HD 2012: "The Milton Wexler Celebration of Life"

August 1-4, 2012
Royal Sonesta Hotel
Cambridge, Mass.

Report prepared by Marina Chicurel, Ph.D.

Table of Contents

Summary

On August 1-4, nearly 300 investigators from around the world gathered at the "HD2012: The Milton Wexler Celebration of Life" meeting to discuss some of the latest research on Huntington's disease (HD). What emerged was a more integrated view of huntingtin's multiple functions, a demonstration of the remarkable potential of several new technologies to interrogate the basic mechanisms of HD pathology, and a sharpened focus on a few promising therapeutic candidates.

The many alterations described in HD and the many functions that have been ascribed to the huntingtin protein have made it difficult to understand the disease in an integrated manner. At the HD2012 meeting, the convergence of some observations on huntingtin's normal functions and the suggestion of its role as a scaffold protein with multiple functions that can be activated or blocked differentially, helped reconcile some of the disparate findings in the field.

The meeting also revealed that many of the key questions that have remained stubbornly unanswered in HD research are now amenable to experimental probing in ways that were previously impossible. For example, several participants described the use of induced pluripotent stem cells (iPSCs) to understand the differential vulnerability of cells in HD, the toxic effects of the HD mutation that depend on cell-cell interactions, and the dependence of different aspects of HD pathology on the length of the HD mutation—the expanded CAG repeat in the huntingtin gene. Participants also noted how these stem cells are expected to shed light on the unfolding of HD toxicity during development, as well as offer a window on the phenotypic variability of HD, including the effects of genetic background on the expression of the disease. As answers to these long-standing questions emerge, the rate-limiting steps of the disease should become increasingly evident, offering a much more reliable guide to therapy development than has been previously available.

Other tools that are accelerating the understanding of HD were also discussed. For example, optogenetics, with its ability to unravel the contributions of different brain cells in the altered circuitry of HD, highlighted the thalamus as an important site of HD pathology. Also, the potential of high throughput techniques to analyze the levels of many proteins at once was underscored in studies identifying altered pathways in HD and candidate biomarkers for tracking HD in clinical trials. Moreover, new transgenic mouse models were described that are helping dissect the different functional domains of the huntingtin protein. And a newly characterized sheep model promises to provide a powerful model to run preclinical tests.

The other important source of excitement at the meeting came from the presentation of therapeutic efforts that are steadily progressing to the clinic. As discussed at previous meetings, using gene silencing techniques to eliminate or reduce the expression of mutant huntingtin has emerged as a particularly promising therapeutic candidate. This year, the field seemed more sharply focused, with increased efforts directed at specifically silencing the mutant copy of the huntingtin gene. Also, several ways of overcoming the obstacles associated with gene silencing, such as new methods to effectively distribute the silencing agents in the brain, were presented. Moreover, modulating protein biogenesis, folding, trafficking and/or clearance—pathways involved in the maintenance of a healthy cellular proteome, or proteostasis—emerged as another promising avenue for therapy development. Recent examples of the development of such therapies for other diseases that involve protein aggregation, helped participants

appreciate the potential of this approach and consider ways of refining previous proposals for modulating these pathways to treat HD.

Participants also recognized the one constant, critical component of the search for HD treatments: the help and inspiration provided by HD families. Nancy Wexler reminded participants of the key role played by the Venezuelan families whose blood and semen samples led to the localization, and subsequent identification, of the HD gene. Moreover, Richard Faull described how his findings on the variable phenotypes of HD depended critically on the families who donated HD brains and helped assemble the associated medical and family histories. In addition, Diana Rosas, who presented recent findings on a pioneering study for evaluating presymptomatic biomarkers and the effects of creatine as a potential therapeutic agent, also thanked the HD families who have been willing to participate in these early clinical trials. Moreover, HD families have also been the source of a growing number of iPSC lines that, as mentioned above, promise to accelerate progress in the field in many ways.

Despair transformed into positive action

One HD family generously contributed their time and experiences at the meeting itself. Hubert and Marie Jessup offered participants a rare view of the courage of a family who did not imagine they would be living with HD. As explained by Mr. Jessup, a charming and articulate 65-year-old man, until only a few years ago, he was living with the near certainty that he would not develop HD. "I was fit and intellectually capable…I took the [HD genetic] test because I thought I wouldn't have it."

Mr. Jessup, a graduate of the Harvard Divinity School, is an accomplished, intelligent, and athletic man who has succeeded at many endeavors during his productive life. For 11 years, he produced a weekly TV program on religion in which he presented one of the first American TV interviews with the Dalai Lama. He also produced a sitcom show which was a precursor of the hit TV series "Cheers." He later became president of Mediascope, a non-profit organization dedicated to raising awareness of the effects of media on society. In 2002, he became the director of external communications at a craft and trade school. Four years later, however, he lost his job, shortly after being diagnosed with HD.

As described by Mr. Jessup, discovering that, like his father, he carried the HD mutation was deeply disturbing. His physical fitness had led him to believe that he would be immune to HD. Mr. Jessup was a serious runner who participated in several marathons, including one in Death Valley. "Wow, I'm a runner," Mr. Jessup recalled thinking. "I can't be sick." Mr. Jessup's sister also developed HD and, as recounted by Ms. Jessup, the family avoided confronting the evidence of her disease. It was not until Mr. Jessup's sister injured herself after falling into a pool that her HD status was recognized. Moreover, the recognition occurred only because the sister accidentally read her own medical chart which included a confidential comment from her sister-in-law noting that she likely had HD.

The courage with which the Jessups are now dealing with the disease was inspiring to all. Mr. Jessup remains very active, and is resourceful and adaptable. He has maintained connections to a large and strong community network, which includes lifetime friends, neighborhood acquaintances, and a church choir. The latter is particularly important for Mr. Jessup as he finds comfort in the joyful singing, as well as emotional support from his fellow singers. Moreover, Ms. Jessup has taken on more household responsibilities (such as managing the couple's finances) and is planning to modify her work schedule to provide her husband with additional support. Although, at times, Mr. Jessup feels deeply depressed and

worried about being a burden, he is not retreating from life. Together, the couple is working to find new ways to cope with HD and support research in the field, as evidenced by their gracious participation at the meeting. The Jessups urged participants to work even harder and faster to find new treatments and a cure. The HDF joined them in this encouragement and all attendees were deeply moved.

Another inspirational example was provided by Nancy Wexler's description of the Casa Hogar Amor y Fé (House of Love and Hope), a clinic and nursing home that provides treatment, food, and care to individuals suffering from HD in Venezuela. Many of the Venezuelans who are tended to at the Casa Hogar are members of families that were instrumental to finding the HD gene. "They endured clinical examinations over many years, gave precious DNA and made it possible to find the HD gene despite skeptics who said it would take centuries to locate, if it could be done at all," noted Wexler. Sadly, these important contributors to the HD field live in extreme poverty and isolation. Located on the shores of Lake Maracaibo, this community has the highest prevalence of HD in the world yet receives very little medical assistance and its members are still shunned from the larger Venezuelan society (individuals with HD are called 'intocables' or 'untouchables').

To alleviate their dire conditions, the Hereditary Disease Foundation (HDF) has been providing support to the community since 1980, donating $17 million dollars in medicines, food, supplies and staff support. In addition, the HDF worked with local Venezuelan authorities to build the Casa Hogar which opened in 1999. "The Casa Hogar is our way of expressing our thanks for these families' invaluable contributions," said Wexler. A unique feature of the Casa Hogar is that its 30-member staff is composed entirely of HD family members, with the exception of its dedicated Venezuelan director, Dr. Margot de Young, who has been working with the HDF since 1991.

Since its opening, however, the home has been repeatedly at risk of closing. Indeed, the day after it opened, it closed briefly because of lack of funding, and in 2011 it almost closed again. However, a generous donation from Lundbeck saved the Casa Hogar and through Lundbeck's Build Hope for HD campaigns, additional funds are expected to allow the clinic/home to continue providing services to over 65 people who now reside there, as well as meals and treatment for hundreds more in the community.

The normal function of huntingtin protein

As noted by Wexler, almost 20 years have passed since the Huntington's Disease Collaborative Research Group identified the HD gene with the help of the Venezuelan HD families. Much has been learned during the past two decades, but the function of the large, ubiquitous protein encoded by the gene has remained elusive. Over 200 different proteins with a diverse array of biological functions have been identified as proteins that interact with huntingtin. Not surprisingly, many hypotheses about huntingtin's biological function have been put forth which have proved difficult to reconcile.

New observations and models presented at the HD2012 meeting, however, offered a window into understanding, in a more integrated way, the many faces of huntingtin. As described by Ray Truant and Frédéric Saudou, huntingtin appears to be a scaffold protein with multiple functional domains. Truant noted that huntingtin has several sites on which protein complexes can assemble and leucine-rich helical structures (HEATs) interspersed throughout most of its length. HEAT repeats have mechanical properties, as described by Nancy Kleckner, that facilitate conformational changes in their host proteins,

exposing or hiding different functional domains in an allosteric fashion. Thus, Truant suggested that huntingtin may have different functions that are activated or blocked under different cellular conditions.

The proposal is appealing because it helps make sense of some of the disparate findings that have emerged over the years. Elaborating on his working model, Truant noted that huntingtin may be tethered to mobile elements in the cell and the mechanical movements of those elements may dynamically affect huntingtin protein-protein interactions. Thus, huntingtin may be like a "Slinky" toy, a helical spring that stretches, revealing different domains, depending on how much it is stretched or compacted. Truant suspects that huntingtin's first 17 amino acids, an amphipathic alpha-helix that associates with cell membranes, is one of the protein's tether points. In addition to mediating the differential activation of huntingtin's functional domains, this configuration may also allow huntingtin to act as a mechanotransducer, Truant hypothesized. If this proves to be the case, huntingtin may be capable of storing a large amount of potential energy due to its large size.

Several participants contributed to filling in the picture of huntingtin's multiple functions. Saudou, for example, presented data suggesting that huntingtin localizes glycolytic enzymes to axonal vesicles, providing an energy supply for vesicle movement. Previous studies from Saudou's group had shown that huntingtin helps mediate the microtubule-based transport of neurotrophic factors, in particular brain-derived neurotrophic factor (BDNF). Because energy deficiencies have been reported in HD, Saudou and colleagues decided to investigate the source of energy for fast axonal transport. Using microfluidic chambers to observe the transport of labeled BDNF in individual axons in culture, the team found that pharmacological and genetic inhibitors of glycolysis, but not mitochondrial function, block vesicular transport. They also observed that the glycolytic enzyme GAPDH localizes to the outer surface of purified motile vesicles in association with huntingtin. Consistent with this observation, huntingtin-deficient cells have reduced levels of GAPDH on their vesicles. Additional experiments revealed that the complete set of glycolytic enzymes is associated with these axonal vesicles, suggesting huntingtin acts as a scaffold for the entire glycolytic machinery. Future experiments will test whether vesicle movement can be achieved in vitro by simply adding glucose.

Participants also discussed other ways in which huntingtin may play a role in cellular transport. For example, huntingtin may help mediate post-Golgi trafficking of several proteins, including lysosomal enzymes, through its association with optineurin and Rab8. In addition, Saudou's team recently demonstrated that wildtype huntingtin regulates the formation of cilia by participating in the retrograde trafficking of PCM1, a protein that ensures the correct localization of several proteins, including microtubules, to centrosomes. Alice Karam added that huntingtin may continue to be important for the function of mature cilia, noting that huntingtin co-localizes with microtubules in all the compartments of the photoreceptor cilia of the retina, and that these cilia become elongated in HD photoreceptors. Furthermore, huntingtin's role in ciliary function is unlikely to be limited to the retina—Karam found huntingtin in cilia from the olfactory epithelium and sperm, and Saudou noted that the motility of ventricular cilia in HD brains is impaired.

Huntingtin also appears to be involved in mediating cellular stress responses. An intricate set of signaling pathways from the endoplasmic reticulum (ER) to the cytosol and nucleus allow cells to respond to the presence of misfolded proteins within the ER. Truant suggested that huntingtin might be a key element involved in coordinating these pathways. Recent studies by his group show that, although wildtype huntingtin is mostly found in the cytoplasm, cellular stress causes it to relocate to the nucleus.

Huntingtin contains both a nuclear export signal (NES) and a nuclear localization signal (NLS), which can be regulated by the binding of huntingtin associated protein 1 (HAP1), phosphorylation, and possibly acetylation. Truant noted that phosphorylation of huntingtin's first 17 amino acids, which contain the NLS, by casein kinase II, increases nuclear targeting. In the nucleus, huntingtin transiently localizes to chromatin and the mitotic spindle, but most strikingly and persistently, associates with nuclear cofilin-actin rods. Truant proposed that huntingtin plays a direct role in stress-induced actin re-organization. This is of particular interest given the importance of actin dynamics in the health of neurons that are particularly vulnerable to HD pathology, the medium spiny neurons of the striatum.

Consistent with huntingtin having a role in cellular stress responses, Joan Steffan noted that huntingtin may also play a role in selective macroautophagy, a lysosomal mechanism involved in the degradation of aggregated proteins which is activated during ER stress. Steffan pointed out that huntingtin has similarities to several proteins required for selective autophagy in yeast. In addition, data presented at the HD2010 meeting by Esther Wong indicate that huntingtin may have a normal role in macroautophagy, possibly in cargo recognition. Steffan further explained that the phosphorylation and acetylation of residues within the N-terminus which affect huntingtin's cellular localization, may also participate in the regulation of huntingtin's role in autophagy. Since activation of autophagy is coupled to the ER's stress response, these observations are consistent with Truant's proposal of huntingtin being a multi-functional stress-response element. Also consistent with this view are a number of observations (see Mutant Huntingtin Toxicity) indicating that, in HD, several processes related to ER stress and the secretory pathway are impaired early in the disease, including axonal transport, ER-associated degradation, ER/Golgi vesicular trafficking, autophagy, and ER calcium homeostasis.

To further elucidate the functions of wildtype huntingtin, participants agreed that a better understanding of huntingtin's N-terminus is required. As noted above, the N-terminus is a target for several post-translational modifications, contains sites for the binding of multiple interacting proteins, is involved in huntingtin's subcellular localization and its interactions with organelles, and influences huntingtin's structure and toxicity when the polyglutamine stretch is expanded. In addition, as noted by Scott Zeitlin, huntingtin's N-terminus evolved more recently than the rest of the protein, suggesting it might be of particular importance to organisms with central nervous systems, as proposed by Elena Cattaneo.

To address this need, Zeitlin's team has generated knock-in mice expressing various versions of huntingtin: constructs that lack the N-terminal 17 amino acids, the polyglutamine stretch, or the proline-rich region, as well as constructs that lack both the polyglutamine and proline-rich regions, and constructs lacking all three regions. Surprisingly, mice expressing huntingtin that lacks the polyglutamine stretch or lacks both the polyglutamine and the proline-rich regions, outperform their wildtype littermates in behavioral tests such as the rotarod and Morris water maze. In addition, deletion of any of the three domains does not appear to impair development. However, as noted by Zeitlin, aging and/or stress might reveal deficiencies that have yet to be discovered, and could be consistent with the findings of Truant and others.

Another model to assess the in vivo effects of deleting a portion of huntingtin's N-terminus was described by William Yang. Yang's team developed a mouse model expressing full-length, wildtype human huntingtin that lacks the N-terminal 17 amino acids. Consistent with Zeitlin's observations, Yang found that the deletion did not disrupt huntingtin's ability to rescue embryonic lethality in huntingtin knockout mice and, so far, mice carrying the deleted version of huntingtin appear to be normal. The

deletion of regions of huntingtin's N-terminus had more visible effects, however, in the context of mutant huntingtin, as described by both Yang and Zeitlin (see Mutant Huntingtin Toxicity).

As investigations proceed to further understand huntingtin's biological functions, Truant noted it will be important to study huntingtin in models that express the protein at low, physiological concentrations, such as those developed by Zeitlin and Yang. Some models overexpress huntingtin more than 1000-fold which could result in misleading data, particularly since excessive full-length huntingtin can be toxic.

Mutant huntingtin toxicity

As pointed out by Marcy MacDonald, the alteration that causes HD is subtle. It is merely the addition of a few extra base pairs (CAG repeats), usually in a single allele, of a single gene. Given the robustness of biological systems and their tolerance of environmental and genetic variability, it is remarkable that such a slight modification could result in such a devastating disorder with a biology that has proven so difficult to unravel.

Of course, it is the context of these few additional CAGs that make this subtle alteration so deadly. As previously discussed, huntingtin is a very large and multi-functional protein and the expansion of its polyglutamine stretch appears to result in both gain-of-function, as well as loss-of-function alterations. Too many glutamines turns huntingtin into a toxic protein that aggregates and disrupts many cellular functions. In addition, the polyglutamine expansion also causes damage by impairing huntingtin's normal biological functions.

A particularly noxious combination of gain- and loss-of-function alterations in HD seems to affect "proteostasis"—a term coined by Jeff Kelly and colleagues to describe the dynamic maintenance of a healthy cellular proteome by pathways which coordinately control the biogenesis, folding, trafficking and clearance of proteins. Not only does mutant huntingtin form aggregates that ultimately overwhelm the proteostatic mechanisms of the cell, but it interferes with the performance of some of huntingtin's normal functions which are themselves associated with proteostasis.

Kelly provided participants with an overview of proteostasis and how it might be possible to harness its mechanisms to treat a variety of diseases, including HD (see Treatments). Kelly explained that newly synthesized proteins have many possible fates: folding, misfolding, aggregation, sorting, clearance. These paths are always competing with each other and must be maintained in balance and aligned with changing cellular and subcellular needs. It is a complicated process of keeping concentrations at the right levels for proper cellular function. A network of over 2500 proteins constantly monitors the levels and conformations of proteins in real time, making appropriate adjustments in individual subcellular compartments. This network is regulated by stress-responsive signaling pathways which transduce signals from stress sensors into transcriptional changes.

Several observations indicate that proteostasis is compromised in HD. For example, at this meeting, Chris Ross presented data indicating that one of the most significant changes in protein levels in HD is the upregulation of proteins involved in folding and degradation (see New Tools and Approaches: *iPSCs*). Some of these changes may result from the activation of protective mechanisms, while others may be pathological. Both of the alterations, however, reflect the strain HD places on cells' proteostatic systems.

Huntingtin aggregates

The most visible and striking evidence of proteostasis dysfunction in HD is the formation of protein aggregates in vulnerable cells. Although aggregates have been the focus of many studies throughout the years, their formation, composition, and effects on cellular function still remain unclear. As explained by Daniel Hatters, one of the challenges is that aggregates are difficult to visualize at the molecular level. In addition, there are multiple types of aggregates that can have very different effects on cellular physiology, ranging from toxic to protective.

To dissect and examine the properties of different mutant huntingtin aggregates, Hatters and colleagues developed a technique using flow cytometry, pulse-shape analysis, and two fluorescently-labeled biosensors to separate and recover cells selectively enriched in huntingtin monomers, oligomers, and inclusions. The technology allows the researchers to monitor aggregation-related phenomena, such as cell stress, while simultaneously tracking other cellular parameters for which biosensors have been developed, such as mitochondrial membrane potential.

One of Hatters's initial observations using this technology is the heterogeneous nature of HD inclusions, which includes a subset of aggregates that bind the biosensor that purportedly binds only monomers. These inclusions appear soon after transfection of a mutant huntingtin construct into cultured cells and decrease steadily over time. One possibility is that some of the huntingtin that co-localizes with these inclusions is loosely associated with the aggregates in its monomeric form. Alternatively, the proteins in the inclusions that bind the monomeric biosensor may be in a particular conformation that has an affinity for the biosensor, even though they are not monomeric. Hatters added that these inclusions are more prevalent in cells expressing constructs with very long polyglutamine stretches.

The heterogeneity of inclusion forms is consistent with findings from Truant's lab. Reviewing observations he presented at the HD2010 meeting, Truant described two types of large aggregates: tight, compact fibrillar inclusions and globular aggregates. Using fluorescence recovery after photobleaching (FRAP), his team found that the exchange of mutant huntingtin in and out of globular inclusions is much more rapid than in fibrillar inclusions. Thus, it is possible, as noted by Hatters, that the inclusions labeled by the monomer-specific biosensor correspond to the globular aggregates observed by Truant. Interestingly, data from Truant's lab indicate that globular aggregates might be more toxic than the more stable, fibrillar aggregates.

Dissecting the huntingtin domains involved in aggregate formation, their structure, and post-translational modifications will be critical to understanding the toxic or protective effects of these aggregates. As described by Ron Wetzel at the HD2010 meeting, however, this task is not easy. The process of huntingtin aggregation is complex, involving at least two different pathways in competition with each other. Also, as mentioned by several participants at this year's meeting, mutant huntingtin might behave as a prion that propagates its misfolded state. Prions act as a template to guide the misfolding of more proteins into the prion form, triggering a chain reaction that produces large amounts of misfolded proteins and fibrillar aggregates. Several participants expressed interest in pursuing investigations to help determine whether the mutant form of huntingtin is indeed a prion-like protein.

Studies to date indicate the polyglutamine stretch and its two flanking regions—the N17 domain and the polyproline domain—are importantly involved in aggregation and toxicity. Thus, several studies

presented at the meeting focused on these domains. For example, Zeitlin and Yang examined the effects of deleting the proline-rich or N17 domains in transgenic animals. Zeitlin generated knock-in mice that express a chimeric mouse/human version of huntingtin with an expanded polyglutamine stretch (140Q) and a deletion of the human proline-rich region. Zeitlin's interest in the proline-rich region stems from previous work indicating that intrabodies which specifically target the proline-rich region appear to reduce polyglutamine toxicity. In addition, the proline-rich region plays an important role in several protein-protein interactions and appears to have co-evolved with the polyglutamine stretch, as they have both lengthened in parallel.

Consistent with experiments in yeast and mammalian cells in culture, Zeitlin observed that the deletion of the proline-rich region rescued some, but not all, of the behavioral and neuropathological HD phenotypes. Of particular interest, Zeitlin was unable to detect any of the large, characteristic HD inclusions in the striata of mice carrying the deleted version of mutant huntingtin, using several aggregation-specific antibodies. And although he did observe small perinuclear and nuclear inclusions using an anti-polyglutamine antibody after treatment with formic acid, their presence was decreased by ten-fold in the cytoplasm. These changes in the presence and subcellullar distribution of aggregates correlated with an improvement in several pathological and behavioral phenotypes. Gliosis was delayed, and lipofuscin accumulation and the expression of DARPP-32—a marker for medium spiny neurons in the striatum—were normalized. In addition, mice lacking the proline-rich domain performed better on a vertical movement test than HD mice without the deletion. However, no improvement was observed in the rotarod task.

Similar experiments to test the in vivo importance of the other domain flanking the polyglutamine stretch, the N17 domain, were described by Yang. As previously mentioned, Yang created BACHD mice lacking this domain with either a normal or expanded polyglutamine tract. Yang explained that N17 has been shown to accelerate mutant huntingtin peptide aggregation, play a major role in the subcellular localization of huntingtin, and affect its clearance. It is highly regulated, being the target of at least 11 post-translational modifications.

Surprisingly, Yang observed that deleting the N17 domain in the context of an expanded polyglutamine resulted in acceleration, rather than the expected reduction, in nuclear aggregate formation. Furthermore, these animals showed HD-like symptoms earlier than HD mice, including depression-like behavior and impaired rotarod performance. They also began suffering at an earlier age from progressive weight loss, progressive and selective forebrain atrophy, loss of DARPP-32 neurons, gliosis, and changes in striatal gene expression similar to those observed in human HD. Interestingly, these mice also exhibit a progressive, abnormal set of movements that are chorea-like, with twisting and upward motions. Although some thought these were similar to the seizure movements of "stargazer" mice, Yang carefully studied the mice's patterns of cortico-striatal activity and behaviors, and established that they do not have the "stargazer" phenotype.

To explain his findings, Yang proposed that N17 may act as a bidirectional molecular switch to critically modify HD pathogenesis in vivo. It is possible that, early in HD, N17 is protective, perhaps by carrying out its important roles in mediating and regulating normal huntingtin functions, including stress-response activities, as described above. Eventually, however, this beneficial role is overwhelmed by the disease process and N17 then becomes a contributor to toxicity, perhaps by accelerating aggregation, and/or interfering with mutant huntingtin clearance and localization.

It is also important to consider the interactions between the N17 and proline-rich regions. As described by Truant, intramolecular interactions between these domains might play an important role in aggregate formation. Based on studies using Förster resonance energy transfer (FRET) microscopy coupled with time-domain fluorescence lifetime imaging (FLIM), Truant reported at the HD2010 meeting that polyglutamine tracts with fewer than 37 repeats appear to act as a flexible hinge allowing the N17 domain to fold back upon itself and interact tightly with the proline-rich region. Constructs with longer polyglutamine stretches or point mutations that destroy the N-terminus alpha helix, however, show marked reductions in interaction, which Truant has now verified to be dominant. These differences appear to be important in determining which types of aggregates will form—fibrillar when the inter-domain interaction occurs easily, and globular (and more toxic) when it doesn't.

Participants also emphasized the importance of post-translational modifications in mutant huntingtin aggregation and toxicity. In 2009, Yang and colleagues reported that mice expressing mutant huntingtin with amino acid replacements that mimic the phosphorylation of serines 13 and 16 are rescued from developing the motor and behavioral deficits associated with HD, as well as from developing mutant huntingtin aggregates and selective brain atrophy. Moreover, at the HD2010 and HD2012 meetings, Truant described that phosphomimicry of these serines in isolated cells, shifts the aggregate population towards fibrillar forms (presumed to be less toxic), whereas kinase inhibition results in more globular aggregates. In addition, the phosphorylation status of N17 seems to be involved in determining the subcellular localization of mutant huntingtin. Using phospho-specific antibodies, Truant's team has observed an increased level of phosphorylated species in the nucleus. Truant is currently examining the kinases involved in regulating the phosphorylation status of N17 (including casein kinase II and IκB kinase) and testing small molecule regulators of these enzymes that are permeable to the blood-brain barrier.

Mutant huntingtin's interactions with other cellular proteins may also be important in huntingtin aggregate formation and toxicity. For example, Gill Bates explained that histone deacetylase 4 (HDAC4), a transcriptional repressor, associates with mutant, but not wildtype, huntingtin and co-localizes predominantly with cytoplasmic inclusions in the brains of HD mice. HDAC4 contains a glutamine-rich domain and interacts with mutant huntingtin in a polyglutamine length-dependent manner. Knocking down HDAC4 reduces and delays aggregate formation, specifically in the cytoplasm. This reduction in aggregates results in several improvements in the HD phenotype, including: enhanced cortical BDNF expression, improved synaptic function in medium spiny neurons of the striatum, enhanced motor performance and increased survival. However, HDAC4 reduction fails to ameliorate HD-associated weight loss or global transcriptional dysregulation. Bates hypothesizes that the beneficial changes she has observed are the result of a dampening of HDAC4's effects on mutant huntingtin's rate of aggregation, rather than on a decrease of HDAC4's enzymatic activity. Consistent with this hypothesis, Bates has observed no change in the acetylation profile of her knockdown mice and, as described above, has evidence to indicate HDAC4 directly associates with mutant huntingtin in inclusions.

The interaction of cellular proteins with mutant huntingtin aggregates is also relevant to determining the aggregates' toxicity. Several proteins have been found to co-localize to huntingtin aggregates and it is likely that the sequestration of at least some of these contributes to HD pathology. For example, Karam

reported the association of optineurin with huntingtin aggregates in the base of photoreceptor cilia, and hypothesized that it might contribute to pathological ciliary elongation.

Downstream Alterations

The expression of mutant huntingtin 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.

Lipid Alterations
At the HD2012 meeting, several participants presented data implicating alterations in lipids, and their associated pathways, as important pathologies of HD. As explained by Suzanne Reid, several of the phenotypic alterations in HD suggest the disease involves early and profound metabolic dysfunction. Energy deficits have been reported in pre-symptomatic individuals, and once symptoms develop, weight loss, insulin resistance, and disruptions in cholesterol metabolism are observed. Given the potential involvement of lipids in all of these alterations, Reid and her colleagues examined the lipid profiles of HD brains. The team used gas chromatography to profile fatty acid methyl esters in post-mortem tissues from the superior frontal gyri of 10 HD individuals and 10 controls. Their results show significant HD-associated changes, both relative and absolute, in 8 of the 36 fatty acid esters they detected. Most of the saturated fatty acids were increased in HD, suggesting alterations in beta oxidation and/or desaturase activity. In addition, two fatty acid esters were found to be decreased in HD brains. Gondeic acid, a dietary lipid derived from olive oil, was the most affected.

To extend these findings, Reid plans to increase the sample size and conduct lipid fractionations. It is possible that these additional studies will provide candidates for dietary lipids that may ameliorate HD symptoms. So far, however, most of the disruptions identified do not involve lipids that are usually obtained from food. As noted by Edward Wild, it may also be necessary to re-think the advice given to individuals with HD to consume high-fat foods. Hopefully, more specific recommendations will be possible in the future.

As reported by Carlos Cepeda, who described the work of Marta Valenza and colleagues, there are already indications that altering the concentrations of one lipid, cholesterol, in the brain may ameliorate HD pathology. Previous findings from this group revealed that cholesterol and cholesterol biosynthesis are reduced in the brains of several mouse models of HD and humans, after HD symptoms develop. Furthermore, the transcription of key genes of the cholesterol biosynthetic pathway is significantly downregulated in HD brains.

Cholesterol is particularly important for brain function as indicated by its enriched presence in the brain as a component of plasma membranes, lipid rafts, myelin, and neurotransmitter vesicles. As described below, Valenza has now obtained data indicating that supplementing the brain with exogenous cholesterol could help lessen HD symptoms (see Therapeutic Candidates: Supplementing decreased metabolites and hormones).

Another lipid reported to have disease-modifying effects in HD mice is the ganglioside GM1. As explained by Simonetta Sipione, gangliosides are glycosphingolipids that are highly enriched in the

brain and are the major components of lipid rafts. Gangliosides bind to membrane receptors and ion channels with high specificity and deliver them to lipid rafts where they activate intracellular signaling pathways. They play important roles, not only in cell signaling, but in cell adhesion and myelin-axon interactions. Interestingly, defects in the ganglioside biosynthetic pathway result in neurodegeneration that shares some similarities with HD. Moreover, the GM1 ganglioside, which activates TrK receptors for neurotrophins, has been found to be decreased in several models of HD.

Hypothesizing that GM1 might provide protection against susceptibility to apoptosis in HD cells, Sipione's team treated knockin HD mice with GM1 and, indeed, observed enhanced survival. Conversely, decreasing GM1 levels in wildtype cells resulted in an HD-like susceptibility to apoptotic stimuli. Sipione suspects that GM1 is enhancing cell survival through its activation of the PI3-kinase/AKT pathway. In addition, Sipione observed that GM1 administration results in phosphorylation of huntingtin's serines 13 and 16, the same serines which have been shown by Yang and colleagues to rescue many HD pathologies in their phosphorylated states. Thus, Sipione's current model is that decreased levels of GM1 in HD brains result in increased apoptotic susceptibility *and* increased mutant huntingtin toxicity. As described below, Sipione is now investigating the therapeutic potential of GM1 to ameliorate the HD phenotype (see Therapeutic Candidates: Enhancing Cell Survival).

Alterations in Gene Expression
Many studies have investigated the transcriptional alterations that accompany HD. But it has been difficult to pinpoint the changes that are most relevant to the disease process and most amenable to therapeutic intervention. New findings and new tools described at the meeting, however, indicate this area of research is advancing in promising directions.

For example, Audrey Dickey showed how studies focusing on a master regulator of transcription that is disrupted in HD are revealing multiple tractable targets for therapy. In previous work reported at the HD2010 meeting, Al LaSpada's team showed that the dysregulation of the transcriptional regulator peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) is linked to mitochondrial and metabolic deficits associated with HD. As described by Dickey, the team also found that PGC-1α can rescue HD proteotoxicity in vitro and, more recently, in vivo. PGC-1α activates several nuclear receptor transcription factors, including PPAR-δ. PPAR-δ is a transcription factor that regulates lipid and glucose metabolism, and is abundantly expressed in the brain. Interestingly, PPAR-δ was also recently found to bind retinoic acid and enhance cell survival. This is in contrast to some of retinoic acid's well known effects inhibiting cell growth and inducing apoptosis through its binding to the retinoic acid receptor (RAR). Moreover, PPAR-δ was identified in an unbiased screen for proteins that interact with both wildtype and mutant huntingtin. Based on these observations, Dickey and colleagues hypothesized that impaired PPAR-δ function might underlie mitochondrial, metabolic, and cell survival alterations associated with HD.

First, Dickey and co-workers used immunoprecipitation to assess huntingtin-PPAR-δ interactions in vivo. The researchers found full-length huntingtin co-precipitating with PPAR-δ in striatal cells of BACHD mice. Using luciferase constructs in neurons in culture, the researchers then monitored the activities of PPAR-δ, RAR and PGC-1α under different conditions. Findings from these experiments indicate that signaling by the RAR was favored in HD neurons over PPAR-δ. Furthermore, when RAR signaling was enhanced in BACHD primary neurons by overexpressing retinoid binding protein, cell death and mitochondrial dysfunction increased, while augmenting PPAR-δ signaling boosted cell

survival and mitochondrial function. To determine whether the expression of genes regulated by PPAR-δ is disrupted in HD, as predicted by the researchers' hypothesis, Dickey performed microarray analyses, RT-PCR, and chromatin immunoprecipitation (ChIP) assays. The findings revealed that, indeed, the expression of nearly all genes regulated by PPAR-δ is altered in individuals with HD, even in the early symptomatic phases of the disease. The researchers are now extending their studies using silencing RNAs to manipulate the levels of PPAR-δ and the targets it regulates.

In addition to these important transcriptional alterations, HD is associated with many other changes in gene expression that occur early in the disease. Indeed, Ernest Fraenkel and colleagues observed transcriptional changes occurring well before symptoms appear in the striata of R6/1 mice. The researchers identified changes in three genes at 4 weeks after birth, and many changes in neuronal genes at 8 weeks. Given that DNA methylation regulates gene expression and can change in response to both normal cellular functions (such as neuronal activity), as well as disease processes, Fraenkel and coworkers evaluated the epigenomic status of R6/1 striata. Using ChIP assays followed by high throughput sequencing techniques to identify histone acetylations and DNA methylations, the researchers found alterations in methylation patterns that were anti-correlated with gene expression levels. Several of the genes with altered methylation profiles corresponded to transcriptional regulators, such as Sox2, Fra2, and JunD.

Another facet of gene expression that may benefit from additional investigation in HD is micro RNA (miRNA) regulation. miRNAs are post-transcriptional regulators that target mRNA molecules causing translational repression or target degradation. It is estimated that roughly 60% of all mammalian genes are regulated by miRNAs. Sharing data from his studies on amyotrophic lateral sclerosis (ALS), Oleg Butovsky emphasized the potential importance of miRNAs in neurodegenerative disorders and their value as therapeutic targets. Butovsky's team identified unique miRNA signatures in inflammatory monocytes in the SOD1 mouse model of ALS, as well as in the monocytes of humans suffering from ALS. The researchers also observed that recruitment of these monocytes to the spinal cord appears to be a key mediator of pathology. At least some of the de-regulated miRNAs, noted Butovsky, might be targets for therapeutic intervention. Indeed, the genetic ablation of miR-155 attenuated the recruitment of monocytes to the spinal cord of SOD1 mice and prolonged survival.

An ambitious effort to integrate transcriptional data with genomic and proteomic data was also presented at the meeting. With the increase in 'omics' data being generated by studies of the biology of various diseases, many models have been created to help make sense of these large, unwieldy datasets. However, as noted by Fraenkel, the data rarely fit neatly into expected signaling pathways. Furthermore, different types of 'omics' data for the same disease process often appear to contradict each other. In addition, the models often result in overly complicated "hairball" networks, too densely populated to provide new insights or understanding. Part of the problem, noted Fraenkel, is that many aspects of biology are still unknown.

To address these issues and attempt to maximize the value of available data, Fraenkel and co-workers are using an advanced computational algorithm to create network models that connect changes across 'omics' datasets (e.g., changes in the transcriptome, phosphorylome, proteome, and protein interactome). The approach is based on the prize-collecting Steiner tree algorithm and is designed to find an "optimum network" that connects genes and proteins detected in experiments through inferred protein-protein and protein-DNA interactions derived from high throughput assays. The resulting

optimum network can then be analyzed with gene ontology enrichment tools. The team has so far applied their approach to the study of a few diseases, including glioblastoma, and found key nodes in the network that describes the biology of the disease. The model appears to be predictive, but does not provide information on whether the key nodes should be upregulated or downregulated to ameliorate the diseased state.

An important feature of Fraenkel's models is that the data used to build them are weighted according to their robustness. This helps minimize the problem, expressed by Mary Kennedy, that some inconsistencies between datasets are not due to a lack of biological knowledge, but to experimental error or incorrect assumptions. Indeed, Fraenkel provided an example of this problem noting that mRNA concentrations are commonly used as indicators of protein levels when, in fact, protein and mRNA levels can deviate from each other by as much as 1000-fold.

Localizing the pathology that causes HD

Which cells and tissues are affected by the expression of mutant huntingtin and why? Despite their apparent simplicity, these questions still lack definitive answers. Huntingtin is ubiquitously expressed in the mammalian body, yet not all cells follow the same fate in the disease process.

It's been long known that the striatum, and in particular the medium spiny neurons of the striatum, are particularly vulnerable to HD. Thus, many investigations have focused on these cells, attempting to identify unique characteristics that confer susceptibility. As explained by John Lee, a recent study by Sol Snyder's team, for example, offered a particularly appealing candidate underlying striatal susceptibility: the small guanine nucleotide-binding protein Rhes. Rhes is selectively localized to the striatum and Snyder's team found that it interacts with mutant huntingtin, inducing its SUMOylation and enhancing cytotoxicity.

To confirm this observation in vivo and test whether it is an amenable target for therapeutic intervention, Lee and colleagues silenced Rhes expression by injecting vectors encoding anti-Rhes RNAi into the striata of BACHD mice. Four months after injection, the researchers observed a normalization of anxiety-like behaviors associated with HD. However, magnetic resonance imaging (MRI) at 7 months revealed acceleration in striatal tissue loss. Lee hypothesized that the silencing of Rhes augmented HD pathology because it interfered with Rhes's role as a modulator of the mTOR signaling pathway which regulates autophagy. This pathway is already compromised in HD and the silencing of Rhes likely exacerbates the deficiency. Consistent with this explanation, Lee observed that overexpressing Rhes delays the onset of motor deficits in HD mice. Thus, Lee cautioned participants about the risks of implementing long-term therapies based on the inhibition of Rhes.

The focus on the striatum and the medium spiny cells in particular, continues to be an important aspect of HD research, but there is an increasing realization that HD affects many other brain areas and these alterations are fundamental to the disease process. Indeed, several researchers like Diana Rosas, who has extensively studied the effects of HD throughout the brain, consider HD to be a disease of the whole brain.

But HD does not affect all brain regions equally, nor does it affect all individuals in the same way. Based on extensive studies of brain tissues from The Neurological Foundation of New Zealand Human

Brain Bank, Richard Faull and his team have found a remarkable variability in HD brain pathology across individuals. Moreover, they discovered that this variability correlates with the symptoms the individuals experienced over the course of their disease. Faull explained that thanks to the very close relationship of the curators of the brain bank with the families who have donated the tissues, he and other researchers worldwide, have access to the detailed histories of individual donors. This invaluable resource allowed Faull and colleagues to make detailed correlations between HD symptoms and brain pathology. Rather than seeing generalized striatal atrophy in all brain samples, Faull and colleagues observed different patterns of pathologies in the basal ganglia, including, but not restricted to the striatum, as well as different cortical pathologies in individuals who had experienced different symptoms during their lifetimes.

Faull began his investigations studying the striatum. As originally described by Ann Graybiel, the striatum is composed of two compartments: the matrix and striosomes. The matrix, which is rich in acetylcholine, receives afferents most directly related to sensorimotor processing. In contrast, striosomes receive many inputs from the limbic system, particularly the amygdala. Examining the striata of 35 HD brains, Faull observed that the striatal matrix of individuals who had mostly suffered from motor symptoms in their lifetime, was much more damaged than that of individuals who had predominantly suffered from mood symptoms. Conversely, the striosomes of individuals with mood-dominant symptoms were more affected than those of individuals with motor-dominant symptoms. Furthermore, individuals with mixed phenotypes had correspondingly mixed pathologies.

These initial studies suggested that pathology in the cortex was also worth examining. Faull observed major and widespread cell loss in the cortex, but it was not uniform. Using unbiased stereological techniques to count cells in specific cortical areas of 12 HD brains, he once again observed pathology patterns that were strikingly correlated with the subjects' symptoms. Individuals who had suffered from a motor-dominant phenotype showed pathology in the motor, temporal and visual cortices, while those who suffered from a mood-dominant phenotype had more damage in the cingulate gyrus and temporal association cortex. Interestingly, both pyramidal cells and interneurons were affected (see Cell Interactions). Faull's group is currently examining other areas of the brain that are also affected by HD. For example, the team has found that the globus pallidus, a principal target of striatal outputs, suffers major cell loss in HD, particularly in the external segment. Faull's group is also investigating the pathology of the thalamus, whose connections with the striatum have been recently found to be significantly altered in HD (see Cell Interactions).

Cell Interactions

A key question highlighted by these results is the degree to which HD pathology is cell autonomous versus non-cell autonomous. Which of the pathologies observed by Faull and others are caused by the expression of mutant huntingtin within the cells that appear affected, and which are caused indirectly, by abnormal interactions between cells? As discussed at the HD2010 meeting, mutant huntingtin appears to mediate both types of effects, with far-reaching consequences that can result in the disruption of entire neuronal networks. New findings presented this year, bolstered this proposal, implicating the dysregulation of both cortical and thalamic inputs in striatal pathology. The possibility of interneuron dysfunction affecting pyramidal cell function was also discussed. Moreover, glial malfunction was identified as a potential source of neurotoxic damage.

Previous studies from several laboratories, including Michael Levine's, George Rebec's and Lynn Raymond's, indicate that corticostriatal communications are disrupted in HD. Furthermore, there is evidence to suggest that cortical alterations result in striatal dysfunction. In particular, William Yang showed that striatal degeneration decreases and striatal gene expression is partially normalized when mutant huntingtin is downregulated in the cortex. Extending these studies, Ana María Estrada Sanchez has now evaluated the electrophysiological properties of cortical and striatal neurons in freely behaving BACHD mice expressing mutant huntingtin at reduced levels in cortical pyramidal neurons. Estrada Sanchez observed that striatal firing rates remain altered when mutant huntingtin is reduced in the cortex, but the bursting properties of striatal cells improve. Thus, some of the electrophysiological alterations of striatal cells mediated by mutant huntingtin appear to be cell autonomous (as indicated by the unchanged firing rates), whereas some of the alterations are, at least partially, non-cell autonomous (as indicated by the observed improvement in bursting patterns).

Estrada Sanchez's experiments also revealed what appear to be non-cell autonomous effects in HD involving interactions between cortical interneurons and pyramidal cells. Bursting activity (spikes per burst and bursting probability) was increased in cortical pyramidal cells despite the very low levels of mutant huntingtin expression in these cells. One possibility, noted Estrada Sanchez, is that cortical interneurons, whose levels of mutant huntingtin expression are not reduced, cause the pyramidal neurons to engage in this disrupted bursting behavior. Interestingly, a link between interneurons and pyramidal neurons was also suggested by Faull. His team observed that cell loss in the motor and cingulate cortices included both pyramidal cells and interneurons. Different types of interneurons (expressing calbindin, calretinin, or parvalbumin) were uniquely affected and, like the pyramidal cells, their losses correlated with HD phenotypes. Faull proposed that interneuron malfunction may cause pyramidal cell excitotoxicity.

Much attention has been focused on corticostriatal connections in HD, but two new studies described at the meeting indicate that thalamostriatal pathways are also importantly disrupted. Taking advantage of the differential expression of glutamate transporters in cortical terminals (VGLUT1) and thalamic terminals (VGLUT2), Anton Reiner and colleagues examined cortico-striatal and thalamo-striatal connections by immunolabeling the striata of HD knock-in mice. The researchers also differentially labeled striatal neurons of the direct pathway (using antibodies against dopamine D1 receptors) and indirect pathway (D1-negative), as well as interneurons (using choline acetyltransferase immunolabeling). Importantly, the researchers used heterozygous Q140 knock-in mice which develop motor symptoms and neurodegeneration more slowly than homozygous mice, enabling them to identify very early disruptions in connectivity, occurring before symptoms or changes in striatal volume are detectable.

Reiner and colleagues observed that at 12 months of age, when motor symptoms just begin to emerge but no changes in striatal volume are detectable, VGLUT1 and VGLUT2 immunoreactivities in the striatum are decreased. The reduction in thalamic inputs (VGLUT2) was detectable in both D1-positive and D1-negative neurons, whereas the reduction in cortical inputs (VGLUT1) was selective for D1 neurons. (This is in contrast to other studies which have identified D2 neurons as being affected earlier than D1 neurons.)

Most interestingly, however, Reiner's team found that at four months of age, and even at 1 month, there were already significant reductions in thalamic, but not cortical, inputs to the striatum. At four months,

cholinergic interneurons, D1-positive and D1-negative neurons showed decreased thalamic inputs. The earliest reductions, at one month of age, corresponded exclusively to terminals synapsing with interneurons. Because thalamic connections are still developing at this age, it appears there is a developmental disruption in the number of thalamic terminals innervating the striatal interneurons, in addition to the later progressive loss in inputs to D1 and D2 cells. The expression of VGLUT transporters is highly regulated, however, such that some the observed changes may be due to changes in VGLUT levels rather than to the presence or absence of specific nerve terminals. Reiner and colleagues plan to investigate this issue in more detail and will continue to count all terminals, including unlabeled ones, to achieve a more complete view of striatal innervation patterns.

Complementing these neuroanatomical findings, Ann Parievsky presented data suggesting functional alterations in thalamo-striatal connections. Levine's team has extensively studied the function of corticostriatal synapses in various mouse models of HD. However, studying thalamo-striatal synapses has proven difficult. One major obstacle has been isolating the effects of thalamic inputs from cortical inputs because the projections onto medium spiny cells are interlaced in the striatum. Using optogenetics, however, Parievsky and colleagues were able to circumvent this problem and monitor medium spiny responses to the specific activation of either thalamic or cortical inputs. The researchers injected AAV2 vectors expressing channelrhodopsin-2, a cation channel that causes depolarization of neurons when activated by blue light, into either the cortex or the thalamus of R6/2 mice. The injections were performed at 4 weeks of age, when the animals were presymptomatic, and patch recordings were performed at 8-10 weeks of age in the striatum to detect responses to blue light stimulation of either cortical or thalamic afferents.

Results from these experiments indicate that both the cortico-striatal and thalamo-striatal pathways are altered in HD animals, but in different ways. Of particular interest, the pathways differed in their evoked NMDA/AMPA response ratios (the ratio of contributions of the two glutamate receptor types, NMDA-R and AMPA-R, to synaptic responses). Stimulation of thalamic afferents resulted in NMDA/AMPA ratios that were reduced in HD mice compared to controls, whereas stimulation of cortical afferents resulted in increased NMDA/AMPA ratios. Moreover, the use of paired-pulse ratios to identify changes in presynaptic neurotransmitter release revealed that the release probability of cortical afferents is similar in HD and wildtype animals, but is increased in the thalamic afferents of HD animals. In subsequent experiments, Parievsky hopes to dissect the underlying causes of these changes in more detail. Kennedy suggested determining whether the absolute numbers of NMDA and AMPA receptors are altered in HD and Paul Rosenberg noted the potential relevance of receptor re-distribution.

Participants agreed that Parievsky's and Reiner's findings are important because thalamic inputs account for a large fraction of striatal inputs, roughly the same as cortical inputs. Moreover, thalamic atrophy is correlated with cognitive impairments in HD. Faull and Reiner added that it will be of interest to examine whether pathology in the thalamus correlates with HD symptoms, as observed by the Faull group in the cortex. Because the thalamus, like the cortex, has specialized areas dedicated to processing different sensory and motor signals, it might also undergo differential atrophy that correlates with particular HD phenotypes.

Although most research in HD has focused on neuronal cells, Michelle Gray reminded participants that other cell types in the brain are also affected and may, in turn, disrupt neuronal function. In particular, Gray discovered that the calcium-dependent release of glutamate by astrocytes in BACHD mice is

enhanced and may contribute to neuronal excitotoxicity. As explained by Gray, astroctyes are critical components of synapses. Among several of their important functions, they help control the fidelity of neurotransmission by releasing and removing neurotransmitters. Previous studies had shown several disruptions in glial function associated with HD, such as reduced levels of expression of a glutamate transporter, and a progressive increase in gliosis in the caudate. In addition, observations of neuron-astrocyte co-cultures have revealed that astrocytes expressing mutant huntingtin are toxic to wildtype neurons.

To investigate how astrocytes are affected by HD, Gray purified cortical astrocytes from BACHD mice and studied their ability to release glutamate. Gray used mechanical stimulation to induce glutamate secretion (a standard technique to elicit transmitter release in astrocytes) and found that it was dramatically increased compared to wildtype cells. After examining several potential sources of the disruption—including calcium levels, glutamate transporter function, and glutamine synthase activity—the researchers pinpointed the likely cause of the alteration: an increase in pyruvate carboxylase activity. Pyruvate carboxylase is a mitochondrial enzyme that can augment glutamate synthesis and is specifically expressed by astrocytes. Gray hypothesized that this enhanced activity results in an increased availability of cytosolic glutamate for vesicular packaging and release. This, in turn, may lead to neuronal excitotoxicity. To test her hypothesis, Gray is now pursuing studies in vivo.

A few cell types outside of the brain have also been implicated in HD pathogenesis. For example, Ed Wild showed that mutant huntingtin levels in peripheral immune cells are significantly correlated with disease burden and caudate atrophy. Whether immune cells importantly contribute to HD pathology is unknown, but evidence from other neurodegenerative disorders suggests it is possible. For example, Butovsky described the recruitment of pro-inflammatory immune cells to the spinal cord in ALS (see Downstream Alterations: Alterations in Gene Expression). Although the immune response is not the primary cause of this disease, it appears to dramatically amplify disease progression.

Even if non-brain alterations fail to emerge as therapeutically attractive targets in HD, their value as accessible windows to monitor and study HD is being increasingly recognized. For example, Wild presented compelling evidence that mutant huntingtin levels in peripheral immune cells could serve as an accessible, quantitative biomarker for HD (see Biomarkers). Moreover, as pointed out by Ai Yamamoto, Karam's observations of HD pathology in retinal photoreceptors may provide a window to study and monitor HD neuropathology, given that the retina is the only part of the CNS that can be visualized non-invasively.

Therapeutic Candidates

Reducing huntingtin production

Blocking the expression of mutant huntingtin altogether is the most direct approach to treating HD, and it has emerged as one of the top therapeutic candidates in the field. As in previous meetings, this year, participants discussed the challenges, successes and future plans for moving this therapy into the clinic. The importance of early intervention, tracking of target engagement, and the choice of animal models for preclinical testing were discussed. In addition, new ways to approach delivery challenges and new silencing agents were presented. The findings and discussions served as encouraging signs of the steady progress being made in the field.

Encouragement also came from Katherine High's overview of gene therapy and some of its successes. High explained that genetic diseases are often thought of as potentially treatable, but fundamentally incurable. However, over the past few years, gene therapy has had a small, but growing, number of victories that may soon upend this point of view. For example, gene therapy efforts to treat cancer, hemophilia B, certain immune disorders, and a rare eye disease known as Leber's congenital amaurosis (LCA), have met with recent success. High described the complex process of developing the therapy for LCA and impressed participants with the long-lived restoration of vision achieved, especially in younger individuals. High also noted that the first gene therapy drug may soon be approved by the European Commission. If approval goes through as predicted, it will be an important milestone, representing the first formally approved gene therapy drug in the Western world.

High explained that gene therapy is a complex, multi-component strategy with the main goals of achieving therapeutic concentrations and long-term expression of the therapeutic agent. In the HD field, two main approaches are being developed to silence mutant huntingtin: antisense oligonucleotides (ASO) and expression-based RNAi. ASOs are small, single-stranded DNAs designed to hybridize with a target RNA and direct its degradation. Expression-based RNAi silencing agents also induce the cleavage and degradation of their target mRNAs, but are generally delivered as encoded constructs by viruses. Both approaches have been shown to ameliorate pathology and symptoms in animal models of HD, each having a distinct set of advantages and limitations.

The delivery of RNAi constructs and the regulation of their expression were two important topics of discussion at the meeting. As reviewed by Paul Patterson and High, adeno-associated viruses (AAV) have emerged as the vectors of choice for delivering RNAi silencers. AAV vectors have been modified to accommodate large stretches of DNA, are non-pathogenic, are naturally replication-deficient, and consist of various serotypes with selective tropisms for different cell types. In addition, manufacturing methods have been optimized to produce reliable, pure batches of viruses.

Despite these advantages, the use of AAV for HD gene therapy still poses some challenges. For example, it is still unclear which will be the most effective site(s) for vector administration. As pointed out by Jodi McBride, gene therapies designed for HD have focused on the striatum because of its known vulnerability and its relative accessibility as a surgical target, but as previously discussed, many other brain regions, and even peripheral tissues, are known to be affected in HD. One possibility is to inject the viruses at various sites, but this increases the invasiveness of the procedure.

An alternative, described by Patterson and McBride is to inject AAV systemically into the bloodstream. Taking advantage of the AAV9 serotype recently described by Brian Kaspar and colleagues which can bypass the blood-brain barrier and efficiently target cells of the central nervous system, both teams are testing this virus for delivering gene therapies to the brain. Patterson's use of an AAV9 carrying a GFP-labeled construct resulted in fluorescent labeling of the choroid plexus, cortex, striatum (including medium spiny neurons), hippocampus, thalamus and spinal cord. Similarly, McBride's injection of AAV9 into the jugular vein resulted in robust, widespread transduction in the brain, with labeling of both neurons and astrocytes, but not microglia. McBride also tested the effects of pre-treating with mannitol, which temporarily disrupts the blood-brain barrier. Her team observed that the pre-treatment enhanced labeling in the striatum, hypothalamus, and motor cortex.

Not surprisingly, systemic administration of AAV9 resulted in the transduction of tissues all over the body. McBride, for example, reported labeling of the liver, heart, pancreas, stomach, testes and muscle. Because HD is known to affect the physiology of many cells in the body, this generalized transduction might prove beneficial. However, if exclusive brain targeting is desired, Patterson noted that it is possible to use brain-specific promoters or miRNA regulatory sequences to repress the expression of the construct in particular tissues. In addition, capsid libraries can be screened for AAV variants that target a particular cell type. Variants that target endothelial cells or neural stem cells, for example, have been identified and used effectively in experimental gene therapies. Screening of capsid libraries may be particularly useful, noted Patterson, if AAV9 fails to target neurons efficiently, as suggested by some reports indicating AAV9 transduces astrocytes more effectively than neurons in primates.

Testing the systemic delivery of AAV9 as a means to deliver silencers of the mutant huntingtin gene, McBride and colleagues used an allele-specific miRNA construct to reduce mutant huntingtin expression in BACHD mice. The reductions were modest in non-brain tissues and failed to reach statistical significance in the brain. McBride suspects that the titer used was too low and is now planning to increase it three-fold. Success using systemic injection of AAV9 in another neurodegenerative disorder, spinal muscular atrophy, lends hope to the future success in HD. As described by Patterson, Kaspar's team used systemic AAV9 administration to replace the affected protein in this disease (survival motor neuron protein), and was able to rescue motor function, neuromuscular physiology and increase lifespan in a mouse model of the disease. The researchers achieved a very high transduction efficiency (42%) in the target motor neurons.

One potential limitation of this approach, however, is the amount of AAV9 that would be required to treat a human. McBride considered it would be feasible, albeit expensive, to manufacture sufficient vectors. High agreed and noted that the more important problem will be dealing with potential immune responses to viral capsids. Although AAV seldom induces an immune reaction, when it does, it can comprise the effectiveness of the therapy. As noted by High, her team detected no immune responses in their trials for treating LCA blindness, nor have such reactions been reported in Parkinson's disease trials which also relied on AAV vectors.

Nevertheless, immune responses to AAV have been reported that strongly interfere with the efficacy of gene therapies. As noted by High, it is often difficult to predict when immune responses will be problematic. In her work developing a gene therapy to treat Hemophilia B, for example, her team encountered problems with immune reactions in humans which had not surfaced in their pre-clinical tests with dogs. After many years of work, the team discovered that in the human, but not in the dog, liver cells were presenting fragments of the AAV capsid protein associated with class I MHC receptors. To circumvent this problem, High now screens individuals for neutralizing antibodies to AAV before administering gene therapy. In addition, the researchers have switched to a more efficient AAV serotype which allows them to use lower viral doses. Furthermore, they monitor individuals very closely after gene therapy injections and provide them with an acute dose of prednisone if they see signs of immune reactivity. High noted that transient immunosuppression might also be worth considering for HD therapies.

Patterson noted that approximately 70% of adult humans test negative for AAV9 antibodies, so gene therapies using this serotype are likely to be effective for most individuals without a need for immunosuppression. Nevertheless, to altogether circumvent the problem of immunity, Patterson

suggested screening capsid libraries to identify antibody-resistant variants. Another potential solution, noted Patterson, is to deliver AAV through a route that avoids contact with peripheral immune cells, such as intrathecal injection. However, a recent study by Lluis Samaranch and colleagues revealed that intrathecal injections do not shield against host antibodies in animals with significant pre-existing anti-AAV antibody titers. Still, Patterson noted that intrathecal delivery may prove useful due to other advantages, such as enhanced transduction efficiency and constrained delivery to the brain.

Yet another challenge associated with the use of AAV vectors has been the inability to control their expression. This is problematic because treatments are essentially irreversible—they cannot be switched off if they prove harmful. Furthermore, treatments cannot be tailored to match symptom severity. Six years ago, at the HD2006 meeting, participants discussed the advantages of generating conditional siRNA constructs and Nicole Déglon reported the successful use of such a construct in a rat model of HD. This year, Patterson described the use of conditional DNA promoters that require very low doses of doxycyline and can be finely controlled in a dose-dependent manner. Patterson is using these promoters in his studies of multiple sclerosis. In a recent paper, his team showed that using AAV to transduce a construct encoding leukemia inhibitory factor (LIF) into mouse ependymal cells, ameliorated several pathologies associated with multiple sclerosis. The ability to conditionally regulate LIF expression should further enhance the potential of this therapeutic candidate.

An important trend revealed at the meeting, was the emergence of a miRNA platform as a top choice for delivering RNAi silencing therapies. At the HD2010 meeting, Ryan Boudreau and Beverly Davidson described the use of siRNAs embedded in a miRNA as a strategy to avoid the saturation of the endogenous RNAi processing machinery that occurs with expression of siRNAs as short hairpin or shRNAs. miRNAs are expressed at much lower concentrations than shRNAs and, hence, are less likely to overwhelm the RNAi machinery and, accordingly, have better safety profiles. This year, three different studies described the use of siRNAs embedded in miRNAs as silencing agents with encouraging results.

An example of how the optimization of siRNA technologies is moving the field forward was presented by Alex Mas Monteys. Monteys is identifying siRNA sequences that preferentially silence mutant huntingtin using a novel in vitro reporter system. Previous work from Davidson's lab demonstrated the benefits of silencing huntingtin expression, in a non-allele specific manner, in mice and nonhuman primates. Although their results revealed no ill-effects from reducing both huntingtin alleles, the long-term effects of this approach remain uncertain. Treatments for HD are likely to last for decades and such a long-lived reduction in wildtype huntingtin levels may result in significant side-effects, as suggested by huntingtin's multiple roles in normal cell physiology (see The Normal Function of Huntingtin Protein).

Thus, Monteys cloned siRNA sequences that target mutant huntingtin specifically, by recognizing either expanded CAG sequences, or single nucleotide polymorphisms (SNPs) that are prevalent in the 3' untranslated region (UTR) of mutant huntingtin alleles. The researchers generated two plasmids expressing full-length cDNAs of wildtype or mutant huntingtin with the 3'UTR of human huntingtin. In addition, the team used epitope tags fused to the C-terminal regions of wildtype and mutant cDNAs to distinguish their expression in vitro. Testing various siRNAs that target either the CAG expansion or one of three SNPs associated with the mutant huntingtin allele, Monteys and co-workers identified sequences and doses that provided the best preferential silencing of the mutant allele. Initial results

yielded a difference in selectivity of 20-30%. However, adding a mismatch to the siRNAs targeting SNPs improved selectivity to 40-45%. The added mismatch resulted in two mismatched nucleotides between the siRNAs and the wildtype sequence, which reduced binding dramatically. (Reduction in binding to the mutant allele was modest because it resulted in a single mismatch between the siRNA and its target.) Monteys's future plans include testing these siRNAs in mice expressing full-length human huntingtin with the relevant SNPs in the 3'UTR and under the control of the human huntingtin promoter.

Updates on the use of ASOs for silencing mutant huntingtin were also discussed at the meeting. At the previous meeting in 2010, important successes were described, including studies by Lisa Stanek and Holly Kordasiewicz indicating that ASOs can have persistent therapeutic effects in both pre- and post-symptomatic HD mice. This year, Amber Southwell added support to the therapeutic potential of ASOs, presenting encouraging results from experiments using ASOs with high selectivity for mutant huntingtin.

As described by Southwell, ASOs have a broad target range (ASOs penetrate the nucleus so they can act upon pre-mRNAs that still contain introns, as well as on exons) and their delivery can be regulated without inducing irreversible gene silencing. However, the latter advantage is also a disadvantage: ASOs must be delivered continuously, or at least repeatedly, resulting in procedures that are more invasive than the single injection of AAV. New data indicate, however, that it may be possible to minimize the number of injections. Southwell considered that as few as two injections per year may be sufficient to keep the disease at bay.

Describing her team's efforts, Southwell noted that they have generated a large collection of ASOs that target HD-associated SNPs, including SNPs located in introns. Testing these ASOs in cultured neurons, the researchers have been able to achieve 39-68% knockdown of mutant huntingtin without significant silencing of the wildtype allele. Using a humanized HD mouse model, Southwell and co-workers have now tested these ASOs in vivo. After a single ASO injection into one ventricle, the researchers observed widespread silencing, including the spinal cord. The silencing was selective, with a 10% knockdown of the wildtype allele and 70% knockdown of the mutant allele. By chemically modifying the ASOs, the researchers increased the specificity even further, to the point that there is no detectable knockdown of the wildtype allele. The modifications also enhanced stability and potency, without diminishing distribution. So far, there is no evidence of toxicity. Moreover, Southwell noted that a single intra-ventricular injection resulted in silencing that persisted for four months. This is consistent with findings from other groups such as Kordasiewicz's, in which the administration of ASOs resulted in effects that lasted 9 months.

Southwell noted that her team is now beginning preclinical testing in nonhuman primates and predicted these tests could translate into human trials reasonably quickly. So far, ASO distribution seems to be widespread, although concentrations are considerably higher in the cortex than they are in structures such as the striatum that are deeper in the brain. Southwell added that her team plans to compare intrathecal infusions, which have been used routinely, with bolus injections, which are expected to yield enhanced distribution. The manufacture of large quantities of ASOs required for treating humans should not be an issue, noted Southwell, because ASOs can be produced inexpensively.

Another application of ASOs was presented by Teresa Gipson. Gipson and colleagues are using ASOs to recruit endogenous methods of translational control to selectively block huntingtin translation. ASOs are

designed to modulate the secondary structure of huntingtin mRNA at its 5'UTR and at the hairpin formed by the CAG repeats. Enhancing the stability of the 5'UTR hairpin should result in the inhibition of ribosome scanning, while stabilizing the CAG repeat hairpin is expected to initiate No-Go mRNA decay.

As a proof-of-concept experiment, Gipson evaluated the in vitro translation of a series of mutant huntingtin constructs engineered to strengthen or weaken the 5'UTR hairpin. Through these genetic manipulations, the researchers were able to drop translation levels to 4% or increase them to 180%, both in vitro and in vivo. As a next step, Gipson and colleagues designed ASOs that were predicted to strengthen the hairpins by spanning the base of the stems, helping maintain the flanking sequences on either side of the hairpin adjacent to each other. Potent stabilizing ASOs were identified for both the 5'UTR and CAG repeat hairpins. Surprisingly, however, some of the control ASOs designed to hybridize with only one or the other side of the hairpins were also inhibitory and, in some cases, actually exceeded the inhibition potency of the bridging ASOs. Canvassing the hairpin regions with a series of ASOs, the researchers discovered that ASOs binding to a region immediately adjacent to the hairpin were most effective at inhibiting translation.

Gipson and colleagues are now testing these potent ASOs in cell lines. To ensure the stability of the oligonucleotides, the researchers are using bridged nucleic acids which confer resistance to nucleases. Furthermore, bridging increases the affinity of ASOs for their targets by fixing the ASOs' conformations so they are poised for hybridization. Eventually, Gipson plans to test the ASOs in R6/1 mice, possibly using exosomes—natural transport vesicles that can be targeted to cross the blood-brain barrier—as delivery vehicles. The feasibility of using exosomes is supported by recent work from Lydia Alvarez-Erviti and colleagues demonstrating that targeted exosomes can efficiently deliver siRNA against a therapeutic target for Alzheimer's disease in mouse brains.

A novel silencing agent, that is neither an expression-based molecule nor an ASO, was described by Jiaxin Hu. At the HD2010 meeting, David Corey showed how duplex RNAs with 1-3 mismatched bases in their central region can be used to block the expression of mutant huntingtin mRNA in a potent and selective manner. The inhibition appears to involve the engagement of endogenous miRNA mechanisms of translational regulation. However, as noted by Hu this year, the duplexes do not distribute in the brain as widely as ASOs. To harness the advantages of both RNA duplexes and ASOs, the Corey lab and collaborators at ISIS Pharmaceuticals have now tested single-stranded anti-CAG RNAs as silencing agents. In vitro studies revealed that these ssRNAs are potent and selective. They also yielded encouraging results when tested in HD mice by intraventricular infusion.

General suggestions for the future development of silencing therapies for HD were also discussed at the meeting. For example, participants agreed on the likely benefits of administering silencing agents early in the disease process. Although Kordasiewicz and Stanek have shown that ASO treatments in post-symptomatic mice are effective, many lines of evidence indicate that it is desirable to halt the disease's progression as early as possible. Drawing on her experience developing a gene therapy for the LCA eye disease, Kathy High urged participants to design clinical trials to include individuals in the early stages of the disease. Her team worked hard to include children in the LCA clinical trials and, indeed, children were the ones who benefitted the most from the therapy.

Participants also agreed on the importance of developing methods to reliably track target engagement. Southwell emphasized that identifying where and when huntingtin is silenced will require preclinical investigation before therapies can move into the clinic. One option, described by Ed Wild, is the quantification of mutant huntingtin in peripheral immune cells (see New Tools and Approaches: Biomarkers). Although measuring such levels in cerebrospinal fluid (CSF) would theoretically provide a more direct assessment of the activities of silencers in the brain, huntingtin is currently undetectable in CSF by antibody-based assays, noted Wild. Still, participants wondered if relevant measures might be extracted from the CSF, particularly through the same catheters or syringes used to deliver the silencing agents. For example, the possibility of isolating exosomes present in CSF to measure endogenous miRNAs (which are more stable than mRNAs) was discussed (see New Tools and Approaches: Biomarkers). Jodi McBride further noted that using gene silencing delivery procedures to co-deliver an imaging marker, might also be helpful.

Another general issue discussed at the meeting was the choice of animal models to carry out pre-clinical tests. Several participants noted the advantage of using sheep to establish therapeutic parameters, run safety tests, and optimize silencer distribution. Sheep brains share important similarities with those of humans, including their large size, which offers the possibility of using them to optimize agent distribution across large areas of tissue. Thus, the recent characterization of a sheep model of HD was welcomed enthusiastically (see New Tools and Approaches: Animal Models). Also, Neil Aronin mentioned that his team is currently using dwarf sheep to study the delivery of HD RNAi silencers. Working in collaboration with surgeons and radiologists, Aronin has identified an AAV subtype that works particularly well in these animals and is now optimizing the dosage. With 40-50 lambs expected to be born in September, the team plans to administer AAV injections in January to begin pre-clinical testing.

In addition, McBride expressed interest in developing a marmoset model of HD. She noted that these animals have a rich behavioral repertoire and her team has the expertise and facilities to develop the model, if they can secure the necessary funding.

Reducing toxic huntingtin forms

Harnessing the proteostasis network to target huntingtin aggregation and clearance emerged as a particularly promising approach. As described by Jeff Kelly, new findings indicate a variety of tantalizing options for the development of new therapeutic candidates for HD and many other diseases characterized by protein aggregation. Kelly explained that there are two types of aggregation-prone proteins: a) those that fold normally but then sample other, partially folded states and b) those that are intrinsically disordered, such as huntingtin's exon 1.

Preventing aggregation of the first type of protein can be approached by generating small molecules that stabilize the nontoxic folded state. Kelly described how his team developed this type of small molecule to inhibit the aggregation of transthyretin, a serum and CSF protein that transports L-thyroxine and holo-retinol binding protein. Transthyretin can cause amyloid disease when mutated or in the course of aging. Kelly's team discovered that transthyretin normally functions as a homotetramer and the rate-limiting step for its aggregation is the dissociation of this tetramer into monomers. Because the weakest link in tetramer association is the dimer-dimer interface where the hormone binds, Kelly and colleagues created small molecules that stabilize the dimer-dimer interface without acting as agonists or antagonists of the

hormone. After screening thousands of molecules generated by structure-based drug design, the researchers homed in on a molecule, Tafamidis, that dramatically reduces transthyretin aggregation and rescues the amyloid disease phenotype, reducing nerve fiber degeneration, muscle weakness, and cachexia. The drug has been approved for use in Europe, and is currently being considered for approval in the US by the FDA.

The results are exciting, not only because of the new treatment now available for a debilitating disorder, but because they more generally suggest that aggregates are feasible targets for structure-based drug design. And although huntingtin is unlike transthyretin in that its N-terminus lacks a highly ordered structure, it may also be amenable to approaches that rely on small molecules to stabilize nontoxic conformations. For example, as previously described, Ray Truant is characterizing small molecules that may help shift huntingtin aggregate populations from the globular to fibrillar forms (see Mutant Huntingtin Toxicity: Huntingtin aggregates).

Kelly suspects that for most aggregate-based diseases, a two-pronged approach will be desirable in which small molecules are used to stabilize nontoxic conformations, as well as to regulate the function of the proteostatic network. In the case of HD, he placed particular emphasis on developing molecules that regulate the proteostatic network because of the relatively disorganized structure of huntingtin's N-terminus (although, as mentioned above, regulating the aggregation status of huntingtin may also be possible).

Kelly illustrated the feasibility of modulating the proteostatic network by describing how his team has used small molecules to manipulate the stress-associated unfolded protein response (UPR). Kelly explained that most membrane or secreted proteins first enter the ER, where they fold and assemble. The ER responds to the burden of unfolded proteins in its lumen (ER stress) by activating intracellular signal transduction pathways, collectively termed the UPR. The UPR has three branches that regulate the expression of numerous genes that maintain homeostasis in the ER, or induce apoptosis if ER stress persists. ER stress activates the stress sensors ATF6, IRE1, and PERK, representing the three branches, which in turn produce specific transcription factors (ATF6(N), XBP1, and ATF4, respectively).

Using small molecules to modulate individual branches, Kelly has confirmed that it is possible to selectively affect the transcription of genes regulated by one branch of the UPR, without affecting the expression of other genes. This opens therapeutic possibilities as exemplified by Kelly's activation of ATF6 in models of transthyretin amyloidosis to reduce mutant, but not wildtype, transthyretin. Kelly explained that ATF-6 controls the expression of genes that distinguish normal from misfolded proteins and target the misfolded ones for degradation. A potential risk of upregulating proteostatic pathways, however, is the possibility of fostering cancer growth. Kelly considers that the problem can likely be circumvented by administering regulators in discontinuous, well-spaced doses.

It is still uncertain how best to modulate the proteostatic machinery to treat HD, but several studies point to the heat-shock response, autophagy and the ubiquitin-proteasome system as potential targets. For example, Steve Finkbeiner recently identified autophagy inducers as highly protective in cells expressing the HD phenotype. Finkbeiner monitored the risk of death of individual cells over time using a high throughput robotic imaging and analysis platform. Finkbeiner's future plans include using a photo-switchable protein to track the effects of different therapeutic candidates on protein clearance.

Moreover, Judith Frydman described the TRiC chaperonin as a particularly promising candidate for mitigating huntingtin aggregation. 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. As Frydman's lab reported at the HD2010 meeting, purified TRiC directly blocks huntingtin aggregation. Furthermore, Frydman's studies using EM tomography show that TRiC caps the tips of huntingtin fibrils, which suggests a mechanism for inhibiting fibril polymerization.

At the HD2012 meeting, Frydman described how her team has now dissected in greater detail how TRiC interacts with mutant huntingtin, offering new options for therapeutic interventions. A surprisingly small portion of a single subunit of the TRiC double-ring complex can remodel huntingtin aggregates. The researchers have found that huntingtin's N17 domain has hydrophobic determinants that interact with TRiC's CCT1 subunit. This suggests that to harness the protective action of TRiC in vivo, it may not be necessary to overexpress all eight subunits of the complex. Indeed, using only a small apical portion of CCT1, the researchers were able to prevent polyglutamine aggregation. In addition, they showed that overexpressing CCT1 in a neuronal model of HD reduced toxicity. Collaborating with Leslie Thompson and David Housman, Frydman is now testing the effects of expressing the apical domain of CCT1 in vivo using AAV. Frydman also plans to continue investigating the molecular underpinnings of the huntingtin-chaperonin interaction, and use this knowledge to redesign the CCT1 domain to increase its specificity for N17, develop small molecules that mimic TRiC, and test the effects of pharmacologically upregulating TRiC activity.

Additional ways of manipulating the proteostatic machinery to treat HD may emerge as the signaling pathways associated with huntingtin processing and clearance are better understood. Kelly noted that the use of genetically-encoded transcription factors with destabilizing domains that can be regulated by small molecules, has proved very helpful for elucidating proteostatic signaling pathways. The method relies on creating a genetic construct consisting of a destabilizing domain fused to a gene of interest. The protein encoded by the gene is rapidly and constitutively degraded when expressed in mammalian cells, except in the presence of a synthetic ligand that binds to the destabilizing domain and shields it from degradation. This technique provides a very fine, rapid and reversible control of the expression of proteins of interest. And as noted by Kelly, it is particularly powerful for studying transcription factors which function at very low concentrations.

Replacing damaged HD cells

Another candidate therapy is the use of transplantation to replace damaged or dead cells. An exciting new approach, described by Lisa Ellerby, is to harvest cells from individuals with HD, correct their CAG mutation in vitro, and then transplant the healthy cells back into the same individual's brain. The key component of this approach is the use of induced pluripotent stem cells (iPSCs). iPSCs are adult cells, such as skin fibroblasts, that have been reprogrammed into a pluripotent state which can then be differentiated to create a particular cell type, such a striatal medium spiny neuron (see New Tools and Approaches: *iPSCs*). Ellerby's plan is to correct the CAG expansions of iPSCs in vitro, re-differentiate the cells to create medium spiny neurons, and then transplant these cells into the striata of their donors.

As a first step, Ellerby electroporated iPSCs, derived from HD fibroblasts, with large BAC constructs designed to correct CAG expansions in the huntingtin gene by homologous recombination. These constructs were able to correct the cells' mutant copy of the gene, without affecting their

pluripotentiality. Ellerby and co-workers were then able to differentiate the cells into striatal neurons and found that the correction persisted. Correspondingly, they observed that all HD phenotypes measured were normalized, including caspase 3/7 activation induced by serum withdrawal, oxygen consumption, BDNF levels, and TGF-β and cadherin signaling. Ellerby has now begun to perform transplantation experiments in mice. So far, the survival rates are promising.

Richard Faull wondered if it might also be possible to mobilize endogenous neural stem cells to areas of cell loss, such as the striatum or cortex. Stem cell proliferation has been reported in the human brain and some of their migratory pathways have been described. If such pathways leading to the striatum or cortex were identified, they could possibly be manipulated to promote repair. Molecules that exert a chemoattractant effect on stem cells have been identified and may have potential for developing such an approach.

Enhancing Cell Survival

As described at previous meetings, creatine has emerged as a promising neuroprotective candidate for treating HD. Creatine is produced endogenously as well as obtained through the diet, and plays an important role in cellular bioenergetics by buffering intracellular energy levels. The evaluation of creatine's effects in humans has yielded promising results, with a phase III clinical trial using high doses of creatine monohydrate (CREST-E) currently underway. As described by Diana Rosas at the HD2008 meeting, high doses of orally administered creatine appear to improve performance in cognitive tests, reduce serum concentrations of 8-OH2'dG (a marker of oxidative stress), reduce thinning of the cortex, and decrease alterations in the white matter of individuals with HD.

This year, Rosas described the PRECREST study, a two-phase clinical trial focusing on presymptomatic HD. The study includes 64 presymptomatic and at-risk individuals and aims to assess the value of potential markers for following disease progression in premanifest HD (see New Tools and Approaches: Biomarkers), as well as monitor the effects of high doses of creatine on presymptomatic individuals. The testing of creatine's effects is being conducted as a random, placebo-controlled, triple-blind phase II study, using markers that include 8-OH2'dG levels, MRI assessment, cognitive testing, and the Unified HD Rating Scale, or UHDRS. This part of the study will last 6 months, followed by an unblinded, open-label phase for an additional year.

So far, Rosas and co-workers have observed a slight increase in 8-OH2'dG and mutant huntingtin levels in the untreated cohort, and a reduction of 8-OH2'dG in response to creatine treatment. The researchers have also observed cognitive deficits that seem to lessen after 6 months of high-dose creatine treatment, although the improvement did not reach statistical significance. Most strikingly, Rosas observed that premanifest individuals have significant and widespread brain pathology that is countered by creatine treatment, as assessed by MRI. In particular, creatine appears to prevent cortical thinning, preserve white matter integrity and may reduce striatal atrophy. The results suggest that creatine is a disease-modifying agent and support the continuation of the CREST-E trial.

Another group of neuroprotective compounds that have received much attention in the field are growth factors, in particular BDNF. HD is associated with reduced levels of BDNF in the striatum, likely because of an inhibition of cortical BDNF gene expression and alterations of the anterograde transport of BDNF from the cortex to the striatum. Furthermore, several studies have described the normalization

of HD phenotypes in response to BDNF treatment. Indeed, at this meeting, Chris Ross presented findings indicating that CAG expansion-dependent susceptibility to cell death in iPSC-derived striatal cells can be rescued by BDNF, or an agonist of the growth factor receptor TrK B.

Yet another target being examined for its neuroprotective potential is the GM1 ganglioside. As described above, GM1 modulates TrK receptor function and is decreased in the brains of several mouse models of HD (see Downstream Alterations: Lipid Alterations). Testing the effects of chronic administration of GM1 in vivo, Simonetta Sipione has observed increased huntingtin phosphorylation, elevated expression of DARPP32, and enhanced performance in a motor task in the YAC128 mouse model. Furthermore, in the R6/2 model, the researchers observed improved motor behaviors, as well as decreased weight loss and reductions in the loss of brain volume, corpus callosum volume, and striatal neurons. Sipione noted that the combination of GM1's general neurotrophic capabilities with its specific effects on huntingtin's phosphorylation state make it a particularly attractive therapeutic candidate. In addition, she noted that the risk of infusing GM1 into the brain seems to be low because excess gangliosides are eliminated by lysosomal degradation. Moreover, a clinical trial in which GM1 was infused over the course of a year to treat Parkinson's disease, resulted in no apparent ill effects.

With the exception of creatine, one of the main challenges of harnessing the neuroprotective effects of growth factors and other neuroprotective agents, is their delivery. At the HD2010 meeting, Leslie Thompson and others discussed the use of stem cell transplantation as a means of delivering these compounds. Although stem cell transplantation was originally conceived as a cell replacement therapy, many experiments have revealed that stem cells' beneficial effects most often arise from their secretion of growth factors and cytokines. Indeed, researchers have created stem cells and progenitor cells that overexpress neuroprotective agents to enhance this effect.

For example, at the HD2010 meeting, Gary Dunbar described transplanting mesenchymal stem cells overexpressing BDNF into the striata of HD mice. The procedure resulted in the almost complete rescue of defects in rotarod performance. This year, Thompson noted that similar experiments have now been performed in non-human primates with encouraging results. In addition, Clive Svendsen and colleagues transplanted neural progenitor cells overexpressing glial cell line-derived neurotrophic factor (GDNF) into N171-82Q HD mouse brains and observed a reduction in the decline of motor function and neuronal loss. Thompson noted that her team is conducting similar experiments in R6/2 mice and has seen improvements in neuronal health, as assessed by increases in synaptic and other neuronal markers.

Delivering neuroprotective molecules through cell transplantation still faces several challenges, however. As noted by Thompson, targeting a broad base of pathology, immune rejection, surgical invasiveness, and uncertainty about the ideal timing and location of delivery are some of the issues that need to be addressed. Nevertheless, the experimental successes in ameliorating HD and other neurodegenerative diseases, suggest this therapeutic avenue is worth pursuing.

Normalizing metabolites and hormones

The levels and activities of a wide range of molecules are altered in HD through direct and indirect pathways. Identifying which of these changes offers therapeutic opportunities has been challenging because the web of HD pathology is complex and predicting phenotypic outcomes is not straightforward. Nevertheless, a few alterations have been identified whose normalization seems to hold

therapeutic potential. These may have effects on disease progression and/or serve as palliative treatments to ameliorate HD symptoms.

One molecule whose normalization may result in therapeutic benefits is cholesterol. Cholesterol is importantly involved in many brain functions and is decreased in HD brains (see Downstream Alterations: Lipid Alterations). To test if exogenous cholesterol ameliorates HD pathology, Marta Valenza and colleagues, as described by Carlos Cepeda, injected biodegradable, polymeric nanoparticles carrying cholesterol intraperitoneally into R6/2 mice. Particles that were modified with glycopeptides to cross the blood-brain barrier localized to interneurons, projection neurons, and glia. After the nanoparticles degraded, the cholesterol persisted for weeks in the brain.

In initial pilot experiments, Valenza administered injections every two weeks and assessed synaptic function with electrophysiological measurements in cortex and striatum at 10-11 weeks. The researchers saw improvements, specifically in inhibitory GABA currents, which were of greater magnitude when the injections were given every week. The researchers expect that increasing the injection frequency even further might provide greater benefits. Cepeda emphasized that their findings not only support cholesterol's potential for ameliorating HD pathology, but illustrate the feasibility of using nanoparticles to deliver a variety of potentially therapeutic molecules in addition to cholesterol (e.g., BDNF), to the brain.

Another molecule that is altered in HD and might ameliorate symptoms through supplementation is melatonin, a hormone involved in regulating circadian rhythms and sleep in mammals. As explained by Maria Bjorkvist, sleep alterations occur early in HD and worsen progressively throughout the course of the disease. Indeed, Sir Michael Rawlins noted that one of the first symptoms of juvenile HD is insomnia and Rosas added that individuals with HD suffer from a variety of sleep alterations, including circadian disruptions, restless legs, hypersomnia and hyposomnia. At the HD2010 meeting, Jenny Morton described the early disruption of sleep-wakefulness patterns in HD mice and an accompanying dysregulation in the expression of genes involved in circadian rhythm regulation in the brain. Moreover, this year, Morton presented data suggesting the emergence of a circadian disruption in a sheep model of HD before other HD phenotypes are detectable.

Consistent with these findings, Bjorkvist presented data from Tom Warner's group indicating that the circadian rhythm of melatonin secretion is disrupted in HD and treatment with this hormone may ameliorate associated sleep disturbances. Bjorkvist explained that melatonin is secreted in a pulsatile manner at night and is involved in regulating sleep in a light-dependent manner. Taking hourly blood samples from control, pre-manifest and HD subjects who experienced standard light/dark conditions for 24 hours, the researchers found that the patterns of melatonin oscillations were altered in individuals carrying the HD mutation. Although the main periodicity of melatonin pulsatility started at 120 minutes in all groups, the strength of pulsatility was reduced and the pulsatility profile was disrupted in both premanifest and symptomatic individuals with HD. Furthermore, the degree of the alterations correlated with disease severity.

Bjorkvist concluded that melatonin may be considered a candidate for treating HD sleep disturbances, as well as a potential biomarker for disease progression (see New Tools and Approaches: Biomarkers). Moreover, melatonin might have neuroprotective effects, in addition to its potential ability to normalize sleeping patterns. A recent publication by Robert Friedlander's group suggests that melatonin delays

disease onset and mortality in a mouse model of HD. Bjorkvist opined that performing a clinical trial to assess the benefits of melatonin supplementation is worthwhile, particularly considering that melatonin's safety has already been established and appears to benefit individuals suffering from other neurodegenerative disorders, including Alzheimer's and Parkinson's disease.

New Tools and Approaches

iPSCs

Several new tools and approaches were described that promise to accelerate both the understanding of HD, as well as the development of new therapies. One of the most powerful and versatile tools discussed at the meeting was the growing collection of HD induced pluripotent stem cell (iPSC) lines. The potential of this relatively new technology was enthusiastically discussed two years ago at the HD2010 meeting. This year, the first examples of concrete applications were presented, indicating the technology is living up to its predicted potential.

As explained by Clive Svendsen, iPSCs are adult cells that have been reprogrammed into a de-differentiated, pluripotent state by a viral vector or episomal plasmid carrying genes that define and maintain many of the properties of embryonic stem cells. The adult cells can be fibroblasts, for example, which are easily removed from a person's skin. Once they have been reprogrammed into iPSCs, they can be differentiated in vitro to create a particular cell type, such a striatal medium spiny neuron. When the cells are derived from an individual suffering from a genetic disorder, such as HD, they can be used to generate both in vitro cellular models, as well as "humanized" in vivo models of disease. These cells also provide a powerful model system for conducting high throughput drug screenings, running pre-clinical tests, and validating human-specific therapies. Examples of each of these applications were discussed at the meeting.

As Leslie Thompson described at the HD2010 meeting, she is leading the HD iPSC Consortium to collect and characterize HD iPSC lines. The lines are now being managed by the Coriell Institute for Medical Research and are listed on their website. The collection includes cell lines with huntingtin alleles spanning a range of 17-180 CAG repeats. In addition, as noted by Ole Isacson, a European consortium is setting up to curate thousands of cell lines. Thus, the combined collections will offer an outstanding resource for HD research.

Most iPSC lines, noted Svendsen, are very stable—only those with the largest repeats show some degree of instability. And because the iPSC field is advancing so quickly—with roughly 100 papers published a week—the process of creating cell lines is rapidly and steadily improving. For example, new ways to identify iPSCs have been recently developed and the use of episomal plasmids as vectors has improved the transduction efficiency of the procedure. Although producing striatal neurons reliably and uniformly is still a challenge, Svendsen considered this will be overcome as protocols for differentiation are fine-tuned.

One of the ways in which iPSC lines promise to move the HD field forward is by offering new ways to prioritize the many phenotypes that have been associated with HD, noted Marcy MacDonald. A fundamental problem in the field is that there is a wealth of data and associated hypotheses about the

mechanistic underpinnings of HD, and identifying which of these aspects are most relevant to the disease process has proven very difficult.

The growing collection of HD iPS cells, with a range of huntingtin CAG repeats, promises to facilitate the identification of these phenotypes. As explained by MacDonald, a recent study showed that the onset of motor symptoms in HD is completely dominant and CAG-dependent. In individuals carrying two copies of mutant huntingtin, the allele with the largest number of CAG repeats is the only one relevant for predicting age of onset of motor symptoms. Neither normal allele CAG length, nor interactions between expanded and normal alleles influence age at onset. In addition, two copies of mutant huntingtin do not appear to be more harmful than one—disease onset tracks with repeat length, rather than mutant huntingtin dose. MacDonald opined that identifying the phenotypes that reflect these fundamental features of HD will be key to understanding and finding effective treatments for HD.

In line with this perspective, an extensive search to identify HD phenotypes in iPS cells, and to test their dependence on huntingtin CAG length, is underway in Chris Ross's lab. So far, his team has characterized alterations in proteostasis (including autophagy), electrophysiological behaviors, cell death, and bioenergetics. Of particular interest, Ross' group identified a susceptibility to cell death that is CAG expansion-dependent and can be rescued by BDNF, or an agonist of the growth factor receptor TrK B, in a dose-dependent manner.

To further investigate the mechanisms of this toxicity, Ross's team used the method known as iTRAQ (isobaric tags for relative and absolute quantification) which enables the comparison of relative amounts of individual proteins in complex protein mixtures. The researchers compared the protein compositions of three cell lines with differing numbers of CAG repeats in their dominant huntingtin alleles: control (under 29), 66 and 180. They found both increases and decreases in protein expression (225 proteins were up-regulated, and 158 were down-regulated) in the HD lines and observed that the changes in the 66 CAG line were a subset of those observed in the 180 line. Moreover, functional network analyses revealed that many proteins related to RNA processing were down-regulated in HD, whereas proteins involved in protein folding and degradation were upregulated.

Another advantage of using iPS cells to study the mechanistic underpinnings of HD is the possibility of observing the emergence of the HD phenotype as cells transform from pluripotent stem cells to differentiated neurons. As noted by Ellerby, iPS cells do not appear to express HD phenotypes, but microarray analyses revealed alterations in the cadherin and TGF-β signaling pathways, as mentioned above. As the cells begin to differentiate, HD-associated phenotypes begin to emerge, some at surprisingly early stages. For example, Ellerby noted that susceptibility to cell death and altered mitochondrial bioenergetics are observed in neural stem cells. Also, Ross and colleagues found CAG-dependent toxicity that was rescued by BDNF in precursors of the medium spiny neurons—cells that were negative for the markers DARPP32 and TCIP2.

Svendsen has also observed a progression in phenotype emergence in iPS cells from other diseases, in particular spinal muscular atrophy. These findings may provide insights into cell vulnerability, offering clues about the cellular characteristics that are necessary for the expression of toxicity. In addition, they may supply new information on the early stages of the disease process, as well as the developmental effects of mutant protein expression. The findings are also of interest from a technical standpoint. As

noted by Ross, at least some HD alterations may be studied without the need of carrying out the full differentiation procedure.

Yet another window being opened by iPSC lines is the possibility of investigating the cellular and molecular sources of the phenotypic variability seen in HD individuals. Just as Richard Faull and colleagues are shedding light on HD variability by correlating individuals' brain pathology with their phenotypes, iPSC lines offer the possibility of making similar correlations at the cell and molecular level. Studying HD variability in this manner might help connect the dots between the disparate collection of HD-associated alterations seen in molecules, cells, tissues, and people.

Svendsen and others are beginning to undertake these studies in HD and other neurodegenerative disorders. For example, Ole Isacson is using iPSCs to study genetic variability in familial Parkinson's disease. Isacson is the leader of the Parkinson's disease iPSC Consortium which is collecting cells from individuals suffering from different genetic forms of this disease. The researchers have observed a convergence of pathological phenotypes in neurons derived from four different iPSC lines, each having a different genetic alteration. So far, the observations highlight the importance of oxidative stress and mitochondrial dysfunction in PD. The researchers also observed a phenotype that is expressed in opposite ways in two different cell lines, providing an unsuspected clue to the mechanistic underpinnings of the disease.

Complementing these approaches, experiments by Ellerby suggest a way to isolate the effects of huntingtin CAG expansion from the genetic background and environmental variations of an individual's lifestyle. Ellerby and colleagues are correcting the CAG expansions in iPS cells using a genetic recombination strategy. In addition to its therapeutic potential described earlier, this technique allows the development of cell models of disease with identical genetic backgrounds and life histories that differ only in their number of huntingtin CAG repeats. The generation of these cell lines will provide a very clean way to dissect CAG-dependent effects in human models of disease and perhaps also help identify genetic modifiers of disease. These cell lines can be studied in vitro, as well as in vivo, by transplanting them into neonatal mouse brains. Ellerby has begun these transplantation studies and, so far, has achieved good survival rates.

Transplanting human neurons derived from HD iPSCs into mice also offers a powerful tool for studying non-cell autonomous effects in HD. Virginia Mattis in the Svendsen group is interested in this application and is using luciferase constructs to track transplanted iPS cells in host brains. The researchers plan to examine several open questions regarding cell-cell interactions in HD, including the proposal that HD is a prion-like disease, as well as the roles of microglia and other non-neuronal cell types in HD.

iPS cells also promise to help drug screening and pre-clinical testing of therapeutic candidates. For example, the humanized models described above may be useful for running pre-clinical tests, as well as validating human-specific therapies, such as allele-specific gene silencing (see Therapeutic Candidates: Reducing huntingtin production). As described by Finkbeiner, iPSC lines are also exceptionally well suited to act as model systems for high throughput drug screens and tests.

Adapting an experimental setup designed to identify mechanisms that promote or hinder the expression of particular phenotypes, the Finkbeiner group is now creating a high throughput platform for evaluating

candidate drugs for HD and other neurodegenerative disorders. The platform includes an automated microscope system that enables the tracking of individual neurons over time and a statistical approach known as survival analysis that provides a quantitative readout of the risk of different parameters on the eventual death of the neuron. The team is now working on expanding the platform's current 96-well format to 384-wells. Furthermore, the researchers have set up assays to monitor many other informative parameters in addition to cell survival, such as neurite length, proteostasis, synapse number and function, mitochondrial function, and interactions between neurons and microglia or astrocytes. The team has also developed a method that relies on a photo-switchable protein to perform pulse-chase experiments and measure the half-life of a particular protein. This method will allow them to track the effects of candidate therapeutic agents on the clearance of huntingtin protein. So far, the researchers have identified BDNF and small molecules that induce autophagy as protective in HD.

New Animal Models

Many animal models for HD have been generated over the years that have proved invaluable for advancing our understanding of the disease process, as well as testing therapeutic candidates. This year, three important new models were discussed at the meeting: mouse models carrying copies of the huntingtin gene with targeted deletions, mouse models with iPSC transplants, and an ovine model of HD.

As described above, Scott Zeitlin and William Yang generated mouse models that carry a copy of the huntingtin gene lacking a specific domain (see The Normal Function of Huntingtin Protein). The models are of particular value because they provide an in vivo context that was previously unavailable to study the complex, sometimes paradoxical functions of huntingtin's multiple domains. Furthermore, the collection of these mutants can be expanded to include deletions of other domains or other deletion combinations, which should further advance the understanding of how huntingtin's various domains function in the wildtype, and mutant, protein. They also promise to help dissect the mechanisms by which the domains interact with each other, and help delineate how they promote or hinder the formation of aggregates.

The iPSC transplant models discussed above, also promise to help advance the HD field significantly. They are expected to allow the study of the dependence of various phenotypes on polyglutamine length, as well as advance the understanding of non-cell autonomous mechanisms of disease. The models should also help screen candidate therapies, run pre-clinical safety and pharmacokinetic tests, and help validate human-specific therapies. Indeed, it is difficult to overestimate the magnitude and variety of potential contributions that iPSCs could bring to the HD field.

Participants were also encouraged by the availability of a transgenic sheep model of HD that carries the full-length human huntingtin gene with 73 CAG repeats. An international team led by Russell Snell and Richard Faull have been working for over a decade to create this model, which is now being extensively characterized by Jenny Morton and colleagues. The model is particularly valuable because the developmental pattern of the basal ganglia and cortex of sheep is similar to that of humans. In addition, as previously mentioned, the size of a sheep's brain is comparable to that of a human's, making it useful for testing interventions that rely on the distribution of therapeutic agents across large areas of brain tissue, such as gene silencing. Moreover, because sheep live for 10 to 15 years, they are expected to

have a lengthy pre-symptomatic window useful for testing therapies intended to stop or slow the progression of HD before it reaches symptomatic stages. .

To study the longitudinal development of HD in these animals and define the pre-symptomatic window, Morton's team is characterizing a wide range of physiological functions and behaviors. Working initially with 0-5 year-old sheep, the team evaluated locomotion, eye movements, cognition, personality, social behavior, brain structure, and sleep. In many cases, Morton's team had to develop new methods and devices to study these features. For example, after confirming that sheep have saccades (despite lacking foveas), the researchers built a sheep saccadometer cleverly designed to stay on the head of an awake, behaving sheep. In addition, Morton and co-workers created backpacks for the sheep to carry various devices to track their motor and social behaviors, including a GPS device, an accelerometer, a magnetometer, and a gyroscope. Studying social networks is of interest, noted Morton, because sheep are highly social animals. Moreover, the team developed tests and built the corresponding props to evaluate the sheep's cognitive functions. MRI scans were also performed (however, the large size of the animals posed a challenge, which will worsen as the researchers extend their studies to include older animals).

So far, Morton has found no overt abnormalities in any of the features described above. The only minor sign of pathology was the appearance of a very small number of brain inclusions, detectable at 3 years of age.

Faull and Snell noted they have hundreds of sheep available and urged participants to contact them with ideas for pre-clinical tests. Several participants expressed interest in using the model to test the therapeutics, safety, and spread of gene silencing interventions which are in a particularly advanced stage of development. As mentioned earlier, Aronin and co-workers have already begun to perform such tests in dwarf sheep model with encouraging results.

Biomarkers

As described by Kurt Fischbeck, to translate experimental interventions into clinical therapies it will be key to have robust, sensitive, and reliable biomarkers. The availability of such biomarkers for tracking multiple sclerosis and its candidate therapies, for example, has been instrumental in achieving recent successes in the field. There are currently a few biomarkers for HD that are emerging as clinically useful, such as the MRI measurements of cortical thinning identified by Rosas and colleagues. However, participants agreed that additional markers are needed to complement and extend their capabilities.

Kathy High noted that the HD field has the advantage of having a substantial, and growing, collection of baseline data on the disease process that should facilitate the identification of robust biomarkers. Indeed, at the HD2010 meeting Sarah Tabrizi reported the detailed monitoring of disease progression in pre-manifest subjects, including imaging, biochemical, clinical, motor, oculomotor, and neuropsychiatric assessments, as part of the TRACK-HD observational study. Among their findings, Tabrizi's team observed alterations in HD brains that can be monitored as they change over a single year, even in pre-manifest subjects more than 16 years away from the predicted onset of disease.

The characterization of these early changes promises to be very useful for therapeutic development, as emphasized by several participants at this year's meeting. Experts who have developed treatments for

other progressive disorders, such as Jeff Kelly and High, for example, stressed the importance of early tracking and intervention. Kelly noted the value of nipping pathological protein aggregation in the bud, and High described how the youngest individuals treated with gene therapy for LCA blindness were those that benefited the most from the intervention.

A pioneering study of early monitoring and early intervention in HD was presented by Diana Rosas. As described previously, the PRECREST study was designed to assess potential biomarkers of premanifest HD, as well as the effects of high doses of creatine in presymptomatic individuals. Rosas explained that biomarkers of the premanifest stages of HD could provide go/no-go evidence of a treatment's ability to modify disease in early phase studies. In addition, these biomarkers could help assess whether treatments that are effective for symptomatic individuals, are also beneficial for pre-symptomatic subjects. Of particular interest, the study uses a novel strategy for recruiting subjects, which includes at-risk individuals who have chosen not to be tested for the HD mutation. As noted by Rosas, this strategy avoids genetic testing coercion and results in trials that are more inclusive. In addition, it automatically provides an unselected, healthy control group. The strategy also greatly increases the pool of potential participants, given that the vast majority of at-risk individuals choose not to be genetically tested.

So far, the PRECREST study indicates that cognitive, biochemical and neuroimaging biomarkers which have proven their value in symptomatic HD (e.g., the oxidative stress marker 8-OH2'dG, MRI, cognitive tests, and the UHDRS) are also effective in tracking premanifest disease. In particular, deficits in cognitive function, cortical thinning, white matter alterations and striatal atrophy (occurring at about 5% per year) were all seen to be present in premanifest individuals and correlate with predicted onset of disease. Moreover, as described above, many of the measured parameters improved in response to creatine treatment (see Therapeutic Candidates: Enhancing Cell Survival). As noted by Rosas, these findings lend support to the feasibility of conducting clinical trials to test presymptomatic therapeutic interventions.

Several new candidate biomarkers for HD, as well as new strategies to identify them, were also presented at the meeting. For example, Wild reported that mutant huntingtin fragmentation in immune cells tracks HD progression. As noted above, Wild provided participants with an update of the use of TR-FRET immunoassays to quantify mutant and total huntingtin protein levels in immune cells. The team's most recent findings indicate that levels of mutant huntingtin protein in monocytes and T cells are significantly associated with disease burden scores and caudate atrophy rates, as assessed by brain imaging in the TRACK-HD study. These results suggest that mutant huntingtin levels in peripheral immune cells reflect pathogenic events in the CNS. To test whether the observed increases are due to mutant huntingtin fragmentation, Ed Wild and colleagues used immunoprecipitation and Western blots with antibodies that recognize different epitopes along the length of huntingtin protein. In support of their hypothesis, the team observed an accumulation of N-terminal fragments in HD monocytes. Wild concluded that quantification of mutant huntingtin in peripheral immune cells by TR-FRET may serve as a non-invasive disease biomarker of HD.

Biochemical and behavioral indicators of the disruption of circadian rhythms were also suggested as potential biomarkers of HD. As noted above, Warner and colleagues observed that the circadian rhythmicity of melatonin secretion is disrupted in HD and correlates with disease severity (see Therapeutic Candidates: Normalizing metabolites and hormones).

Participants also discussed strategies and technologies that promise to help identify additional biomarkers for HD. For example, Richard Perrin and Chris Ross described their use of quantitative proteomics for this purpose. Perrin is applying a sophisticated form of mass spectrometry to analyze CSF samples. As he explained, the CSF is a unique source of molecules that are relevant to brain function—its composition provides a direct, real-time readout of the physiology and biochemistry of the brain. And, although not always recognized, the lumbar puncture required for collecting these samples is relatively simple, safe, and painless. In addition, Perrin noted that standards for collecting, analyzing, and sharing CSF samples already exist for Alzheimer's disease which could be readily applied to HD.

To mine the CSF as a source of protein biomarkers for HD, Perrin and colleagues are performing multi-affinity fractionation of CSF samples, followed by quantitative label-free liquid chromatography tandem mass spectrometry. Some of the advantages of this type of spectrometry include: the ability to obtain quantitative measurements, the absence of a need for protein labeling (specific antibodies are not required), and the ability to obtain hundreds of measurements in a single, small sample (less than 0.5 ml). Perrin explained that, after immunodepleting six of the most abundant CSF proteins, the remaining proteins are trypsinized, followed by peptide analysis, resulting in the identification and quantification of essentially all proteins in the CSF. After excluding the highly variable methionyl peptides, Perrin identified 81 proteins that are present in human CSF with very little intra-individual variability, rivaling the technical accuracy of the enzyme-linked immunosorbent assay (ELISA). As expected, these proteins do vary significantly between individuals. Unsupervised hierarchical clustering of a subset of 24 proteins yielded perfect segregation of pooled and individual samples. The results suggest this approach has potential for the discovery of new HD biomarkers, not only in humans, but in animal models of disease such as sheep.

Ross's approach, on the other hand, relies on iTRAQ—which enables the comparison of relative amounts of individual proteins in complex protein mixtures—to analyze iPS cell samples. As previously described, the team has found 225 proteins that are upregulated in HD and 158 that are downregulated (see Mutant Huntingtin Toxicity: Downstream Alterations). It is expected that a subset of these will emerge as useful biomarkers of disease.

Other biochemical compounds, in addition to proteins, may also prove their value as biomarkers of HD. The cholesterol metabolite 24-hydroxy cholesterol which is produced by the brain and found in plasma, for example, is one such molecule. Furthermore, the characterization of lipids in HD brains by Suzanne Reid and colleagues may help identify additional lipid markers. Moreover, Oleg Butovsky highlighted the potential of miRNAs as novel biomarkers. As described above, Butovsky's team identified unique miRNA signatures in inflammatory monocytes of both humans and a mouse model of the neurodegenerative disorder ALS. Butovsky noted that these unique profiles could be used as a blood biomarker to monitor ALS disease progression, as well as responses to therapies.

Participants agreed that biomarker discovery should remain broad-based, including different technologies and sources of indicators. As noted by Rosas, ultimately, multiple biomarkers will likely be the best way to monitor HD. Jang-Ho Cha agreed and added that biomarkers are needed to fulfill several different needs, including tracking disease status, monitoring target engagement, and measuring the effects of interventions on biological processes that correlate with the clinical manifestation of the disease (surrogate biomarkers). In addition, multiple biomarkers for each function are needed to confirm results and, in some cases, different biomarkers will be needed for humans and animal models of HD.

As emphasized by Fischbeck, biomarkers provide the key data that demonstrate an intervention is likely to work (proof-of-concept or PoC), as well as the tools for monitoring clinical trials, which together are essential for procuring funding.

Standardizing clinical data collection

Complementing the advances in biomarker identification described above is the development of standards to systematically collect, analyze and share data across clinical studies. With its launch of the HD Common Data Elements (CDE) Project, the National Institute of Neurological Disorders and Stroke (NINDS) is spearheading this effort. As explained by Wendy Galpern, the HD CDE working group includes nearly 60 international HD experts, and is divided into 11 subgroups (e.g., motor, cognitive, behavior/psychiatry, etc.). Each of these subgroups seeks to identify CDEs in a standardized format, develop recommendations on the use of instruments in the various stages of HD, and create case report forms. This standardization is expected to make future studies more efficient, reduce their costs, and facilitate comparisons between studies. As noted by Galpern, the process of CDE development is iterative, such that CDE recommendations are posted for public comment, tested in pilot trials, and subjected to annual re-evaluations. Galpern encouraged participants to visit the HD CDE Project website at www.commondataelements.ninds.nih.gov/HD.aspx and noted that academic investigators, and to a lesser extent researchers in industry, are increasingly adopting the emerging guidelines.

<u>**Concluding Thoughts**</u>

As emphasized by Jeff Kelly, sustained funding will likely be a key component to finding successful treatments, and ultimately, a cure for HD. The successes that his team has had in developing therapies for other aggregation diseases, he noted, have hinged on 23 years of sustained support from the NIH. Indeed, Kelly is currently lobbying the NIH to incorporate mechanisms in the funding system that ensures funding continuity.

Moreover, Michael Rawlins suggested that HD warrants increased attention, presenting evidence that HD may be much more prevalent than previously thought. He noted that the prevalence of diagnosed HD is substantially higher in the United Kingdom than suggested by 22 published estimates made over the past 60 years. Examining electronic medical records from the UK's General Practice Research Database, from 1990 to 2010, Rawlins and co-workers identified a total of 1,136 cases of adult HD, indicating that the prevalence has more than doubled in the last two decades, with an estimated 7,500 or more people currently affected by HD. Rawlins described several factors that may explain this apparent rise, including the availability of the HD genetic test, improved clinical diagnoses, and a rise in doctor visits because of the increased availability of palliative treatments. In addition, the increased life expectancy of people with HD, and the decreased stigma of the disease, may contribute to families being more open with their physicians, leading to an increase in the number of cases recorded.

This increased prevalence argues, not only for intensifying efforts to find treatments for HD, but for improving current care practices. In particular, noted Rawlins, the clinical management of juvenile HD is in urgent need for improvement. It currently includes treatments that lack formal evidence of their efficacy or safety. For example, anti-depressants are often prescribed for juvenile HD, yet their effects on children and adolescents are not well understood and possible side effects include suicidal behavior.

Participants left the meeting with much food for thought. As articulated by Anne Young and Nancy Wexler: What comes next? How can research go faster? What is holding it back? How can innovation and creativity be optimally fostered? Today, more than ever, it is important to keep HD research moving forward. The availability of a treatment, or set of treatments, seems to be on the horizon, but much work is still needed to better understand the disorder and optimize the path to a cure.