

Enabling Technologies for Alzheimer Disease Research: Sixth Bar Harbor Workshop, 2006

By Gabrielle Strobel

This past August, Alzheimer disease researchers met with colleagues from other fields and with foundation and NIH representatives in Bar Harbor, Maine, at the sixth annual workshop on Enabling Technologies for Alzheimer Disease Research. The participants were charged with identifying knowledge gaps that hold back progress toward developing new therapeutic strategies, and they discussed opportunities for bridging these gaps. One session dealt with the influence of the immune system on synapses in brain development, animal models, and in aging and AD. The second session emphasized synaptic function in aging and AD; the third highlighted some high-throughput approaches that can be applied to problems of brain aging and neurodegenerative disease. The report below offers a summary of two days of presentations and discussions, followed by a list of recommendations the group compiled. Readers will find a broad description of how the big questions in AD research are changing, as well as plenty of ideas to update their own studies. Comments are always welcome.

Part 1. Alzheimer Disease, Aging, and the Immune System

Clearly, the functions of the immune system in the brain must be better understood. Open questions fall into three areas: 1) What normal roles do immune molecules play in the brain irrespective of inflammation or disease? 2) When immune cells become activated in the aging, degenerating brain, is that good or bad, and how so? 3) What challenges and opportunities does the immune system pose for AD immunotherapy?

Current interest in immune molecules in the brain focuses on the CNS synapse. Mechanisms of synapse maintenance are drawing increasing attention in neurodegeneration research. First, some words on the structure of the CNS synapse. Its protein scaffold is surprisingly stable and is beginning to be better understood in molecular terms. Adhesion molecules are anchored to the cytoskeleton beneath the presynaptic and postsynaptic membranes; they protrude into the synaptic cleft and hold both sides together by homophilic binding. A major class of synaptic adhesion molecules are cadherins, and the brain expresses all 80 known human forms of it. It is thought that the binding specificity of different cadherins helps specify neural connections. N-cadherin clamps excitatory CNS synapses together. At immature synapses it is aligned across the active zone and postsynaptic density, but in highly active mature synapses, it is arranged like the rim of a drum around a central cadherin-free core, which gives the appearance of being "perforated" as the synapse matures. In addition to being tissue glue, cadherins transduce signals and suppress tumors by holding cells in place. Cleavage of their prodomain activates cadherins for their adhesive function, and dysfunction of this process is implicated in metastasis. Prodomain cleavage may also play a role in synapse development; its role in synapse aging, repair, or loss, if any, is not known (Dustin and Colman, 2002).

The field of neuroimmunology is undergoing a shift as a growing list of proteins formerly thought of as strictly immunological is found to occur in the nervous system and to be required for brain function. The complement cascade represents one such set. Current research implicates the complement as a risk factor for age-related macular degeneration. More study is needed to explore their role in other forms of neurodegeneration, with a particular focus on glial cells.

The complement cascade removes infected cells and cellular debris in the periphery by lysing cells or by coating bacteria and waste materials to trigger phagocytosis by macrophages (opsonization). Complement deficiency leads to autoimmune disorders and impairs tissue regeneration; excessive activation leads to tissue damage. In neurodegeneration, the complement is activated but its precise role in AD remains unclear. New research indicates that the complement cascade might serve a fundamental role in brain development. Specifically, complement proteins would create a permissive window of time, in the early postnatal period, during which activity-dependent synaptic pruning in the CNS can occur to refine an approximate projection pattern established in utero. Astrocytes begin releasing the initiating complement protein, C1q, just before pruning begins, and stop producing C1q after it has ended. Both C1q and C3 knockout mice are impaired in desegregating initially overlapping projection patterns of visual neurons coming from either eye. It is unclear whether the complement merely helps remove synapses once they are defunct or plays a more causal role, and how that would relate to synaptic activity. Early AD features both synapse loss and reactive astrocytes, which are known to re-express C1q. Genetic complement defects in humans are known and could be examined for consequences on cognition.

Complement proteins do not appear to hold promise as a CSF biomarker, but they have been reported in the vicinity of amyloid plaques in AD brain tissue. Both A β and tau pathology are thought to activate complement. In genetic studies crossing either APP or P301L tau transgenic mice with complement-inhibited mice, complement deficiency increases amyloid deposition, but also reduces tau pathology and neuron number. Taken together, the new mouse genetics would suggest that the complement in AD brain clears amyloid through the microglial/macrophage opsonization function, and that it can also promote tau phosphorylation and eliminate synapses and neurons through mechanisms still unknown.

Scientists need to clarify how well current amyloidosis and AD mouse strains model the human brain with regard to its immunological component. Scientists are beginning to realize that while APP transgenic mice do upregulate and initiate the complement cascade, at least some strains fail to complete it. This raises the question of whether the absence of the complement's downstream effectors might explain why many APP/PS models do not show the neurodegeneration that marks AD. The role of the complement in immunotherapy remains unclear.

MHC class I proteins are a distinct family of immune proteins that are unexpectedly expressed in the brain. Well-known for their role as ubiquitous antigen-presenting proteins, they have also recently been found to shape synaptic plasticity. Contrary to better-known enhancers of synaptic plasticity, such as BDNF or CREB, MHC class I proteins are emerging as negative regulators of plasticity. The mouse brain during development and in adulthood expresses these proteins in specific patterns, particularly in regions undergoing activity-dependent synaptic plasticity and remodeling, and endogenous activity upregulates MHC class I proteins in vivo. They are required for normal activity-dependent remodeling of initial synaptic circuits, as well as for normal synaptic plasticity in the adult hippocampus. MHC class I proteins appear to influence synaptic physiology by limiting long-term potentiation and favoring long-term depression, which may in turn affect certain forms of learning and memory. These and other recent findings together weaken the old view of the brain as an immune-privileged organ that lacks MHC class I expression, to one where many

different MHC class I genes—and indeed other components of the immune system—are expressed and perform key neuronal functions, although their immune functions are actively suppressed.

In AD, what MHC I does is still speculative. Aging changes MHC class I levels such that they decrease in non-neural tissues, but may paradoxically increase in the brain. Due to the novel neuronal functions of MHC class I, this age-related increase in MHC class I could promote functional and anatomical synaptic weakening, loss of neuronal connectivity, and impaired learning and memory. Since MHC class I in the brain functions to limit plasticity, eventual therapeutic implications could be that drugs lifting this negative regulation might boost synaptic plasticity in remaining circuits. In addition, scientists know that activated T cells (whose receptors bind MHC class I complexes) enter the brain in great numbers, particularly where amyloid has damaged the blood-brain barrier. Activated T cells bind MHC class I, and can kill neurons *in vitro*. This raises the question of whether neuronal expression of MHC class I in specific brain regions, perhaps affected by age or changes in synaptic activity, could render these neurons selectively vulnerable to T cell-mediated damage. Like many proteins involved in AD, neural MHC class I proteins are upregulated after injury, indicating that a secondary wave of neuronal and/or immunological MHC class I-mediated synaptic weakening, remodeling, and damage could occur in regions undergoing active degeneration during later stages of AD.

The molecular details of how MHC class I signals in the brain are still being worked out. One putative neuronal MHC class I receptor has recently been identified (Syken et al., 2006). Part of the innate immune system, paired immunoglobulin-like receptor B (PIRB) binds MHC class I proteins on some neurons and restricts the extent of plasticity that is normally induced by changes in visual input. However, mice genetically deficient for PIRB fail to recapitulate the developmental remodeling defects seen in mice lacking MHC class I, indicating that MHC class I uses multiple binding partners to achieve its neuronal functions. Outside the brain, MHC class I binds dozens of receptors, and ongoing efforts are aimed at identifying additional binding partners for MHC class I in neurons.

Research opportunities in understanding the role of these proteins in AD include identification of the receptors that mediate the effects of MHC class I on normal developmental synaptic remodeling; characterization of expression of MHC class I in normal aging brain, AD patients, and APP/PS transgenic mice; characterization of LTP, LTD, synaptic remodeling, and learning and memory in MHC class I-deficient and overexpressing mice; and crossbreeding of AD mouse models onto MHC class I transgenic backgrounds. More broadly, MHC class I genes exist in many different alleles and are expressed in specific patterns in the brain, offering opportunities for studies in other instances of neurodegenerative or learning paradigms in which other brain regions are affected. Importantly, the peptides that different MHC class I proteins display in different brain areas and normal/disease states are completely unknown.

On the second question of what consequences immune cell activation has in the AD brain, the prevailing view that it is destructive is gradually giving way to a more nuanced realization that aspects of immune/glial cell activation can be protective. Evidence that the immune system contributes to AD pathogenesis includes complement activation, the apparent protection afforded by NSAIDs, cytokine

expression, activated microglia, and the presence of autoantibodies to A β , cholinergic neurons, and neurofibrillary tangles. Evidence for a therapeutic role exists in the immunotherapy results thus far. Different activation states of immune cells need to be characterized and understood functionally. For example, microglia can be activated to be phagocytic, but also to be neurotoxic. Understanding the differences will provide a basis for interfering therapeutically to elicit a helpful, safe immune response.

The relationship of microglial activation to plaque formation needs more study. Recent genetic data indicate that, in CRND8 APP transgenic mice, microglia require the adaptive immune system to clear amyloid. Without functioning T and B cells, microglia failed to become activated, and the mice's plaque and A β burden increased. Genetic ablation of natural killer cells of the innate immune system further increased A β load. This demonstrates a connection between T and B cells and CNS microglia, providing yet another indication that there is no firm blood-brain barrier to separate cellular immune responses. The finding that amyloid pathology increased without microglial activation would suggest that, in future immunotherapy approaches, an A β -specific Th2 response might still be necessary to promote microglial plaque clearance. Because the meningoencephalitis that halted Elan/Wyeth's AN1792 trial probably arose from A β -specific T cells, some current immunotherapy efforts try to avoid an A β -specific T cell response. More effort needs to focus on how to distinguish a microglial clearance function from an inflammatory one. Research also should address the question of whether microglia by themselves can have a role in synaptic function or memory.

The complications encountered in the AN1792 trial have created the need for safer vaccines. An active vaccine is viewed as less expensive and more likely to become widely available than a passive one. Efforts to nudge the immune system toward a humoral response but away from an A β -specific T cell response focus on finding the right combination of adjuvant, route of immunization, and the right immunogen. B cell and T cell epitopes on A β are largely known. Novel immunogens include, for example, a dendrimeric construct that displays 16 copies of A β 1-15 on a lysine tree (Seabrook et al., 2006), or constructs that place A β 1-15 on either side of RGD or lysine spacers (Maier et al., 2006). In general, these immunogens achieve higher antibody titers in mice than full-length A β , and stimulate T cells against the synthetic immunizing peptide but not endogenous A β . Intranasal immunization combined with *E. coli* enterotoxin-based adjuvants boosts the immune response in wild-type and APP-transgenic mice. Some of these novel immunogens reduce cerebral A β levels and pathology and improve spatial memory performance in the mice. They are being tested in Caribbean vervet monkeys, but none have entered clinical trials yet. Immunotherapy studies are limited by the lack of an AD-like mouse model that shows the peripheral T cell infiltration following immunization that occurred in the human trial; however, immunizing mice with both full-length A β and a combination of several adjuvants partially recapitulates this situation.

While second-generation active and passive immunotherapy approaches are being studied in phase 1 and 2 trials, questions about its mechanisms remain open. The human data show patchy clearance consistent with T cell activation of microglia and microglial phagocytosis, but mouse data on efficacy and A β clearance vary among models. Some mouse data support microglial phagocytosis and complement opsonization but others do not. It is also possible that antibodies neutralize toxic aggregates, disrupt toxic oligomers, inhibit their aggregation, or simply cap their

growth, and that multiple mechanisms are at play. According to the peripheral sink hypothesis, peripheral antibody binding would enhance A β efflux from the brain. This hypothesis has been complicated by recent data suggesting that A β binding to antibody in the blood stabilizes A β long past its normal half-life, and that peripheral antibody injections need to be given for extended periods of time before they begin to decrease brain A β levels. To address this issue, A β levels and turnover rates are beginning to be quantified in different body compartments, both in mice and humans.

A novel opportunity in immunotherapy research is emerging around microglia. Independent lines of investigation have reported that the copolymer glatiramer acetate activates microglia to generate an inflammatory state in which they clear amyloid pathology independently of antibodies (Frenkel et al., 2005; Butovsky et al., 2006). Efforts to separate clearance from the attendant inflammation led to intranasal administration of protollin, a proteasome-based mucosal adjuvant that has been tested in humans for shigella and other infections. Protollin alone, without glatiramer acetate, appears to reduce brain A β levels in APP-transgenic mice. Soluble and insoluble brain A β levels, as well as astrogliosis, decreased in a long-term nasal protollin prevention study. Different time courses of prevention and treatment suggest that protollin might boost a transient microglial activation that removes amyloid and then recedes. Of several proposed microglial amyloid clearance pathways, the one triggered by protollin appears to involve Toll-like receptors. The different possible activation profiles of microglia in human brain need to be characterized. Going forward, the challenge of any deliberate microglial activation lies in restraining it to avoid damage to the surrounding milieu (for a recent review, see Weiner and Frenkel, 2006).

Another novel approach toward generating high-affinity A β -binding agents might be to view A β aggregates as a thymus-independent type 2 antigen. These atypical antigens have a polymeric, repetitive structure and activate B cells to produce IgG and IgM antibodies without the help of T cells, which are typically needed to complete B cell activation and antibody production. Single-chain variable fragment (scFv) antibodies against amyloid intermediate structures can be generated using ordered, heterologous antigens that have no sequence homology to A β , and thus preclude autoimmune reactions against A β or APP.

In summary, participants agreed that immunotherapy in mice improves cognition, both through acute amyloid-independent effects and through slower effects secondary to amyloid removal. The questions have shifted from whether A β removal will have cognitive benefits to how oligomers relate to plaques in terms of affecting cognition, and whether oligomers are important in human AD. Mouse models differ importantly from AD. Mice develop a detectable cognitive impairment before plaques, and impairment reverses rapidly with passive immunization. Humans remain asymptomatic until after they have developed a nearly full load of plaques and become symptomatic only once neurodegeneration is well underway. Possible explanations include that dementia represents an end-organ failure that APP/PS1 mouse models do not model, that humans compensate better than mice, and that cognitive tests are too crude to pick up subtle deficits in people.

This issue spawned discussion on diagnostic AD testing. Current tests are inadequate, especially the MMSE, which regrettably remains in wide use. The reason has to do with brain reserve. Family members tend to notice a deficit first, and a CDR-based

clinical diagnosis supported by informant interviews is generally viewed as more sensitive than neuropsychological measures. Highly educated patients report they cannot do what they used to anymore, but even if they do have early AD, they still score in the ninety-eighth percentile of available cognitive tests. At this early stage, cognitive tests pick up a deficit only by the second or third measurement, when comparison to the person's baseline can be made. The key indicator for the AD diagnosis is the change from baseline, and huge baseline variability has stymied development of more sensitive tests despite intensive efforts in academia and industry. Moreover, the disease moves slowly; it has occurred silently for at least four years by the time the patient or relative suspects a problem.

That said, a person's hippocampus and entorhinal cortex have suffered significant cell loss in particular sub-areas by the time MCI or CDR 0.5 can be diagnosed; hence new cognitive tests that specifically call on those pathways could be explored. Spatial memory tests such as maze navigation offer a promising lead.

Another diagnostic option to explore is a neurotransmitter-based pharmacological stress test. It would use a single dose of, for example, an anti-cholinergic drug. In people who are particularly vulnerable to an added decrement in transmitter level, this could unmask an underlying synaptic dysfunction. In some elderly people, anti-cholinergic drugs prescribed for urinary incontinence are known to produce a cognitive deficit that resolves upon discontinuation. Similar tests are available for diabetes (insulin challenge) and depression (serotonin antagonist challenge).

Part 2: Synaptic Function in Aging and AD

To what extent is AD an acceleration of normal aging? This decades-old question receded in favor of the view that AD is a separate process from normal aging when studies showed that patterns of neuronal loss are different in aging and AD. Early AD entails selective loss of neuronal projections, such as the perforant path connecting the entorhinal cortex and the dentate gyrus of the hippocampus. Advanced AD features neuronal loss that far exceeds and differs in its regional pattern from that of normal aging. This anatomical data is undisputed and highlights the selective regional vulnerabilities in AD. But postmortem stereology is limited in that it assesses the brain after the initial pathogenesis has occurred, and therefore says less about cause than about features of disease progression. Hence, the question of aging versus AD continues to be debated. FAD mutations speak to both sides of the issue. They reduce the age at onset, diminishing the importance of age and validating overexpression approaches, but at the same time, even people whose FAD mutations flood their brains with A β from an early age appear healthy until their 40s. The current distinction between aging and AD is called into question by emerging comparisons of gene expression patterns between normally aging people and others with dementia.

One way to address the question is to study synaptic aging. Is Alzheimer disease a problem of synaptic maintenance? When synapses degenerate, is it a problem of "synaptosis" or "synecrosis"? In other words, does an active program inflict death by complement, aberrant reactivation of MCH class 1 proteins, or other outside signals, or does the synapse fall apart as synaptic organizing molecules disappear? GFP- and YFP-expressing transgenic mice allow repeated live imaging of peripheral synapses over time to monitor how synapses age normally and the building of a knowledge base for understanding how they age in AD models. Application of this method to neuromuscular junctions show that as these synapses age, they become fragmented

and the postsynaptic side loses transmitter receptors and nerve contact. Aging nerve terminals sprout, leading to multiple innervations of a given postsynaptic site. Axonal dystrophy and balloon-like distensions of the axon are common. Over time, there is a net loss of innervation. The aging synaptic cleft widens in parallel with changes in the molecular composition of the basal lamina. The aging synapse loses expression of synaptic organizing molecules including agrin and certain types of laminin. Genetic deletion of one laminin form accelerates the morphological changes associated with aging synapses, suggesting that the molecular loss is one cause of the aging, rather than merely a correlate. Together, these results suggest that age-related synapse loss may be quite passive, a form of dedifferentiation where a developmental program rewinds itself as proteins that organize the synapse are progressively lost.

A related question concerns the unit that changes in aging and AD: do individual synapses weaken and disappear one at a time, as happens in activity-dependent synaptic plasticity, or does a given neuronal arbor lose all its synapses just before the axon retracts, much as a winter tree sheds its leaves? The two imply different proximal insults and mechanisms of synaptic loss. The answer to this question is still not clear, but studies of the YFP transgenic mice show that aging muscles tend to have fewer but larger motor units than in young mice, suggesting that some units lose their branches and die back, whereas others gain branches, overextending themselves before they, too, die. Synaptic activity is likely to be an important factor in this process, and could determine selective vulnerability of certain circuits and brain regions.

Technical problems of accessibility and size preclude application of this technique to CNS synapses, so it is unknown whether central circuits age similarly. It is already possible to examine the aging "neural unit," and rapidly improving methods may soon allow imaging of structural details at individual synapses. Further imaging and molecular studies of age-dependent changes in synaptic organizing molecules are needed to address this knowledge gap in AD research.

Live multiphoton microscopy enables prospective brain imaging of the fate of synapses in AD models, albeit at a lower level of resolution. For example, studies monitoring the temporal sequence of events in AD pathogenesis in APP and tau transgenic mouse models are challenging the conventional wisdom that AD represents a steady process of continuous decline. This work suggests instead that AD is punctuated by a series of fast, catastrophic changes. During the 20 years that AD lasts, association cortices lose most of their neurons, and spine and dendritic changes are too numerous to count. Even so, the underlying process may be one of spurts of sudden changes at the cellular and molecular level.

Clinical AD follows a prodromal phase of MCI, during which the brain already contains abundant plaques, tangles, and gliosis, and a majority of neurons have died in certain cortical areas. To identify which changes happen first, scientists monitor over time the same brain area in APP transgenic mice. This work shows that plaques do not grow gradually in size over months, as had been predicted; rather, they appear suddenly from one day to the next in their full size and then remain stable for months. This is consistent with the idea that they might precipitate out of solution. Likewise, within days of the appearance of a plaque, neurites abutting it become crooked and dystrophic. Some neurites stay that way for months; others break. Direct application of anti-A β antibody clears both plaques and neuritic dystrophy within a week.

Dendrites in the vicinity of a plaque rapidly form new spines, but the spines are unstable, leading to net loss of dendritic spines near plaques within days of the appearance of a new plaque. Several studies have documented spine loss and dendritic changes near plaques (e.g., Tsai et al., 2004; Spires et al., 2005). These synaptic changes are a function of the APP transgene and independent of age.

Tangles in humans correlate with cognitive decline and neuronal loss, but neuronal loss exceeds the number of tangles (Gomez-Isla et al., 1996). Tangles can be imaged in Tg4510 mutant human tau transgenic mice, which develop tangle-like pathology and dramatic neuron loss in cortex and dentate gyrus (Spires, 2006). In these mice, individual tangle-bearing neurons remain stable for months even while neurons die in large numbers. Imaging reagents that fluoresce after a specific caspase has cleaved them indicate that tangle-bearing neurons are likely to activate caspases. They cleave tau, and still these neurons survive for months. Caspases remain present in the neuron after transgene expression is turned off. Markers of apoptosis are absent, yet monitoring of a given visual field captures occasional instances of a fluorescent neurite disintegrating over the course of 2 hours. This is seen only in Tg4510 mice, not in APP-transgenic or control mice. Together, these new data suggest that tangles and caspase activation precede additional biological changes that destroy the neuron by a still-mysterious mechanism.

The rapid appearance of fully grown plaques and the sudden collapse of tangle- and caspase-bearing neurites after months of apparent stability raise new questions about which biological changes dominate the long prodromal phase of human AD. Other mouse studies reporting that spine loss and synaptic changes precede plaques (e.g., Jacobsen et al., 2006), and work from multiple labs showing spine loss in response to oligomeric A β , keep alive the related debate about which A β species most damages spines. Imaging labels for diffusing, low-molecular-weight species of A β and tau are needed to resolve these issues in vivo.

Multiphoton microscopy of normally aging mouse brain has generated data to suggest that a large majority of dendritic spines remain stable and could serve to store long-term memories (Grutzendler et al., 2002; Zuo et al., 2005). More recent multiphoton imaging data implicate cortical microglia in dendritic spine plasticity. They show microglia to be highly plastic, reacting to an injury within hours by becoming activated and extending processes toward the injury (Davalos et al., 2005). The time course of this microglial activation parallels changes in spine dynamics following injury. The tools are in place for monitoring the dynamics of activated microglia around amyloid plaques.

Other areas of synaptic biology are expanding rapidly and should be explored for links to AD pathogenesis. They include the study of signaling between synapse and nucleus that enables those forms of long-lasting neuronal plasticity that require gene transcription. These pathways are being established in several laboratories (e.g., Martin and Zukin, 2006) and could be tested for their robustness in aging neurons and AD models. Another area that is already informing the study of AD is that of neuronal endocytosis. Ongoing work in the field is largely focusing on defects in vesicle transport, autophagy, and APP endocytosis and processing, yet additional proteins involved in the membrane traffic that controls synaptic vesicle recycling could expand this research area. Proteins of interest include the phosphoinositide phosphatase synaptojanin, an enzyme overexpressed in Down syndrome brain whose gene lies in

the Down's region of chromosome 21. A cycling synaptic vesicle needs synaptojanin to shed its clathrin coat before it can be reloaded with transmitter. Proteins of growing interest also include the GTPase dynamin, a mechanoenzyme that, together with accessory proteins, pinches invaginated vesicles off the membrane (Roux et al., 2006). Researchers are beginning to understand that some isoforms of dynamin are sufficient for basal endocytosis, whereas the brain-specific version dynamin1 handles increased synaptic demand during intense stimulation/excitation. The field is beginning to generate hypotheses about dynamin's role in AD pathogenesis, but none have yet been independently confirmed and widely accepted. For example, dynamin has been implicated in genetic AD risk (Kuwano et al., 2006), in APP endocytosis, and downstream of A β action on NMDA receptors (Kelly and Ferreira, 2006).

The question of A β effects on postsynaptic receptors of excitatory transmission has become an area of active investigation after a report proposed a negative feedback loop in which synaptic activity would increase APP processing, and A β in turn would restrain activity (Kamenetz et al., 2003). The same group has since expanded this initial data. New results suggest that A β likely destabilizes synapses by recruiting some of the postsynaptic signaling mechanisms that downregulate AMPA receptors during normal instances of long-term depression (LTD). This would, in effect, generate a situation of A β -induced chronic LTD that would weaken first the synapse and then the spine carrying it. How loss of AMPA receptors causes the spines to disappear is unclear, but researchers know that the intracellular tails of these receptors serve as organizing principals for other components of the postsynaptic density. Other labs have similarly implicated A β in glutamate receptor loss (see Chang et al., 2006; Cirrito et al., 2005; Snyder et al., 2005; Almeida et al., 2005). Together, the studies are generating interest in the role of LTD in aging and AD research. Big questions remain. Researchers have not definitively shown with which receptors A β may interact directly as opposed to which ones are affected secondarily, or which forms of A β have these effects in vivo. They also have not yet defined a physiological function of A β on glutamate receptors vis-a-vis an age- or disease-related one, or reached consensus on pre- versus postsynaptic generation of A β . α 7 nicotinic acetylcholine receptors also are likely to play a role in A β -induced synaptic dysfunction, but a clear pathway has not been delineated in vivo.

Another question that is being actively studied concerns whether APP cleavage products other than A β can impair synaptic function. Some research indicates that the cytoplasmic C-terminus generated by BACE and γ -secretase cleavage undergoes further caspase cleavage and then becomes toxic to synaptic transmission. Called C31, this fragment could account for some of the behavioral abnormalities in APP transgenic mice. Evidence for the toxicity of C31 exists in vitro and in vivo. The latter suggests that a mutation abolishing the caspase cleavage site on APP's C-terminus, when crossed with APP-transgenic mice, rescues the deficits in hippocampal synaptic transmission and in the Morris water maze that several labs have established for the APP-transgenics alone. The synaptic rescue occurs in the presence of a full complement of amyloid plaques. These data point to an intracellular pathway by which aberrant APP processing could first lead to synaptic apoptosis and later to the death of the whole neuron (Galvan et al., 2006; also independent, unpublished work). The hypothesis proposes that high levels of A β are necessary but insufficient to cause the synaptic damage and neuron death seen in AD. Rather, A β induces formation of APP complexes that trigger the toxic caspase cleavage (Shaked et al., 2006). This work needs more study, particularly on the precise function of C31. This question,

like many in AD mouse genetics, would benefit from the development of knock-in models, as well as cell-type-specific inducible knockouts of genes of interest in given brain regions, such as the CA1/CA3 synapse in the hippocampus.

Part 3: High-throughput Assays (Application of OMICS to AD)

Genomic/proteomic/metabolomics (OMICS) research in AD remains in its infancy. Most studies stall after discovering lists of hundreds of genes whose expression changes in the chosen comparison. Few groups have been able to validate their results, much less translate to clinical practice. The field needs to devise more focused studies that can advance in this way. General research priorities in the OMICS area include definition of useful algorithms for data mining, comparisons of the relative power of mRNA- versus protein-based approaches in capturing a given biological process, and attempts to characterize the role of post-translational modifications and of non-coding regions. Heterogeneity continues to pose challenges to OMICS, both between brain regions and within a given region. This can partly be addressed by laser capture microdissection.

One line of research that has moved past initial gene expression profiling is establishing a new hypothesis about the biology of brain aging as a way of approaching this old question at a new level of analysis. Classic aging studies have established that people slow down in verbal recall and other cognitive domains even with normal aging. A potential underlying mechanism for those changes is emerging from transcriptome comparisons of people across the human age range. In the prefrontal cortex and other brain areas, gene expression changes early in adult life, around age 40. It changes with a characteristic signature. Certain clusters of genes lose expression; these include synaptic plasticity and memory storage genes such as NMDA, AMPA, GABA, serotonin receptor subunits, calmodulin, calbindins, synaptic organizing molecules such as agrin, vesicle transport genes such as RAB GTPases, dynein, clathrin, kinesin, tau, and energy metabolism genes in mitochondria). Clusters of other genes are induced; this includes genes encoding stress response proteins, inflammatory mediators, antioxidant processes, metal binding, DNA repair, neuronal survival, and myelination. Analysis of this phenomenon has shown that the genes that lose expression have accrued disproportionate oxidative damage in their promoters and failed to repair it, generating the hypothesis that learning, memory, and neuronal survival genes are selectively vulnerable to DNA damage with age. Data are beginning to suggest that old people who maintain exceptional mental acuity have expression signatures similar to middle-aged people (Lu et al., 2004).

Since then, further analysis has indicated that on a given chromosome, DNA damage is not distributed randomly but is most intense in the promoter regions of certain genes, particularly so in promoters of aged samples. The promoters' vulnerability is linked to sequence motifs that bind iron. Iron binding to these promoters appears to increase with age and to lead to double-strand breaks. The working hypothesis coming out of this research holds that how the brain ages depends, in part, on its iron homeostasis, in that increasing iron binding tends to damage the promoters of a small set of genes that are critically important to synaptic plasticity and cognition. Why the affected genes fall into these groups remains unclear but may have to do with high expression in particularly active, that is, plastic, brain regions. (Expression of APP itself does not change with age, but that of some of its binding proteins do [e.g., X11, Fe65], possibly affecting its trafficking or endocytosis.)

A second example of a strategy to move beyond lists of differentially expressed genes lies in exploiting differences between brain areas toward understanding regional vulnerability in a given disease. In one study, high expression of a gene in mouse cerebellum, a region spared in frontotemporal dementia, hinted at a protective function. Follow-up work with fly genetics, in-vitro biochemistry, and human autopsy tissue pinpointed the new tau protease PSA. This aminopeptidase degrades tau protein and is expressed at much higher levels in human cerebellar granule neurons than cortical neurons (see Karsten et al., 2006) The bottleneck in this candidate-gene approach is apparent in that the original microarray experiment identified 30 genes of interest, and establishing the role for this one alone took years of a multidisciplinary and collaborative effort.

For faster identification of functionally important changes in gene expression, the field needs systems biology approaches, that is, techniques to trace higher-order features of the transcriptome. Such features include mechanisms of co-regulation of groups of genes, and expression networks that establish connectivity maps between genes. Microarray data can be analyzed to obtain a connectivity measure for a given gene that captures the sum of its connections and connection strengths. Comparison of gene connectivity between humans and chimpanzees shows that the connectivity of genes in human cortex diverges widely from that in chimpanzee cortex, whereas in other brain areas, gene connectivity in humans and primates are more similar. Measures of gene expression alone do not show this divergence, suggesting that the cortex in particular has undergone a massive expansion in gene connectivity from chimpanzee to human, and that expression data alone therefore cannot capture essential features of human cortical function.

Connectivity measures can point up relationships that expression data alone don't. For example, the new tau protease PSA shares many connections with the new FTD gene progranulin (Cruts et al., 2006; Baker et al., 2006), even though their expression is not correlated. Topologic overlap of connectivity data can identify previously known functional networks of genes. It can identify connectivity hubs that are specific to human cortex, as well as functionally relevant hubs that are linked to neurologic disease, such as ones centered on the PINK1 and UCHL1 genes (for further reading, see Khaitovich et al., 2004; Coppola and Geschwind, 2006).

A separate area of genomics focuses on identifying additional risk genes for AD. Here, the critical need lies in establishing larger sample collections than were previously used in order to power linkage and association studies or scans sufficiently high so that they can detect alleles that exert small effects or occur at low frequency. Underpowered studies have been holding back progress in AD and other diseases, notably psychiatric ones. In AD it is estimated that 3,000 patient samples will be necessary to detect variants that increase the relative risk by 1.25. Sample sizes in the hundreds have been typically used in the past; collaborative data sharing is necessary to move beyond this limitation. The Psychiatric Disease Initiative at the Broad Institute in Cambridge, Massachusetts, aims to use whole-genome scans to find risk genes for schizophrenia and bipolar disorder. In that initiative, collaborating groups have agreed to pool data to increase the number of available cases/controls to several thousand. To expand the sample base further, the initiative is tapping into the Swedish National Cohort Study of Schizophrenia, which aims to draw 7,500 schizophrenia patients and 7,500 matched controls from the country's national registers.

The study of complex disorders needs a better understanding of genetic variation. One novel resource in this regard is the International HapMap Project. Freely available as a public database, it to date has analyzed more than four million SNPs in samples from Africa, East Asia, and the U.S., offering a denser set of markers than previously available for association studies. Genotyping and analytic capabilities must also improve. New SNP microarrays contain all SNPs typed in HapMap samples. By themselves they still miss substantial fractions of all common human DNA variants, but improved genotype-calling algorithms can increase their coverage and accuracy such that they should detect common variants that increase risk for schizophrenia and bipolar disorder. Presently available arrays cannot capture the effect of rare variants. Applied to other diseases, the arrays already have identified new risk genes, for example, common variants of three complement factor genes that together explain half of the heritability of age-related macular degeneration, the leading cause of blindness in the aged (Maller et al., 2006). Until last year, almost none of thousands of prior papers on macular degeneration had mentioned complement, or vice versa. Moreover, new microarrays are more sensitive at detecting copy number variations, which can lead to mass effects at the RNA and protein levels. Mass effects in neurodegenerative diseases have been shown with rare gene duplications (Singleton et al., 2003; Rovelet-Lecrux et al., 2006), and promoter variants can exert similar, smaller effects that together may account for a significant fraction of the genetic variation in AD risk (Lahiri et al., 2005; Singleton et al., 2004). Finally, environmental factors can tip the balance between whether a person develops or escapes a given disease for which they carry a moderate increase in genetic risk. Epidemiology can identify potential new environmental risk factors, and once a disease's risk genes are known, environmental factors can be studied more precisely.

On the proteomics front, a majority of applied studies in AD research attempt to identify panels of proteins that can detect and distinguish the disease better than the clinical diagnosis, and eventually will be able to identify preclinical AD or predict AD in mid-life. For one such study, see the Wyss-Coray presentation in the Alzforum report on the Translational Biomarkers Workshop. For validation, such research needs access to plasma from larger samples and younger cohorts of people. In the U.S., groups at University of California, San Diego; University of Washington, Seattle; Washington University, St. Louis; Columbia University, New York; and University of Pennsylvania School of Medicine, Philadelphia, are currently collecting samples longitudinally. The ADNI initiative funded by NIA is gearing up to bank fluids at participating centers, and will make samples available. A funding shortage and insufficient industry investment in diagnostic tests are additional bottlenecks in this area.

Both genomics and proteomics are areas of active technology development. Following below is an example of each, and both may become useful to the field at large. One is the RNAi Consortium. Formally established in 2004, this group aims to create an openly available lentiviral RNAi library that can be applied to large-scale, unbiased loss-of-function screens in mammalian cells, much as has been possible in yeast for some years. The consortium has created reagents to knock down most human and some mouse genes. It is currently focusing on validation techniques and on exploring how best to use the reagents for extracting biological information. In this area, the group emphasizes development of high-throughput imaging assays rather than lacZ or luciferase surrogate reporter assays; this will render this library attractive

for neuroscience applications. Freeware to analyze thousands of cell images is already available for download.

To date, more than 140,000 reagents have been made, organized by gene groups, such as kinases, proteases, etc. They infect most cell types, including non-dividing cells and neurons, at low multiplicity of infection, and they yield stable expression of the respective shRNA. A robust, automated protocol for how to use the library has also been worked out. Technical hurdles at this stage include how to distinguish true hits from false-positive or off-target effects, obtaining larger numbers of effective hairpins (i.e., individual short RNAi sequences) per gene, knocking down a given gene strongly enough to see a biological effect, and generally validating the library with available funds. The question remains which features of complex diseases can be mimicked in cell-based assays and made amenable to genome-scale RNAi screens. Large-scale screens for a systems approach to pathways involved in a given function of interest are not yet feasible with this library.

Proteomics technologies that could be applied to fundamental questions of neural function and AD include, for example, phosphoproteomics. One uses mass spectrometry-based algorithms to track intracellular signaling circuits by analyzing phosphorylation. This technique allows the scientist to probe a cell's signaling pathways on a global level by quantifying key aspects of phosphorylation, such as the temporal sequence of successively phosphorylated sites, simultaneously for dozens of proteins (see Zhang et al., 2005; Chen and White, 2004). Applied to the research questions in AD, this technology could bring a network perspective to the study of tau phosphorylation. Other questions of interest that could be studied with greater power in this way include APP or ApoE signaling, or a neuron's response to NGF or to a glial signal and vice versa.

Further Topics of Discussion

A topic that was not on this year's workshop agenda generated significant discussion. It is ApoE, a perennially understudied area in AD research. The failure to find a second major risk gene for AD since the discovery of ApoE4 in 1993 only reinforces ApoE's status as the leading genetic risk factor. Yet initial research efforts on ApoE have waned; few groups today investigate it. The role of ApoE in the periphery is better understood than in the brain. It appears to be a stress-response protein; its expression, like that of APP, soars after stroke or injury. The crystal structure of ApoE is available. ApoE shows isoform-specific effects in the brain, but their role in AD pathogenesis is unclear. ApoE4 shows a unique domain interaction, whereas ApoE2 and E3 don't. ApoE2 binds less tightly to the LDL receptor than ApoE3 and 4, which could affect cholesterol recycling and, in turn, synaptic function. Studies examining ApoE in the context of dendritic spines and LTP induction tend to find a favorable effect of ApoE2. Separately from its synaptic effects, ApoE4 enhances amyloid pathology dramatically and is associated with greater damage/poorer recovery in injury models.

ApoE4 carriers have higher levels of corticosteroids and show differences in PET scans even at young ages and without overt cognitive impairments. ApoE2/2 homozygote carriers are rare and can have abnormally low cholesterol levels, but they rarely ever develop AD. This natural form of risk reduction presents an opportunity to understand its mechanism and exploit it for therapies. ApoE is one of the most abundantly produced and released proteins in astrocytes and should be explored for a

potential signaling role. Studies modeling ApoE in animals must be aware of differences between lipid handling in mice and humans, which have made guinea pigs a favored model in the cholesterol field. In the brain, cholesterol and ApoE synthesis and turnover occur mostly locally, without much connection to the periphery. A research area is developing around the discovery that ApoE binds to receptors (i.e., LRP) that also bind APP and that use similar adaptor proteins (i.e., Fe65) to form heterodimeric complexes but, again, relevance for ApoE signaling and AD pathogenesis is not clear.

Participants debated the importance of understanding the biology of AD more fully versus focusing on a given hypothesis for therapy development. An area of common ground lies in the notion that the biology of statins and their effects on lipid lowering and reducing heart disease risk and mortality were not fully worked out before their long-term secondary prevention trials began. Despite the wide use and success of statins, the field of heart disease needs additional drugs. Likewise, anti-amyloid therapy development is timely even while gaps remain in the amyloid hypothesis. Basic research must lay the groundwork for alternative approaches in the event that secretase inhibition and immunotherapy fail. Even if they succeed, there will be ample need for alternative approaches as most researchers expect an effective AD therapy to have to act on multiple components of the disease. In this regard, emerging research on glia, immune system components, DNA repair, and synaptic maintenance open new horizons for AD.

Participants agreed that ways must be found to push the time frame of when people are treated, and experimental drugs tested, back into the preclinical phase from the mild-to-moderate phase of diagnosed AD that is the typical time of treatment today. Statins, for example, have their strongest effect as preventive agents, and researchers feel that a number of promising AD drugs may be failing trials because the patients were too advanced in their disease. Epidemiology is reaching consensus that metabolic factors exert their strongest effect on dementia risk during middle age. Secondary prevention trials are necessary but require validation of an antecedent marker and a surrogate marker that is based on the drug's action and that changes as a function of its dose. Cognitive tests are neither precise enough nor practicable for such trials.

Last but not least, participants agreed on a nagging technical problem that impedes AD research. It is the variability of A β preparations and detection assays used throughout the field, and a lack of precision in how authors describe A β preparations and measurements in publications. To reproduce and compare studied, A β preparations in any publication should be defined using consensus language with regard to their origin (synthetic, cell-secreted) and aggregation state and solubility. Likewise, the field should agree on a set of consensus assays for measuring different kinds of A β species in tissue sections vs. fluids vs. brain extracts, because using the incorrect assay can mask large fractions of A β present in the sample.

Part 4. Recommendations for Future Research

I. Immune mechanisms in aging and Alzheimer disease

- Determine whether endogenous immune mechanisms in the brain can be modulated to alter brain function or disease processes.

- Study differences between immune modulation in mice and humans, and their relevance to AD. How well do mice model the role of immune modulators in the AD process?
- Follow people from younger ages onward to study changes in immune function and cognition; correlate over time. Follow lymphocytes and macrophages separately—can their expression profiles predict who will decline?
- Aging increases human T cell reactivity to A β . Develop a blood-based readout of how the aging immune system is responding to an immunotherapy.
- Identify factors upstream of synapse loss in aging and AD: probe for role of complement factors, of components of immunological synapse, of synaptic organizing proteins.
- Understand why the innate immune system fails to clear amyloid.
- Elucidate the normal function of microglia, and their role in AD and other proteinopathies.
- MHC class 1 and A β peptide loading: do MHC class 1 present A β ? If so, how does age-related proteasome dysfunction, intraneuronal A β accumulation, etc., affect MHC class 1 peptide loading? Does A β presentation affect synaptic function/plasticity, or stay functionally neutral as self-peptide? Does MHC class 1 variability affect A β display?

II. Synaptic function in aging and AD

- Study how synapses disappear in AD: active elimination or dedifferentiation? Loss of individual synapses or collective loss at level of neuron?
- Establish relationship of synaptic loss to other disease measures. Does loss of 20 percent of synapses imply a loss of 20 percent of axonal arbors, 20 percent shrinkage of arbors? At what stage of synapse loss does cognitive function start to fail?
- Develop PET and MRI imaging agents that target markers of neuronal activation and synaptic activity. Use those to track pathogenesis and effect of therapeutics.
- Why does cognitive activity protect against AD?
- Determine causes of DNA damage in neurons.
- Elucidate function of synaptic proteins. Of the 300 proteins known to date, only 5 percent have an identified function.

III. OMICS approaches, other technology development

- Foster conversation among researchers studying synaptic development, function, plasticity, and genomics/proteomics groups. Which synaptic proteins are best candidate genes?
- Use OMICS to understand molecular biology of regional vulnerability.

- Expend a greater effort on proteomic changes with aging.
- Establish networks for APP, tau, ApoE.
- Develop a way to assess synaptic function in live human brain.
- Branch out from amyloid imaging: develop ability to image microglial activation, response to treatment, in human patients.
- Bring together experts in hippocampal memory, e.g., Larry Squire, to devise better cognitive tests for early AD, particularly of spatial memory.
- Develop pharmacological stress test for AD diagnosis.
- Accelerate plasma collection, facilitate distribution to groups that need to validate proteomic biomarkers.

IV. Further priority areas: ApoE, others

- Clarify ApoE's role in brain lipoprotein metabolism. Encourage cross-talk among AD scientists and colleagues who study ApoE in periphery.
- What is ApoE's role in synaptic function? Build on PET data on differences in young ApoE4 carriers during cognitive tasks. Explore interaction of ApoE isoforms with synaptic plasticity molecules.
- What is ApoE's role in repair? Clarify glial vs. neuronal function.
- How are people with ApoE2 protected against AD?
- Focus on role of sirtuins in neuronal function, degeneration, protection.
- Develop best-practice assays for measuring A β .
- Develop consensus protocol for defining A β species in experimental protocols.

The role of the cerebral vasculature in AD remains a research priority (for details, see 2005 Enabling Technologies report).

References

Grutzendler J, Kasthuri N, Gan WB. Long-term dendritic spine stability in the adult cortex. *Nature*. 2002 Dec 19-26;420(6917):812-6. Abstract

Zuo Y, Lin A, Chang P, Gan WB. Development of long-term dendritic spine stability in diverse regions of cerebral cortex. *Neuron*. 2005 Apr 21;46(2):181-9. Abstract