From Science to Therapeutics: The Best Way Forward
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By Esther Landhuis

**Therapeutic Approaches Target Deubiquitinase, Protein Turnover**

Unlike scientific meetings that focus on a particular protein, pathway, or disease, the one that drew some 150 researchers to San Francisco 15-17 April 2013 featured an unusually broad array of topics from nitty-gritty molecular to bird’s-eye conceptual. “From Science to Therapeutics: The Best Way Forward” was the catch-all theme for this annual meeting sponsored jointly by the Gladstone Institute of Neurological Disease, San Francisco, and the German Center for Neurodegenerative Diseases (DZNE), Bonn. Their annual meetings alternate between sites. Last year’s workshop in Germany explored the role of synapses in neurodegenerative disease. The inaugural GIND/DZNE event in 2011 covered tau and tauopathies (see ARF conference series).

**Lennart Mucke** at the Gladstone Institute and **Pierluigi Nicotera** of the DZNE organized this year’s meeting with the complexity of AD in mind. “We deliberately brought together investigators pursuing diverse leads and strategies to comprehensively address the challenges posed by Alzheimer's disease,” Mucke told Alzforum. In addition, the organizers chose to separate presentations on similar themes and approaches, rather than cluster them together. “We did this to keep the audience engaged at all times and to prevent different groups from ‘tuning out’ during talks that fall outside their immediate areas of interest,” Mucke said.

In addition to new data on the physiological function of β-secretases and novel efforts to co-opt a longevity gene, attendees heard a handful of talks on therapeutic approaches that mobilize protein quality control mechanisms to keep amyloidosis in check. One of those promotes ubiquitin-proteasome degradation to counteract pathogenesis. Several others presented at the meeting expand or tweak proteostasis mechanisms to help rid cells of misfolded proteins.

**Li Gan** of the Gladstone Institute explores how acetylation and ubiquitination contribute to tauopathies by controlling tau degradation. Gan and colleagues previously reported that tau can be acetylated in cultured cells and mice that model tauopathies. They detected acetylation in AD brains at early Braak stages and showed that this post-translational modification keeps protein degradation machinery from clearing tau out of cells (see ARF related news story on Min et al., 2010). More recently, Virginia Lee and John Trojanowski at the University of Pennsylvania School of Medicine, Philadelphia, as well as Gan, reported finding
acetylated tau in human tauopathies (see ARF related news story and Irwin et al., 2012).

In San Francisco, Gan discussed her lab’s latest work, showing that acetylated tau can impair dendritic sorting and microtubule dynamics. Her lab generated antibodies to acetylated tau and used them to map three acetylation sites—two in tau’s microtubule-binding region and one in its amino-terminal “projection domain,” which seems to determine microtubule spacing. The researchers identified the acetylation sites from AD brain extract using mass spectrometry. One of them—but not other residues in tau’s microtubule-binding domain—drives proteasome-mediated tau degradation, Gan reported. In collaboration with Dan Finley at Harvard Medical School in Boston, Gan found that a small molecule inhibitor of the deubiquitinase USP14 enhanced proteasome activity and degradation of tau in primary rat neurons expressing human tau. Conversely, the researchers found no such protection in neurons expressing an acetyl-mimic tau that cannot be ubiquitinated at the critical site. Furthermore, transgenic mice expressing this tau mutant in the hippocampus were hyperactive and did not adjust properly to their surroundings in an open field test that measures anxiety, Gan said.

To explore mechanisms underlying the behavioral impairment of this acetyl mimic, the researchers used fluorophores to label and track tau’s movement on microtubules in primary neurons. They found that the acetyl-mimic tau crossed the axon initial segment—a specialized membrane region where neurons initiate axon potentials—more readily than wild-type tau. “We think acetylation at the microtubule-binding domain makes tau hyperdynamic, missorting it to the somatodendritic compartment,” Gan said.

With an emerging picture of how tau acetylation endangers neurons, the Gladstone scientists are also exploring therapeutic approaches that block the histone acetyltransferase p300, an enzyme they determined to acetylate tau in their 2010 study. They are screening for additional p300-blocking molecules, and plan to generate conditional p300 knockouts and cross those with AD and tauopathy mouse models, Gan said.

However, p300 may not be the only tau acetylase. In an intriguing twist, Lee and colleagues at UPenn reported last month that tau can acetylate itself, identifying key cysteine residues in the microtubule-binding domain that are involved in the catalytic activity (Cohen et al., 2013). Gan and others found the data interesting, but said future work is needed to validate the in-vivo relevance of the auto-acetylation, and determine if it correlates with human disease progression.

Exactly what tau’s own acetylase activity contributes is still nebulous, but recent work by Gan and William Seeley at UCSF raises the possibility that tau might require acetylation to become toxic. Using a monoclonal antibody specific for tau acetylated at K274, the researchers probed 22 brain samples from people with AD and eight other tauopathies. They detected acetylated tau ac-K274 in all cases except argyrophilic grain disease (AGD)—a rare condition most often seen in people with long-lasting amnestic mild cognitive impairment that does not progress clinically. “The fact that [the AGD cases] are negative for tau ac-K274 is
consistent with the notion that that tau acetylation may be required to accelerate tau toxicity,” Gan noted in an e-mail to Alzforum.

**Proteostasis to the Rescue?**

The theme of protein degradation also figured prominently in a presentation by Jeff Kelly of Scripps Research Institute, La Jolla, California, who described enlisting the unfolded protein response (UPR), a signaling pathway activated when misfolded proteins accumulate in the endoplasmic reticulum. The concept is simple, in theory: Get the system to degrade mutant proteins while still properly folding the wild-type. In practice, this may be hard to achieve, said Kelly, because the UPR activation turns on three transcription factors that each drive expression of distinct but overlapping sets of regulators that control protein degradation. Researchers are unsure which transcriptional program targets amyloidogenic proteins, such as those implicated in neurodegenerative diseases.

Kelly, together with Scripps colleague Luke Wiseman, devised a way to produce two UPR-associated transcription factors—X-box binding protein 1 (XBP1) and activating transcription factor 6 (ATF6)—at physiological levels within the same cell. They turned on XBP1 using a conventional tetracycline-based promoter. To raise ATF6 levels in the cell, the scientists attached the transcription factor to a mutated variant of *E. coli* dihydrofolate reductase (DHFR), which does not fold properly. Normally, the cell’s proteosomal degradation system makes quick work of ATF6-DHFR fusion proteins because they are highly unstable. However, add some trimethoprim, a molecule that stabilizes the DHFR domain, and the chimera accumulates to high enough levels to trigger transcription of ATF6 target genes.

Using whole-genome arrays and proteomics, the scientists looked in HEK293 cells for genes upregulated after activating XBP1, ATF6, or both transcription factors. XBP1 turned on 180 genes in a variety of ER proteostasis pathways, whereas ATF6 upregulated a smaller subset. Specifically, they found that transthyretin (TTR)—the amyloidogenic protein that causes familial amyloid polyneuropathy (FAP) and related diseases—was controlled principally by the ATF6 arm. Normal cellular secretion of misfolded, toxic TTR dropped 40 percent when ATF6 target genes were activated but held steady when XBP1-driven transcription was turned on. Wild-type TTR and other endogenous proteins were unaffected by activation of either transcriptional program. “Activating ATF6 target genes enhanced the cell’s ability to maintain quality control,” Kelly said.

Kelly hopes the findings will energize drug development by offering a way “to discern whether a given stress response pathway will be useful for ameliorating a given disease,” he said. The system “allows you to express a transcriptional factor at physiological levels and ask if this is something you would like to go after with a drug-like molecule.”

Switching gears from proteasomal degradation of aggregation-prone proteins, Kelly updated the audience on the long-term efficacy of tafamidis—a drug that stabilizes the normal tetramer adopted by transthyretin. Marketed by Pfizer as a treatment for FAP, tafamidis (trade name Vyndaqel®) prevents transthyretin from breaking into monomers, which can misfold and then misassemble into amyloid (see ARF related news story on Alhamadsheh et al., 2011). Others are using
similar approaches to develop therapeutic compounds that target superoxide dismutase 1 (SOD1) in amyotrophic lateral sclerosis (ALS) and apolipoprotein E4 (ApoE4) in AD, Kelly told Alzforum. However, the approach is less likely to work for amyloid-β or tau. “Generally speaking, you need a protein that adopts a well-defined, folded structure to fashion high-affinity stabilizing ligands,” Kelly said.

The European Medicines Agency approved tafamidis in 2011, making it the first therapy to successfully treat a disease by blocking amyloid formation (see ARF related news story). In the U.S., Pfizer submitted a new drug application to the Food and Drug Administration that same year but the agency’s advisory committee did not issue an approvable letter after its meeting in May 2012. Under the U.S. Orphan Drug Act, treatments for rare diseases in principle can gain approval based on positive results in a single trial, if the trial uses a surrogate biomarker that is likely to predict clinical efficacy. This latter point is still being disputed.

Kelly and colleagues have developed and patented an ELISA that they say specifically recognizes transthyretin oligomers, the presumed molecular culprit in FAP. Using this assay to screen human blood samples, the researchers distinguished FAP patients from their spouse controls (100 people total) with 100 percent accuracy. In addition, oligomer levels fell more than 50 percent after six months of treatment with tafamidis, Kelly said. He hopes the new data will enable the FDA to approve tafamidis without requiring a second FAP trial.

Jason Gestwicki, who moved last month from the University of Michigan to the University of California, San Francisco, presented his group’s latest work on small molecules targeting the molecular chaperone HSP70. The ADP-bound form of HSP70 binds tightly to misfolded proteins, preventing them from interacting with, and potentially corrupting, properly folded forms. Gestwicki worked with Chad Dickey at the University of South Florida, Tampa, to see if this strategy might work for tauopathies, which are marked by buildup of pathological tau aggregates. The researchers screened for small molecules that trap HSP70 in its ADP-bound state and, in doing so, promote tau degradation (see Evans et al., 2010).

From those screens came MKT-077, an anti-cancer compound that entered Phase 1 testing in the 1960s but was later abandoned because it caused kidney damage. MKT-077 potently reduced tau levels in neuronal cultures from Tg4510 tauopathy mice. Furthermore, the compound seemed to improve synaptic function, driving up long-term potentiation when administered to brain slices from these animals, said Gestwicki. He reported these data in the April 19 Biological Psychiatry (Abisambra et al., 2013; see also Rousaki et al., 2011).

In a separate study done in collaboration with University of Michigan colleague Andrew Lieberman, Gestwicki reported that an MKT-077 analogue curbs neurotoxicity in a fly model of spinobulbar muscular atrophy, also known as Kennedy’s disease (Wang et al., 2013). In this inherited motor neuron disorder, expanded polyglutamine repeats in the androgen receptor (AR) cause the protein to misfold and aggregate. Similar to MKT-077’s tau-reducing effects, this
compound appears to relieve toxicity by stabilizing AR binding to HSP90 and HSP70, thereby promoting the receptor’s degradation.

While these data demonstrate proof of principle, MKT-077 and its analogues have a major shortcoming—they do not cross the mammalian blood-brain barrier. However, last month Gestwicki and colleagues reported their first brain-penetrant analogue and showed that it reduced levels of phosphorylated tau in cultured brain slices (Miyata et al., 2013). “We are changing the way tau is recognized by the system,” Gestwicki said.

“The big question we need to address next is whether this approach is safe,” Gestwicki noted. “Do other proteins (besides tau and androgen receptor) get degraded by these molecules? And what are the potential side effects?” His lab is working to address these issues.

**Paracrine Signal From BACE1-Clipped Neuregulin Rescues Myelin**

As β-secretase (BACE) inhibitors head into Alzheimer’s disease trials, researchers are paying close attention to what else BACE1 does besides snip amyloid-β from the amyloid precursor protein (APP). Christian Haass fleshed out the proteolytic pathway of another BACE1 substrate, neuregulin, and emphasized that BACE1 function might not be restricted to development at “From Science to Therapeutics: The Best Way Forward,” a workshop held 15-17 April 2013 in San Francisco, California. He also described a role for the related protease BACE2 in melanocytes. Haass leads research at Ludwig Maximilians University, Munich, and oversees the Munich branch of the German Center for Neurodegenerative Diseases (DZNE), a government-funded consortium headquartered in Bonn. DZNE co-sponsors the annual workshop together with the Gladstone Institute of Neurological Disease, San Francisco.

When Haass and others reported that peripheral nerves are poorly myelinated in BACE1-deficient newborn mice (Willem et al., 2006), they noted the striking resemblance to animals lacking neuregulin 1. This transmembrane protein signals through its epidermal growth factor (EGF) domain to regulate myelination, which raised the prospect that BACE1 and neuregulin 1 might act in the same pathway. The accumulation of unprocessed neuregulin 1 in BACE1 knockout mice lent credence to the idea and suggested neuregulin 1 as a natural substrate for the secretase. Neuregulin 1 also undergoes cleavage by ADAM (a disintegrin and metalloproteinase) proteases, which include ADAM10, the α-secretase that cuts within the amyloid-β domain of APP.

Scientists had assumed that neuregulin 1 type III signals as an autocrine factor, because they believe its extracellular EGF domain remained tethered to the cell membrane. However, in San Francisco, Haass reported that BACE1 and ADAM17 each cut neuregulin 1 on either side of the EGF motif, releasing it to drift away in the extracellular space.

To map neuregulin 1 cleavage sites, the scientists expressed the sheddases in HEK293 cells containing flag-tagged neuregulin 1 chimeras. They analyzed any cleaved neuregulin by mass spectrometry after immunoprecipitation with anti-flag antibodies. Notably, the BACE1 and ADAM17 sites on the C-terminal side of
neuregulin’s EGF domain occur on the N-terminal side as well, Haass reported. The flanking sites suggest that BACE1/ADAM17 can solubilize the EGF domain. BACE1 cleaved neuregulin 1 “much more efficiently than expected,” said Haass. His group discovered that the N-terminal BACE1 site on neuregulin shares the same sequence as its counterpart in APP with the Swedish mutation. That genetic variant dramatically increases BACE1 processing.

To test if the EGF domain was indeed released from neuregulin, the researchers generated antibodies to it and used them to locate the peptide. Immunoblotting experiments showed that primary rat neurons make soluble EGF peptide (sEGF), and that BACE inhibitors wipe out its production. Given that neurons excise the peptide in such a fashion, “it is likely that neuregulin 1 signals through its soluble EGF domain,” Haass said. Graduate student Daniel Fleck and others in the lab did the work with Ludwig Maximilians University colleague Michael Willem.

The next big question was whether sEGF signals in a paracrine fashion. To address this, the researchers knocked out the BACE1 orthologue in zebrafish. Then, to visualize myelination, they mated the BACE1-deficient fish with a transgenic line expressing green fluorescent protein in myelin-forming Schwann cells and oligodendrocytes. Recapitulating the mouse BACE1 knockout phenotype, nerves in the peripheral nervous system were undermyelinated in fish missing BACE1, Haass reported. He showed they could partially restore the defect by injecting the fish with mRNAs for β-EGF. Importantly, central nervous system neurons were unaffected. The researchers published much of this data in the May 1 Journal of Neuroscience.

![Paracrine Signaling](image)

**Paracrine Signaling**
A soluble, BACE1-cleaved piece of neuregulin rescues hypomyelination in the peripheral nervous system of zebrafish. Green: myelin; dpf: days post-fertilization. *Image courtesy of Christian Haass*

This work settles several longstanding questions about BACE1 and neuregulin function, Haass said. Since the 2006 reports of peripheral nerve hypomyelination in BACE1 knockout mice, scientists wondered if the enzyme also supports
myelination in the CNS. “There has been uncertainty about this, but now it is crystal clear. At least in zebrafish, BACE1 deficiency has no effect on CNS myelination,” Haass told Alzforum.

Moreover, scientists have debated whether the downstream effects of neuregulin 1 processing depend on which protease cuts the substrate. After all, this is the case for APP. Some believed that BACE1 cleavage would produce a product that is competent to signal, whereas ADAM cleavage would not. However, “we showed it does not matter—either protease produces a molecule fully capable of signaling,” Haass said.

In a separate study published online February 14 in the Journal of Neurochemistry, Haass and LMU colleague Bettina Schmid found that BACE1 and BACE2, though catalytically similar, have different biological functions (van Bebber et al., 2013). First author Frauke van Bebber, a postdoctoral fellow in the group, counted neuromasts, the mechanoreceptive organ of the fish’s lateral line, in BACE1 and BACE2 knockouts. The number of neuromasts the fish develops depended on neuregulin 1 processing. Their numbers rose in BACE1-deficient animals but not in BACE2-deficient fish. BACE1/BACE2 double knockouts had as many as BACE1 knockouts, suggesting that the two enzymes have distinct functions with respect to neuregulin signaling.

Moreover, BACE2 deficiency did not affect myelination. It did, however, compromise melanocytes. Whereas these skin pigment cells normally cluster at the tip of the tail, they migrate past their normal destination in BACE2-deficient animals, Haass reported. This jibes with color effects that Bart de Strooper and colleagues at KU Leuven, Belgium, see in BACE2 knockout mice (see Dominguez et al., 2005). Their fur is silverish instead of the usual black, de Strooper told Alzforum. Together with Guillaume van Niel of Institut Curie in France, de Strooper’s group recently determined that BACE2 is responsible for the color change. Other research suggests that BACE2 regulates pancreatic β-cell function (Esterházy et al., 2011).

Toward the end of his talk, Haass presented new data on yet another BACE1-dependent neuregulin pathway that suggests the secretase could play an important role not just during development but also in adulthood. This new role concerns maintenance of muscle spindles. These studies were done in collaboration with Walter Birchmeier’s lab at the Max Delbrück Center for Molecular Medicine in Berlin. Details of this study are submitted for publication, Haass told Alzforum.

Other scientists at the meeting found Haass’ data compelling. Some suggested that BACE inhibitor trials should look carefully for phenotypes that might reflect changes in neuregulin 1 processing—for example, early hints of a neuropathy. At this point, however, researchers do not know whether the biology Haass reported in zebrafish occurs in humans, how strongly BACE1 and BACE2 would need to be inhibited in people to block it, and whether the drug doses in the current trials come anywhere near those levels of inhibition.—Esther Landhuis.

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Could Longevity Factor, Epilepsy Med, Treat AD One Day?
At a San Francisco conference addressing Alzheimer’s and related disorders, the agenda featured a diverse mix of topics—occasionally even from the same lab. Lennart Mucke of the Gladstone Institute of Neurological Disease, which hosted the meeting 15-17 April 2013, discussed two fundamentally different treatment strategies—searching for new compounds and repurposing existing drugs. “We likely need to attack AD, like other multifactorial diseases, with a variety of different compounds,” said Mucke. Other speakers also pushed for a multipronged approach. Some discussed ways to improve the predictive power of experimental models and proposed strategies to nurture academic-industrial partnerships to speed drug discovery.

Mucke’s screening approach centers around Klotho, an anti-aging factor named after the Greek goddess who spins the thread of life (Kuro-O et al., 1997). In people, Klotho gene variants modulate longevity, though researchers are unsure how. When overexpressed in mice, Klotho extends their lifespan, possibly by suppressing insulin signaling and oxidative stress. The kidney expresses high levels of Klotho, which binds to ion channels and regulates their assembly. The brain’s choroid plexus also churns out a lot of this protein. “We know next to nothing about Klotho’s function in the nervous system,” Mucke said. Research in Carmela Abraham’s lab at Boston University indicates that Klotho expression falls not only with normal aging in monkey, rat, and mouse brain, but also in the brains of AD mice and people with AD (see ARF related conference story; see also ARF related news story).

To determine if systemic Klotho overexpression could treat mouse models of AD, Mucke and colleagues crossed Klotho transgenic mice (Kurosu et al., 2005) with his J20 line that expresses mutant human amyloid precursor protein (APP). APP/Klotho mice lived longer and did better in tests of spatial learning and memory than J20 controls, Mucke said at the meeting. “Of all genetic manipulations in our APP mice, this was one of the most striking phenotypes,” he said, noting that Klotho overexpression improved cognition in non-APP animals, too. Electrophysiology and pharmacology studies in the double transgensics yielded preliminary evidence that Klotho might exert these behavioral benefits through changes in particular neurotransmitter receptor levels.

Emerging human data support these results from mice, said Mucke. Together with Dena Dubal, Bruce Miller, and other colleagues at UCSF, Mucke examined data obtained in more than 700 seniors from three independent cohorts at the University of California, San Francisco; Rush University Medical Center in Chicago, Illinois; and the University of California, Los Angeles. In their analyses
thus far, people with higher serum levels of Klotho had better cognition, Mucke told meeting attendees. The Klotho benefit did not depend on apolipoprotein E status. “We looked carefully at this and were surprised to find no interaction between Klotho and ApoE4,” he said.

Collectively, the data suggest that “augmenting Klotho could counteract the development of AD and related diseases of aging,” Mucke said. Since they see some effects in younger mice, he noted that Klotho may work through mechanisms that do not modulate the aging process per se. Abraham’s lab is screening novel compounds that boost Klotho expression (King et al., 2012), and Mucke is collaborating with her group to test potential candidates in mice. Other therapeutic approaches could include blocking Klotho turnover, stimulating its activities, activating downstream mediators, or quelling counteracting pathways.

In the second part of his talk, Mucke pivoted to a completely different strategy that he hopes will pay dividends more quickly. The anti-epilepsy drug levetiracetam suppressed seizures and improved performance in tests of behavior and cognition in J20 mice through a mechanism involving binding to the synaptic vesicle component SV2A (see ARF related news story on Sanchez et al., 2012). AD patients are prone to spontaneous seizures (Imfeld et al., 2012; Scharfman, 2012), and growing evidence from various labs supports a connection between AD and epilepsy (see ARF related conference story). As reported recently by Michela Gallagher of Johns Hopkins University, Baltimore, Maryland, levetiracetam quieted hippocampal activity and improved memory in a small sample of people with amnestic mild cognitive impairment (aMCI) (see ARF related news story on Bakker et al., 2012).

Mucke noted that cognitive improvements occurred in both mice and humans at similar plasma levels of levetiracetam. Mucke and Gallagher hope to test this drug in a Phase 2 AD trial but have not designed it yet. In working toward a next trial, they are weighing many considerations. For instance, is the drug effective only in people who actually have epileptiform activity, meaning enrollment would have to ascertain that? Is it effective only in certain disease stages or subtypes? Should other SV2A-binding molecules be tested?

In the meantime, Mucke and UCSF colleague Keith Vossel are enrolling people with AD, MCI, and other diseases including dementia with Lewy bodies (DLB) and frontotemporal dementia (FTD) into a clinical observational study to determine if they have epileptiform activity. At the research hospital, participants undergo a 36-hour electroencephalography (EEG) test as well as magnetencephalography (MEG), another high-resolution measure of human brain activity. So far, the scientists have results for four MCI and 17 AD patients, as well as 11 controls. Almost half the AD patients had non-convulsive epileptiform activity, Mucke reported.