2012 Keystone Symposium Series
(http://www.alzforum.org/new/detail.asp?id=3102)

By Tom Fagan

Keystone: Symposium Emphasizes Key Aspects of ApoE Biology
Why has it been such a challenge to pinpoint the exact role in pathology of apolipoprotein E, which surpasses all other genetic risk factors for late onset Alzheimer's disease? In short, because ApoE does so many things. It transports cholesterol, regulates cell signaling, promotes Aβ aggregation and/or clearance, and even acts as a neurotoxin by poisoning mitochondria. At "ApoE, Alzheimer's and Lipoprotein Biology," a Keystone symposium held 26 February-2 March 2012, scientists shared the latest research and brainstormed about its meaning. One take-home message: Research now seems focused on Aβ clearance, synaptic plasticity, and neurotoxicity as the three main aspects of ApoE biology. The jury is still out on which, if any, is most important. As one researcher put it, "the camps can be dogmatic and we need to integrate to keep the dialogue moving." The cross-disciplinary nature of the meeting seemed to do that, with talks on neurophysiology, tau biology, genetics, and some of the latest clinical trials complementing presentations by the card-carrying ApoE researchers. Conference attendees uniformly found the meeting informative, and those who were unable to go can pick up highlights in this news series.

ApoE and Aβ Clearance
There is now extensive evidence, much of it from **David Holtzman's** lab at Washington University, St. Louis, Missouri, that ApoE perturbs clearance of Aβ from the brain, and that ApoE4 is the most nefarious of the three isoforms in this regard. The scientists showed, for example, that Aβ has a longer half-life in PDAPP mice expressing ApoE4 than in those endowed with ApoE2 or 3 (see ARF related news story). If this is so, then reducing ApoE in the brain could prove beneficial, and, indeed, that was recently reported for all three isoforms (see ARF related news story). Do lipoprotein receptors, which bind ApoE, influence this dynamic? At Keystone, Holtzman reviewed new data on how low-density lipoprotein receptor (LDLR) plays into Aβ clearance.

Of the lipoprotein receptors regulating cholesterol, LDLR is the main one. It binds ApoE. It promotes endocytosis of apolipoproteins and their signaling. Does LDLR influence the half-life of ApoE? To explore this question, Holtzman and colleagues used a technique that WashU's Randall Bateman (see ARF related news story) pioneered to measure Aβ turnover, that is, isotopic labeling with carbon-13 leucine followed by mass spectroscopy. Holtzman reported that, in mice that overexpress LDLR, production of ApoE is the same, but it turns over 2.5 times faster than normal, and the total pool of ApoE shrinks compared to control mice. Merely doubling the expression of LDLR increased Aβ clearance from the brain interstitial fluid (see ARF related news story).

Does the LDL receptor accelerate Aβ clearance because it clears ApoE, which in turn binds Aβ? To address this question, the scientists turned to cell models. Holtzman reported that the medium of cultured astrocytes that overexpress LDLR contains less ApoE, which fits with greater uptake of ApoE into these cells. In addition, the LDLR-overexpressing cells more readily subsumed Aβ added as part of conditioned medium from other cells. But as Holtzman pointed out, that alone did not clarify whether uptake of ApoE explained the cells' appetite for Aβ, or whether the peptide might be taken up by some other pathway. Hence, his group turned to 125I-labeled Aβ to directly probe the sequence of events.

In this experiment, labeled Aβ colocalized with the LDLR in the lysosomal pathway, and LDLR stimulated not just Aβ's uptake, but also its degradation. Intriguingly, this process seems to be independent of ApoE, Holtzman told the audience. LDLR stimulated uptake and degradation of Aβ equally in ApoE-positive and negative astrocytes. Since then, Jacob Basak in Holtzman's lab determined that Aβ binds directly to LDLR. All told, the data from the lab suggest that LDLR acts independently of ApoE to clear Aβ, Holtzman said (see Basak et al., 2012). This is in keeping with a recent study from Spiros Georgopoulos' group at the University of Athens, Greece. Georgopoulos found that LDLR effects on Aβ deposition in a transgenic mouse model of AD (5xFAD) are independent of ApoE (see Katsouri and Georgopoulos, 2011).

Seem straightforward so far? Don't get used to it—no story about ApoE stays simple for long. A different interpretation of the role of ApoE4 in Aβ clearance came from **Gary Landreth**, Case Western Reserve University, Cleveland, Ohio. In a widely publicized study, Landreth and colleagues last month reported that bexarotene, a retinoid X receptor (RXR) agonist, boosts brain levels of ApoE in transgenic mouse models of AD while dramatically lowering soluble and insoluble Aβ and amyloid plaques (see ARF related news story and commentary). At Keystone, the data were still fresh enough to create a stir. While most in the audience seemed impressed with the findings and eagerly await the results of the first clinical trial, some niggling loose ends inevitably came into debate.

One discrepancy that raised eyebrows was that, while bexarotene seems to work wonders over the short term (two weeks), over the longer term (three months), the mice show no change in amyloid burden despite a drop in soluble Aβ in the brain. Landreth believes that plaque clearance requires a change in the activation state of microglia, and that the drug dose used might not achieve that over the long term. He told this reporter that the dose of bexarotene, which was developed as a cancer treatment, in his mouse study may be so high that it eventually desensitized glial RXRs and/or the PPARγ and liver X receptors (LXRs) with which RXRs form heterodimeric complexes. Landreth said he is planning to study the activation state of microglia after chronic treatment.

How the bexarotene data fit with ApoE as an impediment to Aβ clearance was another topic for discussion. There was consensus that the lipidation state of ApoE is crucial in this regard. Bexarotene induces a plethora of genes related to lipid metabolism, and the treated mice produce dramatic quantities of lipidated ApoE, reported Landreth. But that very multitude of RXR targets had some researchers questioning whether bexarotene works through ApoE at all. For

example, at the meeting, **Cheryl Wellington**, from the University of British Columbia, Vancouver, Canada, presented evidence that LXR agonists reduce Aβ in ApoE4-negative mice. Landreth agreed the drug's actions may be complex. He said he plans to tease out the relative contributions of ApoE and microglia by using ABCA1 knockouts, which fail to lipidate ApoE, and toll-like receptor 4 mutants, which do not activate microglia.

C2N, a diagnostics company founded by Holtzman and Bateman, is collaborating with Case Western on a randomized, placebo-controlled clinical trial for bexarotene that will begin enrolling volunteers in the second quarter of 2012. Landreth told this reporter that it is a small pilot study to look at CSF changes in Aβ and ApoE. Participants must be ApoE4 negative. "If we see an effect in CSF, then we will consider pursuing this further," he told ARF. A notice on his lab's home page says that no new patients can be enrolled for this small trial.

In her presentation, Wellington emphasized the role of ApoE lipidation. Wellington reminded the audience that ABCA1 (ATP-binding cassette A1), a cell-surface cholesterol and phospholipid transporter that transfers lipids onto ApoE, plays a key role in formation of lipoprotein particles. She noted that ABCA1 knockouts retain much less ApoE in the brain, and deposit more Aβ than controls (see ARF related news story), whereas overexpressing ABCA1 in PDAPP mice protects them against Aβ accumulation. Poorly lipidated ApoE slows Aβ clearance, while lipidating the protein "greases the wheels," she concluded. Wellington's take was that liver X receptor agonists, which, like bexarotene, induce ABCA1 and therefore promote lipidation of ApoE, may help clear Aβ from the brain. Lipidation, then, could resolve some of the contradictions on whether lowering or elevating brain ApoE would be therapeutic.

This story being about ApoE, it's now time to add another layer of complexity. What about the role of ApoA1, the major apolipoprotein in the body's periphery. ApoA1 is the major protein in high-density lipoproteins (HDL) that carry the "good cholesterol" in the bloodstream. HDLs protect against cardiovascular disease, and there is considerable interest in understanding whether circulating HDL may also protect against AD, said Wellington. Researchers previously reported that ApoA1 inures the vasculature against cerebral amyloid angiopathy (CAA), aka deposition of Aβ in the brain's blood vessels. Overexpressing ApoA1 in transgenic AD mouse models prevents this vascular pathology, while ApoA1 knockouts are more susceptible to it (see ARF related news story). Like ApoE, ApoA1 receives lipids from ABCA1, suggesting that LXR agonists might boost vascular clearance of Aβ through lipidating ApoA1 and boosting HDLs.

While that may turn out to be true, there is a twist. Wellington reported that the commonly used LXR agonist GW3965 increases ApoA1 levels in brain and plasma of APP/PS1 mice; however, in ABCA1-negative animals, it only boosts brain ApoA1, not plasma. The finding suggests that distinct mechanisms regulate ApoA1 in the brain and in the periphery, said Wellington. It is unclear how LXR agonists boost brain ApoA1, but the mechanism might offer new insight into Aβ clearance and explain the link between cardiovascular health and AD, she said.

If a predominantly peripheral apolipoprotein may move Aβ in the brain, what about the role of ApoE in the plasma? In a short talk, **Huntington Potter**, University of South Florida, Tampa, showed how he addressed this using parabiosis. In this technique, two different animals are surgically connected so that they share a circulatory system. Potter revealed that when APP/PS1 ApoE+/- mice are conjoined with APP/PS1 ApoE nulls, the number of Aβ plaques in the latter, even though low to begin with, drop significantly. The parabiosis corrected hypercholesterolemia in the transgenic mice as well. Potter said that none of the ApoE in circulation gets into the mouse brain. He concluded that increasing plasma ApoE could be a potential strategy for reducing Aβ in the brain.

Continuing the vascular theme, **Guojun Bu** at the Mayo Clinic, Jacksonville, Florida, outlined a potential role for blood vessels' smooth muscle cells in Aβ metabolism. Bu studies the role of low-density lipoprotein-related protein 1 (LRP1), a member of the LDLR family. His lab previously reported that conditionally knocking out LRP1 in the mouse forebrain neurons sparks a plethora of bad events. Synaptic markers go, as do dendritic spines; neuroinflammation flares up, and motor control and cognition decline. The working hypothesis is that these deficits are due to altered lipid metabolism (see ARF related news story). Strong evidence also implicates the lipoprotein receptor in Aβ clearance (for a review see Zlokovic et al., 2010). This could be relevant to AD, suggested Bu, because people with the disease appear to have too little LRP1 in blood vessels (see Bell and Zlokovic, 2009). If cells of the vasculature take up Aβ via LRP1, then loss of the receptor could impede Aβ's removal from the brain through the vessels. However, which cells of the blood vessels might employ that mechanism is unclear.

To get a handle on this, Bu and colleagues conditionally knocked out LRP1 only in smooth muscle cells of the vasculature. In wild-type knockouts, endogenous Aβ40 and Aβ42 rose in the brain. In APP/PS1 transgenic mice, total Aβ40 and 42 rose, as did the plaque burden. The findings suggest that smooth muscle cells in the vasculature contribute to clearance of Aβ, Bu told the audience. The finding set the audience abuzz. Some researchers said it helps clarify how Aβ is transported out of the brain. On that, scientists have debated whether endothelial cells are prime movers of the peptide. But, in fact, Bu hinted at new evidence that endothelial LRP has little impact on Aβ clearance from the brain. In any event, the mouse genetic data indicate that LRP1 in brain vasculature smooth muscle cells plays an important role in Aβ clearance. Bu said that Aβ may downregulate LRP1 in smooth muscle cells, potentially setting off a vicious cycle that blocks Aβ clearance. Bu, Holtzman, and meeting co-organizer Joachim Herz co-wrote a chapter on ApoE for *The Biology of Alzheimer Disease* on the current state of research in the field (see ARF book review).

Probing the Function of Lipoprotein and Related Receptors

On the face of it, lipoprotein receptors may not sound like they have much to do with Alzheimer's disease, or even the central nervous system. But as scientists are finding out more about these multifaceted cell-surface proteins, they are discovering just how intimately involved they are in the care and maintenance of neurons and their synapses. At "ApoE, Alzheimer's and Lipoprotein Biology," a Keystone symposium held 26 February-2 March, 2012, presentations reflected the

breadth and depth of the biology of these receptors. The conference drove home to attendees how the receptors' functions dovetail with neurobiology and, potentially, neurodegeneration.

One member of the low-density lipoprotein receptor family that is familiar to AD researchers is SorLA (short for the unfortunate mouthful, sortilin-related receptor, low-density lipoprotein receptor class A repeat-containing protein). In the last decade, researchers, including those in **Thomas Willnow's** lab at the Max Delbruck Center for Molecular Medicine, Berlin, Germany, discovered that SorLA (aka SORL1 and LR11) regulates processing of amyloid-β (Aβ) precursor protein (see ARF related conference story). Variants in the SorLA gene subsequently emerged as risk factors for late-onset AD (see ARF related news story). Using overexpression and knockout models, researchers gradually built a picture of the protein sequestering APP and keeping it away from endosomes, where β- and γ-secretases would process it to release Aβ. That is the simple view, Willnow said at the Keystone symposium. In fact, he said, exactly how SorLA regulates APP processing is still being worked out. The overexpression and knockout models are probably too drastic to reflect what happens in a physiological setting. In a talk that stood out for detailing a rigorous biochemical approach, according to meeting co-organizer Joachim Herz of the University of Texas Southwestern Medical Center, Dallas, Willnow reported how even slight tweaks in levels of APP and/or SorLA profoundly affect their choreography, suggesting that modest changes in levels of SorLA may be meaningful in AD.

Willnow used the tetracycline (tet-off) system for controlling gene expression to alter transcription of both SorLA and APP by small increments in a cell-based system. He mathematically modeled the relationship between the two proteins and the production of sAPPα and sAPPβ. His data basically boiled down to a major kinetic finding, namely, APP processing in the absence of SorLA does not follow Michaelis-Menten kinetics. For those who remember their biochemistry, that predicts a simple enzyme-cleaves-substrate type of reaction. Instead, the data fit Hill kinetics, which assumes cooperativity between APP molecules, said Willnow. In fact, the Hill coefficient for APP processing is 2.0, which implies that secretases preferably process APP as a dimer. In the presence of SorLA, the coefficient reverts to 1.0, indicating non-cooperativity and APP monomer processing.

How could SorLA alter kinetics? Willnow's data indicate the lipoprotein receptor and APP together form a dimer, and SorLA prevents APP dimerizing with itself, at least in Chinese hamster ovary cells. Western blots revealed an APP dimer on native gels, which disappeared upon coexpression of SorLA. Mouse brain showed a similar pattern, whereby extracts from wild-type mice contained big and small APP species, but extracts from SorLA knockouts only the larger.

"This work really takes us back to basic principles and gives us the molecular details we need to understand how these receptors work," Herz told Alzforum after Willnow's talk. The kinetic data are particularly relevant to normal physiology, said Willnow, because if there is cooperativity in processing, then a small change in APP concentration can have a large change on Aβ production. It could also explain how non-coding genetic variants that modestly perturb SorLA

expression might affect risk for dementia. Researchers have been struggling to interpret how non-coding genetic variants uncovered by genomewide association studies alter risk.

Not just SorLA, its relatives, too, came up for discussion at the symposium. SorLA is part of a family of Vps10p domain receptors (named after the vacuolar protein sorting 10 protein domain that they all share) that also contains SorCS isoforms and sortilin. **Anders Nykjaer**, Aarhus University, Denmark, together with Stephen Strittmatter at Yale University, New Haven, Connecticut, found that sortilin binds progranulin and carries it to the lysosome for degradation (see ARF related news story on Hu et al., 2010). Genetic variants near the sortilin gene are also risk factors for frontotemporal lobar degeneration (FTLD) (Carrasquillo et al., 2010). Reducing sortilin could, therefore, elevate levels of progranulin, which is essential to stave off FTLD (see ARF related news story). But as Nykjaer explained in his talk, the picture is not so simple.

Nykjaer and colleagues found that sortilin regulates the balance between long-term potentiation (LTP), and long-term depression (LTD) because it controls levels of brain-derived nerve factor (BDNF). Working in cooperation with tyrosine receptor kinase B (TrkB), BDNF promotes LTP, while the immature proBDNF, working through p75, induces LTD (see ARF related news story on Woo et al., 2005). Nykjaer showed that sortilin stabilizes proBDNF, and that without the receptor, proBDNF quickly degrades and LTD dwindles, while short-term LTP escapes unscathed. However, late-phase LTP, which depends on localized conversion of proBDNF to BDNF at synapses, is weakened in sortilin knockouts.

What could this mean for synaptic activity in a physiological setting? Nykjaer showed that mice without sortilin have behavioral problems. They respond to some environmental challenges in a similar fashion to people who suffer from bipolar disorder or schizophrenia, said Nykjaer, and in Denmark, genetic screens uncovered single nucleotide polymorphisms that are linked to such disorders.

Neuromuscular Junction as a Model System

Steve Burden, New York University, also addressed synaptic roles for lipoprotein receptors. Burden looked to the neuromuscular junction (NMJ) to identify functions for these receptors, and he emphasized that some of the same molecules once thought to exist solely at NMJs have since been discovered in synapses in the central nervous system. Several researchers at the meeting agreed that looking at NMJs may help scientists understand synapse maintenance and loss in the CNS. This is germane to Alzheimer's pathology, he noted, in the sense that the disease is widely believed to be one of synaptic failure. Lipoprotein receptors turn out to be essential for the maintenance of NMJs, raising the possibility that they may also be indispensible for synapses in the brain.

Burden's talk focused on low-density lipoprotein receptor-related protein 4 (Lrp4), which coordinates the clustering of acetylcholine (ACh) receptors on the muscle side of the neuromuscular synapse. Burden noted that Agrin, a protein released by motor neuron axons, binds Lrp4. The lipoprotein receptor in turn activates MuSK kinase, setting off a signaling cascade that upregulates expression

of ACh receptor genes on the post-synaptic side of the junction. This cascade plays out as developing motor neurons seek out muscle tissue to innervate. Early in development, Lrp4 binds and activates MuSK independently of Agrin, reported Burden. This sets up an initial incorporation of ACh receptors into the muscle in anticipation of the arrival of the motor neuron. Agrin then boosts the Lrp4-MuSK interaction 50-fold, stabilizing the NMJs. Burden's lab found that only the ectodomain of Lrp4 is essential for these interactions. He rescued the Agrin response in Lrp4-negative cells by simply expressing this external domain of Lrp4, or a chimera with the intracellular domain substituted with one from the CD4 receptor (see Gomez and Burden, 2011).

So far so good—but what happens on the motor neuron side of the equation? That is mostly unknown, said Burden. Without MuSK or Lrp4, motor neuron axons do not stop when they reach muscle cells, but keep growing around and pass them. Burden wondered if Lrp4 corrects this by activating MuSK and setting off signals solely within the muscle tissue, or if it somehow signals directly to the developing axon. To test this, Burden and colleagues co-cultured motor neurons with fibroblasts engineered to express Lrp4. Lo and behold, these cells induced clustering of presynaptic vesicle and active zone proteins in the motor neuron axons. Lrp4-coated beads did, too. The scientists found that the Lrp4 ectodomain binds to motor neurons, supporting the idea that the lipoprotein receptor directly signals the cells. He concluded that the lipoprotein receptor controls both the muscle and the neuron side of the developing NMJ. The related Lrp1 and Lrp6 had no such effects, suggesting the property may be unique to Lrp4.

How could this be relevant to the brain, or to AD? CNS expresses Agrin, Lrp4, and MuSK, noted Burden, and their roles there are unclear. But given that Agrin prevents synapse loss in the cortex (see Ksiazek et al., 2007), its signaling might be relevant not just to the neuromuscular system, but also to neurodegenerative disease.

ApoE Receptors and Ligands in Memory and AD

Of the many facets of ApoE's biology, the most complex one may be its activation of cell surface receptors. ApoE binds to a family of low-density lipoprotein receptors (LDLRs), and scientists are just beginning to parse how those interactions influence cell and neurobiology in adult animals. At "ApoE, Alzheimer's and Lipoprotein Biology," a symposium held 26 February-2 March 2012 in Keystone, Colorado, the functions of those receptors in neural plasticity emerged as a major theme.

A quintessential example of LDLR signaling is in development. Reelin, an LDLR ligand, is a de-facto competitor of ApoE and, hence, inextricably entwined with its effects on receptors and downstream signaling in neurons. Reelin has long been known to play an essential role in migration of neural cells and pattern formation in the brain. In reeler mice, a naturally occurring mutant strain that completely lacks this protein, Purkinje cells fail to navigate to their proper place in the cerebellum and the mice develop severe ataxia. The cerebral cortex and other laminated structures of the brain develop abnormally as well, and the mice die soon after birth. While reelin was traditionally viewed as a developmental

regulator, in recent years scientists have taken a second look at the protein's role in the adult brain.

At Keystone, **Gabriella D'Arcangelo**, Rutgers University, Piscataway, New Jersey, noted that reelin expression in the brain changes soon after birth. Rather than being produced solely by the Cajal Retzius cells that orchestrate embryonic neurodevelopment, reelin occurs throughout the brain in adult mice. What is its function? To test this, D'Arcangelo has been studying mice heterozygotes for reelin-null genes. She noticed a paucity of dendrites in these animals shortly after birth, but by postnatal day 21, the dendrite complement looked the same as in wild-type animals. The data indicate that maturation of brain connections in reelin-deficient animals may just take a little longer than usual. D'Arcangelo and colleagues also found that fewer spines decorate dendrites in postnatal reelin heterozygotes, and that recombinant reelin from conditioned medium restores spine numbers to cultured hippocampal cells. Their data suggest that well after birth, reelin helps the maturation of synapses.

If reelin is still important in the postnatal period, what about in adults? In 10-month-old reelin heterozygotes, the numbers of both dendrites and spines appear normal, again suggesting that the brain eventually matures even with less-than-normal reelin. However, on closer inspection, D'Arcangelo and colleagues did find that not all is quite right with dendritic spines. For example, while the total amount of the important synaptic scaffold protein post-synaptic density 95 (PSD95) appears the same as in normal mouse brain, considerably less of it makes its way into spines. Similarly, spines contain too little of the NR2A and NR2B glutamate receptor subunits and the PTEN kinase (see Ventruti et al., 2011). These proteins form a complex with PSD95, perhaps explaining why all three are deficient in reelin heterozygotes, said D'Arcangelo. These molecular differences may help explain synaptic plasticity and learning and memory deficits in these animals. They may also relate to Alzheimer's, D'Arcangelo said, since postmortem analysis indicates less reelin is produced in the brains of people with AD, and spine and synapse losses are characteristics of the disease. Transgenic APP mice make less reelin that do wild-type animals (see Chin et al., 2007 and ARF related news story).

How might reelin loss play into AD pathology? The protein is a major ligand for ApoE receptor 2 (ApoER2) and the very low-density lipoprotein receptor (VLDLR). Signaling through these receptors supports synaptic plasticity and may be antagonized by ApoE4. Working with **Edwin Weeber**, University of South Florida, Tampa, D'Arcangelo found that reelin heterozygotes underperform in certain learning and memory paradigms and have synaptic deficits, including weaker long-term potentiation. Weeber has been testing if reelin can rescue deficits in adult rodents. At the conference, he wowed the audience with dramatic effects of reelin on learning and memory, not only in reelin heterozygotes, but even in wild-type.

Weeber previously reported that reelin rescues spatial navigation deficits driven by RAP, a protein that binds and blocks all ApoE receptors. Reelin also corrects learning and memory deficits in reeler heterozygotes, which are haploinsufficient (see ARF related conference story).

Weeber and colleagues use thin tubes, or cannulae, to deliver reelin into brain ventricles. From there, the ligand reaches the hippocampus, where it activates the downstream kinase Dab1 and induces CREB phosphorylation. Weeber showed that in wild-type mice, a single shot of reelin to the ventricles boosts both spine density and long-term potentiation in the hippocampus. The enhancement seems confined to the post-synapse, since paired-pulse facilitation, which relies on presynaptic strengthening, was unchanged. Mice that are missing ApoER2 fail to respond to reelin, singling out that receptor as one that initiates synaptic changes in response to this ligand, said Weeber.

Do these molecular and electrophysiological effects amount to any behavioral changes? Weeber showed that a single shot of reelin to the ventricle substantially improved spatial learning and memory in wild-type mice. The researchers used the Morris water maze. It involves several days of training, during which mice get progressively faster at finding an underwater platform. Typically, differences between control and treated animals emerge after a few days; in this case, however, the treated mice did so much better that the difference was statistically significant on day one. Each day's training involves four trials in the water bath, said Weeber, and the treated mice already outperformed controls by the third trial.

Researchers at the meeting seemed impressed by how fast and robustly the learning improved. But lest anyone considers popping reelin pills, the long-term effects are not clear. During question time, it emerged that if animals are given reelin daily, they become quite dumb, falling far behind untreated controls in the water maze.

Nevertheless, researchers at the meeting wondered if reelin might rescue deficits in disease models. Weeber said he is currently testing AD mice in the same experimental design and is planning to look at mice expressing human ApoE isoforms. He reported that reelin rescues spatial memory in a model of Angelman syndrome, a developmental disease where loss of a ubiquitination factor essential for regulating synapse architecture causes motor and cognitive deficits.

In his talk, meeting co-organizer **Joachim Herz**, University of Texas Southwestern Medical Center, Dallas, noted that reelin and Aβ seem to antagonize each other. While the ApoE receptor ligand boosts LTP, and learning and memory, Aβ suppresses the latter and strengthens long-term depression (LTD). Herz previously outlined how ApoE isoforms play into this dynamic. On binding to receptors, ApoE4, a major risk factor for AD, induces their uptake and sequestration inside the cell, thereby limiting reelin signaling at the cell surface (see ARF related conference story). This ultimately retards incorporation of glutamate receptors on the cell surface, said Herz. It also prevents reelin from rescuing against Aβ-induced LTD. ApoE3 and E2, in contrast, do not perturb ApoER2 distribution in the cell or limit reelin signaling.

Could ApoER2 sequestration by ApoE4 be prevented or reversed? Herz and colleagues screened for small molecules that can do just that. They identified several compounds that Herz says are promising and awaiting patent protection. He showed that, in an ApoE4 background, one of the compounds normalized ApoER2 on the cell surface and restored the ability of reelin to rescue Aβ-induced

synaptic deficits. How the compound works is not exactly clear, but Herz hinted that it perturbs intramolecular domain interactions that are unique to ApoE4.

How does Aβ antagonize reelin signaling? Researchers are still trying to understand the toxic effect of the peptide, but in his short talk **Steve Barger**, University of Arkansas for Medical Sciences, Little Rock, reported that both ApoE4 and some forms of Aβ are competitive antagonists of ApoER2 and/or VLDLR ligands, and thereby block signaling. ApoE2 and 3 activate these receptors, Barger said, because they have at least one cysteine and form disulfide dimers that span the divide between individual receptors and thereby stabilize receptor dimers. Because ApoE4 lacks a crucial cysteine residue, it cannot form dimers. Instead, it appears to bind the receptors as monomers, blocking dimeric ligands and suppressing signaling. Barger used a luciferase reconstitution assay, with N- and C-terminals of the enzyme on different ApoE receptors, to demonstrate ApoE receptor dimerization by reelin and ApoE2/3. These agonists also enhanced NMDA receptor activity in a manner sensitive to RAP, or knockdown of ApoER2 expression. Interestingly, while he found that fibrils of Aβ did the same, Aβ oligomers bound to the receptors but did not induce dimerization; instead, they blocked the effects of reelin on dimerization and NMDAR activation. Barger concluded that both ApoE4 and Aβ oligomers suppress reelin signaling in the same manner.

Reelin perturbations have been recorded in Alzheimer's disease, though human genetic data at present are sparse (see reelin on AlzGene). Could these have ramifications unrelated to synaptic signaling? In addition to being downregulated in the AD brain, reelin has been reported to associate with amyloid plaques in AD and in transgenic mouse models. In Keystone, **Irene Knuesel**, University of Zurich, Switzerland, wondered how it ends up there. Does it have to bind to Aβ, or can it accumulate on its own? Previous work suggested that reelin with the C-terminus aggregates, said Knuesel (see de Bergeyck et al., 1997), but the proteases that generate those fragments and how they relate to AD pathology are completely unknown. Reelin is itself a protease. Knuesel described how neuroinflammation may exacerbate Alzheimer's pathology by triggering proteolysis and aggregation of reelin.

Knuesel and colleagues searched for reelin proteases in p19 carcinoma cells. While undifferentiated, these multipotent cells express no reelin protease, but when pushed toward neurogenesis with retinoic acid, they cleave reelin into several fragments consistent with N- and C-terminal proteolysis, said Knuesel (see Ducharme et al., 2010). Knuesel and colleagues used these cells to identify ADAM metallopeptidase with thrombospondin type 1 motif, 4 (ADAMTS-4) and tissue plasminogen activator (tPA) as the enzymes that cut reelin near the C-terminus.

To test if reelin proteolysis dovetails with AD pathology, Knuesel injected the virus simulator polyI:C into pregnant mice to induce neuroinflammation in their offspring. In the process, reelin became cut, and its proteolytic fragments accumulated in axon terminals and neurites, said Knuesel. When she treated prenatally exposed mice with a second shot of polyI:C in adulthood, they formed intraneuronal aggregates that Knuesel thinks may contain Aβ precursor protein.

Some researchers wonder if these are Hirano bodies, which contain actin and are sometimes associated with AD. In a transgenic mouse model of AD, polyI:C elevated soluble Aβ and tau phosphorylation, and led to a dramatic increase in amyloid plaques. All told, Knuesel said the believes that proteolytic fragments of the ligand may form seeds for aggregation of proteins, including reelin itself and Aβ.

Does ApoE Fragmentation Drive Pathology?

Fragmentation can bog down your hard drive, and some scientists think it also wreaks havoc on processing in the brain. At "ApoE, Alzheimer's and Lipoprotein Biology," a Keystone symposium held 26 February-2 March 2012 in Keystone, Colorado, researchers discussed whether cleavage of the notorious lipoprotein explains why it is the strongest genetic risk factor for Alzheimer's disease. Fragments of ApoE have been reported to poison neurons, and ApoE4, the isoform that confers AD risk, gives itself up to proteolysis more readily than does ApoE2 or E3. Stopping that proteolysis, then, might seem a therapeutic approach, and several presentations at the symposium focused on that idea. But as the AD field has come to acknowledge over the years, nothing about ApoE is straightforward.

Fragmentation represents but one way in which ApoE4 may increase risk for AD. Many researchers in the field believe it may be secondary to ApoE's more widely accepted meddling with Aβ and synaptic plasticity. At Keystone, a hypothetical scenario emerged to tie these nefarious properties together. The hypothesis has Aβ and plasticity effects driving early pathology; proteolytic fragments would emerge later as cells begin to ramp up ApoE production in an attempt to repair ongoing neuronal damage. (ApoE, being the major lipid and cholesterol carrier in the brain, plays a crucial role in the repair of cell membranes.)

Robert Mahley, from the Gladstone Institute for Neurological Disease, San Francisco, California, said in his keynote address that ApoE4 is a risk factor for other neurodegenerative diseases as well, such as Parkinson's, multiple sclerosis, and traumatic brain injury. (ApoE is listed in PDGene and MSGene, though not among the Top 10.) Mahley suggested that ApoE might play a role beyond AD because it sets the stage for multiple "second hits" that promote different downstream pathologies. Blocking ApoE proteolysis, then, might stem deterioration of neurons quite broadly in neurodegenerative and brain injury conditions.

Why does ApoE4 more readily undergo fragmentation than the other isoforms? Together with Gladstone colleagues Karl Weisgraber and **Yadong Huang**, Mahley reported years ago that ApoE4 assumes a different tertiary structure from ApoE2 and E3, rendering it vulnerable to proteases (see ARF related news story). In ApoE4, substitution of an arginine for a cysteine at position 112 frees up other amino acids in the N- and C-termini of ApoE4 to interact, and this domain interaction exposes amino acids to proteases that are hidden in ApoE2 and 3. One of the criticisms researchers have voiced of this scenario is that lipidation of the protein protects it from proteolysis, and ApoE is mostly lipidated.

Nevertheless, if proteolytic susceptibility is ApoE4's Achilles' heel, Mahley and colleagues would like to save it from protease arrows. For about a decade, the scientists have worked on developing "structural correctors." As outlined in a paper published last month (see Chen et al., 2012), a high-throughput, fluorescence resonance energy transfer (FRET) assay identified small molecules that disrupt ApoE4 domain interactions in cultures of neuronal cells. These molecules reduce ApoE fragmentation in neuronal cells and prevent ApoE4-induced mitochondrial toxicity. They also unblock stalled endoplasmic reticulum/Golgi trafficking of ApoE4 to levels seen in cultured neurons expressing ApoE3. Intracellular sequestration of cell-surface receptors by ApoE4 attenuates receptor signaling and weakens synaptic signaling.

In the NSE-ApoE4 mouse model, structural correctors given daily for 10 days reduced ApoE4 fragments in the whole brain, including the hippocampus, by about a fifth, said Mahley. The treatment also boosted levels of the mitochondrial enzyme cytochrome c oxidase 1 by half. Its loss indicates damage to the organelles, and its levels are lower in NSE-ApoE4 mice than controls. Researchers at the meeting were intrigued by the cytochrome c oxidase rescue, and felt that other groups may want to replicate the effect. In response to questions about clinical development, Mahley said the molecules are being modified as potential therapeutics, and suggested they may be ready for testing in the clinic in two years.

While Mahley and colleagues have identified the most toxic of the known ApoE4 fragments—they are those containing the C-terminal lipid-binding domain—which protease generates them remains a mystery despite years of effort to identify it. The main proteolytic sites are methionine 272 and leucine 268, and the protease(s) responsible seem unique to neurons, noted Mahley. Fragments are not found in other brain cells or in peripheral cells.

ApoE fragments are found in the human brain, as **Yadong Huang**, also from the Gladstone Institute, noted in his talk. His group examined tissue samples from 41 human volunteers—25 AD patients and 16 controls of different ApoE genotypes. Huang reported that there are very few fragments in the brain tissue from controls, twice as many in AD cases, and that homozygote ApoE4 carriers had more fragmentation than heterozygote carriers. ApoE4 carriers had more fragments regardless of whether they had dementia, and AD patients homozygous for ApoE3 showed more fragmentation than did homozygous ApoE3 controls. This is an important point, Huang said during questions time. "ApoE3 can get proteolytically cleaved," he said, "and in some AD cases, perhaps the protease responsible is elevated." Huang said that he also sees ApoE fragments in plaques and in tangles.

In general, the AD field at large has generated little independent evidence to corroborate findings on ApoE fragmentation. Researchers debate whether, and under what circumstances, neurons express ApoE, and data on neuronal ApoE fragmentation have not caught on in a broad way. However, researchers in Bradley Hyman's lab at Massachusetts General Hospital, Charlestown, did report seeing more ApoE N-terminal fragments in plaques in Alzheimer's cases than controls (see Jones et al., 2011).

The human brain appears to have three major ApoE fragments of 29, 14-20, and 12 kDa, respectively, Huang said. His group has since looked in cerebrospinal fluid and found these three fragments among a small number of cases. Huang is now working with collaborators to test a larger number of CSF samples. If the fragments reliably turn up in the CSF, then they may form the basis of a future diagnostic test for neuronal damage, he suggested. At present, CSF tau and phospho-tau are the leading CSF markers for neuronal damage.

Given that ApoE fragmentation only seems to occur in neurons (see Brecht et al., 2004), are all neurons equally at risk? Huang's group reported at Keystone that hilar GABAergic interneurons seem particularly vulnerable. In a mouse model expressing a truncated ApoE found in human brain, these neurons are decimated by the time the animals reach 12 months of age (see Andrews-Zwilling et al., 2010), and spatial memory deficits in the mice correlate with the loss. While neuron loss is a hallmark of AD, many established animal models do not recapitulate it. Some researchers at the symposium were intrigued that it occurs in this model. Tau pathology emerges in these neurons as well, noted Huang, and genetically removing the microtubule-binding protein protected against both neuron loss and learning deficits.

To test what ApoE fragments and tau might have to do with the health of GABAergic neurons in people, Huang is now studying induced pluripotent stem (iPS) cells generated from non-demented older individuals. When generating neuronal cultures from these iPS cells, he saw fewer GABAergic neurons from ApoE4/4 than ApoE3/3 donors, indicating ApoE4 stem cells have a hard time making this particular type of cell; total neuron production was normal. Looking more closely at the E4 cells, the scientists found more ApoE fragmentation, and more tau phosphorylation as judged by binding of the AT8 antibody. Again, the structural correctors rescued both phenotypes and boosted expression of the GABAergic neuron marker GAD67, Huang said.

Huang believes that, rather than block differentiation of GABAergic neurons, ApoE4 brings on their premature death. Cell culture stresses the cells, he said, and that makes them produce a lot of ApoE. In this sense, the culture may mimic what is going on in the brains of people with AD or other neurodegenerative diseases. Huang plans to use this system to study why tau gets phosphorylated. So far, it appears there was no change in the kinases typically suspected of modifying tau, including GSK-3β and Cdk5; instead, Huang thinks tau phosphorylation may be kick-started through the reelin/ApoE receptor signaling pathway.

Therapies Around ApoE—Has Their Time Come?

Even though ApoE stands head and shoulders above other genetic risk factors for late-onset Alzheimer's disease, few therapeutic strategies aimed at ApoE have made it to clinical trials. That may be about to change. At "ApoE, Alzheimer's and Lipoprotein Biology," a five-day Keystone symposium held 26 February-2 March 2012, researchers delved into ApoE biology and touted potential remedies to match. Boosting lipidated ApoE with retinoid X receptor agonists, elevating signaling through ApoE receptors, and blocking proteolytic fragmentation of the apolipoprotein were among the ideas that could be put to the test. Here are some

more, as well as updates on solanezumab and a BACE inhibitor presented at Keystone.

In her presentation, **Kelly Bales** of Pfizer in Groton, Connecticut, outlined a screening program to search for small molecules that raise ApoE levels. Bales uses an ApoE promoter-driven luciferase gene to measure expression changes in a human astrocyte cell line. Hits included RXR and liver X receptor agonists, and also histone deacetylase (HDAC) inhibitors (see ARF related news story). At Keystone, Bales talked about delving deeper into the role of HDACs to find specific deacetylases that regulate ApoE. While there are five classes of HDACs, astrocytes primarily express class I and II. A small interfering RNA that suppressed expression of all class I enzymes modestly raised ApoE expression, said Bales, whereas knocking down class II HDACs achieved more dramatic results. However, she noted that HDAC inhibition modulates expression of other genes as well. Blocking class I HDACs stimulated production of ABCA1, a cholesterol and phospholipid transporter. ABCA1 keeps ApoE lipidated, which seems crucial for ApoE-mediated clearance of Aβ from the brain. Blocking class I HDACs also suppressed astrocytic interleukin 6 (IL6), a proinflammatory cytokine. With these pleiotropic effects, HDAC inhibition might be a novel way to tackle neurodegenerative diseases, suggested Bales.

Keeping with the inflammation theme, **Michael Vitek**, Duke University Medical Center, Durham, North Carolina, outlined a strategy for tamping it down with ApoE mimetics. Vitek cited lines of evidence that point to ApoE3 being anti-inflammatory, while ApoE4 falls short in that respect. For example, macrophages from ApoE4 targeted replacement (TR) mice generate much more of the proinflammatory protein tumor necrosis factor α (TNFα) and IL6 than those from ApoE3 TR mice. Vitek founded the biotech company called Cognosci, Inc., to develop compounds that mimic ApoE3 anti-inflammatory properties. The company is evaluating candidate drugs for AD, multiple sclerosis, and traumatic brain injury.

The compounds are short peptides that correspond to the receptor-binding domain of ApoE3. The prototype, COG133 (amino acids 133-149 of the protein), reduced inflammatory responses in human blood ex vivo and in the CNS and the periphery of mice treated with lipopolysaccharide, which induces inflammation.

Two analogs, COG112 and COG1410, protected the CVND transgenic mouse model of AD developed by Vitek and Carol Colton at Duke (APPSwDI/NOS-/-; see ARF related news story). These mice develop robust plaques by 12 months of age and show substantial neuron loss, something other models recapitulate less successfully. Vitek and colleagues gave subcutaneous COGs to these animals at nine months. Three months later, the animals made less IL6 in the brain, maintained more of their neurons, grew fewer plaques and neurofibrillary tangles, and navigated better in the radial arm water maze than did untreated littermates (see Vitek et al., 2012). Vitek and colleagues also tested compounds in a model of traumatic brain injury. Given two hours after injury, the ApoE mimetics improved survival, strength on the rotarod, and cognition in the Morris water maze as compared to untreated animals, Vitek said.

What is the mechanism of action of these compounds? Researchers at Cognosci used biotin-labeled compounds to fish out binding partners and found strong binding to the protein SET/I2PPA, short for inhibitor No. 2 of protein phosphatase A (PP2A). The compounds appear to relieve inhibition of PP2A, said Vitek, because they reduce the amount of phosphorylated MAP kinase, p38, and JNK kinase in cells. The mechanism intrigued researchers at the meeting, who wondered whether the reductions in plaque and tangle pathology are due to toning down inflammation, or are more directly due to activation of PP2A. Vitek said that was not known, though he noted that PP2A inhibitors boost tau phosphorylation.

Though their candidates are not directly related to ApoE or lipoproteins, researchers from Lilly and Merck reviewed the status of some of their drugs in clinical trials. **Eric Siemers** from Lilly first spoke about the failure of semagacestat, the company's γ-secretase inhibitor (see ARF related news story). Siemers emphasized how important it is to learn as much as possible from that trial. His take-home message was that, while the company had struggled with getting optimal dosing of the drug in Phase 2, it eventually took appropriate doses into Phase 3, where biomarker analysis confirmed that the drug indeed reached its target in the brain. CSF levels of Aβ1-16 rose in patients taking the drug, a finding in keeping with predictions from Kaj Blennow's lab at University of Gothenburg, Sweden. This short fragment appears when β-cleavage of APP is followed, unusually, by α-, not γ-cleavage, which could happen when the latter is blocked (see ARF related news story). Despite engaging its target as predicted, the drug caused a decline in cognition. Siemers believes that was likely due to blocking γ-secretase cleavage of substrates other than APP, of which some 50 are known. Companies are now pursuing γ-secretase modulators that tweak APP cleavage while allowing processing of other substrates, including Notch.

Lilly's solanezumab, a humanized mouse monoclonal antibody to Aβ, is in Phase 3 (see ARF related news story). Noting that the trials will end shortly, Siemers presented no new data at Keystone. Reviewing some pre-Phase 3 biomarker data, he noted that in mouse models and humans, the antibody dose-dependently raised plasma and CSF Aβ, implying that the antibody pulls some Aβ out of the brain (see ARF related conference story). In mice, it reduced plaque load. What about humans? Tantalizingly, Siemers said that pyroglutamate-modified Aβ appears in plasma of people who received the antibody. "You don't normally see pyroglu-Aβ in the blood, so this is an indication that bits of plaque are ending up there," said Siemers.

Eric Parker of Merck Research Laboratories, Kenilworth, New Jersey, gave an update on his company's BACE program. BACE has been a tough nut to crack from a pharmaceutical perspective. Parker said the key is saturating BACE in the brain. Merck has achieved that goal now with the drug MK-8931, Parker claimed. In a two-week-long Phase 1 trial in healthy volunteers, MK-8931 reduced Aβ in the CSF by 90 percent. The company presented those data at an investors' meeting in November 2011 (see slides 152-155 of the presentation).

So far, the drug appears to be safe enough for further human testing, though other BACE inhibitors have failed in late Phase 1 (see ARF related conference story).

In mice, rats, and monkeys, MK-8931 does not affect nerve conductance or prepulse inhibition, which are suppressed in BACE knockout animals and are likely related to myelination defects during development, Parker said. The company is planning a Phase 2 trial to start this year. For that, they are using a modeling strategy for dose finding since the drug's inhibition of BACE during Phase 1 was too strong for appropriate dose ranges to be determined. Merck is currently recruiting for a second Phase 1 dose-finding trial in Alzheimer's patients to determine a mean inhibitory concentration. This will overlap with the planned Phase 2 study. The company hopes this parallel-phase strategy will save development time, according to a company spokesman.