE3 and E4 at residue 112 (Mahley et al., 2006). Such "structure correctors" would make the ApoE4 protein E3-like in its biochemical interactions with protein and lipid binding partners. The institute last November created the Gladstone Center for Translational Research. The center will pursue this and other ApoE-related therapeutic targets in a collaboration with the pharmaceutical company Merck.
- A modified A $\beta$ 12-28 peptide has been shown to abolish ApoE4's promotion of A $\beta$  fibril formation by virtue of blocking interaction between ApoE4 and A $\beta$  (Sadowski et al., 2006).
- The wide-open field of ApoE receptor signaling and endocytic pathways may yield therapeutic approaches in the future. ApoE receptors are specific for ligands, and drugs could interfere at this level to block particular responses. For example, ApoE mimetic peptides are under study for their neuroprotective and anti-inflammatory effects in brain injury models (Laskowitz et al., 2007).

In concluding the ApoE sessions, the workshop group came to consensus that ApoE exerts multiple different effects. An effect through A $\beta$  is without question, but a separate effect on neurobiology that is distinct from A $\beta$  likely occurs, as well.

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### **Part 3: Protein Folding and Degradation**

In Alzheimer's and related neurodegenerative diseases, misfolded proteins that normally are degraded instead accumulate and form aggregates. Therefore, understanding how neurons selectively destroy misfolded proteins can offer insight into the pathophysiology of AD. This section of the Bar Harbor 2007 report summarizes recent progress and points out major gaps in present-day understanding of how defects in protein folding and degradation might contribute to neurodegeneration.

Several different sensing and degradation systems for misfolded proteins exist in the human brain. Most are not well characterized, and few are known to respond strongly to an accumulation of toxic oligomeric proteins in neurodegenerative diseases. The ubiquitin proteasome system is one such system (see more detail below). It generally is not functioning as an effective protective response in these diseases, even though mutant forms of tau protein can become ubiquitinated and directed toward the proteasome in models of tauopathy. Numerous serum proteases degrade extracellular A $\beta$ , but only the UPA-TPA system is induced by its oligomeric forms and can degrade it; other serum proteases clear primarily monomeric A $\beta$ . Inside neurons, the ER-associated system of chaperones and proteasome-targeting factors (ERAD) does become activated in AD, as does the autophagy/lysosomal system. On the other hand, cytosolic chaperones that are part of the heat shock response do not. Additional intracellular proteolytic systems exist whose normal function and possible role in neurodegeneration are poorly understood. They include the calpains, the caspases, and cytosolic peptidases that work downstream of the proteasome. As a general rule, cytosolic and nuclear degradation systems are insufficiently upregulated in human neurodegenerative disease.

Another general theme emerged at the workshop. It is becoming accepted that a given protein does not exist merely in one structure, the native state, but assumes many different structures, some of which have biological functions and different interactions with degradation systems. As a rule, partly folded structures tend to penetrate membranes and unfolded structures get degraded. Biological processes regulate those states. There are equilibria between them, but the rate constants and fundamental kinetics and thermodynamics of the transitions are unknown. It is known that small changes in concentration of a given state can profoundly change its solution state and deposition. Some workshop participants felt that the field had accumulated enough data to begin quantitative modeling of the transitions.

Basic biochemistry research, particularly in bacteria, has built a body of knowledge in the past 30 years that established rapid degradation as the cell's means to remove abnormal proteins of different sorts, including incomplete proteins, proteins resulting from missense mutations, free subunits of multimeric complexes, and oxidatively damaged proteins. Much of this degradation occurs in the ubiquitin proteasome, a soluble proteolytic system whose discovery and basic biology won the 2004 Nobel Prize in Chemistry. The proteasome differs fundamentally from other cellular proteases in that it depends on ATP for its function. Many neurodegenerative diseases feature pathologic inclusions of abnormal proteins with covalently linked ubiquitin, and even proteasomes associated with them. Ubiquitination of a target protein to mark it for degradation in the proteasome requires the sequential action of four types of enzyme, E1 through E4. Specificity arises partly from ubiquitin-carrying E2 proteins, but more so from the great variety of E3 ubiquitin ligases. (CHIP is an E3 of note in AD—it targets misfolded proteins attached to molecular chaperones, presumably as a fallback option after proper folding by CHIP has failed.)

The core 26S proteasome comprises a 20S core cylinder with dense walls that allow no lateral entry of ubiquitinated protein, and a less well-understood 19S component. Inside the cylinder, three different active sites (chymotrypsin-like, trypsin-like, caspase-like) cleave target proteins into peptides two to 24 amino acids long. Beyond their known role in antigen presentation, these scraps are understudied and may be

important to neurodegenerative disease. The 19S complex consists of six ATPases and controls entry into the core cylinder. The ATPases may hold clues to neurodegenerative diseases, and their characterization in mammals requires much more study. This much is known, partly from research of homologous structures in *Archae bacteria*: the ATPases restrict entry of ubiquitinated target proteins into the 20S core. They act both to open the gate of the proteasome so the ubiquitin conjugate can bind, and to unfold the marked protein so it can be injected into the core. In this sense, the 19S complex is a chaperone. The ATPases form a ring on top of the proteasome, and their C-termini can align to fit into proteasome pockets to allow the gate to open. A conserved tyrosine residue is needed for this key-in-lock mechanism (Smith et al., 2007). The structural determinants and dynamics of this interaction should be studied further to explore the therapeutic potential of modulating gate-opening with small molecules designed to enhance protein degradation in neurodegenerative disease (Horwitz et al., 2007).

On the flip side, proteasome inhibitors are approved for the treatment of various forms of cancer. Some 50,000 cancer patients have undergone treatment with bortezomib to date, the first in this new class of drugs. The development of this drug was accompanied by questions about potential risks of neurodegenerative protein accumulation. This drug generally does not enter the brain, but one side effect that limits its application is a peripheral neuropathy with characteristic protein inclusions. Its effectiveness against cancer rests the twin findings that cancer cells are dependent on NFkB, whose activation requires proteasome degradation of Ikb, and that cancer cells produce large numbers of misfolded proteins, which activate the unfolded protein response and cell death when the proteasome fails to degrade them.

Various hypotheses link proteasome dysfunction to neurodegenerative disease. They invoke, for example, binding of the proteasome to inclusions, rapid aggregation prior to degradation, or stopping up of the proteasome. None have been conclusively proven to date. One separate line of investigation has pointed to a new player in neurodegeneration. Surprisingly, the three active sites inside eukaryotic proteasomes are unable to cleave either normal or pathologically expanded polyQ sequences. The proteasome degrades most of a polyQ-containing protein; however, it ejects the polyQ tract undigested back into the cytosol, where those polyQ peptides either aggregate or become subject to degradation by a cytoplasmic enzyme. New research suggests that among all cytoplasmic proteases, only one is able to digest polyQ fragments. It is puromycin-sensitive aminopeptidase (PSA), a largely obscure enzyme (Bhutani et al., 2007). PSA is upregulated in polyQ disease and has drawn attention for its expression pattern in human brain areas resistant to tauopathy and for its ability to degrade tau and protect against neurodegeneration in a fly model of tauopathy (see ARF related news story; Sengupta et al., 2006; Karsten et al., 2006). Typically, fragments leaving the proteasome are destroyed within seconds. By contrast, PSA is a slow and inefficient way of degrading aggregation-prone or potentially toxic proteins. (Cathepsins in the lysosomal/autophagy pathway are able to degrade polyQ fragments, as well.)

This meeting session continued a prior discussion of protein misfolding in neurodegenerative disease. Besides Alzheimer's, some 20 diseases of protein deposition are known, each of them characterized by a given protein that forms cross- $\beta$  fibrils and other associated pathogenic intermediates. They are unrelated by

sequence or native fold, raising the question of what it is that makes this particular set of proteins aggregate and cause disease. Recent research into the characteristics of aggregation showed that many more than those 20 disease proteins form amyloid fibrils as a default structure if given enough time. Indeed, the amyloid structure was found to be a generic polymer that is determined by the intrinsic properties of the polypeptide backbone. By contrast, the highly varied native structure of proteins is determined by the primary sequence and specific packing of amino acid side chains under regulated conditions (Fandrich and Dobson, 2002; Auer and Dobson, in press).

Like the ability to form fibrils, the toxicity of amyloid aggregates is also a generic feature. Amyloid aggregates from proteins that do not cause human disease, e.g., SH3 domains, can penetrate cells and cause similar toxicity as seen with known disease proteins. Furthermore, early aggregates, i.e., oligomers, of such generic proteins show the highest toxicity of the range of different species generated during aggregation, suggesting that oligomeric toxicity, too, is intrinsic (Baglioni et al., 2006). The reason why early forms of non-disease protein aggregates are most toxic is under intense investigation, but appears to lie partly with an increased exposure of hydrophobic surface while oligomers grow and convert to structures with a cross- $\beta$  core (Cheon et al., 2007).

By contrast, while the ability of proteins to form fibrils is generic, their relative propensity to do so is not. Propensity varies dramatically with thermodynamic and kinetic parameters of a given context. In vitro, algorithms exist that can predict the intrinsic aggregation propensity of a given protein based on physicochemical principles such as charge and hydrophobicity (Chiti et al., 2003; Pawar et al., 2005). In vivo, the interconversions between different states of a protein underlie active control by molecular chaperones working in concert with quality control and degradation mechanisms. One hypothesis holds that when these protections fail, the proteins causing amyloid diseases revert to the stable, generic amyloid fibril and in the process generate a variety of toxic species.

A recent finding in this regard is that in vivo, proteins tend to occur in the cell at concentrations that put them close to the limit of their solubility. This arose from experiments plotting in-vitro aggregation rates of human proteins against their in-vivo concentrations derived from mRNA levels measured in tissue, which fell onto a near-perfect correlation of 0.97. This implies that even a subtle increase in concentration could drive up a given protein's propensity to aggregate. In this way, small changes in protein concentration, in aggregation propensity, in quality control, or environmental factors could combine with longevity to cause disease (Tartaglia et al., 2007). This hypothesis is currently undergoing testing in fruit flies that express different forms of A $\beta$ 42 in brain and show amyloid deposition, movement deficits, and curtailed lifespan. Prior in-vitro work enables prediction of how specific mutations will affect the physicochemical properties and hence the intrinsic aggregation propensity of the resulting A $\beta$  peptide. Mutations designed to either slow or speed up the aggregation rate generated fly strains whose histology, movement deficit, and survival improved or worsened accordingly. Even small changes in aggregation rate affected the flies' survival drastically, and a given mutation's propensity to form oligomers along the way correlated most strongly with survival (Luheshi et al., 2007). This system could be used for drug screening, as the ability to generate and test large numbers of flies renders statistically robust data.

Broadening research beyond A $\beta$ , approaches at the systems biology level hold promise in addressing how the cell detects and responds to misfolded proteins globally. Systems biology can ask how protein homeostasis changes with age, and how it responds to the chronic stress of having a pathogenic, aggregation-prone species in the mix. These include proteins implicated in neurodegenerative diseases as well as a larger group of some 200 known diseases of protein conformation. There is consensus around the notion that these proteins adopt multiple states, some of which are toxic, and that the toxic states over time impair common pathways of cell function—folding, translocation between compartments, nuclear import-export, gene expression, etc. That is why studies trying to identify the toxicity mechanism of a given neurodegenerative disease protein have implicated virtually all aspects of cell function without converging around any one proposed mechanism. It is also widely agreed that cells have evolved the unfolded protein response (UPR) and the heat shock response to cope with the flow of aberrant proteins through the ER and cytoplasm, respectively. One open question in this area is why neurons in these diseases tend to activate the former but not the latter to remove toxic proteins from the cytoplasm.

Systems biology approaches to address these questions are feasible using genetics in model organisms. For example, recent studies of polyglutamine repeat disease in the *C. elegans* worm model have pointed to a group of some 350 genes that together form a "protein quality control proteome." These genes can either enhance or suppress polyQ aggregation toxicity, and SOD1 aggregation toxicity, through effects on global mechanisms of protein homeostasis, or proteostasis. The current hypothesis holds that these genes make up a network of chaperones and clearance machines that maintain proteostasis. During normal biology, they support proper folding of polymorphic, mild folding variants, and clear misfolded forms. But the chronic presence of a mutant, highly aggregation-prone protein overwhelms the capacity of the proteostasis network such that even mild folding variants end up misfolding. This would lead not only to sequestration and loss of function of a variety of proteins from essential cellular processes, but also to toxic gains of function from the misfolded and accumulating species (Morimoto, 2006).

Supporting data for this hypothesis have come from studies using temperature-sensitive (ts) mutants as "folding sensors" to monitor protein homeostasis in *C. elegans*. Ts mutations are essentially mild folding mutations; prior developmental biology research has made many well-characterized examples available for study. The function, or absence of function, of a given ts protein reflects a fragile balance between its folding and degradation, making ts proteins suitable reporters of the global folding environment in the cell. Experiments coexpressing a given ts mutation with a polyQ protein at the threshold length of causing symptoms in human disease (i.e., Q40) showed that the presence of Q40 tracts abolished function of the ts mutant protein at the normally permissible temperature and killed the worms. Vice versa, the added presence of a ts mutation also rendered the Q40 protein more toxic. Ts mutations of many different proteins behaved in this way, showing that the enhanced misfolding is specific neither to cell type nor protein. In essence, the polyQ protein exposed the folding vulnerability of the ts protein, suggesting that it is competing for other proteins that are essential to maintain proteostasis. The presence of Q40 in the cell pushed the cell against the limit of its proteostasis capacity, presumably

exhausting its ability to absorb additional misfolded proteins. Otherwise a single ts mutation could not markedly enhance polyQ toxicity (Gidalevitz et al., 2006).

The components and modifiers of a proteostatic network remain to be fully elucidated. One component is known modifiers of lifespan, i.e., genes in the insulin signaling pathway, such as *daf-16*. In genetic experiments, they both enhance proteostasis and suppress polyQ aggregation toxicity. (Insulin signaling is increasingly being implicated in AD, as well.) Heat shock factor-1, the upstream regulator of chaperones, is essential for this effect of the insulin signaling pathway (Morley et al., 2002; Morley et al., 2004; Hsu et al., 2003). Taken together, data from several labs suggest that Hsf-1, i.e., the classic stress response, does not merely protect the cell from acute damage but is critical for day-to-day protein homeostasis.

By contrast, aging works in the opposite direction in *C. elegans*. The ts mutations alone, without coexpressed Q40, aggregate at an accelerating rate as the animal aged, illustrating an age-dependent collapse of proteostasis in a vulnerable system. Both Hsf-1 and *daf-16* slowed this aging phenomenon in genetic experiments. This suggests that aggregation-prone proteins lose function during aging because protein homeostasis fails. This research also suggests therapeutic interventions targeted to small-molecule enhancers of key proteostasis factors, such as Hsf-1, or more specific members of chaperone networks. A commonly proposed mechanism holds that age-related changes in mitochondria lower ATP levels sufficiently to starve proteostatic systems such as chaperones and degradative enzymes. However, this has not been shown in vivo in mammalian models. Moreover, mammalian neurons have significant reserve capacities and alternative sources for ATP generation. They can maintain fairly stable ATP levels even under prolonged fasting conditions. The interaction of A $\beta$ 42 with proteostasis and aging remains to be investigated.

Global changes in proteostasis notwithstanding, any given neurodegenerative disease is marked by a selective vulnerability of specific sets of neurons. Studying which normal functions of the disease protein a given cell type loses can give insight into the pathogenic process of the disease at hand. Recent progress on the polyglutamine disease spinocerebellar ataxia 1 (SCA1) is a case in point. This progressive, fatal, autosomal-dominant disease is caused by GAC repeats in the gene encoding ataxin-1, and Purkinje cells in the cerebellar cortex are primarily affected. While the disease features characteristic nuclear inclusions that are positive for ubiquitin, proteasome subunits, and chaperones, research is increasingly shifting from studying toxicity of misfolded aggregates to a newer focus on lost normal functions. Ataxin-1 is widely expressed throughout the brain, yet Purkinje cells degenerate selectively early on even though they are not even among the highest expressors of the gene. Series of mouse models have led to consensus that the disease is a consequence of the expanded protein, not the RNA, and that the large microscopic inclusions of misfolded ATXN1 play less of a role in causing the disease than does the normal function of ATXN1.

ATXN1 is a nuclear protein that interacts with transcription factors. Developmental studies yielded clues to their importance. They showed that transgenic mice developed much more severe disease phenotypes if the ATXN1 polyQ transgene began to be expressed during a specific window of 3 postnatal weeks, when Purkinje cells grow and mature their dendritic trees. If the Purkinje cells were allowed to develop normally during these 3 weeks without expressing the transgene yet, the mice

later on were protected from its effects. Subsequent microarray studies examining gene expression during this time window pointed to a selective loss of the ROR $\alpha$  gene in Purkinje cells in mutant ATXN1-182Q mice (Serra et al., 2006). ROR $\alpha$  forms part of a transcriptional regulation complex that also contains the protein TIP60. This protein interacts with ataxin-1 to destabilize the ROR $\alpha$  complex. Further study led to the hypothesis that ataxin-1 normally is part of a transcriptional complex at the promoter sites of various ROR $\alpha$ -mediated genes, and that the interaction of mutant ataxin-1 with TIP60 somehow leads to the eventual degradation of this complex so that the respective target genes cannot be expressed. ROR $\alpha$  is more highly and specifically expressed in Purkinje cells than is ataxin-1, and the loss of its function might explain part of the characteristic neuronal vulnerability in this disease. The idea is that loss of ROR $\alpha$ -mediated gene expression during a time of intense postnatal development stunts the growth of this class of neurons and makes them vulnerable to insults later in life.

This could be explored as a possible general principle in other neurodegenerative diseases, as well. The hypothesis holds that, more generally, alterations in folding due to expanded polyQ tracts or other aggregation-prone molecular characteristics can affect the ability of the protein to function normally, and these functional changes occur long before microscopic pathologies and symptoms show up. These changes can be developmental, leaving neurons prone to age-related insults later.

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#### **Part 4: New Vistas in Brain Imaging**

The technological limitations to studying Alzheimer disease remain major. For example, the ability to image changes in brain needs to move to a new level of analysis. In vivo-imaging, functional imaging, imaging of behaving animals, deep brain imaging, and 3D structural imaging of tissue are all priorities. On some of those, technical innovations in basic neuroscience have reached a point where they can now be imported into AD research. Below are three examples.

##### **Channelrhodopsin-Transgenic Mice**

The field of in-vivo imaging of neural activity and neural circuitry advanced with the development of transgenic mice expressing channelrhodopsin-2 (ChR2). This membrane protein conducts non-selective currents in response to illumination with blue light. Several mouse lines now exist that express ChR2 fused to yellow fluorescent protein (YFP) selectively in the brain, such that in a given transgenic line, individual neurons fluoresce in a particular cortical area, the retina, motor nerves, etc. Both in brain slices, and in anesthetized mice, illuminating a respective area of cortex evokes light-activated action potentials from the ChR2-expressing neurons. Light intensities as low as 1 milliwatt per square millimeter depolarize the ChR2-expressing neurons, and milliseconds later produce recordable currents from those neurons. The operator can control the frequency of the neurons' spike trains; that is, light pulses of up to 30 Hz produce corresponding spike trains of up to 30 Hz. Sustained illumination of up to several seconds generates sustained firing (Wang et al., 2007).

As a proof of principle that this technique is suitable to map circuits in the brain, an initial study applied it to trace the projections from mitral neurons in the olfactory bulb to the piriform cortex of the mouse brain, where olfactory stimuli undergo

higher-order processing. A line of transgenic mice expressing ChR2 only in mitral neurons served this purpose. Illuminating olfactory bulb patches of varying diameter while recording from piriform cortex suggested strongly convergent mitral inputs onto piriform cortex neurons, rather than one-to-one connections (Arenkiel et al., 2007; Feng lab page).

This technique is robust enough to be applied to questions of changes in neural activity and circuits in AD research. One ongoing technical refinement involves the use of specific promoters to target ChR2 expression to specific subtypes of neuron (i.e., GABAergic, glutamatergic, etc.). Another involves combining ChR2 with additional genetically encoded calcium- or voltage-sensitive fluorescent proteins to improve the functional readout of exactly what is going on in neurons when they are stimulated. A current limitation of this technique is that, as with multiphoton imaging of AD mouse models, the light has to be delivered through a cranial window. Another is that light scattering and attenuation place any tissue deeper than 600 micrometers from the surface beyond reach. One option to overcome this limitation is to stereotactically implant a pinpoint source of light into transgenic mice, essentially attempting deep brain stimulation using light.

### **Fluorescence Microendoscopy**

Besides seeing cellular dynamics in deeper layers, a second goal in brain imaging is to do so in freely moving animals, not sedated mice immobilized onto a microscope stage. Fluorescence microendoscopy uses minimally invasive micro-optic and fiberoptic probes from 1,000 down to 350 micrometers in diameter. The tiny objective lenses at their tips now exist as doublets or triplets and provide micron-scale lateral resolution. This technology can image capillary blood flow in the hippocampal CA1 region of mice (see prototype movie on Schnitzer lab page). It visualizes pyramidal neurons expressing YFP and could be applied to image transgenic mice of interest to AD research.

Two basic forms of microendoscopy are complementary to each other. Epifluorescence microendoscopy is similar to conventional epifluorescent microscopy, except it uses a long, thin probe to reach into the brain. The probe sends light into the brain and projects the image back onto a camera. It is simple and fast but prone to light scattering and does not generate true 3D images. In the two-photon laser-scanning form of microendoscopy, the focus of an ultrafast laser beam scans across the top face of a micro-optic probe, and the scan pattern is projected into the brain. The excited fluorescence passes back through the system and is captured by a photodetector that allows optical sectioning through 3D data stacks. This method allows deeper penetration into the brain, focal excitation, and is robust to scattering. Examples of high-quality imaging with this technology include pictures of the rows of outer and inner hair cells in the cochlea of live guinea pigs, excised human cochlea, as well as gentamycin-induced damage to those cells (Monfared et al., 2006).

More recent refinements to this technology have made possible long-term neuronal imaging in the same animal for up to a year. Guided tubes mounted on the heads of mice ensure that repeatedly inserted microendoscopy probes image the same hippocampal region, with the same 10 to 20 fluorescent neurons and dendrites in the visual field, day after day. As an initial experimental application, this technology has enabled the observation of tumor growth over time.

The long-standing goal of imaging the brain of freely moving rodents had an early breakthrough with a portable microscope mounted on the heads of rats (Helmchen et al., 2001), but this prototype is too heavy for mice. The wide availability of disease models makes mice the animal of choice for biomedical applications, therefore further engineering effort focused on miniaturizing all components. A mouse prototype weighing 3.9 grams and hanging slightly over the mouse's shoulders achieved a lateral resolution of 1.2 microns (Flusberg et al., 2005; see movie). Further micromachining and electrical engineering led to a second-generation microscope that has a smaller footprint, weighs 2.5 grams, as well as optical improvements (Piyawattanametha et al., 2006). This instrument took a month to assemble once all parts were made.

The most recent device weighs 1.8 grams and does not extend past the mouse's head. It features three micro-lenses with a lateral resolution of 0.9 micrometers, enough to see dendrites though not dendritic spines. This device enables imaging while the mouse carrying it walks about the cage. A calcium-sensitive indicator images spikes as the mouse moves. This is the first device that combines the concepts of chronic imaging and imaging of freely moving mammals. Motion artifacts so far have proven minimal.

In principle, this microscope can be used to introduce light to excite neurons in ChR2-expressing and similar transgenic mice, provided separate spectra can be used to excite the channels and the fluorophore. Quantum dots would become attractive sources of fluorescence once their targeted delivery could be worked out. For more on technologies to illuminate genetically targeted brain circuits, see Deisseroth et al., 2006. This freely downloadable review features a picture of the portable microscope.

### **Array Tomography**

A third imaging method discussed here has similarly completed proof of principle and is established enough to be applied to questions of AD research. It is currently undergoing technical refinement to make it cheaper, smaller, and more readily accessible to investigators either at their own respective institutions or through a service core. Array tomography is a structural imaging technique that combines advanced features of optical fluorescence and electron microscopy to render exquisitely detailed, high-resolution, 3D views of synapses in blocks of cortical tissue. Prototype movies are at Smithlab, but the method has been greatly enhanced since then.

With array tomography, the experimenter fixes a specimen—e.g., a piece of fresh human cortex excised during aneurysm surgery, or a piece of APP/PS-transgenic mouse brain—in acrylic resin and cuts it with a specially devised technique into bands of hundreds or thousands of consecutive serial sections 50 to 200 nm thick. Two innovations in these initial steps lie in automating the cutting of the series without losing any, and gathering them on coated glass microscope slides where they are amenable to repeated future manipulations and are generally much more robust than serial sections cut for conventional electron microscopy. Another innovation lies in optimizing the resin toward post-embedding staining with fluorescent antibodies, not immunogold as used in conventional TEM.

Next, hundreds of consecutive sections on a slide are labeled with antibodies, and then a fluorescent microscope is fully automated to take individual pictures of the

same view section after section. Software is available to align the images into data stacks that can either be analyzed quantitatively or visualized in 3D.

For example, array tomography resolved, and then counted, 280,000 individually visible synapses stained with synapsin-1 in the synaptic neuropil of a sample of mouse whisker barrel cortex. This tissue block represented 1/300th of the entire mouse whisker barrel structure, and the data was collected in 1 hour of fully automated imaging. The density of synapses rendered in this way corresponds to estimates made previously with unbiased stereologic counts. In an example relevant to AD, array tomography imaged within 2 days an amyloid plaque with a halo of missing synapses in a Tg2576 mouse. A previously generated multiphoton image of the same plaque had taken months to generate computationally (Skoch et al., 2005). Array tomography could be combined with multiphoton microscopy to assess in more detail how plaques affect nearby synaptic architecture as a function of time.

Array tomography offers two further advantages. First, antibody stripping and repeated application of different labeling antibodies work robustly, having been tested in nine cycles of antibody staining, imaging, and elution on the same band of sections (Micheva and Smith, 2007). This allows for multiplexing and detailed immunohistochemical characterization of a given cortical area without degrading the antigens in the sections. Second, after fluorescent imaging, the same sections can be imaged with other modalities, e.g., backscattered electron detector scanning electron microscopy (BSE-SEM). Voxel registration provides images of a given synapse with fluorescent microscopy side-by-side with an SEM image that approaches high-magnification transmission electron microscopy (TEM) in its resolution.

These two features open up the possibility of classifying individual synapses, i.e., by their neurotransmitter or postsynaptic receptor subunits. Used in transgenic mouse models, array tomography can classify which synapses are most susceptible to toxicity by A $\beta$ . It can also tackle more broadly the problem of differential sensitivity of neuronal populations to AD pathology in molecular ways. A given tissue block can be sequentially probed for the presence of GluR2, of GABA, ChAT, specific forms of hyperphosphorylated tau, etc., and rendered in a joint image.

In contrast to array tomography, optical sectioning with confocal imaging is widely available. It can approach similar questions to a rough approximation. However, poor penetration of antibodies into deep tissue, as well as incontrovertible physical limits imposed by the point spread function, both curtail its efficiency of imaging along the Z axis, i.e., into the depth of tissue. Physical sectioning, post-embedding staining, and automated microscopy and image processing open up a new level of analysis in tissue imaging.

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## Part 5: Research Priorities

- Study how ApoE4 enables conformational changes in A $\beta$  to occur.
- Characterize human ApoE4 effects independent of A $\beta$ .
- Explore what role ApoE receptors play in its metabolism.

- Study CSF lipoprotein particles in normal people, MCI, AD. How much A $\beta$  is free, how much is in ApoE particles, does this vary by isoform?
- Resolve whether there is an ApoE isoform-specific effect on learning and memory that makes E4 carriers more vulnerable to AD. This question is old, but human studies on learning and memory have not definitively answered it. ApoE genotype appears not to influence intellect in young adults, but FDG PET does show strikingly different activation patterns in adult ApoE4 carriers; age-related effects of cognitive maintenance are not known yet.
- Study whether ApoE plays a role in other dementias (e.g., FTD, PSP, CBD) that goes beyond an amyloid- $\beta$  component in the pathology of these diseases. At present, ApoE4 is thought to have a 20 percent effect on age of onset of PD, but besides that, little is known.
- Develop chemical probes that are selective for soluble A $\beta$  oligomers for use in imaging in normal brain, disease models, under treatment monitoring.
- Develop chemical probes of neuronal function, e.g., chemicals to image real-time neuronal activity, synaptic activity in different states, glial cell activity.
- Begin quantitative modeling of A $\beta$  aggregation. Build from known rate constants and published literature to work toward deriving mathematical equations for successive aggregation reactions.
- Quantify A $\beta$  in different compartments in vivo, under normal, disease, and treatment conditions. Deploy biological mathematics to create equations that capture the interdepartmental dynamics of A $\beta$ .
- Relate ongoing in-vitro research characterizing structure, stability, kinetics of A $\beta$  to in vivo quantification studies.
- Link quantitative A $\beta$  manipulation to therapeutic outcome. By how much can A $\beta$  be reduced safely with secretase modulator or inhibitor? Is that reduction enough to slow cognitive decline in the disease?
- Innovate clinical trial design. Bring biomarkers to bear, reduce trial duration, cost.
- Develop better voltage probes, better calcium probes. For example, the new probe GCaMP2 improves brightness, but not temporal resolution (Tallini et al., 2006; Diez-Garcia et al., 2007).
- Develop better ways of fixing lipid for microscopy. Osmium destroys the antigenicity of lipids. In general, better chemical fixatives that preserve antigenicity are needed.
- Expand study of lipid biology, lipid biomarkers in CNS and its diseases, lipidomics analysis.
- Determine extent of non-convulsive epileptic seizures in AD. Develop robust clinical measures for them.
- Integrate expanding knowledge of microRNA into study of neurodegeneration.
- Understand mechanisms behind lifestyle changes shown to protect against cognitive impairment.
- Study how proteasome in neurons differs from proteasome in other cells, especially in terms of its many associated proteins.
- Investigate feasibility of using drugs to keep proteasome gate open longer to facilitate degradation.
- Study role of puromycin-sensitive aminopeptidase in neurodegenerative diseases.
- Why is heat shock response not effective in neurodegenerative diseases?

- Explore generic protection by factors not specific to AD. For example, Hsf protects generically in models of cardiac disease, cancer, other diseases; PGC-1 raises mitochondria and free radical defenses; sirtuin induction appears to be neuroprotective. Can these pathways be harnessed for AD therapy?

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