

# **HD 2014: “The Milton Wexler Celebration of Life”**

August 6-9, 2014  
Royal Sonesta Hotel  
Cambridge, Massachusetts

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Nearly three hundred participants attended the “HD2014: The Milton Wexler Celebration of Life” Symposium bringing into focus the many avenues of research directed at finding treatments for Huntington’s disease (HD). A diversity of approaches, ideas, and findings were presented. In addition to bringing participants up-to-date on the many aspects of current HD research, the meeting shined a light on what needs to be done in the future and how best to translate this wealth of information and creativity into treatments for HD.

### **Living with HD: family perspective**

Nancy Wexler kicked off the meeting thanking participants for their dedicated efforts to solve HD. She likened the HD research community to a family with a common goal. Following these opening remarks, Anne Young introduced Allen Goorin and his wife Linda who generously shared their experiences battling HD. Goorin was a professor of pediatrics at Harvard University specializing in osteogenic sarcoma. He cared for many patients and his work contributed to patient survival rates rising from 40% to 70% during the 35 years he worked in the field. However, when his HD symptoms set in, he had to give up his license, one of the hardest things he’d ever done in his life. Goorin also talked about the many other challenges that have arisen over the nearly ten years he has lived with HD. He said he is increasingly unable to do the things he once loved and is losing his independence (he can no longer drive, for example). He has suffered from insomnia, dangerous falls, chorea, depression and cognitive decline. In addition, the family has been through a lot of tension as Goorin’s daughters didn’t learn of their father’s genetic disease until they were adults with kids of their own. Combined with the deep sadness for their father’s condition, they are now distressed about their own fates and those of their children’s.

Despite these challenges, Young noted that Goorin is an exemplary patient who exercises regularly, takes all his medications, and faces pain and hardship with strength and a positive attitude. Moreover, the Goorins are committed to help the HD research community advance their efforts towards finding a cure. In addition to sharing their story at the meeting, they recently helped set up a successful fundraising event for the Hereditary Disease Foundation featuring a long-time friend of Goorin’s, the renowned cellist Yo-Yo Ma. Participants were inspired by Goorin’s story and motivated to continue working hard towards developing effective treatments for HD.

### **Clinical trials: current status and future possibilities**

Ray Dorsey and Diana Rosas provided participants with an update of the clinical trial landscape for HD and discussed challenges and opportunities that lie ahead. Dorsey noted the steady increase over the decades in both publications of HD clinical trials and average number of participants enrolled. As examples of current trials, Dorsey pointed to the Reach2HD trial sponsored by Prana Biotechnology to test the metal chaperone PBT2, Raptor Pharmaceuticals’ Phase 2/3 trial to test cysteamine, and the testing of the SirT1 inhibitor Selistat for safety, tolerability and pharmacodynamic characteristics. In addition, David Stamler presented an update of Auspex’s studies of SD809, a deuterated form of tetrabenazine (an anti-choreic medication) that is expected to enable less frequent dosing,

improve tolerability and reduce interpatient variability. As explained by Stamler, the substitution of deuterium for hydrogen at specific positions in tetrabenazine attenuates the breakdown of the drug's active metabolites.

Two clinical trials are currently underway in patients with SD809. The first is a Phase 3, randomized, double-blind, placebo-controlled, parallel-group trial is designed to evaluate the safety, tolerability and efficacy of SD-809 for treating chorea associated with HD. In parallel, Auspex is conducting another study which includes an open-label four-week "switch" trial in approximately 36 patients with chorea associated with HD adequately controlled with tetrabenazine. The objectives of this trial are to evaluate the safety of switching subjects from tetrabenazine to SD-809 and to provide guidance to physicians on how to do it, although it is not powered to compare the treatments' relative efficacies. Future plans include rolling over patients from both trials into a 52-week open-label, long-term, safety clinical trial. So far, the conversion to SD-809 seems to be generally well tolerated and able to maintain chorea control (at one and four weeks after switching to SD-809, the mean total chorea score decreased by approximately one point from baseline). However, the study is not powered to compare the relative efficacies of tetrabenazine and SD-809.

Looking ahead, Rosas described other compounds that might be clinically tested for HD in the near future, including new antagonists for glutamate receptors (such as mGluR5 antagonists recently tested for Fragile X syndrome and Parkinson's disease), phosphodiesterase 10 inhibitors, anti-dopaminergic neuroleptic agents, and inhibitors of kynurenine 3-monooxygenase activity. Of particular interest are clinical trials planned for 2015 to test the safety and tolerability of gene silencing agents (see below).

Noting the potential to ameliorate HD symptoms with compounds being developed for other neurodegenerative disorders, Sharon Mates from Intracellular Therapeutics presented her team's findings of ITI-007, a compound under development for the treatment of schizophrenia and other neuropsychiatric disorders. As explained by Mates, at low doses, ITI-007 acts primarily as a 5-HT<sub>2A</sub> receptor antagonist. In this dose range, ITI-007 has been shown to restore natural sleep patterns in patients with primary insomnia and is currently being studied for the treatment of behavioral disturbances (e.g. agitation, aggression, psychosis and sleep disturbances) associated with dementia. As the dose is increased, additional pharmacologic activity is recruited, including inhibition of serotonin reuptake and interactions with dopamine receptors (as a pre-synaptic partial agonist and post-synaptic antagonist at dopamine D<sub>2</sub> receptors) and NMDA glutamate receptors (as a GluN2B receptor phosphoprotein modulator).

Testing ITI-007 at the higher dose range, in a large, randomized, double-blind, placebo- and active-controlled clinical trial, showed that the drug is safe and effective in reducing schizophrenia symptoms, including hallucinations, delusions, and co-morbid depression and possesses an overall response profile consistent with improved social function. Mates added that ITI-007 is also being tested for behavioral disturbances in dementia, including Alzheimer's disease. In the context of HD, the compound may be particularly useful in reducing behavioral symptoms and sleep disturbances.

As noted by Rosas and Dorsey, both challenges and opportunities to improve clinical trials for HD lie ahead. Rosas noted that it often takes 15 years (or longer) to take a compound from the lab to the clinic, so there's a need to streamline this process. Challenges include the limited nature of studies of bioavailability in animal models, the length of HD progression, and the variability of the HD phenotype in humans. Dorsey added there are several barriers to patient participation in clinical trials, a major one being the expense and logistical difficulty for patients to travel to study sites. To address this problem, his team is developing internet-based studies in which participants can provide information remotely from their homes. Also, Dorsey noted the need to increase the involvement of patients in all aspects of clinical trials, including the planning stages.

The need for additional and better biomarkers to track disease was also emphasized. As noted by Bill Kaemmerer, biomarkers for both prodromal and early symptomatic HD are needed. Rosas noted that several alterations detected by brain imaging techniques appear to occur very early and are very robust. Other biomarkers currently under development include metabolic signatures, markers of oxidative stress, and reporters of huntingtin levels (see below). The value of these individual indicators will greatly depend on the specific goals of the study (e.g., the experimental compound's mode of action, its expected effects on the HD phenotype, and time of intervention), noted Rosas. Also, a combination of different measurements will likely be needed in many clinical studies because the dynamic range of changes that occur over the course of HD is very large, leading to confounding ceiling and floor effects.

### **Reducing mutant huntingtin**

Eliminating the root cause of HD, mutant huntingtin, is the most direct approach to developing a treatment for HD. As in previous meetings, several approaches to accomplish this goal were presented. The findings and discussions served as encouraging signs of the steady progress being made in the field.

#### Knocking down mutant huntingtin production: Advances

As explained by Frank Bennett, there are many different approaches, at different stages of development, for silencing genes. And given the recent progress in the field, several are already being tested in clinical trials. According to Diana Rosas, there are currently more than 50 clinical trials, most in phase I or II, using RNA/DNA-based therapeutics to treat a variety of genetic diseases.

Highlighting the use of antisense oligonucleotides (ASOs), Bennett reported that he and his collaborators at Isis Pharmaceuticals have optimized the chemistry of these molecules to increase resistance to degradation, improve affinity and enhance specificity, thereby increasing potency and reducing undesirable off-target effects. ASOs are readily distributed throughout tissues and, when delivered intrathecally, they disseminate widely (although the striatum is less well targeted than other brain regions).

As an example of the potential for using ASOs to treat neurodegenerative disorders, Bennett reported that trials testing the safety and tolerability of ISIS-SMNRx, an ASO

designed to treat spinal muscular atrophy (SMA), have been completed with encouraging results. ISIS-SMNRx targets the gene encoding survival motor neuron 2 (SMN2) and modulates alternative splicing to produce a full-length SMN protein which can alleviate the SMN deficiency characteristic of SMA. So far, the researchers have observed increased SMN protein levels in human cerebrospinal fluid (CSF) and noted that multiple doses are well tolerated. Most children with SMA who received a single dose of ISIS-SMNRx (6 mg or 9 mg), showed increases in muscle function scores up to 14 months after injection and a study in infants yielded similar results. A Phase III study of ISIS-SMNRx was initiated at the beginning of August and will evaluate the efficacy and safety of a 12 mg dose of ISIS-SMNRx with a primary endpoint of survival or permanent ventilation.

Several silencing agents are currently under development for HD and the prospects for testing in clinical trials are encouraging. For example, Julia Alterman described work performed in the labs of Anastasia Khvorova and Neil Aronin, in which hsiRNAs, heavily modified oligonucleotides (2'-O-methyl, 2'-fluoro, sterol, etc.), are being optimized for increased stability, reduced ability to induce inflammatory responses, and efficient cellular uptake that does not require a delivery vehicle and does not interfere with normal RNA cellular functions. So far, the team has obtained efficient huntingtin silencing (passive delivery EC50 ~ 80nM; lipid-mediated delivery EC50 ~ 9 pM) in primary cortical and striatal neurons. The silencing is potent (85% protein levels), occurs within minutes of exposure, and lasts for several days. In vivo experiments, involving unilateral intrastriatal injections, yielded encouraging results with some knockdown occurring even in the contralateral side.

As noted by Bennett, Isis has also developed ASOs that target huntingtin mRNA. They hope to test their lead compound, ISIS-HttRx, in humans in 2015. The current plan is to administer the drug once a month with a single lumbar push expected to induce knockdown activity with a delay of 4-6 weeks and a predicted knockdown duration of 4-6 months. Data from animal models indicate that ASO effects persist beyond the period of huntingtin knockdown (Kordasiewicz et al, 2012), so a yearly regimen of drug administration may be sufficient for efficacious, continuous treatment.

ISIS-HttRx does not discriminate between wildtype and mutant huntingtin, however. So far, mice, rats, dogs and non-human primates have tolerated ISIS-HttRx well, but the non-allele selectivity of the approach could be problematic. The complete loss of wild-type huntingtin expression is lethal in embryonic mice, and several studies have identified functions of wild-type Htt that may be critical for various cellular processes. Bennett noted that Isis has plans to also test allele-specific ASOs (which target specific single nucleotide polymorphisms (SNPs)) in the future.

Another strategy to accomplish allele selective knockdown was presented by Steve Zhang from Sangamo. Zhang and colleagues are using engineered zinc-finger protein transcription factors (ZFP TFs), which can be designed to bind virtually any DNA sequence and regulate gene expression, to specifically target the mutant huntingtin gene. In particular, they have designed ZFP repressors that recognize the CAG repeat and significantly repress mutant Htt alleles (CAG40-70), while minimally affecting normal

Htt alleles (CAG15-21). The specificity of the repressors is remarkable—alleles with 21 repeats can be distinguished from those with 38 repeats over a 100-fold dose range.

So far, the repressors have been tested in several cultured cells (fibroblast lines, human embryonic stem cells, and neural stem cells) and HD mouse models (R6/2, Q175, HdhQ7/Q60) using viral vectors for delivery into the striatum. In vivo, mHtt protein levels were knocked down by 60-65% and improvements in nuclear aggregates, behavior (clasping test), and neuroprotection (elevated striatal medium spiny cell markers) were observed. Encouraged by these results, Zhang and colleagues hope to test the treatment in clinical trials soon.

#### Knocking down mutant huntingtin production: Challenges

Despite the important strides that have been recently made in the field, several challenges remain. More work is needed to develop methods to monitor target engagement, deliver therapeutic agents, and identify key target tissues (see “Localization of pathogenesis”) Also, as noted by Rosas, the stability and safety of huntingtin-suppressing agents and their vectors need to be evaluated further and optimized.

It is hoped that through efforts that bring researchers together to share information and brainstorm solutions, like the HD2014 meeting, these challenges will be overcome. Bill Kaemmerer noted that a new organization, the CGTA Research Group, may also help. The goal of this group is to “bring gene therapies for rare CNS diseases into clinical trials and use as quickly and efficiently as possible.” Their first project is focused on developing a gene therapy for HD. Kaemmerer hopes the CGTA group will become a seed organization, bringing together other groups working on HD gene therapies.

One approach to monitoring target engagement in huntingtin-suppression therapies was presented by Ed Wild. His team has developed a new method to quantify mHtt in cerebrospinal fluid. The method, based on a novel single-molecule counting (SMC) immunoassay, is selective for mHtt in the femto-molar range and can detect the mutant protein in both premanifest and manifest individuals. In addition, a significant association was found between mHTT level and disease burden score. As noted by Wild, the source of mHtt is unknown, but might represent protein released from dying neurons since mHTT concentrations were significantly associated with the concentrations of two intracellular neuronal proteins (neurofilament light chain and tau), but not with hemoglobin. Wild and colleagues are now working on an assay specifically designed for clinical trials. In addition to serving as a tool for assessing gene therapy effectiveness, the assay may serve to track disease progression and help advance the understanding of HD pathobiology in humans.

Participants also presented several approaches to overcome the challenge of delivering huntingtin knockdown therapies. As Gai Ayalon discussed, the efficacy of many therapeutic candidates is limited by their inefficient uptake across the blood-brain barrier and, as panelists in the session on Animal Models pointed out, the large size of the human brain poses a serious challenge for the effective distribution of therapeutic compounds once they are in the brain.

Kaemmerer commented on his team's efforts testing adeno-associated virus (AAV) vectors in sheep. So far, his studies have revealed the importance of selecting appropriate serotypes for targeting specific brain areas, neuronal circuits, and cell types. He also noted that it will be important to increase the safety of these agents by designing constructs that can be switched on or off, as well as optimizing parameters to reduce costs. Neil Aronin pointed out that many labs and companies are working on identifying useful AAV serotypes (there are over 100 types that have yet to be examined for their potential value as vectors) and modifying them to improve delivery—a single point mutation can radically change a virus' behavior.

As an example of such efforts, Ben Deverman, working in the lab of the late Paul Patterson, presented his work developing new AAV serotypes that efficiently transduce CNS neurons and glia after intravenous administration. Deverman has generated highly diverse libraries of AAV capsid variants and selected for those with the most desirable properties by serial *in vivo* biopanning. Using this approach, he has developed an AAV variant that can transfer genes to CNS neurons and glia via the vasculature in adult mice with an efficiency that is 40-90-fold greater, depending on the brain region, than AAV9 (a vector considered to be particularly efficient at transducing these cells). As emphasized by Deverman, the results are of interest not only because of the new vector's high transduction efficiency, but because of the possibility of using intravascular administration to widely distribute therapeutic agents to the many areas that are affected by HD (see "Localization of pathogenesis").

Additional delivery methods were described by Julia Alterman and Juan Sanchez-Ramos. Alterman noted that the most serious limitation of oligonucleotide-based therapies is the inefficient transit of oligonucleotides into neurons. Describing work in Aronin's lab, Alterman noted that the natural mechanism by which exosomes, natural-occurring lipid nanoparticles that traffic small RNAs across cellular boundaries, can be exploited. The researchers developed a novel approach to load exosomes with hsiRNA and obtained efficient, dose-dependent silencing of Htt in mouse primary cortical and striatal neurons. Moreover, they also observed neuronal uptake after *in vivo* striatal injection of hsiRNA-exosome complexes with anterior and posterior spread, also including the non-injected side.

Another nanoparticle, a manganese-containing particle made of chitosan (mNP), was also put forth as a potentially efficient and non-invasive vector for delivering gene silencing therapies. As explained by Sanchez-Ramos, manganese can get into the brain, by inhalation through the nose, where it reaches the olfactory bulb and distributes widely throughout the brain with no signs of being harmful at low doses. Using these mNPs loaded with anti-GFP siRNA, the researchers were able to significantly decrease GFP fluorescence in mouse fibroblasts expressing GFP. Toxicity to cells using mNPs was similar to that seen with the commonly used reagent lipofectamine. Following intranasal instillation, mNPs were visualized by MRI (manganese is visible using this imaging technique) throughout the brain within 24h. mNPs were effective in significantly reducing GFP mRNA expression (>50%) in olfactory bulb, striatum, hippocampus and

cortex at 48h. Intranasal instillation of mNPS loaded with dsDNA encoding RFP also resulted in expression of the RFP in multiple brain regions.

### Reducing huntingtin protein

Finding ways to modulate the clearance of mHtt, the full-length protein or its toxic fragments, is another approach to target the root cause of HD. At the HD2014 meeting, Eric Reits presented his group's latest findings on the role of the proteasome in HD and how it might be modulated to enhance clearance of mHtt. Previous studies have suggested that proteasomes are irreversibly sequestered into aggregates and unable to cleave within polyQ repeats, possibly becoming clogged with mHtt fragments. However, using a fluorescent pulse-chase assay, Reits and colleagues have found that proteasomes are reversibly recruited into aggregates, where they remain accessible and active. Moreover, mHtt proteins are completely degraded when targeted to proteasomes.

Interestingly, targeting of mHtt to proteasomes is regulated differently across subcellular domains. Reits noted that adding a nuclear localization signal to mHtt-exon1 alters its ubiquitylation pattern. Solubilization of cytoplasmic and nuclear inclusion bodies containing mutant Htt-exon1 uncovered a ladder of poly-ubiquitylated Htt species in cytoplasmic, but not nuclear, inclusion bodies. Reits is interested in examining the mHtt interactome in these two locations to pinpoint the protein(s) underlying this difference.

Reits's team has also examined whether proteasomal modification could enhance clearance of mHtt. They observed that introducing the PA28 activating cap, which normally serves to open the 20S proteasome core to improve peptide degradation and appears to be reduced in HD, was ineffective. However, immunoproteasomes, proteasomes with alternative catalytic subunits that are expressed in hematopoietic cells in response to pro-inflammatory signals, improve mHtt degradation *in vitro*.

Autophagy is another endogenous clearance pathway that could be modulated to increase mHtt removal. Previous studies have tested whether enhancing autophagy ameliorates HD pathogenesis, but it is difficult to draw clear conclusions because of the many cellular proteins and functions affected by these manipulations. A more selective modulation of autophagy, however, may provide better opportunities for therapeutic intervention.

At the HD2014 meeting, Leora Fox, working in Ai Yamamoto's lab, presented work examining the role of the large PI3P-binding protein Alfy, required for selective autophagy of mHtt aggregates, in HD brains. Fox and colleagues created inducible Alfy knockout mice and crossed them with inducible HD mice to examine how removal of Alfy affects clearance of mHtt *in vivo*. These experiments, which are currently in progress, should also help test whether the phenotypic reversal observed when mHtt expression is switched off, is dependent upon aggregate clearance and provide insight into aggregates' potential as therapeutic targets (see Aggregates). The researchers plan to monitor biochemical, immunohistochemical and behavioral changes under four conditions (with both mHtt and Alfy switched on, both proteins switched off, or either protein singly switched on). So far, they have observed that after Alfy knockout, there's



an increase in p62, a protein which appears to normally interact with Alfy to organize ubiquitinated proteins into protein bodies for degradation by autophagy.

Another study revealing the potential for manipulating proteins involved in the selective clearance of mHtt was presented by Boxun Lu. Based on a high throughput screen of patient fibroblasts designed to identify genes that alter mHtt levels, Lu and colleagues identified an orphan G protein-coupled receptor, Gpr52, as a potent modifier of mHtt levels. As explained by Lu, Gpr52 expression is enriched in the striatum and knocking it down reduces Htt levels in HD cells. Treatment with the adenylyl cyclase activator forskolin or with cyclic-AMP analogs that do not activate PKA increased the baseline level of Htt and completely abolished Gpr52's effect, suggesting that the modulation of mHtt levels by Gpr52 is mediated via a cAMP-dependent but PKA-independent mechanism, likely through small GTPases. Gpr52 locates in the intron region of Rabgap1L, which is a putative GTPase-activating protein that balances the Gpr52 mediated modulation of Htt. Moreover, knocking-down Gpr52 suppresses toxicity in a human neuronal model as well as in a fly HD model.

#### Reducing toxic mutant huntingtin forms

Yet another approach to reducing mHtt's toxic effects is to specifically block or eliminate mHtt's toxic forms. Mutant Htt is highly post-translationally modified and there is evidence that these modifications could be important mediators or modulators of HD pathogenesis. As explained by Leslie Nucifora, who delivered Tamara Ratovitski's talk, identifying and studying these modifications, some of which are highly druggable, could offer important new therapeutic options. Ratovitski and her colleagues in Chris Ross's lab are conducting a comprehensive study of post-translational modifications occurring on endogenous Htt expressed in HD mouse models and HD human brains. Using quantitative mass spectrometry to analyze samples of purified Htt, the researchers have so far identified previously reported modifications, plus 29 novel ones, most of which are phosphorylations in the C-terminus.

To validate and prioritize these modifications, the team is: 1) using quantitative proteomics (iTRAQ) to determine whether the stoichiometry of modifications is altered by the polyglutamine expansion, 2) using biochemical and biophysical techniques to characterize how the modifications affect oligomerization and aggregation of Htt constructs, 3) using circular dichroism to study how Htt secondary structures are affected, and 4) using cell toxicity assays in primary neurons to evaluate the effects of different modifications on cell viability. Of particular interest, the researchers observed that the phosphorylation state of S116 is a modifier of toxicity—a lack of phosphorylation at this site is protective.

As in years past, the role of mHtt aggregates in HD pathogenesis and their potential as a therapeutic target were also discussed. Although aggregates have been the focus of many studies throughout the years, their formation, composition, and effects on cellular function still remain unclear. Two approaches that could help clarify the link between aggregates and toxicity were presented by Kevin Drombosky and Leora Fox.

Working in Ron Wetzel's lab, Drombosky designed an experiment to distinguish between Htt-exon 1's ability to aggregate and its absolute polyglutamine length as inducers of toxicity. Drombosky generated a short polyglutamine (22-24 Qs) Htt-exon 1 construct containing  $\beta$ -hairpin enhancing mutations which make it aggregate faster than Htt-exon 1 constructs containing the same or even longer polyglutamine stretches (as high as 37). When expressing these constructs in PC12 cells and *Drosophila*, he observed that the beta-hairpin enhancing mutations were associated with HD-like toxicity. PC12 cells had perinuclear inclusion bodies similar to those observed with constructs containing 46 glutamines, and *Drosophila* exhibited shortened lifespans, signs of neurodegeneration, and an age-dependent decline in locomotion. Thus, Drombosky concluded that the ability of a polyglutamine sequence in HTT-exon1 to bestow an HD-like phenotype is more associated with aggregation than with expanded repeat length.

Using a different approach, Fox is also trying to determine the relationship between aggregates and toxicity. Yamamoto's team had observed that inducible transgenic mice overexpressing mutant human exon1-Htt develop Htt inclusions and progressive decline in motor coordination, but when the transgene is switched off, both motor deficits and aggregates are eliminated or drastically reduced. To test whether the removal of aggregates is required for this disease reversal, Fox and colleagues are creating inducible *Alfy* knockout mice (which are expected to be deficient in aggregate clearance) and crossing them with inducible HD mice, as described above. It will be interesting to see if the results from these experiments also support the aggregation hypothesis.

A strong link between aggregation and toxicity was provided by Judith Frydman who presented an overview of how the chaperonin TRiC/CCT modulates Htt aggregation and toxicity, and described its potential as a therapeutic target. TRiC is a chaperone that is essential for folding many proteins, especially those with complex topologies, high beta-sheet content and a propensity to aggregate. Unlike other modulators of Htt toxicity, TRiC interacts with many different mHtt species, capping the tips of mHtt fibrils, as revealed by cryo-electron microscopy tomography.

Frydman further explained that a surprisingly small portion of a single subunit of the TRiC double-ring complex, the CCT1 subunit, interacts with Htt's N17 domain and can remodel huntingtin aggregates. In addition, exogenous administration of the substrate-binding apical domain of CCT1, ApiCCT1, can penetrate cell membranes, reduce both oligomers and insoluble mHtt species, and ameliorate cellular toxicity. Moreover, administration of ApiCCT1 in HD brain slices, rescued axonal trafficking defects. Preliminary evidence from animal models (using viral delivery, direct application, or cell-based delivery of ApiCCT1) is consistent with these positive, *in vitro* findings. Frydman plans to continue investigating the molecular underpinnings of the Htt-chaperonin interaction, and use this knowledge to develop small molecules for therapeutic intervention.

## Downstream Alterations

The expression of mHtt and its aggregates results in many downstream alterations. Thus, a key goal is to identify the disruptions that are most significantly involved in the disease process—those that emerge the earliest, are rate-limiting, and/or have the most phenotypic impact—as well as those that are most amenable to therapeutic intervention.

### Cellular Stress

Alterations in cellular stress responses emerged as a particularly important feature of HD that could be amenable to therapeutic intervention. Rick Morimoto explained that many neurodegenerative disorders involve chronic stress in which cellular stress-response mechanisms become maladaptive. In particular, the network of processes that modulate protein biogenesis, folding, trafficking and clearance—pathways involved in the maintenance of a healthy cellular proteome, or proteostasis—is often disrupted.

As an example of how this might happen in HD, Morimoto described recent findings from his lab showing that heat shock cognate protein 70 (HSC70)—a chaperone involved in several cellular processes including clathrin-mediated endocytosis (CME)—co-localizes with visible aggregates of several different expanded polyglutamine proteins in cell lines and primary neurons. The sequestration correlated with reduced CME, which could also be induced by RNAi depletion of HSC70. Overexpressing HSC70 in cells with visible aggregates improved CME. Because CME plays a central role in trafficking of vesicles and receptors in neurons, the inhibition caused by the sequestration of HSC70 (and possibly other proteins involved in CME) could play an important role in HD and other polyglutamine disorders.

Another way in which stress responses and endosomal trafficking might be disrupted in HD was presented by Siddharth Nath from Ray Truant's lab. Using live cell imaging, Nath observed that huntingtin appears to be involved in a very rapid cell stress response, which occurs in seconds, before the heat-shock response is activated. The response is characterized by the formation of cytosolic puncta, and the reversible localization of huntingtin to early endosomes. Formation of these puncta, or huntingtin stress bodies (HSBs), is associated with arrest of energy-intensive endosomal trafficking. Thus, HSB formation may divert high ATP use from vesicular trafficking, and mobilize canonical stress responses without relying on increased energy metabolism. Nath added that the critical domains for this response have been mapped to two regions flanking the Htt polyglutamine tract. When the polyglutamine tract is expanded, it appears to interfere with cells' ability to recover from the stress response.

A variety of approaches indicate other ways in which cellular stress responses play an important role in HD. For example, working in William Yang's lab, Xiao-Hong Lu has found that a kinase that functions in the DNA damage response triggered by oxidative stress, ataxia telangiectasia mutated (ATM), is consistently elevated in cells derived from HD mice and patients, as well as in HD postmortem brains. Lu has also observed that ATM knockdown protects against mHtt cytotoxicity in cell culture and, crossing *Atm*

heterozygous null allele with BACHD mice, results in the amelioration of multiple behavioral deficits and the reduction of aggregates. As noted by Lu, ATM is highly druggable and of interest as a target not only for HD, but for several neurodegenerative disorders, including Alzheimer's disease. Lu has shown that small-molecule ATM inhibitors can reduce neuronal death in mHTT-transfected rat striatal neurons and human striatal cultures differentiated from HD patient iPS cells.

Another stress-related target was described by Myriam Heiman. Using a powerful new method to screen for *in vivo* modifiers of toxicity known as SLIC (*Synthetic Lethal In the Central nervous system*—see *New Tools*), her team identified a gene that rescues HD motor alterations and DARPP-32 levels when overexpressed. The gene is GPX6 which encodes a protein from the glutathione peroxidase family that functions in the detoxification of hydrogen peroxide.

As noted by Christian Neri, the ability of neurons to cope with the chronic stress induced by mHtt may determine the course of their decline and eventual demise. Focusing on the earliest stages of mHtt pathogenicity and how it disrupts the pathways that regulate neuronal homeostasis, Neri presented robust findings pinpointing key factors that appear to disrupt the normal development of neuronal survival responses in HD. Neri's team has found that the Wnt receptor Ryk, a protein important for neurogenesis, is increased in several models of HD. Ryk represses FOXO transcription factors, which play an important role in cell survival/longevity and neuronal homeostasis, through its intracellular domain, a gamma-secretase cleavage product. Consequently, neurons are unable to develop an efficient FOXO-mediated survival response very early on, before neuronal dysfunction is detectable and prior to the early phases of motor decline.

As noted by Neri, there are several ways in which these findings may lead to therapeutic opportunities. Screens could be performed to identify compounds that stimulate FOXO3, or existing compounds could be tested for their capacity to enhance FOXO activity. An even more powerful approach, however, is to use systems biology to identify specific groups of FOXO targets that are consistently altered across different data sets from different HD models and human patients. Indeed, Neri's team is developing a network-based data integration framework including transcriptomic and gene perturbation data from 16 publically available HD datasets to gain such insights.

Several participants pointed out that these stress-associated alterations indicate, not only the importance of stress pathways in HD, but the likely importance of aging as a modifier of the disease process. Heiman and Lu noted that aging is a major risk factor in neurodegenerative diseases and Morimoto pointed out that chaperone activity in the human brain is known to decline with age. Moreover, recent data from Morimoto's group in *C. elegans* revealed a dramatic shut down of all stress responses between 8 and 12 hours after these organisms generate germ cells and become adults. The switch seems to occur as a result of signaling from germ-line stem cells and involves changes in DNA methylation patterns. In animals devoid of germ-line stem cells, the stress responses don't decline and the animals' lifespans are extended indefinitely.

Specific links between stress responses, aging, and HD pathogenesis were also discussed. For example, Neri noted that the alterations in Ryk signaling that his team has identified suggest HD neurons might be prematurely aged given FOXO's role in survival and longevity. In addition, Heiman noted that the upregulation of GPX6 in HD mice is particularly significant in the aged striatum. Heiman's team hopes to understand in greater depth the role of normal aging in HD and other neurodegenerative diseases using SLIC, as described above, as well as cell-type specific translating ribosome affinity purification (TRAP—see New Tools) to profile gene expression changes occurring in vulnerable cell types, in young versus old animal tissues.

Another important facet of understanding stress responses in HD is the recent discovery by Morimoto and colleagues of how proteostasis is regulated by cell nonautonomous signaling and by the process of transcellular chaperone signaling. Morimoto explained that metazoans appear to employ multiple modes of cell-nonautonomous signaling across tissues to integrate and transmit the heat-shock response (HSR), for example. The transcellular signals affect the expression of molecular chaperones in other cells, possibly preventing the spread of proteotoxic damage. For example, in *C. elegans*, Morimoto's team has found that a moderate increase in cholinergic signaling at the neuromuscular junction induces calcium-dependent activation of heat shock factor-1 (HSF-1) in post-synaptic muscle cells resulting in suppression of protein misfolding.

#### Epigenetic and transcriptional dysregulation

As described by Ferah Yildirim, who presented collaborative work from Ernest Fraenkel's and David Housman's labs, transcriptional dysregulation has long been known to be an early and key pathogenic alteration in HD. Seeking to get at the root cause of this dysregulation, Yildirim and her colleagues conducted systematic, genome-wide evaluations of epigenetic alterations and transcriptional changes in HD brain samples collected at very early stages of disease, before symptom development. In the striata of R6/1 and CHL2 knockin mice, the researchers observed transcriptional dysregulation of many key neuronal genes, including genes encoding neuronal receptors and signaling factors, as well as immediate-early-response genes.

Genome-wide analysis of histone H3K27 acetylation, an active transcription and enhancer mark, revealed highly coordinated histone acetylation changes along with the detected transcriptional changes. By studying the DNA sequences within the altered H3K27 acetylation regions, the researchers identified transcriptional regulators whose disrupted activity may be responsible for the earliest transcriptional changes they detected. One of the regulators, the Elk-1 transcription factor, regulates most of the neuronal immediate-early-genes found to be altered.

Yildirim added that similar analyses are being conducted by several collaborating labs in human iPS cells. Experiments targeting histone modifications (H3K4me3, H3K27ac, and H3K36me3) have shown clear differences between disease versus control differentiated iPSCs and these differences are consistent with observed transcriptional alterations. The team is also examining the relationships between diverse epigenetic features. Based on

the analysis of 39 human cell types, the researchers found a high level of coordination among various epigenetic features that they call “epigenetic ensembles.” Interestingly, one of these ensembles is disproportionately affected in HD. This ensemble is marked by the presence of both active and repressive histone modifications and is especially important in brain tissues and largely present at genes associated with neuronal functions such as axonogenesis, synaptic transmission, and neuron differentiation.

Approaching transcriptional dysregulation in HD from a different angle, Al LaSpada presented his team’s work validating a specific transcriptional factor, peroxisome proliferator-activated receptor delta (PPAR- $\delta$ ), as a potential therapeutic target for HD. Previous studies from LaSpada’s group and others revealed that the activity of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), a regulator of mitochondrial biogenesis and oxidative stress, is decreased in HD. PGC-1 $\alpha$  regulates lipid and glucose metabolism by activating PPAR- $\delta$  which is abundantly expressed in the brain. Moreover, PPAR- $\delta$  interacts with Htt and LaSpada found that a dominant-negative mutation of PPAR- $\delta$  results in an HD-like phenotype.

Given these observations, La Spada considered that modulation of the PPAR  $\delta$  pathway could be an attractive therapeutic strategy for HD. Moreover, since compounds that modify the activities of PPARs are being tested for their potential benefits in other neurodegenerative diseases, La Spada decided to investigate the effects of some of these drugs in HD. For example, he tested bexarotene, a retinoid X receptor (RXR) selective agonist that activates PPAR  $\delta$  /RXR heterodimers and has been reported to ameliorate symptoms and pathology in Alzheimer’s disease mouse models. When administered to primary HD mouse neurons, bexarotene resulted in the improvement of PPAR  $\delta$  transcriptional function, mitochondrial function, and neuron survival. In addition, bexarotene treatment of HD N171-82Q mice ameliorated motor symptoms (rotarod, ledge, clasping, gait), and overall slowed disease progression.

La Spada is now testing other more potent compounds that target PPAR- $\delta$  more selectively. Of particular interest, KD3010, a drug currently in phase II clinical trials for diabetes, has proven to be very effective at ameliorating several HD alterations. La Spada is now performing preclinical tests in HD mice and proposed that direct PPAR- $\delta$  agonist therapy, alone or in combination with bexarotene, might be a promising approach to counter neuronal dysfunction and neurodegeneration in HD.

Another approach to ameliorate HD pathogenesis by modulating the activity of a transcription factor was presented by Aleksey Kazantsev. Kazantsev and colleagues have identified a novel small molecule scaffold, MIND4, which targets KEAP1 (Kelch-like ECH associated protein 1), a negative regulator of the nuclear transcription factor Nrf2 (NF-E2 p45-related factor 2). Nrf2 regulates a wide range of cellular processes in different cell types, including immune cells, neurons, microglia, and astrocytes. It is induced by oxidants and electrophiles, resulting in increased expression of antioxidant enzymes and the repression of inflammatory molecules. It also regulates the degradation of misfolded proteins, mitochondrial biogenesis and the production of neurotrophic factors.

As described by Kazantsev, the MIND4 analogs potently enhance expression of NRF2-responsive genes by inducing the rapid accumulation and nuclear translocation of Nrf2. The team's lead-compound is ~60 times more potent than dimethyl fumarate, another Nrf2 activator which has been approved for the treatment of multiple sclerosis. Treatment with MIND4 compounds reduced toxicity in various HD models (primary neurons, slice cultures, and *Drosophila*). They reduced reactive oxygen species (ROS) levels in primary HD neurons and ROS and RNI (reactive nitrogen intermediates) production in activated microglia. Moreover, pro-inflammatory responses were prevented in activated primary microglia and astrocytes from HD and wild-type mice, and in primary monocytes from HD patients and healthy subjects.

A particularly potent Nrf2 activator, MIND4-17, however, proved to be less effective at protecting primary neurons from toxicity than the less potent and less selective original agonist MIND4. As explained by Kazantsev, in addition to activating Nrf2, MIND4 activates Hsp70 and dramatically enhances the clearance of mHtt which requires more than only Nrf2 activation. Recent data from Vanita Chopra and colleagues indicate MIND4 reduces mHtt levels by up to 50% in HD mice brains. Moreover, MIND4 seems to also be protective in other neurodegenerative disease models. Kazantsev suggested that compounds that target multiple alterations may ultimately prove more useful for therapy development than highly specific drugs.

#### Other multi-functional modulators

A regulator of protein translation and a multi-functional ganglioside were also discussed as potential targets for developing HD treatments. As explained by John Lee, who is working in Bev Davidson's lab, mTOR is a master regulator of protein translation that modulates a wide variety of cellular functions, including mitochondrial function, myelination, autophagy and axonal growth, and whose activity appears to be impaired in the HD striatum. Lee noted that introducing a constitutively active form of the mTORC1 regulator, Rheb, into HD mouse brain, alleviates mitochondrial dysfunction, aberrant cholesterol homeostasis, striatal atrophy and reduced DARPP-32 staining, and increases autophagy.

Lee also noted that the expression of Rhes, a striatum-enriched mTOR activator, is reduced in HD mouse brain and that exogenous addition of Rhes alleviates motor deficits and improves brain pathology. However, Rhes's effects are complex. Rhes not only activates mTOR, but also appears to interact with mHtt independently of its mTOR-regulating activity, inducing mHtt SUMOylation and enhancing its cytotoxicity in vitro. Also, cellular conditions are important factors to consider. Under normal conditions, mTORC1 acts as a nutrient/energy/redox sensor and its activation is dependent on the status of these cellular parameters. Thus, Lee proposed that efforts to target Rhes/mTOR for therapy development should focus on restoring the homeostatic balance of mTORC1 activity, while avoiding manipulations that could result in toxic side-effects.

Another downstream alteration which may serve as a therapeutic target is an HD-associated deficiency in GM1, a ganglioside essential to neuronal cell survival and

function. As explained by Vanita Chopra, GM1 is involved in many processes, including neurotrophin signaling, calcium homeostasis and aggregate modulation, and its levels and synthetic enzymes are reduced in HD cells, as well as in HD mouse and human brain tissues. Several lines of evidence suggest GM1 is protective in HD—it rescues HD cells from apoptosis, reverses motor deficits in various mouse models of HD, increases DARPP-32 labeling, and enhances the phosphorylation of serines 13 and 16 in Htt.

However, GM1 has very poor brain permeability. To improve on the pharmacology of natural GM1, Chopra and colleagues have developed several GM1 analogs using a semi-synthetic approach. Performing in vitro screening tests, they have identified five GM1 analogs that are 100-1000 times more potent than natural GM1. The researchers also discovered that the analogs reduced huntingtin in HD transgenic mice and have favorable pharmacological properties, including blood-brain permeability. Except for one analog that was found to be hepatotoxic, the others appear to be well-tolerated even at fairly high doses. Motor and behavioral tests are currently underway, and the researchers have found that at least one analog improves rotarod performance in R6/2 mice.

Some of these studies are expected to yield new targets with very specific functions which could be modulated in a directed manner. But others suggest developing treatments based on targeting multi-functional cellular components that regulate a wide variety of basic cell functions. The challenge of these latter approaches will be to develop drugs that can be used over long time frames, as required for treating a chronic disease, without eliciting serious side-effects. Intermittent dosing or combining multiple drugs (to lower the doses of individual compounds), as suggested by LaSpada, might prove helpful.

### **Localization of HD pathogenesis**

To make the most of all the potential therapies described above, ranging from reducing mHtt levels to modulating complex signaling pathways, it will be critical to identify the tissues and cell types where interventions will be most effective. As described by William Yang, HD is characterized by the degeneration of striatal and cortical neurons, however, increasing evidence indicates that other cells and tissues are also importantly involved. The concept of selective vulnerability in HD is expanding and the importance of cell-cell interactions, beyond the cell-autonomous alterations caused by mHtt, is being increasingly recognized. Studies of transgenic animals that selectively express mHtt in a subset of cells are providing information about sufficiency—is expression of the mutant protein in a particular cell type sufficient to see a particular phenotype? Conversely, studies in which mHtt has been selectively turned off in a subset of cells are providing information about necessity—is it necessary to switch off mHtt in a particular cell population to eliminate a phenotype? This latter question is expected to be of key importance for the development of optimal HD therapeutics.

Until now, most studies have been of the first kind, assessing sufficiency using Htt fragment models of HD. One of the first hints of HD pathogenesis being non-cell autonomous emerged from studies by Yang and collaborators showing that selectively



expressing a mHtt fragment in striatal medium spiny neurons (MSNs) failed to induce key HD phenotypes, including locomotor deficits and striatal degeneration. Also, the team discovered that progressive motor deficits and cortical neuropathology were only observed when mHtt was expressed in multiple neuronal types, including cortical interneurons, but not when mHtt expression was restricted to cortical pyramidal neurons. Other labs, including Xiao-Jiang Li's, Michelle Ehrlich's and Chris Glass's, have assessed sufficiency of mHtt expression in astrocytes, MSNs and microglia, respectively, suggesting roles for all these cell types in HD pathogenesis.

To address the question of sufficiency, Yang recently generated conditional transgenic mouse models of HD expressing full-length human mHtt in all cells, but at reduced levels in neuronal populations in the striatum, cortex or both. The data revealed that reduction of cortical mHtt expression in these mice partially improves motor and psychiatric-like behavioral deficits but does not improve neurodegeneration, whereas reduction of mHtt expression in both neuronal populations consistently ameliorates all behavioral deficits and selective brain atrophy. Furthermore, whereas reduction of mHtt expression in cortical or striatal neurons partially ameliorates corticostriatal synaptic deficits, further restoration of striatal synaptic function can be achieved by reduction of mHtt expression in both neuronal cell types. The study demonstrates distinct but interacting roles of cortical and striatal mHtt in HD pathogenesis and suggests that optimal HD therapeutics may require targeting mHtt in both cortical and striatal neurons.

Several presentations provided new clues as to how the striatum seems to be particularly vulnerable to HD pathogenesis. As previously mentioned, modulating the expression and/or functioning of some striatal-enriched proteins (e.g., GPX6, Gpr52, and Rhes) can result in the amelioration of HD phenotypes. For example, GPX6, identified by Heiman as a modifier of mHtt toxicity, rescues open field performance and DARPP-32 expression when overexpressed in HD striatum. Also, Lisa Ellerby reported the dysregulation of genes involved in the formation of the dorsal striatum, including DARPP-32, in a neural stem cell (NSC) model derived from HD patient induced pluripotent stem cells (iPSCs). The dysregulation was specifically associated with Htt CAG repeat expansion as revealed by comparisons between NSCs derived from the same patient with or without correction of the CAG expansion using the CRISPR/Cas9 system (see New Tools). Interestingly, CAG repeat-dependent phenotypes were observed at the NSC stage, but not the iPSC stage, and the number of differentially expressed genes associated with CAG repeat number was over 10 times greater in NSCs versus iPSCs.

Extending current understanding of how cortical-striatal connectivity is affected in HD, Carlos Cepeda, working in Michael Levine's lab, reported on the differential contributions of GABAergic interneurons in the striatum and cortex of HD mice. Previous work indicated the presence of altered inhibitory responses in HD. To investigate the underlying causes for these changes, the researchers used whole-cell patch clamp recordings and optogenetics to selectively activate or silence interneurons expressing parvalbumin (PV), somatostatin (SOM), and neuropeptide Y (NPY), and dual patch clamp recordings to study feedback inhibition mediated by MSN axon collaterals.

In both R6/2 and BACHD mice, feedback inhibition was reduced compared to wildtype mice.

The contributions of the different interneurons, however, were varied. In symptomatic R6/2 mice, activation of PV-expressing interneurons induced larger evoked inhibitory postsynaptic currents (IPSCs) in more MSNs and pyramidal cortical neurons compared to those evoked in wildtype littermates. In contrast, optically activating SOM interneurons in the striatum produced evoked IPSCs in MSNs similar in amplitude, but with faster kinetics, to those in wildtype MSNs; and in cortex, the amplitudes of evoked responses in cortical pyramidal neurons were smaller than in wildtype with no significant difference in kinetics. Subsequent experiments silencing SOM interneurons showed that these interneurons contribute to the increased frequency of spontaneous IPSCs observed in striatal MSNs. Activating NPY interneurons in the striatum resulted in very large IPSCs in both wildtype and R6/2 MSNs that displayed similar amplitudes and kinetics. Interestingly, average MSN IPSC responses from NPY interneurons were 10 times larger in amplitude than SOM-activated IPSCs suggesting a larger inhibitory contribution from these interneurons in the striatum. Cepeda's future plans include examining the role of TH+ interneurons, dissecting how each class of interneurons modulates cortical and thalamic inputs, and testing whether in vivo optogenetic manipulations can ameliorate HD phenotypes.

Another clue into cortico-striatal pathogenesis was provided by Dan Wilton working in Beth Stevens's lab who described aberrant interactions between the complement system and microglia which may drive early loss of cortico-striatal synapses. Stevens's team recently identified components of the classical complement cascade as mediators of synapse elimination during development of the visual system. Their data suggest a model in which less active synapses are selectively labeled with complement and then engulfed by microglia expressing complement receptors. Hypothesizing that aberrant reactivation of this mechanism might occur in HD, Wilton measured the levels of complement cascade components (C1q and C3) in BACHD and Q175 mice and found they are elevated in striatum and motor cortex, and increase with disease progression. Using high resolution microscopy, he showed that, as early as 3 months, complement localizes specifically to cortico-striatal synapses prior to their loss from the striatum. Also, microglia appear more phagocytic in the striatum, but not the hippocampus. In addition, complement and microglial activation were observed in HD human brain tissue (stage 4).

Participants also provided new insights into HD pathogenesis beyond the striatum and cortex. Malvinder Singh-Bains working in Richard Faull's lab, for example, reported HD-associated neurodegeneration in three major striatal projection targets, the internal globus pallidus (GPi), the external globus pallidus (GPe), and the ventral pallidum (VP). Using stereology methods to analyze tissue and cell volume, as well as neuronal loss in GP tissues from the Human Brain Bank of the Neurological Foundation of New Zealand, Singh-Bains found that volume changes are largest in the GPe and smallest in the VP, consistent with previous imaging studies. She also found a strong correlation between the extent of GPe, GPi and VP degeneration and striatal neuropathology. The data are the first to suggest an involvement of VP in HD pathogenesis. Also, with access to detailed

family and clinical histories, Singh-Bains was able to determine that GPe and VP volume appear to correlate with motor impairment (but not chorea), GPi volume with irritability, and VP neurodegeneration with both motor and cognitive deficits.

Consistent with the importance of cell nonautonomous processes in HD pathogenesis, Chris Ross described connections between atrophic regions of human striatum and cortex that support a “circuit” model of HD progression in which degeneration reflects the anatomic connections of affected neurons. The study used structural and diffusion tensor imaging data from the PREDICT-HD dataset to assess whether the heterogeneous atrophy seen in the striatum of pre-manifest individuals correlates with regions predicted to receive projections from cortical regions with greatest atrophy. The initial data are consistent with a circuit degeneration model of HD. As Yang’s findings in mice, such studies should help identify seed regions of pathology. If the time course and directionality of pathology can be determined, it may be possible to infuse gene silencing reagents to these focused seeds, early in the premanifest period, and block further spread of pathology. To extend his findings, Ross plans to include in future studies detailed assessments of regional cortical atrophy.

Pathogenesis beyond the nervous system, involving peripheral tissues, was also discussed at the meeting. For example, Gill Bates noted that muscle atrophy is an important component of HD pathology observed both in humans and mouse models (e.g., R6/2 and HdhQ150). The adult neuromuscular junction appears to develop normally in HD, but a progressive dysregulation of homeostatic feedback mechanisms regulating anabolic and catabolic pathways ensues. To test whether modulating myostatin signaling could improve this alteration, Bates and colleagues inhibited myostatin in R6/2 mice using an ActRIIB receptor decoy (weekly dosing, starting at 5 weeks of age). The treatment completely rescued body weight and muscle mass (quadriceps, tibialis, gastrocnemius) loss and improved grip strength impairment, but had no effect on rotarod or open field deficits (suggesting these latter alterations may be caused by CNS pathogenesis). The researchers also observed improvements in neuromuscular function (twitch kinetics, tetanic force, rescue of motor unit loss). In addition, aggregation was reduced and the transcription of several genes was normalized, although there was no change in myogenic gene expression suggesting myogenesis remains unchanged. Bates noted her team is now using RNAseq genomic analyses to further examine the transcriptional changes. Of particular interest, several therapies that regulate myostatin are currently under clinical development for a number of disorders.

#### Mechanisms of cell nonautonomous effects

As summarized by Yang, several mechanisms have been proposed to explain cell nonautonomous pathogenesis in HD. Mutant Htt-mediated dysfunction of a particular cell can affect the function of cells it directly interacts with as noted above. Alterations in cortical pyramidal cell electrophysiology, for example, affect target MSNs, and interneuron dysregulation in both striatum and cortex appears to affect MSN and cortical pyramidal cell behaviors. In addition to synaptic signaling, alterations in the production and transport of brain-derived neurotrophic factor (BDNF) from cortex to striatum have been proposed to mediate cell nonautonomous effects.

Glial cells also seem to importantly contribute to cell nonautonomous effects. Shihua Li, for example, noted that transgenic mice expressing mHtt exclusively in astrocytes have delayed, HD-like phenotypes. In addition, Li presented data suggesting that oligodendrocytes are also involved in HD pathogenesis. Working with Xiao-Jiang Li, Shihua Li established a line of transgenic mice (PLP-150Q) that selectively expresses mHtt in oligodendrocytes. PLP-150Q mice show progressive neurological symptoms, including unsteady gait and deficits in rotarod performance, nuclear aggregates, weight loss and early death. The researchers also observed age-dependent demyelination and reduced expression of myelin genes regulated by myelin regulatory factor (MRF), a transcriptional regulator expressed in postmitotic oligodendrocytes. A mechanism underlying this alteration was suggested by the additional finding that mHtt binds abnormally to MRF and affects its transcriptional activity.

Another mechanism to explain cell nonautonomous effects is the transcellular propagation of misfolded proteins. As described by Marc Diamond, multiple studies have shown cell-to-cell spread of A $\beta$  aggregates,  $\alpha$ -synuclein, mutant SOD1, and tau protein. Sharing his experience characterizing the transcellular propagation of tau, Diamond noted that, ten years ago, he hypothesized that tau might behave like a prion protein. Imaging data from Alzheimer's disease patients indicated that tau pathology involves interconnected brain regions with nodes of most connectivity exhibiting the most atrophy.

Consistent with these observations, Diamond showed that tau moves across synapses and stably propagates aggregates. In addition, he discovered that, like prions, tau maintains unique conformations or "strains" in vivo that appear to link structure to patterns of pathology. Diamond first observed that distinct amyloid conformations could be indefinitely propagated in culture and introduced in vivo to induce distinct pathologies. Subsequently, he was able to isolate tau strains from patients with 5 different tauopathies, indicating that different diseases are associated with different sets of strains. The findings suggest a new way of classifying and diagnosing disease.

As explained by Diamond, studies aimed at understanding the release and uptake of aggregates have implicated several different mechanisms in the trans-cellular propagation of prion-like proteins such as tau, including the release of aggregate seeds that are free or encapsulated in exosomes, the binding of these seeds to heparan sulfate proteoglycans (HSPGs) on the cell surface, the stimulation of macropinocytosis that results in seed internalization, the movement of aggregates across long cytoplasmic extensions called tunneling nanotubes, and the direct translocation of seeds across plasma membranes. This understanding is useful for therapy design. Indeed, Diamond noted that small molecule heparin mimetics are being developed to block tau seeding and propagation, as well as blockers of HSPG synthesis and anti-tau blocking antibodies.

Whether mHtt also propagates between cells is currently unknown, but a subject of ongoing research. Data from Ross and colleagues (described above), supports a circuit model of HD progression which would be consistent with transneuronal propagation of toxic mHtt aggregates. However, Diamond noted his team has so far been unable to identify such a phenomenon in cells and added that, if it occurs, the mechanism is likely

to be different from that of tau. In line with data reported by Ron Kopito's team, Diamond has observed extracellular mHtt exon 1 aggregates entering cells in culture. However, he has seen no co-localization of these aggregates with HSPGs and no sensitivity to heparin. Also, studies in which BACHD mice have been vaccinated with mHtt aggregates, producing robust immune responses, have failed to induce a detectable amelioration of behavioral deficits, neuropathology or biochemical alterations. However, as pointed out by Anne Messer, other types of immune vaccinations (using plasmids, for example) might prove more effective and Diamond noted that longer mHtt constructs may yield different results.

Of particular interest, just days before the meeting, Pecho-Vrieseling and colleagues published a paper describing transneuronal propagation of mHtt from cortical to striatal cells in two brain slice models and an in vivo model of HD. The uptake of mHtt aggregates resulted in neurite degeneration and reduced DARPP-32 staining in brain slices. The researchers also reported that mHtt propagation appears to require synaptic vesicle release, at least in a co-culture model as assessed by botulinum toxin sensitivity. As noted by Diamond and Faull, if mHtt does indeed behave like a prion, it could generate different aggregate forms helping explain the wide variety of aggregates described in the literature, as well as the heterogeneity of HD clinical phenotypes. Also, as noted by Gai Ayalon, the therapeutic potential for treating a prion-like agent in HD is particularly attractive given that treatments (such as antibodies) could be administered, at least in principle, early in the disease process, before symptoms arise.

But much remains to be understood. Even if mHtt is found to propagate in human HD, its pathogenic relevance will need to be established since Htt is expressed in all cells. Also, mHtt might be dispersed by any of a number of mechanisms, including paracrine propagation and long-distance dissemination. Indeed, Francesca Cicchetti described the presence of extracellular mHtt aggregates within fetal allografts in post-mortem brains of three individuals with HD and suggested the possibility of paracrine dissemination. The aggregates were slightly less numerous, but similar in size and shape to those seen in the host putamen, and notably restricted to the extracellular matrix (in both striatal-like neuronal areas and areas lacking striatal markers). Previous studies failed to detect aggregates within grafts, but this new study examined transplants exposed for a longer time to the disease (approximately a decade) and used more sensitive detection techniques. The exclusively extracellular location of these aggregates suggests that mHtt might not propagate transneuronally and instead involve paracrine mechanisms. Cicchetti proposed that mHtt could have been transported into the graft by host immune cells in the bloodstream. Electron microscopy revealed mHtt aggregates in perivascular macrophages, which might gain access to the brain via an HD-compromised blood-brain barrier.

## New Tools and Approaches

### Cell and animal models of HD

Participants recognized the importance of developing, characterizing, and optimizing cell and animal models of HD. Lisa Ellerby, for example, described her work using the CRISPR/Cas9 system to generate isogenic allelic series of HD iPSCs (CAG repeats of 45, 72, 100). Of particular interest, Ellerby noted that the use of the Cas9 nuclease variant D10A enhanced the frequency of desired homologous recombination events. In addition, a novel antibody screening assay (based on the epitope for the polyQ expansion) allowed her team to measure the relative rate of gene targeting events and streamline the process of generating of allelic series. The optimization of this process is of value for several reasons. It provides a model system in which the effects of CAG repeat length can be studied independently of other genetic factors both in vitro and in vivo (when cells are differentiated and transplanted into animal models). It also offers the possibility of testing the effects of transplanting corrected, differentiated cells into animal models to answer mechanistic questions about pathogenesis (including studies of the localization of pathogenesis). Ellerby and colleagues have observed that correction of iPSCs in vitro rescues mHtt-associated alterations that manifest when iPSCs are differentiated into neural stem cells. Although several challenges are yet to be resolved, the possibility of using this same system for therapeutic purposes in the future has also been considered.

The status of new and large animal models of HD was reviewed by a panel including experts on bird, pig, sheep and primate models of the disease. Jenny Morton set the framework for the discussion by asking panelists to describe the features of their models that make them useful for HD research. Morton urged participants to avoid focusing on the limitations of the models, which all models have, and instead describe their unique advantages. Quoting the British mathematician George E.P. Box, she said, “Essentially, all models are wrong, but some are useful.”

A major advantage of several new animal models of HD, including pigs, sheep and non-human primates, is the size of their brains. As noted by Morton, rodent models have provided invaluable information on the pathogenesis of HD, but as an increasing number of new candidate therapies come closer to being ready for evaluation in humans, animals with larger brains, in which the distribution of therapeutic agents can be studied, are sorely needed. Russell Snell described a transgenic sheep model carrying a full length human Htt gene with 73 CAG repeats which should prove useful for such studies. The researchers are now characterizing histological and molecular signatures of disease in these animals and have found cortical mHtt aggregates, as well as changes in the metabolite and transcriptional profiles of different brain areas and the liver. Snell noted that, given the mild phenotype of these animals, indicating a slow progression of disease, the molecular changes may help identify useful presymptomatic biomarkers of HD and provide a window for the exploration of early therapeutic interventions.

Morton, who has been characterizing the behavioral phenotypes of these sheep, added that these animals are also advantageous because they live long, are affordable and are easily managed. Her group has been studying the gait, cognitive abilities and circadian

rhythms of HD sheep. So far, they have not observed any overt motor or cognitive deficits in 5 year old sheep, but have seen early circadian abnormalities. The team is now setting up to perform long-term, longitudinal EEG recordings to detect sleep disruptions or seizure activity. In pilot experiments with another sheep model (Batten's disease), Morton has found that sheep EEGs during sleep are similar to humans'. Her team also discovered that sheep ruminate not only when awake, but also during sleep. Because rumination is a highly structured motor behavior, Morton speculated it could be a read-out for dysphasia in HD.

Sharing his experience using sheep to optimize the delivery and distribution of AAV vectors in the brain, Bill Kaemmerer reiterated the benefits of sheep's large brains and noted the benefits of being able to use the same neurological tools and techniques used in humans, including MRI to track interventions. As examples of the technical lessons he has learned working with sheep, he noted that, whereas infusion flow rates for delivering AAV into the brain do not seem to be critical, the volume of injection is. His work in sheep has also led to insights into the unique advantages and limitations of different AAV serotypes.

Anthony Chan and Jodi McBride, on the other hand, are developing and characterizing nonhuman primate models of HD. As explained by the two researchers, their aim is to establish models that recapitulate many of the phenotypes in humans that are not present in other animal models (e.g., cognitive and psychiatric alterations). McBride is generating these models by performing MRI-guided injections of AAV1 carrying mHtt fragments with either 82 or 16 CAG repeats into the caudate and putamen of rhesus macaques. Compared to control-injected hemispheres, experimental hemispheres showed a loss of DARPP-32 expression and, to a lesser extent, NeuN-positive neurons in the caudate and putamen. In addition, the researchers observed increased numbers of GFAP-positive astrocytes, IBA-1-positive microglia and cellular inclusion bodies. The investigators are now training macaques on several motor, cognitive and temperament tests. As summarized by McBride, the main advantages of this model are its rapid time course (as compared to human disease), the ability to target discrete brain regions, the availability of a large number of animals at the Oregon National Primate Research Center, the scalability of the generation process, and the opportunity to use different constructs to create different models.

Another nonhuman primate model was described by Chan who has developed transgenic monkeys carrying constructs encoding the N-terminal 508 amino acids of human Htt with 67-72Q under the control of the human *HTT* promoter. So far, the researchers have observed progression of HD-like phenotypes in this model as assessed by longitudinal studies conducted over 5-6 years, including imaging (MRI), cognitive, behavioral, and motor assessments, as well as molecular profiling of blood and CSF samples. The findings reveal the presence of progressive dystonia and chorea, as well as a decline in motor skills. Moreover, changes in MRI scans, a decrease in striatal neurons and the appearance of inclusions have also been observed. The team's goal is to establish a time course of disease progression in HD monkeys that will facilitate future pre-clinical studies.

A transgenic HD model is also being developed in pigs whose organ morphology and size is comparable to that of humans, are relatively easy to care for, and have large litters. As summarized by Jan Motlik, his team has generated transgenic minipigs using a lentiviral vector carrying a construct encoding the N-terminal 548 amino acids of human Htt with 126 CAG/CAA repeats under the control of the human Htt promoter. The expression of this transgene is similar to that of the wildtype gene. Analyzing phenotype progression, the researchers have so far observed impairments in sperm function (e.g., motility, mitochondrial physiology, egg penetration), increased microglial activation in the caudate, increased monocyte activation and interleukin-8 production, alterations in BDNF levels and elevated levels of ACBD3, a protein involved in maintaining the structure and function of the Golgi apparatus and which has been implicated in mHtt cytotoxicity.

Richard Mooney, on the other hand, has generated songbirds that express mutant Htt and display alterations in their singing patterns. The model is of interest because vocal learning in songbirds requires a brain circuit called the anterior forebrain pathway (AFP) which is very well understood and is homologous to cortico-basal ganglia-thalamo-cortical loops in mammals. Area X, a brain region that is part of this pathway and is necessary for juvenile song learning and adult modulation of learned songs, is similar in many ways to the mammalian striatum. In addition, bird song is a complex motor output that can be quantitatively analyzed and monitored over time. Also, birds have short reproductive cycles, there are methodologies for performing targeted viral gene transfer in these animals, and birdsong analysis could be relevant to HD phenotypes such as speech impairment and learning deficits. Mooney and colleagues injected a lentivirus containing a mHtt exon 1 (Q94) construct into Area X. In such birds, increases in both syllable sequence variability and song length were observed. In addition, the researchers found perseveration, introduction of new syllables and elevation in the number of repeated syllables. In contrast, the spectral features of individual syllables remained stable, indicating that mutant Htt selectively disrupts the neural control of vocal sequences.

As participants discussed the challenges and opportunities that lie ahead, they noted several important questions: 1) Are more models needed, and if so, which types? 2) How can models be used more effectively? 3) Should a set of standardized mHtt constructs be compiled? 4) How can research needs (e.g., understanding of pathogenesis, testing of efficacy and safety of candidate therapies, recreating specific human phenotypes, etc) be best matched to particular model systems? 5) What are the FDA's and the pharmaceutical industry's views of the value of the different models for pre-clinical testing? 6) How can the models help track disease progression in new ways (e.g., pig sperm is accessible, birdsong may reveal more subtle dysfunctions)? 7) How can the models best help assess the risk of futility and safety concerns before taking a candidate treatment into the clinic?

#### New technologies for basic research and therapeutic development

Several participants presented new techniques that are helping advance HD research. Steve Finkbeiner, for example, noted that his laboratory has established a system for "deep phenotyping in a dish." With a library of 100-200 biological sensors, using



automated microscopy to longitudinally follow individual cells' behaviors, the team is able to correlate a wide array of cell phenotypes with genetic and environmental factors. Using iPSCs, the researchers can also correlate their findings with clinical information from patients. The large amounts of data generated are challenging to handle, but collaborating with Google, Finkbeiner and colleagues are finding new ways to extract valuable information using sophisticated pattern recognition algorithms. The approach promises to help identify new drug targets, contribute to the validation of candidate drugs and targets, aid in the identification of genetic modifiers of disease, and enable testing of candidate modifiers. In the future, it could also be used to stratify patients for clinical trials based on complex cellular signatures.

In addition, Christian Neri briefly described a systems biology approach that his group is spear-heading to aid in the extraction of information from large HD datasets. Neri and co-workers have developed Biogemix, a network-based, data integration framework for rule/pattern extraction across models and species. The framework has allowed the researchers to integrate more than 14 publically available HD datasets, including compilations of transcriptomic and gene perturbation data. In addition to identifying new HD-associated alterations, the team has been able to determine the functional distances between experimental models of HD, assess their biological relevance across different parameters, obtain functional profiles of biological modifiers, and elucidate the shared/unshared properties of pathological and compensatory/survival genes.

Pointing to the need for methods that allow a higher throughput and sensitivity to assess neuronal morphology, William Yang described his team's development of a new, unbiased procedure to label single neurons sparsely. The technique yields Golgi-like images, but is much more versatile and powerful than the classic staining protocol. It is based on generating transgenic mice expressing a GFP construct with an out-of-frame mononucleotide repeat which stochastically slips into frame in about 1% of cells. Using this method to specifically label MSNs expressing dopamine D1 receptors, and crossing these animals with Q175 knockin mice, the researchers have been able to accurately measure HD-associated alterations in the length and diameter of MSN D1 dendrites.

Another new promising technique was described by Ed Wild. Working with Rainer Kuhn's and Sarah Tabrizi's teams, Wild reported the development of a single-molecule counting immunoassay (using the 2B7/MW1 Htt antibody) that can measure femtomolar concentrations of mHtt in the CSF of HD patients. Wild noted that the assay is capable of detecting significant differences between premanifest and manifest subjects. In addition, a significant association was found between mHtt levels and each one of the following indicators: disease burden score, 5-year probability of onset, UHDRS motor score, and cognitive test results. Given that CSF mHTT concentrations were significantly associated with the concentrations of neurofilament light chain and tau, but not with hemoglobin, the researchers reason that the mHtt detected by the assay is of neuronal origin, possibly being released by dying neurons. The assay could aid the study of HD pathogenesis in living patients and may serve as a biomarker of disease progression and/or response to huntingtin-suppression therapies.

Calling attention to a fundamental problem in the development of therapeutics for neurodegenerative diseases, Gai Ayalon shared his and his Genentech colleagues' experiences developing new ways to deliver large molecules across the blood-brain barrier. The removal of toxic protein aggregates is a common goal shared by researchers working on neurodegenerative disorders and antibodies, noted Ayalon, offer a promising tool to advance this goal. Antibodies have high selectivity for their target, are cleared very slowly, and are widely used for other therapies. However, antibodies are very large—roughly 375 times larger than small molecules.

To overcome this problem, Genentech researchers led by Ryan Watts, co-opted the transferrin receptor pathway which carries transferrin across the blood-brain barrier via receptor-mediated transcytosis. They created bispecific antibodies with one end that recognizes the transferrin receptor and the other a target protein (such as beta-secretase 1 (BACE1) which produces amyloid-beta--antibodies against other target proteins are also under development). In initial studies, the engineered antibodies bound to the endothelial cells, as expected, but a large proportion remained stuck on the transferrin receptor failing to reach their ultimate target. Testing various engineered antibodies with mutations that resulted in differing affinities for the transferrin receptor, the researchers were ultimately able to generate an antibody that binds to the transferrin receptor with low affinity such that it is quickly released into the brain after transcytosis and able to bind to BACE1. Testing the therapeutic antibody in mice and monkeys, the researchers found that it spreads throughout the animals' brains, decreasing levels of amyloid- $\beta$ .

These results are of general importance because they show that the bispecific antibodies appear to be safe, can successfully enter the brain, and suggest that the method could be modified to carry any number of antibodies or drugs across the blood-brain barrier. Looking ahead, Ayalon noted that a few open questions still remain, however. For example, the mono-valency of the antibodies results in low avidity, a feature that can significantly affect their binding to oligomeric proteins (an issue of potential importance for HD). Another challenge is that antibodies can induce or aggravate neuroinflammation via their effector regions. Antibody backbone effector regions are recognized by microglia leading to clearance of the antibodies attached to their targets (a beneficial event), but also causing microglial activation (a potentially harmful event). Thus, the researchers compared the effects of an anti-tau bi-specific antibody with and without its effector region, and found that effectorless antibodies were capable of blocking the spread of tau and less neurotoxic than antibodies with an effector region. Further studies are warranted to more fully understand the fate and effects of antibodies with and without effector domains.

The relevance of these findings to HD remains uncertain. As has been well established, mHtt is an intracellular protein and, consequently, not directly accessible to extracellular antibodies. However, as discussed earlier (see "Localization of Pathogenesis: Mechanisms of cell nonautonomous effects"), some findings suggest mHtt may be capable of traveling through the extracellular space via transcellular propagation. If this indeed occurs, then anti-Htt antibodies could be helpful, at least in theory. However, the pathogenic importance of this spread (if it occurs) would need to be assessed. Given Htt's

universal expression, it could be of less pathological relevance than the spread of other toxic proteins.

#### China and its potential for advancing HD research

Even more encouraging than the development of new tools, is the potential of recruiting more talented experts to tackle HD. Zhi-Ying Wu prepared a talk (delivered by Xiao-Jiang Li) highlighting the growing opportunities for HD research in China. Introducing the talk, Chenjian Li noted that a full quarter of the world's population is Chinese and China's economy is the second largest in the world, growing at a remarkable pace for the last 20 years. Biomedical funding and positions for new researchers have been increasing dramatically, and with an increase in the population's life expectancy, interest in neurodegenerative disorders is rising sharply.

Li noted that Dr. Wu is highly regarded in the field of clinical genetics and her research interests encompass several neurological disorders, including HD. Reviewing the literature on HD in China, Wu identified 92 published studies since 1980, including 279 patients. Although the disease appears to express similarly in Asian versus Western populations, there are some differences worth noting. The prevalence of HD in Caucasians is 5~7/100, 000, whereas in Asian populations (Japanese) it has been reported to be 0.5/100, 000. The reason for this disparity is unknown, but may be due to under-diagnosis and/or differences in haplotype distribution. Summarizing Wu's work in China, Li noted that age of onset is, on average 35.8 yrs old and age of death 45.6 years old. Clinical manifestations appear to be similar to those reported in Western populations (99.6% involuntary movements, 67.9% cognitive impairment, and 35% mental disorder). Very few juvenile cases have been examined, but interestingly, these have involved much less chorea and higher levels of cognitive impairment than typical adult-onset cases. Moreover, haplotype analysis of the CAG and CCG repeats revealed that whereas in Caucasians larger CAG repeats are often linked to (CCG)7, in Chinese populations they appear to be linked to (CCG)10.

As evidence of the growing awareness and interest in HD in China, Li noted the establishment of the Chinese Huntington's disease network in 2011 which includes seven medical centers. Also, he pointed to the recent Chinese translation of HD Buzz--an internet portal for the rapid dissemination of HD research news. But much remains to be done. Li noted a need for improved HD diagnosis, including greater access to genetic testing, improved access to genetic counseling and prenatal diagnosis, and better medical and psychological care for pre-symptomatic and symptomatic patients. In addition, he noted that further study of genotype-phenotype associations and increased basic research into HD pathogenic mechanisms is critical for the development of HD research in China.

## **Concluding thoughts**

In addition to bringing participants up-to-date on some of the most exciting advances in HD research, the meeting provided insights into what is needed to move the field forward. With the wealth of reported alterations associated with HD, one of the most difficult and important challenges is to identify and validate those mechanisms and targets that are most central to the disease process and that will most likely lead to effective disease-modifying treatments. As Steve Finkbeiner noted, lack of drug efficacy is currently estimated to be responsible for over 50% of the failures of Phase II and Phase III clinical trials. The situation is particularly challenging for neurodegenerative disorders where, as noted by Gai Ayalon, very few targets and drugs have been properly validated. To address this challenge, the Hereditary Disease Foundation supports research and the exchange of ideas that will lead to the robust validation of targets and/or pathogenic mechanisms for the development of HD treatments. Visionary researchers like the late Paul Patterson, whose life and work were honored at the meeting, promise to serve as models and inspiration for the hard work that lies ahead.