

## **Enabling Technologies for Alzheimer's Disease Research: Fifth Bar Harbor Workshop, 2005**

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In August 2005, a group of researchers from inside and outside the field of Alzheimer disease met in Bar Harbor, Maine, with foundation and NIH representatives for two days of presentations and discussion at the fifth annual workshop on *Enabling Technologies for Alzheimer's Disease Research*. The participants sought to identify current knowledge gaps that need to be bridged to provide a foundation for rational therapeutic strategies. They proposed research opportunities for filling these gaps, involving both new technical approaches and fresh ways of looking at old questions. This year's meeting focused on APP function; vascular components of AD and the blood-brain barrier; and mechanisms driving the overlap between aging and AD. The report below is a brief summary of the presentations and discussions, followed by a list of recommendations made by the group. Readers will find a broad description of how open questions in the AD field are evolving, as well as ideas to update their own research programs.

### **Summaries of Talks and Discussions**

#### **APP Function**

Although a topic of intense interest, it is still not clear what the normal function of APP is and where, when, or why it is processed. Several studies have described a role for APP in controlling neurite outgrowth and cell adhesion, which appears to be activated in the context of the brain's response to injury. Ongoing research investigates a possible role for APP in the migration of newly differentiated neurons to their proper place in the developing cortex. More research is needed to explore the interplay between membrane-bound and soluble fragments of APP and to reconcile seemingly contradictory results in this field.

A fundamental knowledge gap in AD research today is where inside a neuron most A $\beta$  is made. It is clear that A $\beta$  gets deposited in the terminal fields of neurons. Rodent experiments in which the perforant pathway was cut showed subsequent loss of A $\beta$  deposition in the terminal field. These studies are widely considered evidence for APP's axonal transport, processing at the nerve terminal, and presynaptic release of A $\beta$ . However, recent studies exploring a possible physiological role of A $\beta$  in regulating synaptic activity argue that intense neuronal activity drives APP processing to A $\beta$  postsynaptically ([Kamenetz et al., 2003](#)). Transecting the perforant pathway would silence its synapses and in this way reduce A $\beta$  release from the postsynaptic side of its terminal field. Reconciling these two views, FRET imaging of presenilin suggests that APP processing may occur near the membrane on both sides of synapses ([Tesco et al., 2005](#)).

A physiological role for the processing of APP to A $\beta$  in response to synaptic activity has been suggested by studies demonstrating that A $\beta$  can depress neighboring synapses, thus creating a feedback cycle which could essentially cool down excess synaptic activity.

Some data suggest that A $\beta$  causes synaptic depression through a mechanism involving the internalization of excitatory receptors ([Synder et al., 2005](#)). Intense research continues to focus on A $\beta$  and the effects of particularly its oligomeric forms on synaptic function. A broad question is how A $\beta$  oligomers affect learning and memory in rodents, and whether they act through a synaptic depression model as just described.

### **APP and Axonal Transport**

It is widely accepted that at least a fraction of APP is actively transported along the axon to the nerve terminal and does not return to the cell body. Current points of debate on APP and axonal transport include whether APP binds directly to the motor protein kinesin or indirectly as part of a complex with scaffolding proteins, how the transport of APP relates to its processing, and whether axonal transport deficits are an early and critical event in AD pathogenesis.

Evidence is also now emerging that APP can impair retrograde transport. For example, studies with Down syndrome models indicate that elevated levels of APP, particularly certain cleavage fragments, can disrupt the proper fusion and subsequent retrograde transport of NGF-containing vesicles (see [ARF conference news](#)). In addition to the obvious connection this makes between APP and neuronal survival, this raises the question of whether there exists a wider system of signaling endosomes in neurons that may be disrupted by APP or other proteins implicated in neurodegeneration.

Given that anterograde and retrograde axonal transport occurs along microtubules, the question arises whether this context offers a connection between the microtubule-binding protein tau and APP. In cell-culture experiments, increasing the levels of tau can disrupt transport, perhaps because tau and kinesin compete for the same binding sites on microtubules. It is not clear yet whether this happens *in vivo* in AD; imaging methods to probe this process *in situ* are a priority. (For more on a dynamic model of tau in axonal transport, see [ARF conference news](#).)

Overall, the cause and significance of transport deficits in AD remain a topic of debate. How large a transport deficit a neuron can tolerate over time should remain a focus of active research.

### **BBB/Brain Vasculature**

After release, some A $\beta$  is degraded locally, a second fraction leaves the brain through interstitial fluid drainage and along brain arterioles, while another fraction is actively transported by proteins, such as LRP and glycoprotein-P, across the blood-brain barrier (BBB) into systemic circulation. Recent studies binding blood A $\beta$  with antibodies have raised the prospect that brain levels of A $\beta$  could possibly be lowered by a therapy that removes the peptide from the periphery, thus creating a peripheral sink.

Cerebral amyloid angiopathy (CAA) commonly accompanies Alzheimer disease and makes blood vessels prone to hemorrhages. Recent studies indicate that both parenchymal and blood vessel amyloid are released from neurons, not smooth muscle or endothelial cells (glia have not been exhaustively tested), and the site of deposition

depends on the species of A $\beta$ . Specifically, A $\beta$ 40 aggregates more slowly and tends to do so only after it has accumulated near the vessel wall on its way out of the brain, whereas A $\beta$ 42 aggregates more readily and tends to do so on the way through the parenchyma to the nearest blood vessel.

The brain vasculature is now amenable to study with new imaging methods, for example, corrosion casting combined with scanning electron microscopy and computer tomography. This method requires perfusing the brain vasculature with a resin that hardens and leaves behind a cast of the "vascular brain" once all organic tissue is removed. Such studies open up new modes of studying the role of cerebrovascular insufficiencies in the pathogenesis of Alzheimer's and related diseases. It can also address whether vascular changes precede parenchymal changes in given mouse models (see [Krucker et al., 2004](#)).

The brain's vasculature is sealed off from its neurons and glia by the blood-brain barrier, which keeps the vast majority of proteins and most small molecules out of the brain and controls the active transport of selected molecules. The role of this system in neurodegeneration remains largely unknown. However, the BBB remains a formidable obstacle to drug development efforts and needs to be taken into account by the increasing number of academic drug discovery efforts. Generally speaking, only lipophilic compounds smaller than 400 Daltons cross effectively. The majority of biologically active small molecules, and certainly promising proteins, remain untapped as drugs due to BBB permeability or efflux problems.

The microvasculature is so dense that a nearby capillary feeds each neuron, meaning that overcoming the BBB is not only necessary but also sufficient to reach neurons effectively. In addition to efforts to design small molecule drugs with favorable BBB properties, an alternative approach to overcoming the permeability problem of the BBB involves making chimeric molecules to exploit BBB transporter proteins. Gene therapy approaches using lipid vectors also show promise. A better understanding of the BBB may come from basic genomics and proteomics research. This area is beginning to open up as new proteomic tools are enabling the profiling of purified BBB cells that are isolated via mechanical means or laser capture microdissection ([Shusta, 2005](#)). Improved understanding of the molecular basis of the BBB may also improve the ability to design and test drug candidates.

The choroid plexus is a membrane system at the interface between blood and the cerebrospinal fluid, sometimes called the blood-CSF barrier. Made of a capillary bed surrounded by secretory epithelial cells, the choroid plexus is known primarily for producing the CSF. This poorly understood arm of the BBB requires further study in the context of AD because age-related changes in its function may influence transport of A $\beta$  and other CSF components.

### **Aging and AD**

Clearly, the greatest risk factor for AD is advanced age, but a satisfying explanation of

the link between aging and AD remains uncertain. To understand this connection, it is argued that a better understanding of "normal" aging is needed.

An emerging area of overlap between aging and AD lies in synaptic atrophy. Loss of synapses is generally accepted as an underlying process of AD, but it also may explain a more general drop in people's cognitive ability with age. One theory is that chronic, mild inflammatory activation of glial cells may lead to synaptic atrophy with aging. Inflammatory processes increase with advancing age, and conversely, anti-inflammatory drugs reduce amyloid load in transgenic mouse models. Epidemiologic evidence in humans also supports this theory.

To study neuronal aging, a recent study compared gene expression of normal humans ranging from people in their twenties to centenarians. Gene expression changes that correlated with age revealed a group of genes whose expression starts to drop around age 40. More precisely, this group of genes showed a uniformly high expression level in young people and a uniformly lower expression level in most very old people, but great variability from person to person in the middle decades of life, perhaps yielding clues as to why some people age "better" than others. Among these down-regulated genes were genes that function in mitochondria, synaptic plasticity, and vesicle transport. A cell-based assay revealed that these down-regulated genes sustained high levels of DNA damage in their promoter regions when exposed to mild oxidative stress and were less able to repair that damage. In essence, DNA damage results in transcriptional silencing of a subset of genes important for cognition ([Lu et al, 2004](#)). This study was recently confirmed and extended ([Fraser et al., 2005](#)).

The reasons why particular promoters are more susceptible to oxidative damage, and the connection of this phenomenon to AD pathology are unclear. It remains an open question whether similar expression changes could be detected in peripheral cells around mid-life, yielding perhaps a biomarker for elevated risk. Broadly, DNA sequence determinants as well as epigenetic changes during life should be analyzed for how they affect expression of these genes. For now, it appears as if aging processes are heterogeneous. Different people may be impacted by different genetic and epigenetic factors, some combinations of which will be more likely to result in AD.

The link between oxidative stress and AD and Parkinson's pathology has inspired tests of antioxidants as therapeutic agents. The free radical scavenger coenzyme Q10 has shown some promise in the clinic and is expected to enter a phase 3 trial of Parkinson's in 2006. Preclinical CoQ10 tests in APP transgenic mice also show promise. That said, as a group, antioxidants have disappointed in the clinic so far. Their weak effect may be partly due to the BBB, which makes it difficult to achieve effective concentrations in brain while keeping levels safe in the rest of the body.

Caloric restriction has been shown to increase longevity in multiple systems from yeast through worms and flies to rodents. Molecular biological approaches to the study of aging have focused on the genetic programs that become active under conditions of caloric restriction. The biochemical function of some of these genes, called sirtuins, is to

remove acetyl groups from components of the DNA-packing material chromatin. When that happens, certain genes become silenced. Intriguingly, the sirtuins require a cofactor, NAD, whose supply rises and falls with an organism's nutritional state. This makes certain sirtuin genes a nexus between diet and gene expression, and it suggests that sirtuins might relate a person's metabolism to his or her pace of aging. Sirtuins are one player in a web of molecular pathways, and though it grows rapidly, this field has to date gained little more than a foothold into a complex area of metabolic regulation. Scientists need a much larger body of knowledge about exactly how diet and other epigenetic factors that drive gene expression can influence the molecular processes of aging and age-related diseases.

### **Enabling Technologies**

This workshop has an enduring interest in facilitating the import into AD research of new technologies developed in other areas. This year an approach was presented that makes it possible to watch neurons grow and change over time inside living mice. Research on diseases that have a long prodromal phase of asymptomatic pathology and then slow progression could benefit especially from *in situ* biology. Frequently in AD research, interpretation of a given result is hobbled by circular arguments about whether the result is a cause of pathogenesis or a consequence of it. This problem can keep old questions unsettled despite large amounts of research. In such instances, it could be tremendously useful to have tools to observe a disease process over time in a living, behaving animal.

The green fluorescent protein of the jellyfish species *Aequorea* has become widely used in various genetic constructs throughout cell biology. Few people realize, however, how much more powerful this technology has become with the production of a wealth of different protein variants that fluoresce with different wavelengths. Jeff Lichtman of Harvard University gave the audience a taste of what these proteins can do. In collaboration with Joshua Sanes, Lichtman's laboratory have made more than 90 lines of mice that express GFP and similar fluorescent proteins (XFPs) in different subsets of neural cells. Most use a regulatory element from the Thy-1 promoter that drives neural expression, and indeed these mice are already in use in AD studies ([Brendza et al., 2003](#)).

In the peripheral nervous system, it is possible to counterstain the neuromuscular synapses of Thy-1/XFP mice, and then watch a nerve as it grows to innervate its muscle. One can crush the nerve and observe how single axons abandon their synapses and withdraw through their myelin tube ([Walsh and Lichtman, 2003](#); [Bishop et al., 2004](#)), and then watch regenerating axons cross the injury site and grow back through the inside of their original Schwann cell tube to re-innervate their old target ([Nguyen et al., 2002](#)). Moreover, scientists can image changes in the branching pattern and other characteristics of a given nerve as the animal ages normally or with disease ([Schaefer et al., 2005](#)).

In a more recent strain called "Brainbow" mice, different neurons within a given nerve will light up in a multitude of rainbow colors depending on how each cell recombines a complex transgenic construct encoding four different-colored fluorescent proteins (manuscript in preparation). It is up to clever Alzheimer researchers now to devise meaningful experiments for this brilliant resource. This summary closes with a nudge and

cheers to card-carrying technology geeks who'd like to develop ways to image fluorescent neuron dynamics in the CNS of live, aging mammals, preferably through the intact skull.

## Recommendations

I. Address gaps in our knowledge of APP processing, movement, and signaling.

- A. Questions to be addressed include:
  - 1. Where in a neuron is A $\beta$  made? This is an old question that should be settled.
  - 2. Why is APP processed? What is the physiological role, if any, of A $\beta$  (e.g., does it play a physiological role in synaptic function)?
  - 3. What triggers APP processing through different pathways?
  - 4. With regard to the axonal transport of APP:
    - a. Is the interaction with kinesin direct or indirect?
    - b. Is the transport of APP related to its processing?
  - 5. Do transport deficits play a key role in the pathogenesis of AD?
- B. To study these issues most productively, a central source to support tool sharing and make carefully developed and isolated material uniformly available (e.g., oligomer-producing cells) is needed.

II. Analyze neuronal circuits in aging and disease "connectopathies." To do this, we need better ways to visualize and study neuronal circuits.

- A. Develop tools and methods to image central synapses in real time, in live animals. The goal is to map structure, function, and connectivity in a given circuit simultaneously.
- B. Training on the use of imaging tools is needed.
- C. Computational capacity and speed are rate-limiting factors for in situ imaging. The development of technologies at the interface of biology and computing is needed.
- D. It was suggested that perhaps there could be support for a few sites that could build and house new tools as well as provide training and expertise on their use.

III. Using improved imaging and computational capabilities in the CNS, exploit Brainbow mice to address AD questions (e.g., examine structural and functional changes with aging, amyloid deposition, etc).

IV. Explore why age is the most important risk factor for AD.

- A. Study how the biological processes of brain aging intersect with prodromal AD. Priority areas:
  - 1. Image inflammatory processes in live mice.
  - 2. Study epigenetic shifts in gene expression.

- 3. Examine how chronic exposure to low-level environmental toxins affects the aging process and disease.
- 4. Document changes in protein turnover.
- 5. Study phylogenetic differences in brain aging as clues to why humans are uniquely susceptible to many neurodegenerative diseases.
- 6. Look for the signature of age-related changes in lymphocytes as peripheral markers of AD during its prodromal period.
- 7. Conduct longitudinal studies of aging and disease at the cellular level.
- B. Look for protective factors in humans. Project ideas:
  - 1. Identify high-performing sibling pairs and compare synaptic gene variants in highly concordant and highly discordant pairs.
  - 2. Mine the literature for protective alleles other than ApoE2.
  - 3. Consider doing forward screens for genetic modifiers that mitigate the phenotype in mouse, *Drosophila*, and nemotode models of AD.

V. Expand the study of glial cells. They outnumber neurons nine to one and appear to express AD-relevant genes at high levels. Explore their normal functions and their role in neurodegenerative processes including A $\beta$  generation, inflammation, synaptic dysfunction, and axonal degeneration.

VI. Explore novel ways of getting small and macromolecular drugs across the BBB.

- A. Study the ability of macrophages/microglia to transport substances into the brain. Explore exploiting this route for drug delivery.
- B. Continue studying the exploitation of active transporters as routes of delivery.
- C. Consider novel ways to bias small-molecule libraries for properties that would be favorable to BBB permeability.